

Co-encapsulation of HNF4a Overexpressing UMSCs and Human Primary Hepatocytes Ameliorates Mouse Acute Liver Failure

Defu Kong

Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital

Huiming Xu

State Key Laboratory of Oncogenes and Related Genes, Renji-MedX Clinical Stem Cell Research Center, Renji Hospital, Shanghai Jiao Tong University

Mo Chen

Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital

Yeping Yu

Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital

Yongbing Qian

Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital

Tian Qin

Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital

Ying Tong

Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital

Qiang Xia

Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital

Hualian Hang (✉ hanghualian@shsmu.edu.cn)

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Abstract

Background: Acute liver failure (ALF) is a complicated condition that is characterized by global hepatocyte death and often requires immediate liver transplantation. However, this therapy is limited by shortage of donor organs. Mesenchymal stem cells (MSCs) and hepatocytes are two attractive sources of cell-based therapies to treat ALF. The combined transplantation of hepatocytes and MSCs is considered to be more effective for the treatment of ALF than single-cell transplantation. We have previously demonstrated that HNF4 α -overexpressing human umbilical cord MSCs (HNF4 α -UMSCs) promoted the expression of hepatic-specific genes. In addition, microencapsulation allows exchange of nutrients, forming a protective barrier to the transplanted cells. Moreover, encapsulation of hepatocytes improves the viability and synthetic ability of hepatocytes and circumvents immune rejection. This study aimed to investigate the therapeutic effect of microencapsulation of hepatocytes and HNF4 α -UMSCs in ALF mice.

Methods: Human hepatocytes and UMSCs were obtained from liver and umbilical cord separately, followed by co-encapsulation and transplantation into mice by intraperitoneal injection. LPS/D-gal was used to induce ALF by intraperitoneal injection 24 h after transplantation. In addition, Raw 264.7 cells (a macrophage cell line) were used to elucidate the effect of HNF4 α -UMSCs-hepatocyte microcapsules on polarization of macrophages. The protein chip of conditioned mediums (CMs) of UMSCs and HNF4 α -UMSCs were used to define the important paracrine factors and investigate the possible mechanism of HNF4 α -UMSCs for the treatment of ALF in mice.

Results: HNF4 α -UMSCs can enhance the function of primary hepatocytes in alginate–poly-L-lysine–alginate (APA) microcapsules. The co-encapsulation of both HNF4 α -UMSCs and hepatocytes achieved better therapeutic response in ALF mice by promoting M2 macrophage polarization and inflammatory resolution effect mainly modulated by the paracrine factor HB-EGF from HNF4 α -UMSCs.

Conclusions: The present study confirms that the co-encapsulation of HNF4 α -UMSC and hepatocytes could exert an efficient effect on ALF mainly by HB-EGF and provides a novel therapeutic strategy for the treatment of ALF.

Background

Orthotopic liver transplantation for patients with acute liver failure (ALF) has improved their survival rates as well as quality of life, but this surgery is too expensive, complex and limited by donors(1). Transplantation of hepatocytes has emerged as a potential candidate to prolong the survival rate of patients with ALF waiting to undergo liver transplantation.(2) Nevertheless, the scarcity of donor hepatocytes and the quality of hepatocytes isolated from marginal livers have limited the development of this technique. Furthermore, primary hepatocytes forfeit their proliferative ability and undergo phenotypic de-differentiation and metabolic regression after isolation and *in vitro* culture.(3) Therefore, a feasible

approach to improve the quality as well as vitality of human hepatocytes after isolation is urgently needed.(4)

Mesenchymal stem cells (MSCs) are characterized by self-renewal, no tumorigenicity, low immunogenicity and immunomodulatory ability, and have a great potential in regenerative medicine.(5) Among different types of MSCs, human umbilical cord MSCs (UMSCs), isolated from Wharton's jelly of umbilical cord, have high proliferation ability and easy availability, and have drawn much attention of the researchers.(6) More importantly, the UMSCs were reported to ameliorate ALF, hepatic ischemia and reperfusion injury.(7, 8) Furthermore, a recent study revealed that MSCs co-transplanted with hepatocytes is regarded as a more effective treatment for ALF.(9) In a co-transplantation system, hepatocytes were found to temporarily support the metabolic function, whereas MSCs ameliorated the inflammatory reaction and improved the viability and the functioning of both the recipient liver and the donor hepatocytes in ALF mice.(10)

Hepatocyte nuclear factor-4 alpha (HNF4 α) is an important transcription factor of the nuclear hormone receptor family, and is essential for maintaining a normal liver architecture.(11) Moreover, it also plays a crucial role in hepatic differentiation, metabolic function and formation of a polarized hepatic epithelium and cell-cell contact.(12) Previous studies have shown that the overexpression of HNF4 α in hepatocytes or hepatocyte-like cells derived from stem cells or adults cells enhanced the differentiation and function of these cells.(13, 14) Interestingly, our previous study showed that HNF4 α -overexpressing UMSCs (HNF4 α -UMSCs) has significantly improved the differential status of hepatocyte-like cells by activating the expression of genes related to hepatocyte function.(15)

More efforts have been made to improve the viability and function of hepatocytes. Of note, microencapsulation permits exchange of nutrients, oxygen, and small molecules, forming a protective barrier to the transplanted cells. According to a previous report, the microencapsulation of hepatocytes promoted hepatocyte viability and improved albumin and urea synthesis, and also facilitated the circumvention of immune rejection.(16, 17) Hence, the present study would investigate the therapeutic potential of co-encapsulation of HNF4 α -UMSCs and human primary hepatocytes on LPS/D-gal-induced ALF mice.

Materials And Methods

Source of human liver specimens

Adult human liver specimens were collected from patients who were undergoing partial hepatectomy or liver transplantation and immediately stored at 4 °C in UW solution (the University of Wisconsin solution, Netherlands) to isolate hepatocytes. This study was approved by the Institutional Ethical Review Committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University, and all participants gave informed consent for the collection of their liver specimens.

Cell isolation and culture

Isolation of human hepatocytes was performed as described previously.(18) Briefly, the liver tissue was perfused through intrahepatic vein with PBS for 15 min at 37 °C and then digested with collagenase IV (Sigma, MO, USA) for 25 min. Next, mechanical destruction and filtration of the liver tissue were performed through a 70-um cell strainer to obtain hepatocytes suspension. Finally, the cell pellet was washed twice using GBSS (Gibco, MA, USA) and centrifuged at 80 g for 10 min. The isolated hepatocytes were cultured in Williams' Medium E (Gibco, MA, USA) with 10% Fetal bovine serum (FBS, Gibco, MA, USA).

The primary mice hepatocytes were isolated from the livers as previously described.(19) Isolation and cultureof UMSCs were performed as described in the previous report.¹⁵ UMSCs were cultured in DMEM/F12 supplemented with 10% FBS. UMSCs at the 3rd passage to the 5th passage were used in the following experiments.

Encapsulation of cells in alginate–poly-L-lysine–alginate microcapsules

Hepatocytes and UMSCs were immobilized into alginate–poly-L-lysine–alginate (APA) microcapsules by syringe-extrusion technique as reported previously.¹⁴ Briefly, the hepatocytes and the UMSCs were suspended in 0.9% sodium chloride containing 1.5% alginate (Sigma, MO, USA) at a density of $2\text{--}5 \times 10^6$ cells/ml. The cell suspension was forced out through an encapsulator (NISCO, Zurich, Switzerland), and the beads were allowed to gel in a hardening bath buffer containing 100 mmol/L of CaCl₂ (Sigma-Aldrich, MO, USA), and 10 mmol/L of MOPS (Sigma-Aldrich, MO, USA). After 10 min of hardening, the beads were washed thrice with PBS, and the microspheres were coated with 0.05% (w/v) poly-L-lysine for 10 min, followed by washing with the buffer containing 0.1% CHES, 1.1% CaCl₂ and 0.9% NaCl. Furthermore, the microspheres were exposed to 0.15% sodium alginate for 4 min to form the outer layer of the membrane, and then the droplets were washed with saline. The diameter of the microcapsules was approximately 250–350 μm.

Measurement of albumin and urea production

Microcapsules of human hepatocytes (1×10^5 cells) alone or with UMSCs at a ratio of 10:1 or 5:1 or 2.5:1 were seeded in a 24-well plate. The supernatant was collected from day 2 to day 10. The concentration of albumin and urea were measured using an albumin ELISA kit (Bethyl Laboratories, TX, USA) and a urea assay kit (Bioassay System, CA, USA) following the manufacturer's instructions.

Experimental design and animal groups

Male 8–10 weeks old C57BL/6 mice were purchased from Shanghai SLAC Co. Ltd (Shanghai, China). All animal experiments were approved by the Institutional Animal Care and Use Committee of the Shanghai Jiao Tong University School of Medicine (No. SYKX-2008-0050). For intraperitoneal (i.p) transplantation of microcapsules, the mice were randomly divided into four groups with eight mice per group. The groups were as follows: control (APA), HEP (hepatocytes, 5×10^6), UMSC-HEP (UMSCs 2×10^6 and hepatocytes 5

$\times 10^6$ cells), and HNF4 α -UMSC-HEP (HNF4 α -UMSC 2×10^6 and hepatocytes 5×10^6 cells). ALF was induced by i.p injection of the combination of LPS (50 μ g/kg) and D-gal (800 mg/kg) dissolved in PBS for 24 h after microcapsule transplantation. The HB-EGF-neutralizing antibody (anti-HB-EGF, R&D, MN, USA) and control IgG (AB-108-C, R&D, MN, USA) were reconstituted in sterile PBS and administered to ALF mice (10 μ g/injection, i.p)

The enzyme activities of aspartate aminotransferase (AST) and alanine transaminase (ALT) were measured using a standard clinical automatic analyzer (Dimension Xpand; Siemens Dade Behring, Munich, Germany). The levels of TNF- α , IL-8, and LDH were detected by ELISA kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

Histopathology, immunohistochemistry and immunofluorescence analyses

Liver tissues were fixed in 10% formalin and embedded in paraffin, and then were cut into 5 μ m thick liver sections. The liver sections were then stained with hematoxylin and eosin for histopathological examination. The histopathological changes were given scores according to a classic liver injury score standard.¹⁹ Immunohistochemistry analyses were performed as previously described.(20) Next, the liver sections were incubated with primary antibodies against MPO for overnight at 4 °C. After washing with PBS, the sections were incubated with horseradish peroxidase-conjugated secondary antibodies.

For immunofluorescence analyses, the liver sections were incubated with the following primary antibodies: anti-CD68, anti-CD86 and anti-CD206 (all from Abcam, Cambridge, MA, USA). After washing in PBS, the tissue sections were incubated with corresponding conjugated secondary antibodies. The images were then visualized by using an inverted fluorescence microscope. The quantification results were evaluated in at least six representative visual field for each group in a blinded manner by an experienced pathologist. Image-Pro Plus 6.0 (Media cybernetics, Silver Springs, MD, USA) was employed for image analysis.

Protein chip

Protein antibody array was performed to test the secretion factors in the conditioned medium (CM) of HNF4 α -UMSCs and UMSCs. The expression levels of 1070 human target proteins in cell supernatant were detected. The procedure was done as described by the manual of the manufacturer (Aksomics, Shanghai, China). In brief, the protein concentration was firstly tested by a BCA Protein Assay Kit (Kang Chen Corporation, KC-430, China), followed by adding the CM onto the blocked protein array membranes, and then incubating at room temperature (RT) for 2 h. Next, the membranes were washed and incubated with biotin-conjugated antibodies at RT for 2 h, and then reacted with HRP-conjugated streptavidin at RT for 2 h. Finally, the membranes were exposed to X-ray films and were developed using a film scanner. The intensities of the signals were quantified by densitometry.

Dual-luciferase reporter assay

The cells were cultured into 96-well plates, and were transfected by Lipofectamine TM 3000 (Invitrogen, CA, USA) with the plasmids of HB-EGF promoters, pWPXL or HNF4a, and internal control PRL-TK reporter plasmid after 24 h. After culturing for 48 h, the luciferase activities of the cells were measured by dual-luciferase reporter assay kit (Promega, WI, USA). The experiment was repeated at least three times.

Flow cytometry analysis and cell sorting

Mice were sacrificed and the livers were minced and tamped by using 70 μ m filter, the liver leukocytes were purified with 35% Percoll gradient (GE Healthcare), and then the red blood cells were lysed with RBC lysis buffer (ebioscience, MA, USA). The leukocytes were stained with fixable viability dye (ebioscience) and then incubated with Fc block (BD Biosciences), followed by staining with the antibodies against F4/80 (mouse PE T45-2342), CD11b (mouse FITC M1/70) and CD206 (mouse CD206 Alexa 647 MR5D3) (all from Biolegend, CA, USA). Finally, flow cytometry was performed by Cyto FLEX flow cytometer (Beckman coulter, Fullerton, CA, USA) and analyzed with Flowjo software (Treestar, OR, USA).

Statistical analysis

Data were presented as means \pm SEM ($n \geq 3$ experiments), and statistical significance were determined using Student's t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, and **** $P < 0.001$; # $P < 0.05$ and ## $P < 0.01$.

Results

HNF4a-UMSCs enhances the function of human primary hepatocytes in APA microcapsules

The trophic factors secreted by MSCs were found to be efficient in improving the hepatocyte function in ALF.(21) To assess whether HNF4a-UMSCs exert a beneficial effect on human hepatocytes in the microencapsulation system, human hepatocytes from liver specimens were isolated and the phenotype of hepatocytes was confirmed by glycogen staining (Fig. 1A). Overexpression of HNF4a in UMSCs was validated by western blotting and fluorescence (Fig. 1B). To explore a better ratio of human hepatocytes and HNF4a-UMSCs, hepatocytes and HNF4a-UMSCs were encapsulated at ratio of 10:1, 5:1, and 2.5:1, followed by culturing for 10 days. The albumin and the urea levels of hepatocytes were detected by ELISA kits from day 2 to day 10. As shown in Fig.S1, when hepatocytes and HNF4a-UMSCs were encapsulated at a ratio of 2.5:1, higher levels of albumin and urea were produced, suggesting that the hepatocytes have exerted a better function at this ratio. Therefore, human hepatocytes alone (HEP), or with UMSCs (UMSC-HEP) or with HNF4a-UMSCs (HNF4a-UMSC-HEP) at a ratio of 2.5:1 were encapsulated (Fig. 1C), cultured for 10 days and then the supernatant was harvested every two days for albumin and urea assay. The results revealed that albumin secretion and urea production were increased gradually and peaked on day 4, and then gradually declined from day 6 to day 10 (Fig. 1D). In addition to, UMSCs promoted albumin secretion and urea production of hepatocytes. More interestingly, the HNF4a-UMSC-HEP more efficiently promoted synthesis and secretion of hepatocytes than HEP or UMSC-HEP. To further confirm the effect of HNF4a-UMSCs on hepatocytes, the hepatocytes and the HNF4a-UMSCs were co-cultured in different

layers of transwell-chambers, and the hepatocytes were harvested on day 4. Real-time PCR was performed to examine the expression levels of hepatocyte genes in different groups. The expression of Albumin, CYP3A4 and CK18 separately present hepatic maturation, the activity of hepatocytes and as a liver specific marker. As shown in Fig. 1E, the expression levels of ALB, CYP3A4, and CK18 were higher in HNF4a-UMSC-HEP group than those in the HEP or UMSC-HEP group, suggesting that the HNF4a-UMSCs exerted a more beneficial effect on hepatocytes than HEP or UMSCs alone. Taken together, these results indicated that HNF4a-UMSCs in APA microcapsules could significantly improve the viability and function of primary hepatocytes.

Co-encapsulation of HNF4a-UMSCs and human hepatocytes ameliorated mouse ALF

To assess whether HNF4a-UMSC-HEP have a protective role in ALF mouse model, the microcapsules were cultured for 4 days based on the data mentioned above (Fig. 1D-1E). First, HNF4a-UMSC-HEP, UMSC-HEP, HEP or APA alone were transplanted into mice by intraperitoneal injection(i.p). 24 h later, LPS (50 µg/kg) and D-gal (800 mg/kg) were then used to induce ALF by intraperitoneal injection (i.p) (Fig. 2A). During the 7-day follow-up period, after transplantation of the microcapsules, all mice in the control group progressed to death, with a mortality rate of 100%. In contrast, only three mice died during the observation, indicating a better survival rate of mice in HNF4a-UMSC-HEP group (Fig. 2B). As depicted in Fig. 2C, there was an evident increase of the hepatic necrotic areas in the control (CON) mice after 6 hours of injection of LPS and D-gal. Interestingly, a reduced hepatic necrotic level was observed in the other groups. Moreover, HNF4a-UMSC-HEP more significantly reduced hepatic necrotic areas compared to HEP or UMSC-HEP. Immunofluorescence staining of TUNEL (apoptosis marker) indicated a remarkable reduction of hepatic apoptosis in the group of UMSC-HEP and HNF4a-UMSC-HEP (Fig. 2D). Moreover, histopathological changes were quantified by a classic liver injury score (Fig. 2E).(22) Of note, the HNF4a-UMSC-HEP pretreated mice demonstrated a more significant improvement of the architecture of the liver, and were characterized by decreased levels of edema, necrosis, and neutrophil infiltration when compared to those in the mice of other groups. Consistent with these histological alterations, HNF4a-UMSC-HEP pre-treatment also considerably decreased the levels of serum AST and ALT (Fig. 2F). These results demonstrated that HNF4a-UMSC-HEP significantly attenuated the liver injury in ALF mice models.

Co-encapsulation of HNF4a-UMSCs and human hepatocytes alleviated inflammatory responses in ALF mice

Acute inflammatory response is a detrimental hallmark of ALF, thus enhancing inflammation resolution can greatly protect liver injury by inhibiting acute inflammation.(23) Here we wondered whether the attenuated liver injury in HNF4a-UMSC-HEP group was attributed to the enhanced inflammation resolution effect. As shown in Fig. 3A and Fig. 3C, much fewer myeloperoxidase (MPO, a neutrophil marker) positive cells were observed in the mice of HNF4a-UMSC-HEP group compared to those in the other groups, indicating a decreased neutrophil infiltration. Similarly, a relatively few F4/80 (known as a

marker for macrophages) positive cells were observed in the group of HNF4a-UMSC-HEP (Fig. 3B and Fig. 3D). The levels of proinflammatory cytokines such as TNF- α and CXCL15 in the serum of mice decreased in HEP, UMSC-HEP and HNF4a-UMSC-HEP groups compared to those of CON group, while the lowest levels of TNF- α and CXCL15 were shown in the group of HNF4a-UMSC-HEP compared to those of other groups (Fig. 3E). Furthermore, the mRNA levels of proinflammatory cytokines in the livers were detected by real-time PCR. Consistent with the protein levels, the mRNA levels of TNF- α and IL-8, remained the lowest in the liver tissues of mice in HNF4a-UMSC-HEP group when compared to those in the other groups (Fig. 3F). These data indicated that HNF4a-UMSC-HEP relieved liver injury induced by LPS/D-gal by alleviating the inflammatory responses in mice.

Co-encapsulation of HNF4a-UMSCs and human hepatocytes promoted M2 polarization of macrophages both in vitro and in vivo

LPS is known to induce M1 polarization of macrophages, as a novel mechanism inducing sequential inflammatory response of ALF.(24) To determine whether the therapeutic effect on ALF achieved by HNF4a-UMSC-HEP was related to the M1 polarization of macrophages induced by LPS/D-gal, immunostaining analysis of liver tissues with the antibodies against CD68 (a total macrophage marker) and CD86 (M1 macrophage marker) was performed. As shown in Fig. 4A and 4B, higher infiltration of M1 macrophages (CD86 and CD68 double positive cells) was observed in mice subjected to LPS/D-gal treatment in the control group. However, there was a significant reduction in the number of M1 macrophages in HNF4a-UMSC-HEP group when compared with those in the other groups. Moreover, flow cytometry indicated that HNF4a-UMSC-HEP profoundly increased the number of M2 macrophages (CD206 and CD68 double positive cells) when compared with that in the other groups (Fig. 4C-4D). These results suggested that HNF4a-UMSC-HEP promoted M2 polarization of macrophages, protecting the liver from LPS/D-gal-induced injury. To further elucidate the effect of HNF4a-UMSC-HEP on polarization of macrophages, LPS was used to induce the murine macrophage cell line Raw264.7 cells to polarize towards M1 phenotype and the cells were then separately co-cultured with conditioned medium (CM) of HEP, UMSC-HEP, and HNF4a-UMSC-HEP. As shown in Fig. 4E, the mRNA levels of M1 macrophage markers such as TNF- α , CD86, and inducible nitric oxide synthase (iNOS) were significantly higher in the control group after exposure to LPS. However, the levels of M1 macrophage markers were significantly reduced when the cells co-cultured with the CM of UMSC-HEP or HNF4a-UMSC-HEP. Moreover, the CM of HNF4a-UMSC-HEP exerted a stronger capacity to downregulate the expression of M1 macrophage markers and upregulate the expression levels of M2-macrophage markers, such as CD206, peroxisome proliferation-activated receptor-gamma (PPAR- γ), and Arginase-1 (Arg-1), than those in UMSC-HEP and LPS control group (Fig. 4F). Taken together, these results suggested that HNF4a-UMSC-HEP could more efficiently promote polarization of macrophages towards M2 type both *in vivo* and *in vitro*.

Secretions of HNF4a-UMSCs prevented hepatocytes from D-gal-induced injury

To further illustrate the protective effects of hAECs on liver injury, we applied D-gal (10 mg/mL) to induce injury of primary mouse hepatocytes and co-cultured the injured cells with the CM of cells in the different groups. The cell viability and the levels of LDH and AST in the supernatant were then measured. As shown in Fig. 5A and 5B, high cell viability and low levels of LDH and AST in HNF4a-UMSC-HEP group were observed than those in the other groups. To further illustrate what factors were responsible for protecting the injured hepatocytes, a protein array of 1080 factors was performed with the CMs of UMSCs and HNF4a-UMSCs respectively. Relative expression levels of 1080 soluble proteins can be simultaneously detected. Next, we mainly focused on the neurotrophic factors, growth factors, cell adhesion molecules and anti-inflammatory factors which have been implicated previously involved in the repair of liver injury (20). The heatmap shows that the relative expression levels of these secretory factors in the HNF4a-UMSCs were higher than those of UMSCs (Fig. 5C, Table S2). Growth factors, such as FGF9, HB-EGF, HGF, PDGF and TGF- β were of high concentration in the CM of HNF4a-UMSCs. In addition, the levels of anti-inflammatory factors, such as IL-1ra, IL-10 and IL-11, also remained higher in the CM of HNF4a-UMSCs, suggesting that the CM of HNF4a-UMSCs exerted an enhanced inflammatory resolution effect on injured hepatocytes both in *in vivo* and *in vitro*. Next, we further performed gene ontology (GO) function enrichment analysis of biological process (BP). The ERK and AKT (protein kinase B) signaling pathways were activated in HNF4a-UMSCs (Fig. 5D). More interestingly, the ERK signaling pathway was proven capable of promoting cell proliferation and migration,(25) improving anti-apoptotic effects and attenuating liver injury.(26) To further explore which secreted factor mainly play essential role on injured hepatocytes, antibody neutralizing experiments using blocking antibodies against FGF9, HB-EGF, HGF, PDGF and TGF- β on injured hepatocytes were performed. The primary mouse hepatocytes were injured by D-gal and co-cultured with the CM of HNF4a-UMSC-HEP containing a corresponding blocking antibody for 12 h. The levels of LDH and AST in the supernatant were then measured. As shown in Fig. 5E, the levels of LDH and AST were significantly increased in the presence of neutralizing antibody against HB-EGF, while no significant effect was observed in the groups with other neutralizing antibodies. These results indicated that HB-EGF secreted by HNF4a-UMSC-HEP might exert a protective effect on the ALF mice.

Activation of HB-EGF by HNF4a mediated the protection of HNF4a-UMSC-HEP on ALF mice

To evaluate the therapeutic potentiality of HB-EGF *in vivo*, HB-EGF neutralization antibody was intraperitoneally injected into the ALF mice. 6 hours later, the liver injury was assessed. As shown in Fig. 6A-6D, the enhanced LPS/D-gal-induced liver injury were observed in the present of neutralizing antibody against anti-HB-EG, as evidenced by increased hepatic necrosis, hepatic apoptosis (Fig. 6A-6C), and ALT/AST levels (Fig. 6D). In addition, HB-EGF plays a critical role in the polarization of macrophages. (27) To explore whether HB-EGF secreted by HNF4a-UMSC-HEP affects the polarization of macrophages in the livers of ALF mice, flow cytometry was performed. As shown in Fig. 6E and 6F, the proportion of M2 macrophages was obviously decreased in the present of neutralizing antibody against anti-HB-EGF

group, indicating that HB-EGF exerts an important role on the relief of liver injury and the promotion of macrophages M2 polarization mediating the therapeutic effects of HNF4a-UMSC-HEP on ALF mice.

On the other hand, HNF4a is regarded as an important transcription factor that regulates genes expression in liver as well as others cells.(28) To explore the mechanism of HNF4a on the expression of HB-EGF, the HB-EGF promoter region was analyzed by JASPAR database (<http://jaspar.genereg.net/>) and the potential binding sequence of HNF4a was predicted, which is located at about - 839 ~ - 848 bp relative to the promoter site.(Fig. 6G-6H). To verify the effects of HNF4a on HB-EGF expression, HNF4a and HB-EGF promoter luciferase reporter plasmid (HB-EGF promoter) of 4848 bp with HB-EGF promoter region was transfected in HEK 293 cells. As shown in Fig. 6I, HNF4a significantly activated the expression of HB-EGF reporter. As a control, no effect on PGL3 basic was observed in the HNF4a group without HB-EGF promoter. These results indicate that HNF4a binds to HB-EGF promoter and activates the expression of HB-EGF. HB-EGF, playing an essential role on the therapeutic effects of HNF4a-UMSC-HEP on ALF mice.

Discussion

Cell-based therapies have emerged as potential alternatives to liver transplantation for the treatment of ALF due to their feasibility and low-invasive nature.¹⁰ Among the cells, human hepatocytes were isolated from the donor organ livers that are either unused surplus or rejected for human transplantation. Although the isolation techniques have been optimized to improve the viability and metabolic function, the cell yield after thawing still remained low, and the detrimental effect of the freezing process on metabolic function and cell attachment property is still inevitable.(29) Recently, the novel therapeutic strategies have been developed to promote the function of transplanted hepatocytes, including the co-transplantation strategies.(30) Previous studies have demonstrated that co-transplantation of hepatocytes and MSCs exerted a significant therapeutic effect on ALF animal models, in which the co-transplanted MSCs exerted differentiation abilities towards hepatocytes in PLGA (poly-lactic-co-glycolic acid) scaffolds to support hepatocellular metabolism and stabilization in ALF. However, there seem on the effect of MSCs on ameliorating inflammatory response during ALF is rarely investigated.⁹ Furthermore, paracrine factors secreted by MSCs were documented to be responsible for preventing the deleterious cytokine/chemokine/receptor responses in ALF.(21, 31) Of note, HNF4a is a key regulator of morphological and functional differentiation of hepatocytes.(32) Therefore, in the current study, HNF4a was overexpressed in UMSCs to investigate the protective effects of UMSCs in ALF mice. The results showed that HNF4a-UMSCs promoted the synthesis and secretion of human primary hepatocytes and enhanced the viability and function of hepatocytes in ALF(**Fig.1D-1E**). Furthermore, to prevent possible immune reaction induced by transplanted cells, the microcapsules were used to construct an immuno-isolation membrane to eliminate the entry of immunocytes into the host immune system, tolerant from immune recognition and immune attack. Microcapsules have a porous structure that allows oxygen and other nutrients to nourish the encapsulated cells. Moreover, they also provide a diffusive control of surrounding vasculature or tissue by the paracrine factors secreted by the encapsulated cells.¹⁷ A

previous study showed that MSCs in the microcapsules with porcine hepatocytes enhanced the viability and function of porcine hepatocytes.(33) Therefore, we used APA to microcapsule HNF4a-UMSCs with human primary hepatocytes and transplanted these microcapsules into ALF mice to observe their effects on ALF in the present study (**Fig.1C and Fig.2A**). The results showed that transplantation of HNF4a-UMSC-HEP has significantly attenuated liver injury, resulting in improved survival rate of LPS/D-gal-induced ALF mice (**Fig.2B-2E**). Moreover, hepatocytes co-transplanted with HNF4a-UMSCs in APA microcapsule can compensate the function of injured liver, preserving as substitute cellular source of large scale of quantities for liver function.

ALF is characterized by hepatic encephalopathy, coagulopathy, and progressive multiorgan failure.(34) Acute inflammation along with dysregulation of immune cells contributes to the pathogenesis of ALF.(35) For better simulation of the acute inflammatory condition, LPS combined with D-gal was often used to induce liver failure model in mice.(36) LPS is known to drive the process of inflammation by activating Toll-like receptors (TLRs) on macrophages and following a promotion on the secretion of inflammatory cytokines and infiltration of inflammatory cells.(37) D-gal triggers oxidative stress in hepatocytes, causing lipid peroxidation.(38) Of note, MSCs have been reported to suppress oxidative stress and neutrophil inflammatory response in rats with hepatic ischemia-reperfusion injury(8) and ALF.(39) In this study, HNF4a-UMSC-HEP significantly alleviated neutrophil and macrophage infiltration in the liver (**Fig.3A-3D**), and the inflammatory cytokine secretions in the serum as well as mRNA levels of inflammatory cytokines in the liver (**Fig.3E-3F**).

In addition, polarization of macrophages was also critically involved in the inflammatory responses of ALF.(35, 40) Macrophages are highly plastic, and has the ability to switch between two functional phenotypes, pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages, in response to microenvironment changes.(41) The activated M1 macrophages exert phagocytic activity and act as initiators of inflammation, accompanied by the release of pro-inflammatory cytokines, including IL-1 β , iNOS, and TNF- α . In contrast, M2 macrophages upregulate the expression of anti-inflammatory factors and other factors, such as IL-10, TGF- β , and other molecules, that are involved in cell proliferation, wound healing, tissue remodeling, and immuno-modulation,(42, 43) and these in turn contribute to the resolution of M1 macrophage-mediated inflammatory response. Therefore, a phenotypic switch from M1 to M2 has been suggested as a novel therapeutic approach to reduce inflammation in spleen, adipose tissue and liver.(44-46) We herein found the dramatically increased portion of M1 macrophages in the livers of LPS/D-gal-induced ALF mice. Moreover, the number of M1 macrophages was reduced and the number of M2 macrophages was increased in the livers of ALF mice in HNF4a-UMSC-HEP group (**Fig.4A-4D**). The CM of HNF4a-UMSC-HEP decreased the LPS-induced M1 polarization and enhanced M2 polarization of macrophages in Raw264.7 cells *in vitro* (**Fig.4E-4F**). Taken together, HNF4a-UMSC-HEP switched the macrophage functional phenotype from pro-inflammatory M1 to anti-inflammatory M2 to alleviate ALF.

Mounting evidence showed that MSCs secretions exerted a beneficial effect by reducing the inflammatory response, promoting the survival and proliferation of injured cells, and ameliorating liver injury, by the mediation of paracrine factors including IL-10, TIMP-1, and MCP-1.(47-49) In the present

study, microcapsules were used as carriers to encapsulate UMSCs and hepatocytes to persistently release the secretory factors contributing to the repair of impaired liver in ALF. Firstly, the transplanted microcapsules of HNF4a-UMSC-HEP significantly reduced the inflammatory response, repaired the liver injury and improved the survival rate of mice with ALF (**Fig.2** and **Fig.3**). Secondly, HNF4a-UMSC-HEP decreased the LPS/D-gal-induced M1 polarization and enhanced M2 polarization of macrophages both *in vitro* and *in vivo* (**Fig.4**). Thirdly, the CM of HNF4a-UMSC-HEP promoted cell viability and reduced liver injury induced by D-gal (**Fig.5A** and **5B**). To further determine the secretory proteins of HNF4a-UMSCs, a protein chip assay was performed. The results showed that 38 proteins were significantly upregulated in HNF4a-UMSCs when compared with those of UMSCs. Analysis of GO categories revealed that the proteins in the CM of HNF4a-UMSCs are more closely associated with some pathways that regulate fundamental cellular processes, such as proliferation, differentiation, motility, stress response, apoptosis, and survival (**Fig.5D**).⁽⁵⁰⁾ Of note, heat map analysis showed that the levels of growth factors (FGF-9, HGF, HB-EGF, PDGF and TGF-β) and anti-inflammatory cytokines (IL-1ra, IL-10, IL-11 and IL-13) were considerably higher in the CM of HNF4a-UMSCs compared to those in UMSCs (**Fig.5C**). Thus, the proteins including FGF9, HB-EGF, HGF, PDGF and TGF-β were selected to perform antibody neutralization experiments to determine their protective effects on injured hepatocytes. The results showed that only HB-EGF has significantly relieved hepatocyte injury, and no significant effects were observed in other groups (**Fig.5E**). Furthermore, previous studies have shown that HB-EGF could protect intestine tissues from inflammatory damage by promoting the M2 polarization of macrophages in necrotizing enterocolitis injury.²⁷ In addition, HB-EGF combined with HGF obviously inhibited BDL-induced cholestatic liver injury by exerting acute cytoprotective effects and enhancing the anticholestatic effects and liver regeneration during the chronic phase.⁽⁵¹⁾ Interestingly, in the present study, we found that HB-EGF neutralization antibody in mice has reversed the protection effects against ALF (**Fig.6A-6D**) and the switch of polarization of macrophages from M1 to M2 that was exerted by HNF4a-UMSC-HEP on ALF mice (**Fig.6E-6F**). These data suggested that HNF4a-UMSC-HEP played the therapeutic effects mainly mediated by HB-EGF. More importantly, HNF4a binds to the HB-EGF promoter and directly upregulates the expression of HB-EGF (**Fig.6G-6I**). Together, these results indicated that HNF4a-UMSC-HEP ameliorated the ALF mice, mainly mediated by activating the expression of HB-EGF by HNF4a.

In conclusion, the present study revealed that the overexpression of HNF4a in UMSCs has effectively increased the synthesis and secretion of human primary hepatocytes. Co-encapsulation of HNF4a-UMSCs and hepatocytes attenuated LPS/D-gal-induced liver injury and improved the survival rate of ALF mice. The molecular mechanism includes the reduction in the inflammatory response and promotion of macrophages polarization switch from M1 to M2, and the paracrine, in which HNF4a-UMSC-HEP promoted survival, proliferation, and metabolism of hepatocytes. In addition, HB-EGF upregulated by HNF4a plays a key role in macrophages polarization and a direct protection from the cytoprotective effects. These findings provide novel insights into the approach of cell-based therapy for ALF(**Fig.7**).

Abbreviations

ALF: acute liver failure; MSC: Mesenchymal stem cells; UMSC: Umbilical cord mesenchymal stem cells; HNF4α: Hepatocyte nuclear factor-4 alpha; APA: alginate–poly-L-lysine–alginate; LPS: Lipopolysaccharides; D-gal: D-galactosamine; ALT: Alanine aminotransferase; AST: Aspartate transaminase; TNF-α: Tumor necrosis factor-α, IL-8: Interleukin-8, iNOS: inducible nitric oxide synthase; PPAR-γ: peroxisome proliferation-activated receptor-gamma; Arg-1: arginase-1.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Renji Hospital, Jiao Tong University.

Consent for publication

No consent was involved in this publication.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions

DFK, HMX and MC designed research; DFK, MC and HLH performed related experiments; YBQ, TQ, YZ and YT analyzed data and prepared the figures; HMX, HLH and QX supervised the research; DFK, HMX and MC wrote the main manuscript. All authors read and approved the final manuscript

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Figures

Figure 1

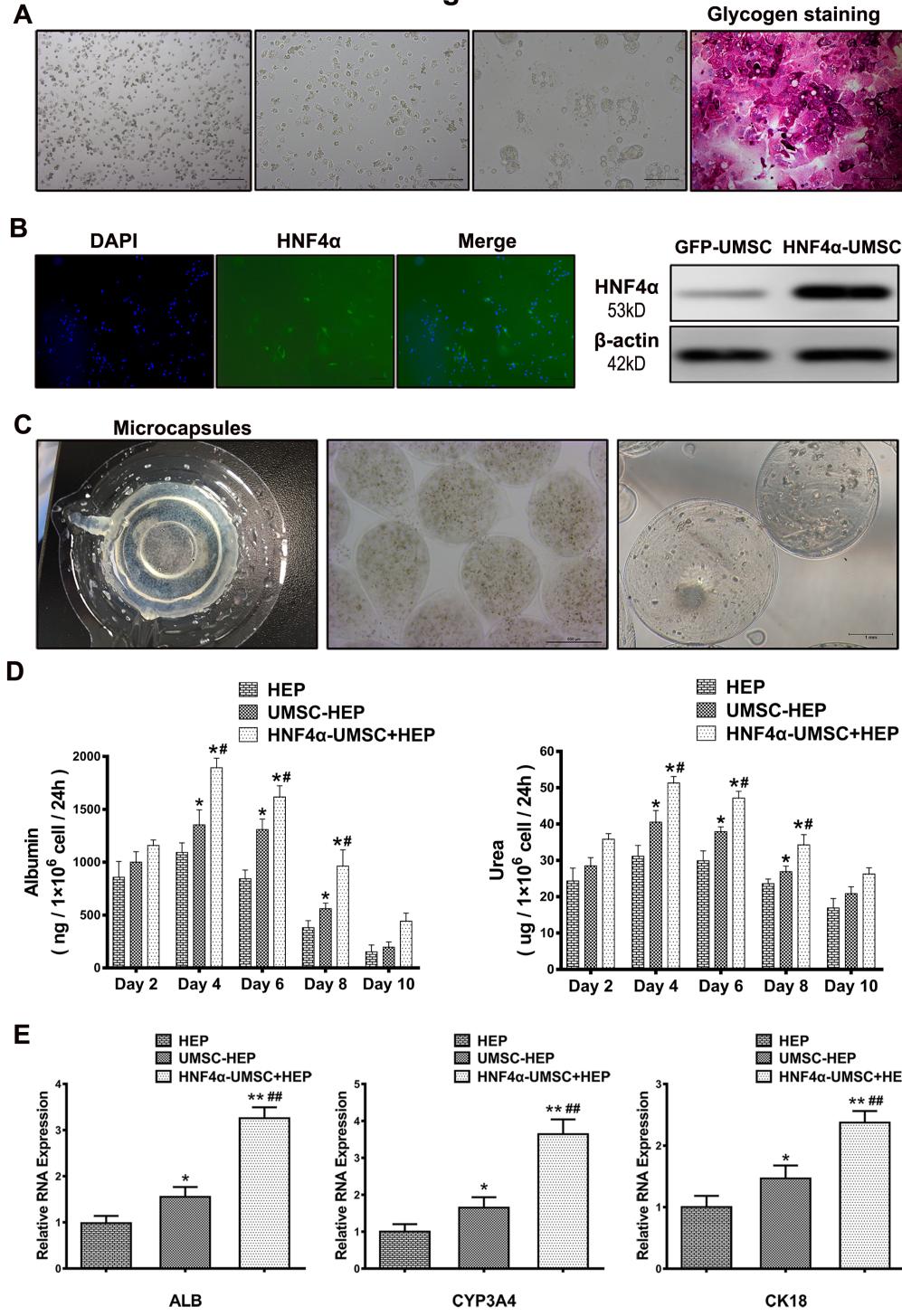


Figure 1

HNF4α-UMSCs enhance the function of human primary hepatocytes in APA microcapsules. (A) Phase contrast of human primary hepatocytes and image of glycogen staining. (B) Over-expression of HNF4α in UMSCs was confirmed by fluorescence and western blotting against HNF4α antibody. (C) Phase contrast of microcapsules of primary hepatocytes with/without HNF4α-UMSCs or UMSCs. (D) Measurement of the levels of albumin and urea in the supernatant of microcapsules in the groups of HEP, UMSC-HEP and

HNF4 α -UMSC-HEP at various time points. (E) The mRNA expression levels of hepatocyte-specific genes, ALB, CYP3A4 and CK18 by qPCR analysis. Total RNA was extracted on day 4. The levels of mRNA in HEP was set as 1. Data were collected from at least six separate experiments and are presented as means \pm S.E.M. Statistical significance was tested by student's t test. *P < 0.05 and ** P < 0.01 compared with CON. # P < 0.05 and ## P < 0.01 compared with UMSC-HEP.

Figure 2

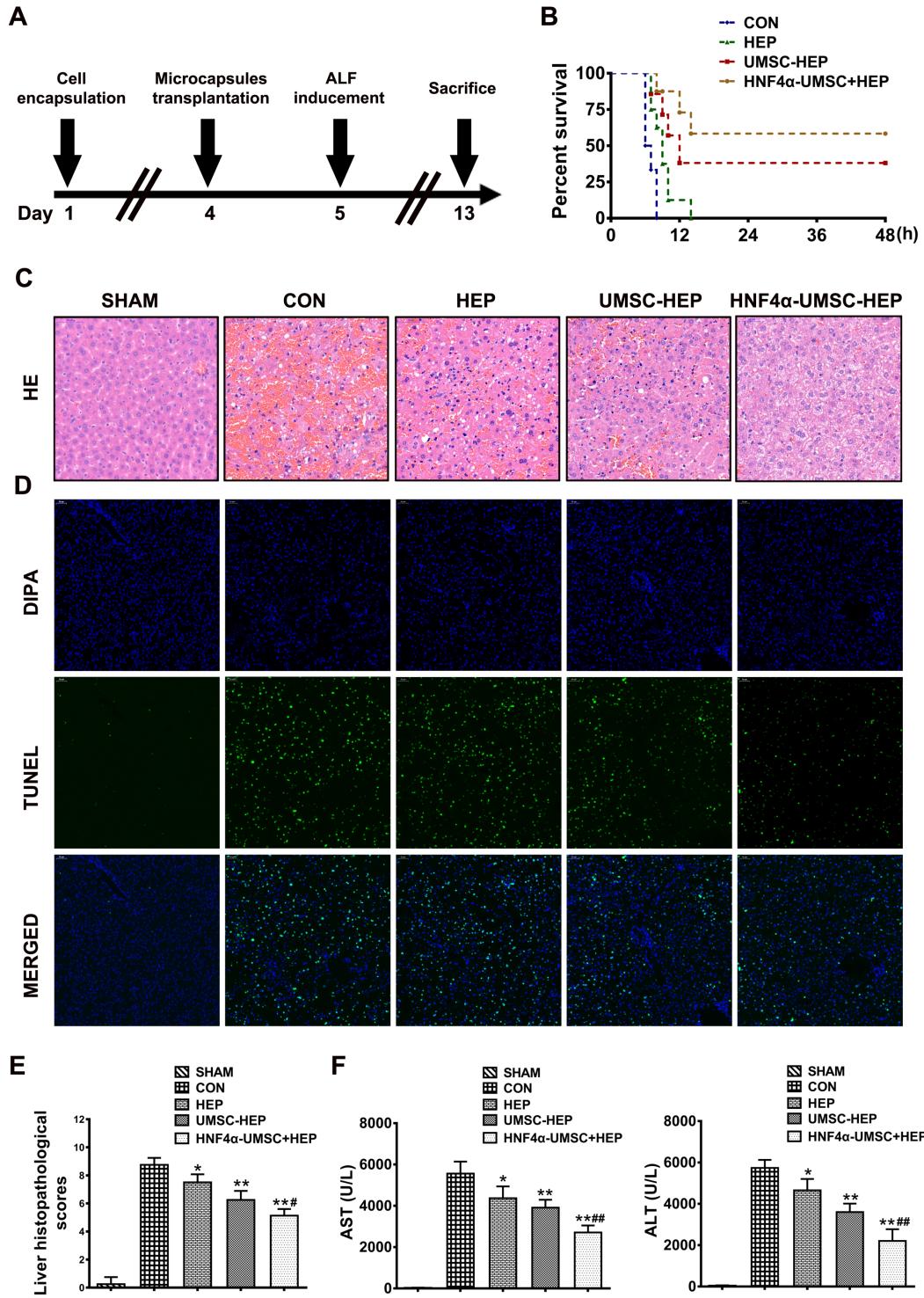


Figure 2

Co-encapsulation of HNF4 α -UMSCs and human hepatocytes ameliorated ALF in mice. (A) Schematic representation of experimental procedure design. Mice were transplanted with Control or HEP, UMSC-HEP, and HNF4 α -UMSC-HEP 24 h before LPS/D-gal challenge. (B) Survival rate of mice injected with LPS/D-gal in Control (CON), HEP, UMSC-HEP and HNF4 α -UMSC-HEP groups ($n = 6\text{--}12$). (C) Liver tissues were harvested 6 h after LPS/D-gal challenge for histopathological examination using H&E staining (Original magnification $\times 200$). Representative images are displayed. (D) Immunofluorescence staining of liver sections of ALF mice with antibodies against TUNEL with DEPI. (E) Liver histopathological scores. Data were collected from at least six separate fields of different mice. (F) The serum ALT and AST activities were measured 6 h after intraperitoneal injection of LPS/D-gal ($n = 6$). Data were collected from at least six separate experiments and are presented as means \pm S.E.M.* $P < 0.05$ and ** $P < 0.01$ compared with CON. # $P < 0.05$ and ## $P < 0.01$ compared with UMSC-HEP.

Figure 3

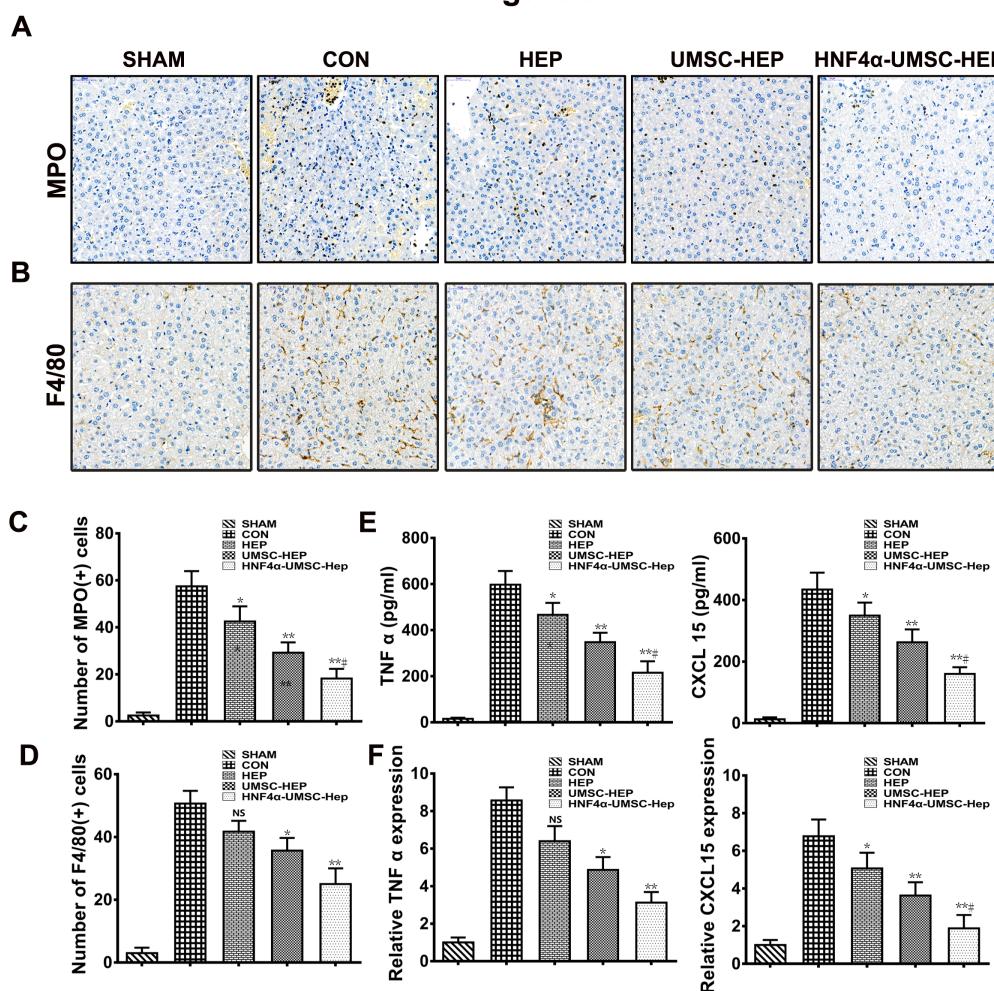


Figure 3

Co-encapsulation of HNF4 α -UMSCs and human hepatocytes alleviated inflammatory responses in ALF mice. (A) Immunohistochemistry images of liver sections with MPO antibodies (original magnification, $\times 200$). (B) Immunohistochemistry images of liver sections with F4/80 antibodies (original magnification, $\times 200$). (C) Quantification of MPO positive cells in livers. (D) Quantification of F4/80 positive cells in livers. (E) ELISA analysis of TNF- α and CXCL15. (F) The mRNA levels of TNF- α and IL-8 levels in the serum of ALF mice by

real-time PCR analysis. Data were collected from at least six separate experiments and are presented as means \pm S.E.M.* P< 0.05 and ** P< 0.01 compared with CON. # P< 0.05 and ## P< 0.01 compared with UMSC-HEP.

Figure 4

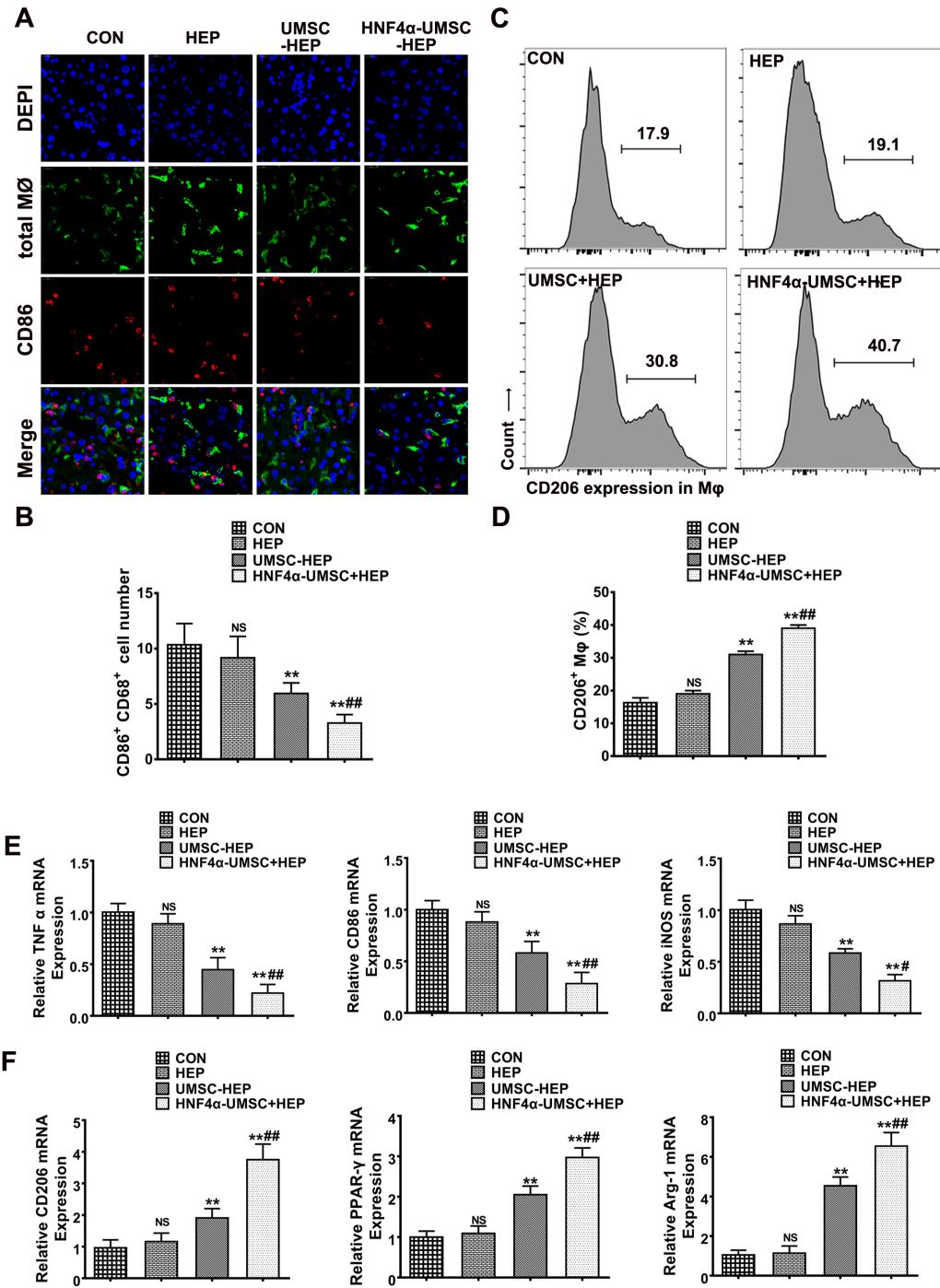


Figure 4

Co-encapsulation of HNF4 α -UMSCs and human hepatocytes promoted M2 polarization of macrophages.
(A) Immunofluorescence staining of liver sections of ALF mice with antibodies against CD68 and CD86.

(B) Quantification of CD86+ CD68+ macrophages under a microscopic field. Eight sections were analyzed for each liver and counted each section at least in four fields. (C) Flow cytometry of CD206+ macrophages (Mφ). (D) Quantification of CD206+ macrophages (Mφ) ($n = 4-6$). (E-F) The mRNA expression levels of TNF- α , CD86, iNOS, CD206, Arg-1 and PPAR- γ in Raw264.7 cells treated with CM of different groups after challenged with LPS. Data are expressed as means \pm S.E.M. ($n = 6$), * $P < 0.05$ and ** $P < 0.01$ compared with CON, # $P < 0.05$ and ## $P < 0.01$ compared with UMSC-HEP.

Figure 5

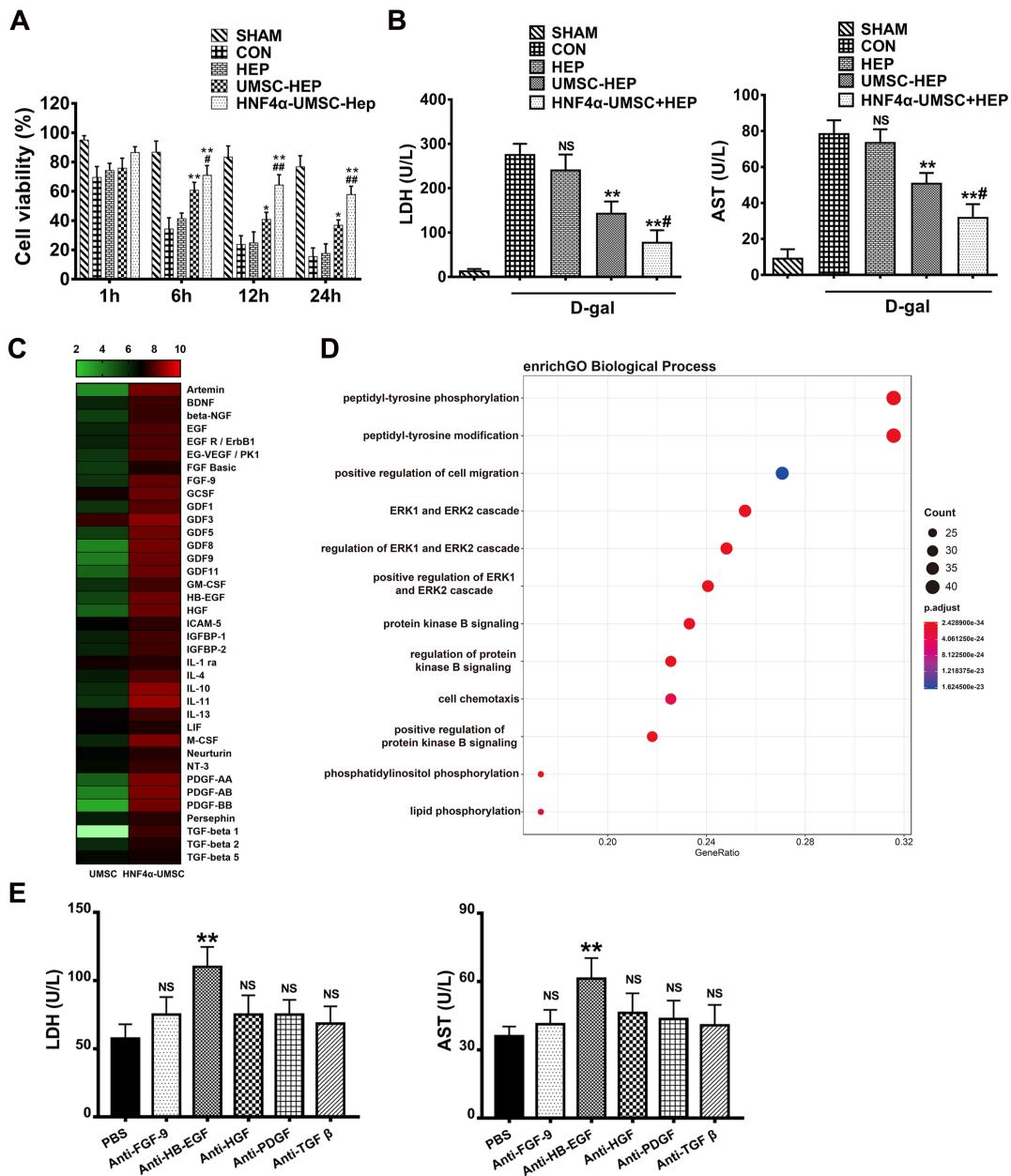


Figure 5

Conditioned medium of HNF4α-UMSCs prevented hepatocytes from D-gal-induced injury. (A) Analysis of viability of mouse primary hepatocytes in different groups at different time points by CCK-8. (B) Analysis of LDH and AST levels in different groups. (C) The relative concentrations of proteins of the condition mediums of UMSCs and HNF4α-UMSCs that obtained a significant score (q-value <0.001%) are presented in a “heatmap”. Low concentration is shown in green, medium concentration is shown in black and high concentration is shown in red. (D) GO enrichment analysis of biological processes of high-level proteins in CM of HNF4α-UMSCs. Normalized array data of proteins were analyzed by SAM to detect differences in the concentrations between HNF4α-UMSCs-CM and UMSCs-CM. (E) The levels of LDH and AST in the medium of hepatocytes injured by D-gal with CM of HNF4α-UMSCs-CM, and neutralizing antibodies against different proteins. Data are expressed as means ± S.E.M. (n = 6), * P< 0.05 and ** P< 0.01 compared with CON, # P< 0.05 and ## P< 0.01 compared with UMSC-HEP.

Figure 6

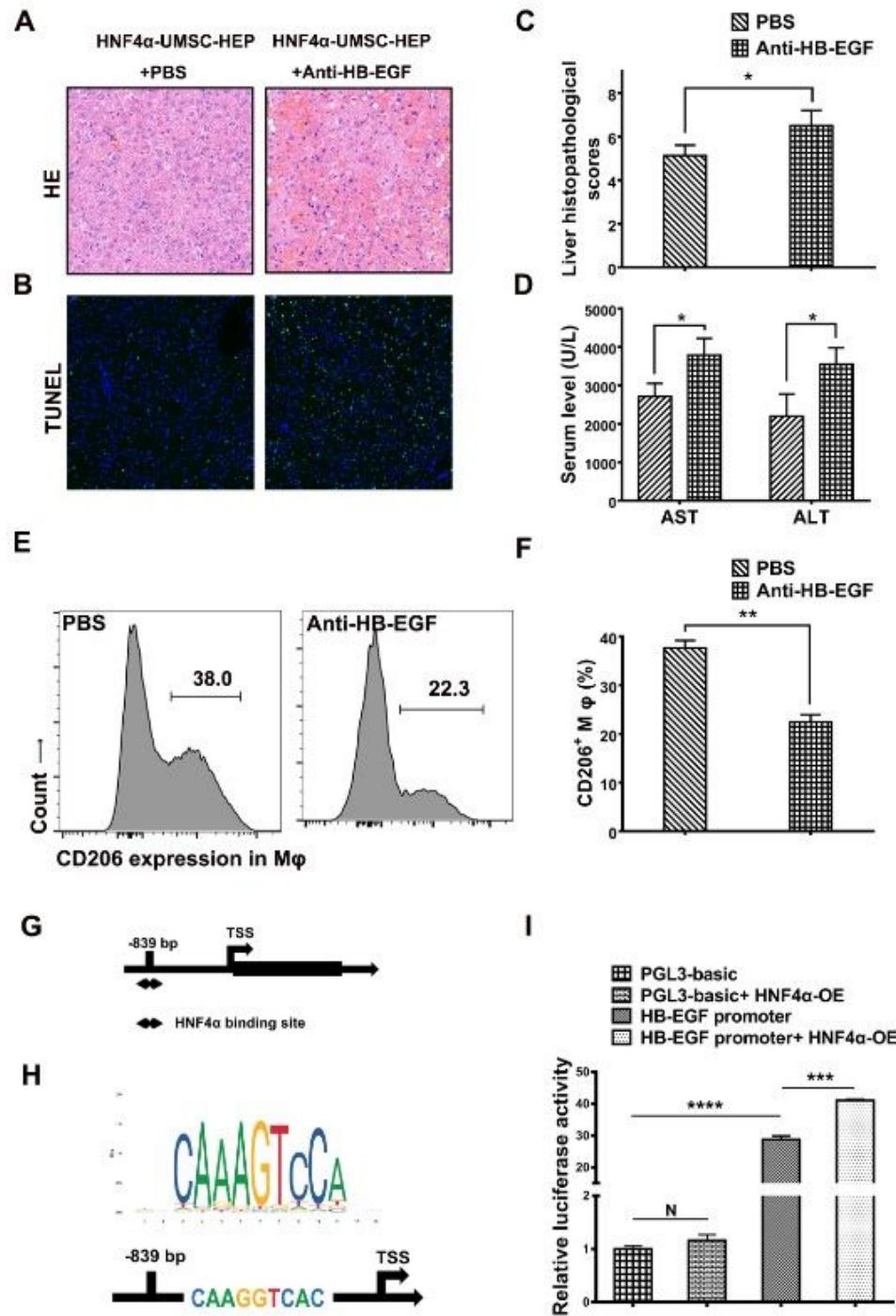


Figure 6

HB-EGF induced by HNF4α accounted for HNF4α-UMSC-HEP-mediated protection. Mice in HNF4α-UMSC-HEP group were treated with LPS/D-gal and with or without neutralizing HB-EGF antibody. (A) Liver samples were harvested 6 h after LPS/D-gal challenge for H&E staining, and the representative images are displayed. (B) Immunofluorescence staining of liver sections of ALF mice with antibodies against TUNEL. (C) The liver histopathological scores were collected from the data of at least six separate

experiments. (D) The serum ALT and AST activities were measured in HNF4 α -UMSC-HEP group with or without neutralization of HB-EGF antibodies. (E-F) Representative FACS plots and statistical quantification of CD206+ macrophages ($M\phi$) upon anti-HB-EGF in HNF4 α -UMSC-HEP group ($n = 4-6$). (G) HNF4 α -targeted sites on HB-EGF promoter were predicted by the JASPAR website. (H) The sequence of HNF4 α potential binding site on HB-EGF promoter by JASPAR (upper panel) and a diagram of HNF4 α predicted the binding site (lower panel). (I) The relative luciferase activities of HB-EGF promoter with or without HNF4 α expression vectors upon transfection in 293T cells (NC stands for the control of HNF4 α plasmid, and PGL3-basic stands for the control of plasmid of HB-EGF promoter). Data are presented as means \pm S.D. ($n \geq 3$), * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$, **** $P < 0.001$.

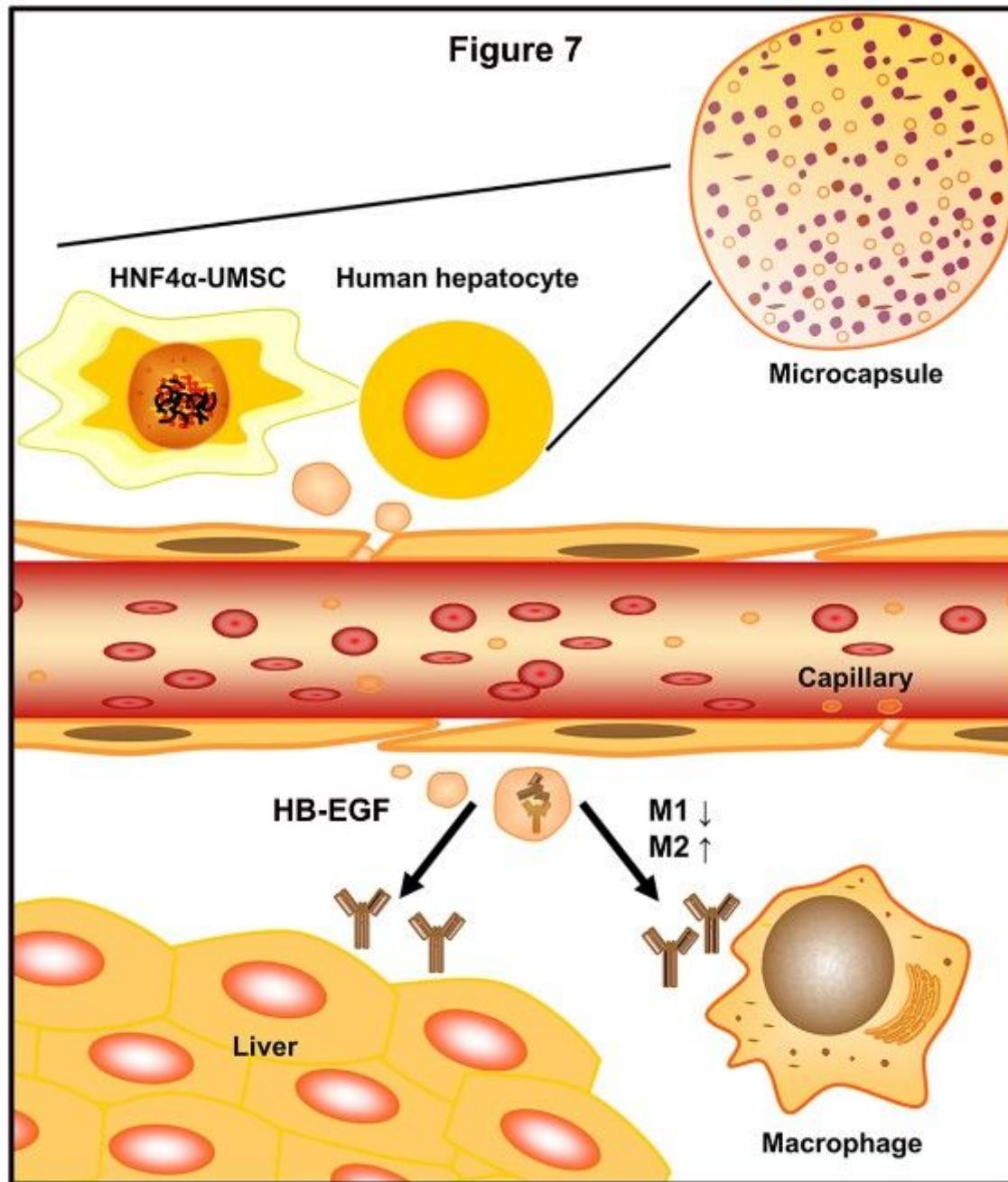


Figure 7

A schematic diagram illustrating the mechanisms of co-encapsulation of HNF4α-UMSCs and human hepatocytes in ALF mice injured by LPS/D-gal. “–” indicates promotion.

Supplementary Files

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