Int J Physiol Pathophysiol Pharmacol 2020;12(1):11-24
www.ijppp.org /ISSN:1944-8171/IJPPP0103223

Original Article

Amniotic mesenchymal stem cells derived hepatocyte-like cells attenuated liver fibrosis more efficiently by mixed-cell transplantation

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Received October 7, 2019; Accepted February 2, 2020; Epub February 25, 2020; Published February 28, 2020

Abstract: Background: Cell transplantation is a promising treatment for the patients with end-stage liver diseases. Stem cells derived hepatocyte-like cells (HLCs) attenuated liver injury upon transplantation in animal models for liver fibrosis. However, only a small portion of the transplanted cells propagated in the recipient liver. Aim: We hypothesized that the efficiency of cell therapy could be improved by transplanting amniotic mesenchymal stem cells (AMSCs) derived HLCs along with human umbilical vein endothelial cells (HUVECs) and undifferentiated AMSCs. Methods: Briefly, we used a two-step protocol to generate induced HLCs. We confirmed organoids formation of HLCs in 3D collagen scaffolds with HUVECs and AMSCs. To determine whether the HLCs can migrate into the liver tissue and perform in vivo function, we transplanted the cells to mice with liver fibrosis. Results: Co-culture of HLCs with HUVECs and AMSCs demonstrated improved function of HLCs within the organoids. Furthermore, transplantation using non-homogeneous cells, i.e. HLCs mixed with HUVECs and AMSCs, exhibited better graft survival in the host animals with liver fibrosis. Our experiment results suggested that compared to mock transplantation or HLCs transplantation groups, liver fibrosis was reduced significantly in mixed-cell groups. The AST levels in the plasma of transplanted mice were markedly decreased only in the mixed-cell transplantation group. The engraftment of HLCs in mice liver was better in mixed-cell transplantation group, compared with HLCs-only transplantation group. Conclusions: The HLCs attenuated liver fibrosis more efficiently when transplanted along with HUVECs and AMSCs, and this suggested that we could improve the efficiency of cell therapy by transplanting functional cells partially along with stromal cells.

Keywords: Amniotic mesenchymal stem cells, hepatocyte-like cell differentiation, umbilical vein endothelial cells, liver fibrosis, cell therapy

Introduction

As a major public health issue, liver fibrosis is characterized by the excessive accumulation of extracellular matrix in the liver parenchyma in response to chronic inflammation and injury. These injuries may be caused by viral infections, autoimmune responses, cholestatic and metabolic diseases, and alcoholic or nonalcoholic steatohepatitis. Advanced liver fibrosis leads to cirrhosis, portal hypertension, and eventually it irreversibly developed into liver failure as end stage disease state. Orthotopic liver transplantation is considered to be the most effective treatment for the patients with advanced liver diseases or end stage liver disease. However, liver transplantation is limited by the shortage of suitable donor liver, the risks of post-transplant rejection, infection, lifelong immunosuppression, and the recurrence of pre-existing liver diseases. Frequently, liver fibrosis develops in the liver grafts as early as one year after transplantation when the previous iteological causes are not eliminated.

Cellular transplantation to treat liver fibrosis can be much less invasive. If successful, this procedure represents a promising alternative to liver transplantation. However, because most grafted cells die upon transplantation, a cell-based therapy generally requires large number of differentiated cells for colonizing the target

tissues. Human iPS cell-derived hepatocyte-like cell and organoid based functional human liver bud (LB) might be the better candidate. However, the incomplete differentiation of iPSCs may poses a serious safety risk in engraftment of functional iPS cell-derived cells in vivo. As undifferentiated iPSCs exhibit intrinsic tumorigenicity properties, iPSC-derived hepatic cells containing residual undifferentiated iPSCs may cause tumor formation following transplantations, and lentiviral and retroviral based iPS cells still need further detailed studies before it comes to clinical application. Mesenchymal stem cells (MSCs), however, attract a lot of attention due to the low-immunogenicity, low-oncogenicity, and availability. Further, amniotic mesenchymal stem cells can be retrieved easierly than the MSCs derived from other tissues, such as bone marrow and adipose. It has been reported that MSCs have broad therapeutic effects on various injury and diseases in animal models, and the possible therapeutic mechanism may include all or some of the paracrine factors with anti-apoptotic, anti-scarring, proangiogenic and immunomodulatory functions [1, 2]. Previous reports have shown that MSCs can differentiate into hepatocyte-like cells (HLCs) under so-called committed condition, and the derived HLCs can ameliorate liver injury when transplanted into the animal model [3, 4]. However, only a small portion of the transplanted cells were found to propagate in the recipient liver, suggesting a great loss of the transplanted cells [5]. Further, the transplanted HLCs seemed less durable, withaconcomitantandprogressivelossoftheobserved *in vivo* effects. Thus, in order to develop a clinically relevant cellular transplantation for liver fibrosis, it is crucial to improve the transplantation efficiency and *in vivo* durability of MSC-derived HLCs.

MSCs are one kind of adult stem cells. The biological functions of MSC include tissue scaffolding and stromal constituents, as well as differentiation into various cell types, functional maturation upon differentiation and integration into different organ tissues. It has been confirmed that MSCs, also known as precursor for stromal cells, promote hematopoietic cell engraftment and immune recovery after hematopoietic stem cell transplantation [6]. Hepatocyte precursor and hematopoietic stem cells share the same location during fetal development, and apparently require the same stromal cell support [7]. For treating the liver injuries, MSCs have been used as an effective source of hepatocytes for repairing liver tissues, and regeneration through transdifferentiation and cell fusion. More importantly, MSCs can inhibit hepatocyte apoptosis and stimulate hepatocyte proliferation by paracrine regulation [8, 9]. Furthermore, co-culture of human hepatocytes with MSCs demonstrated both improved function and viability of the hepatocytes [10]. These findings suggest that MSCs are promising candidate for facilitating survival, integration and function of hepatocyte after cell transplantation.

The liver development during embryogenesis is a complex process, requiring the endodermal, mesenchymal, and endothelial cells those are differentiated with hepatic characteristics to form the vasculature and intricate signaling processes and interactions between these components are prerequisite [11, 12]. MSC-like cells can localize to the pericyte niche in microvasculature, where they make close contact to endothelial cells. Purified perivascular cells exhibit decisive advantages over conventional MSCs, including higher proliferative potential and multilineage differentiation capacity [13]. Therefore, it is plausible that direct cell-cell contact and paracrine effects between MSCs and endothelial cells play a critical role in maintaining principle characteristics of MSC-like cells.

Liver sinusoidal endothelial cells (LSECs) are endothelial cells specifically differentiated for establishing the functional tissue structure [14]. The orientation of hepatocytes around liver sinusoid is essential for the restoration of liver microarchitecture. When hepatocytes are injured, a subset of LSECs survives and serves as “guide rails” for regenerating hepatocytes [15]. In addition to the role in establishing functional liver microarchitecture, LSECs also coordinate hepatocyte proliferation during liver regeneration [16].

Since the parenchymal cells require certain stromal cells to survive and function, and new hepatocytes may benefit from interacting with endothelial cells during the hepatocyte-sinusoid alignment, we hypothesized that co-transplantation of AMSCs and HUVECs together with AMSCs-derived HLCs may facilitate hepatocyte incorporation into the liver microstructure, thus improving the engraftment efficiency and dura-

bility of the graft. We aim to develop a therapeutic treatment for liver fibrosis, and the transplantation of AMSCs-derived HLCs with supporting cells may be an efficacious and cost-effective method for preventing the liver failure caused by liver fibrosis.

Materials and methods

Characterization of AMSCs

The collection of samples and their use for research purposes were approved by Beijing Mary's Women and infants Hospital. All participants provided written and informed consent prior to sample collection. Placentas were collected from women who were free of medical, obstetrical, and surgical complications and delivered at term (≥ 37 gestational weeks). As described previously, AMSCs were harvested and cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (FBS; HyClone) and 10 ng/mL epidermal growth factor (EGF) (PROSPEC) at 37°C with 5% CO_2 [17]. Using flow cytometry analysis, we first detected the expression of cell surface markers of AMSCs: CD44, CD90, CD73, CD34, CD45 and HLA-DR. Meanwhile, we evaluated the capacity of differentiation into osteocytes and adipocytes as standard characteristics of mesenchymal stem cells by Oil-red-O and Alizarin Red staining, respectively.

Characterization of HUVECs

HUVECs were isolated from human umbilical cord by enzymatic digestion [18]. Briefly, umbilical cord was rinsed twice in Phosphate Buffer Solution (PBS) until the cord blood was cleared, and then human umbilical cord vein was perfused with DMEM containing 1% collagenase I (Sigma). After enzyme digestion for 15 minutes at 37°C, the collagenase solution in human umbilical vein was diluted by M199 medium (GIBCO). The isolated cells were washed with M199 medium for three times. Then the HUVECs were harvested, resuspended and seeded to a 0.1% gelatin-coated T25 flask in M199 medium containing 20% fetal bovine serum, 10 mg/ml ECGS, 40 U/ml heparin and 1% penicillin/streptomycin. To characterize the HUVECs, we detected the expression of CD54, CD31, vWF and HLA-DR in HUVECs by flow cytometry analysis.

Differentiation of AMSCs into HLCs

The purified AMSCs (passage 3-5) were cultured until the cells were 90% confluence, and then the cells were treated with 25 μM 5-Azacytidine (5'-AZ, Sigma) for 24 hours in propagation medium. The differentiation of AMSCs into HLCs includes two steps. Step I: induction, IMEM (GIBCO) with 2% FBS (GIBCO), 20 ng/mL fibroblast growth factor-2 (FGF2, PROSPEC), 20 ng/mL hepatocyte growth factor (HGF, PROSPEC) and 1.25 mM nicotinamide (Sigma) for 7 days. Step II: maturation, IMEM with 2% FBS, 20 ng/mL HGF, 20 ng/mL oncostatin M (OSM, PROSPEC) and 1 μM dexamethasone (Dex, Sigma) for 14 days. The medium were changed every 3 days.

Total RNA isolation and reverse transcription pCR

The total RNA was extracted from MSCs 0, 7, 14 and 21 days after cytokines induction using RNeasy Kit (Qiagen, Stanford, Valencia, CA) under the manufacture's introduction. The mRNA was reverse transcribed to cDNA and the cDNA was amplified with Albumin, α1-anti trypsin (α1-AT), CK18, CYP3A4 and β-actin primers using the Advantage RT-for-PCR (Clontech, Palo Alto, CA) under the manufacture's instruction. The sequences of the primers were listed as follows: Albumin, forward, 5'-TCT ATC CGT GGT CCT GAA CC-3', reverse, 5'-CCT AAG GCA GCT TGA CTT GC-3'; α1-AT, forward, 5'-CCA CGA TAT CAT CAC CAA GTT CC-3', reverse, 5'-TGG TCA GCA CAG CCT TAT GC-3'; CK18, forward, 5'-GAG ATC GAG GCT CTC AAG GA-3', reverse, 5'-CAA GCT GGC CTT CAG ATT TC-3'; CYP3A4, forward, 5'-CCT TAC ATT ACA CAC CCT TTG GAA GT-3', reverse, 5'-AGC TCA ATG CAT GTA CAG AAT CCC CGG TTA-3'; β-actin, forward, 5'-GAG AAA ATC TTG CAC CAC AC-3', reverse, 5'-CTC GGT GAG GAT CTT CAT-3'.

Western blot analysis

Total protein was extracted from MSCs 0, 7, 14 and 21 days after cytokines induction by RIPA lysis (Dingguo, Beijing, China). Equal amounts of proteins were resolved with SDS/PAGE (10% gel) and transferred on to 0.2 μm PVDF membranes. After blocked with 5% skimmed milk for 1 h at room temperature, the membranes were incubated in the mouse anti human AFP and β-ACTIN primary antibodies at 4°C overnight.

Then the protein bands were detected by chemiluminescence (Thermo).

Periodic Acid-Schiff (PAS) staining for glycogen

Cell slides were immersed in 4% PFA for fixation and cells were stained with PAS under the manufacture's instruction (Baihao, Tianjin, China). Briefly, the slides were oxidized in 1% periodic acid for 5 minutes, rinsed 3 times in deionized water, treated with Schiff's reagent for 15 minutes and rinsed in deionized water for 5-10 minutes. Then the slides were counterstained with Mayer's hematoxylin for 2 minutes, rinsed in deionized water and assessed under light microscope.

3D cell culture

Cells were maintained in a humidified 37°C, 5% CO_2 incubator and passaged at 70-80% confluence. Trypsinized cells were resuspended in 3D culture medium (Hepatocyte-like cells culture medium and HUVEC culture medium in 1:1 ratio). 1×10^6 AMSCs-derived HLCs, 7×10^5 HUVEC and 3×10^5 MSCs were mixed in 1 ml 3D culture medium. The mixed cells were diluted 1:1 with collagen, added to the non-coated 96-well plates and further cultured in 3D culture medium. AMSCs-derived HLCs only in collagen were cultured as negative control. After 10 days, 3D collagen were fixed in 4% PFA for 24 hours at 4°C, then dehydrated in 15% and 30% sucrose solution overnight.

Immunofluorescence analysis

Cell slides were immersed in 4% PFA for 20 minutes at room temperature for fixation. The cells were permeabilized with 0.1% Triton X-100 for 10 minutes, and blocked in 2% BSA for 30 minutes at 37°C. Slides were incubated with mouse primary antibodies anti human α-fetoprotein (AFP, abcam, 1:100) at 4°C overnight, followed by goat anti mouse 488 secondary antibody (Zhongshan, 1:400) for 1 hour at 37°C. The nucleus was stained with DAPI (Vector). 3D tissue slides were incubated with mouse primary antibodies anti human vWF (Santa cruz 1:100) and rabbit primary antibodies anti human albumin (Abcam, 1:100), followed by goat anti mouse PE-conjugated secondary antibody (BD, 1:100) and donkey anti rabbit FITC-conjugated secondary antibody (Jackson immunoresearch, 1:200).

CCl_4-injured liver mouse model and transplantation

Eight-week-old male CD1 mice (Vital River) were maintained in an air-conditioned animal house under specific pathogen-free conditions. Liver fibrosis was induced by intraperitoneal injection of CCl_4 (1.0 ml/kg) dissolved in corn oil twice a week for 5 weeks. Control mice (n=6) were injected with an equal volume of corn oil alone. To further validate the in vivo function of our derived HLCs, we transplanted AMSCs-derived HLCs to the liver fibrosis mice. To reduce the immune rejection of donor cells, these mice were treated with an immunosuppressant, cyclophosphamide (60 mg/kg), 1 day before transplantation by tail vein injection. We divided the mice into three groups. The mice in group 1 (n=7) were transplanted with HLCs (2×10^6 cells/animal) alone by intrasplenic injection. The mice in group 2 (n=7), the mixed-cell transplantation group, were transplanted with HLCs (1×10^6 cells/animal), along with AMSCs (3×10^5 cells/animal) and HUVECs (7×10^5 cells/animal). The mice in group 3, without cell transplantation (n=6) were treated with PBS as a sham control by intrasplenic injection. Mice were anesthetized with pentobarbital sodium and plasma was obtained by collecting blood into heparinized tubes 2 and 4 weeks post-transplantation. Plasma was collected by centrifuging blood at 3000 rpm for 30 minutes at 4°C and stored at -80°C until analysis. Meanwhile, liver and spleen tissues were collected from all mice after heart perfusion with PBS.

Sirius red staining

The liver and spleen were fixated in 4% PFA, processed for paraffin embedding and sectioned into 5-μm sections. To analyze the extent of liver fibrosis, the sections were stained with commercial Sirius red staining kit (Leagene) according to standard protocols. Typical photos were taken for data show and analysis of collagen accumulation in liver.

Immunohistochemistry

The liver sections were first deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 for 10 minutes. Antigens were retrieved by microwave treatment in 0.1 mol/L citrate buffer (pH 6.0). The

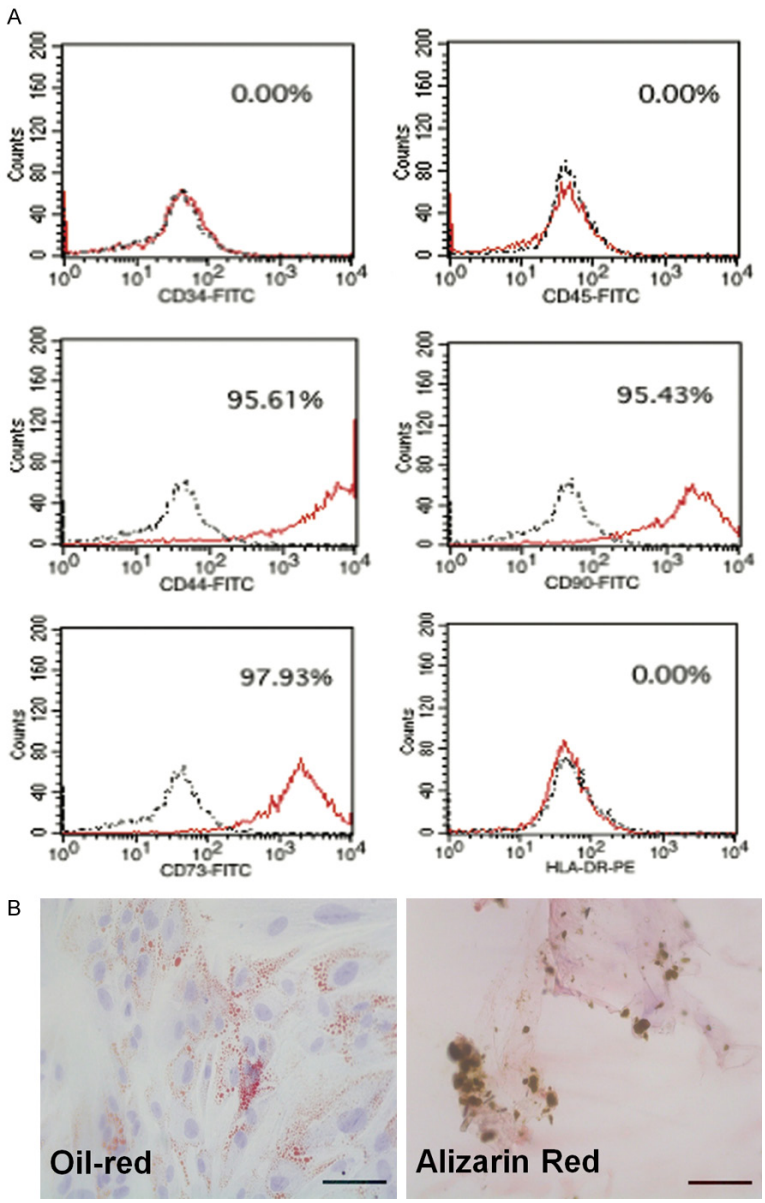

Figure 1. Characterization of amniotic mesenchymal stem cells. A. Immunophenotype of AMSCs by flow cytometry analysis. B. Oil-red and Alizarin Red staining of AMSCs after adipogenic and osteogenic differentiation. Scale bars, 50 μm.

tissue sections were then blocked with 10% normal goat serum for 30 minutes at room temperature. Tissues were incubated overnight at 4°C with rabbit anti-mouse antibody against α-SMA (1:500; Epitomics). Detection was performed after incubating the sections with peroxidase-conjugated goat anti-rabbit secondary antibody (Zhongshan). Peroxidase activity was revealed by diaminobenzidine. The nucleus was stained with Mayer's hematoxylin for 10 minutes. Images were randomly chosen for analyzing collagen accumulation in liver.

Liver function analysis

Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in mice blood were measured with an automated biochemical analyzer.

Detection of engraftment of HLCs in recipient livers

The engraftment of transplanted cells in recipient livers was further confirmed by genomic PCR for human-specific Alu DNA sequences. The sequences of primers were listed as follows: forward, 5'-AAT ATG GCC CAA CTG CAG AA-3', reverse, 5'-CAT CGC ATT TTC ACA TCC AA-3'.

Statistics

All data were expressed as mean ± standard deviation (SD). SD was illustrated by error bars in Figures. The Student's *t* test was applied for calculating statistical probability in this study. *P* values less than 0.05 ($P < 0.05$) were considered to be statistically significant difference.

Results

Characteristics of AMSCs

Consistent with previous reports, AMSCs exhibited a homogeneous population of spindle fibroblast-like cells after 3-4 passages [19]. Further analysis by flow cytometry revealed that these cells were positive for CD44, CD90 and CD73, which are generally taken as markers of MSCs, but negative for the expression of hematopoietic markers, CD34, CD45 and also HLA-DR (**Figure 1A**).

The pluripotency of AMSCs was verified by inducing their osteogenic and adipogenic differentiation. We found the intracytoplasmic lipid droplets were observed by Oil-Red staining in AMSCs after adipogenic induction, and the cal-

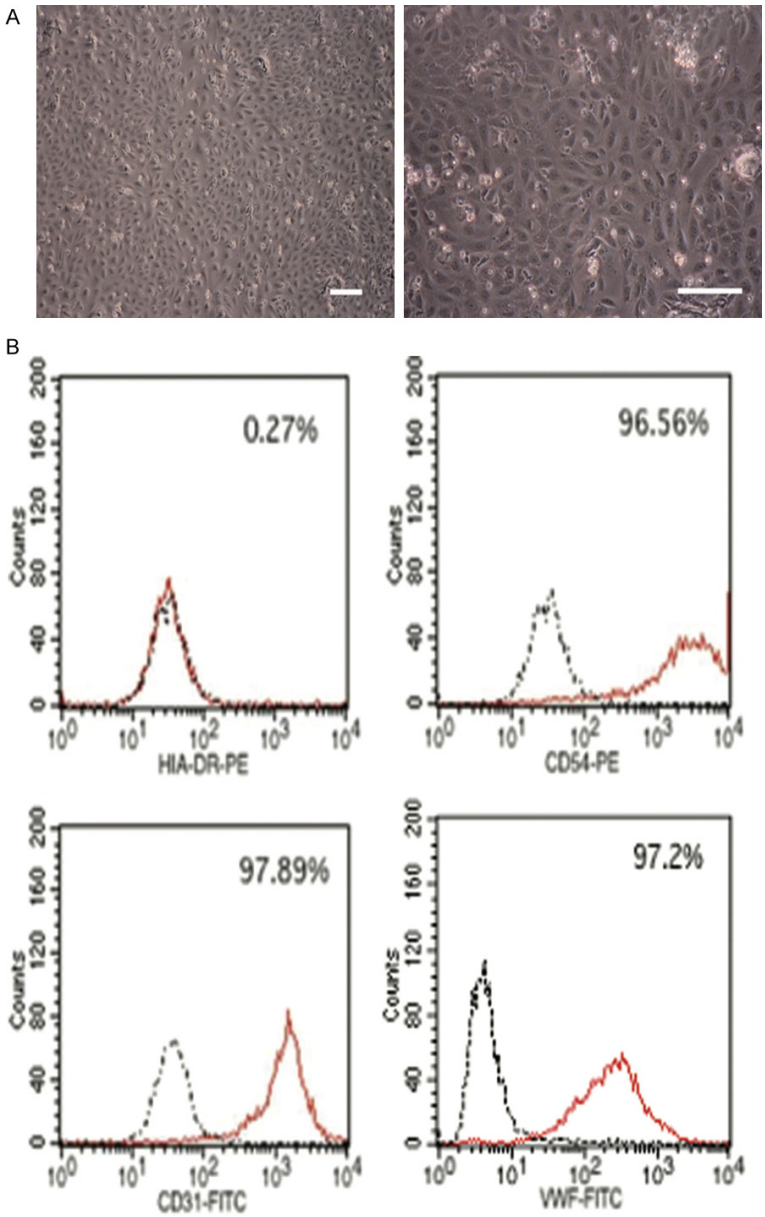

Figure 2. Characterization of human umbilical vein endothelial cells. A. Morphology of HUVECs. B. Endothelial cells-specific phenotype of HUVECs by flow cytometry analysis. Scale bars, 100 μm.

cium mineralization nodules were formed by alizarin red staining after osteogenic induction (**Figure 1B**).

Identification of HUVECs

As shown in **Figure 2A**, HUVECs exhibited a cobblestone-like morphology in a single cellular layer at confluence. Moreover, these cells were positively stained for CD54, CD31 and vWF, which are generally considered endothelial cells markers, but were negative for the expression of HLA-DR by flow cytometry analysis (**Figure 2B**). Therefore, HUVECs showed typical morphology of endothelial cells and EC-specific phenotype.

Differentiation of AMSCs into hepatocyte-like cell lineage

With our two-step protocol **Figure 3**), we found that the morphology of AMSCs changed gradually. As shown in **Figure 4A**, the AMSCs showed a remarkable transition from a bipolar fibroblast-like morphology to a round epithelial-like shape during the hepatic differentiation process. Furthermore, the cells exhibited a polygonal and a cobblestone-like morphology 21 days after hepatocyte-like induction, which was similar to that of primary adult hepatocytes.

Expression of hepatic-lineage markers in AMSCs-derived hepatocyte-like cells

To identify whether the phenotype of induced AMSCs was similar to that of hepatocytes, we confirmed hepatocyte-specific gene expression of HLCs, such as albumin, α1-AT, CK18, CYP3A4 and AFP. We found that albumin, α1-AT and CYP3A4 (markers of mature hepatocytes) were up-regulated gradually during differentiation. However, the expression of CK18 was decreased (**Figure 4B**). In addition, the expression of AFP, a marker of hepatocyte precursors, was also detected in the nucleus of AMSCs-derived HLCs 21 days after induction by immunocytochemistry staining (**Figure 4C**). This result was further verified by western blot analysis (**Figure 4D**). From above data, we concluded that AMSCs could differentiate into hepatocyte-like cells by our two-step protocol, and the derived cells were non-homogenous in phenotype.

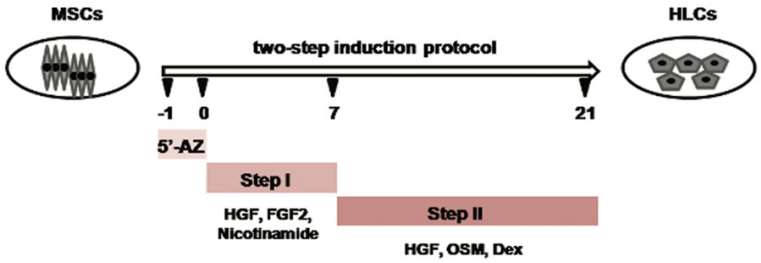

Figure 3. New method for hepatocyte-like cells generation from AMSCs. This schematic figure represents two-step protocol to induce AMSCs differentiation into HLCs. In the first stage of induction, AMSCs were treated with 20 ng/mL HGF, 20 ng/mL FGF2 and 1.25 mM nicotinamide in IMEM with 2% FBS for 7 days. In the second stage of maturation, these cells were treated with 20 ng/mL HGF, 20 ng/mL OSM and 1 μM Dexamethasone for a further 14 days.

Glycogen storage of AMSCs-derived hepatocyte-like cells

To prove whether our derived HLCs were as functional as primary hepatocyte, we detected the glycogen storage ability of these cells by PAS staining. As shown in **Figure 4E**, the glycogen storage was gradually increased during differentiation, which was very weak in undifferentiated AMSCs. This result demonstrated that AMSCs-derived HLCs after 21 days of induction performed the glycogen storage function similar to mature hepatocyte.

Generation of liver organoids in vitro

To test whether the three cell types can self-organize in a three-dimensional collagen hydrogel scaffold, we detected the morphology of HLCs mixed with HUVECs and MSCs. The volume of the collagen hydrogel were gradually reduced, which self-assembled into a compact structure (**Figure 5A**). Mixed cells formed an organoid structure, which was not observed in HLCs-only control group (**Figure 5B**, **5C**). To show the distribution of HLCs, HUVECs and MSCs, immunofluorescent staining using antibodies against cell-type specific markers was performed. The results demonstrated that HLCs (stained for albumin) arranged in adherent clusters, whereas vWF-positive HUVECs were mainly localized at the central regions. In addition, CD44-positive MSCs were distributed at the periphery of the liver organoid or randomly between HLCs and HUVECs (**Figure 5D**).

To further clarify the liver-specific phenotype of HLCs in collagen hydrogel, we compared the albumin expression of HLCs in HLCs-only and mixed-cell-culture systems. The results showed that higher albumin expression in mixed-cell-culture system was observed, compared to HLCs-only control (**Figure 5E**). HLCs in mixed-cell-culture system retained a more mature phenotype.

The effect of HLCs transplantation on liver fibrosis

After detecting the specified markers and function of hepatocyte in our derived cells *in vitro*, we further validated the *in vivo* function by transplantation of AMSCs derived-HLCs to mice with liver fibrosis induced by CCl_4. Transplantation was performed with HLCs alone or mixed with HUVECs and undiferentiated AMSCs (**Figure 6**). To assess the liver function, we first observed the gross anatomy changes of fibrotic livers. Compared with the normal mouse liver with a smooth surface and soft textures, the livers of mice in group 3 (mock transplantation) appeared with fibrotic conformation, with no obvious attenuation 4 weeks after PBS injection. We observed fibrotic tissues of these mice livers with palpable hard textures (**Figure 7A**). Compared to the HLCs-only transplantation group, mice livers of mixed-cell transplantation group showed less visible fibrosis, and were smoother on the surface 4 weeks post-transplantation (**Figure 7A**).

To assess the liver fibrosis after transplantation, we determined the accumulation of collagen I and collagen III in mouse liver by Sirius red staining. In addition, we also determined the expression levels of α-SMA by immunohistochemistry. We found that by these parameters the liver fibrosis was attenuated in both HLCs-only transplantation group and mixed-cell transplantation group. Moreover, compared with the HLCs-only transplantation group, the liver fibrosis was further attenuated in mixed-cell transplantation group 4 weeks after intrasplenic transplantation (**Figure 7B**, **7C**).

The effect of HLCs transplantation on liver function

To evaluate the recovery of liver function after transplantation, we quantified the levels of AST and ALT in mice plasma by automatic clinical

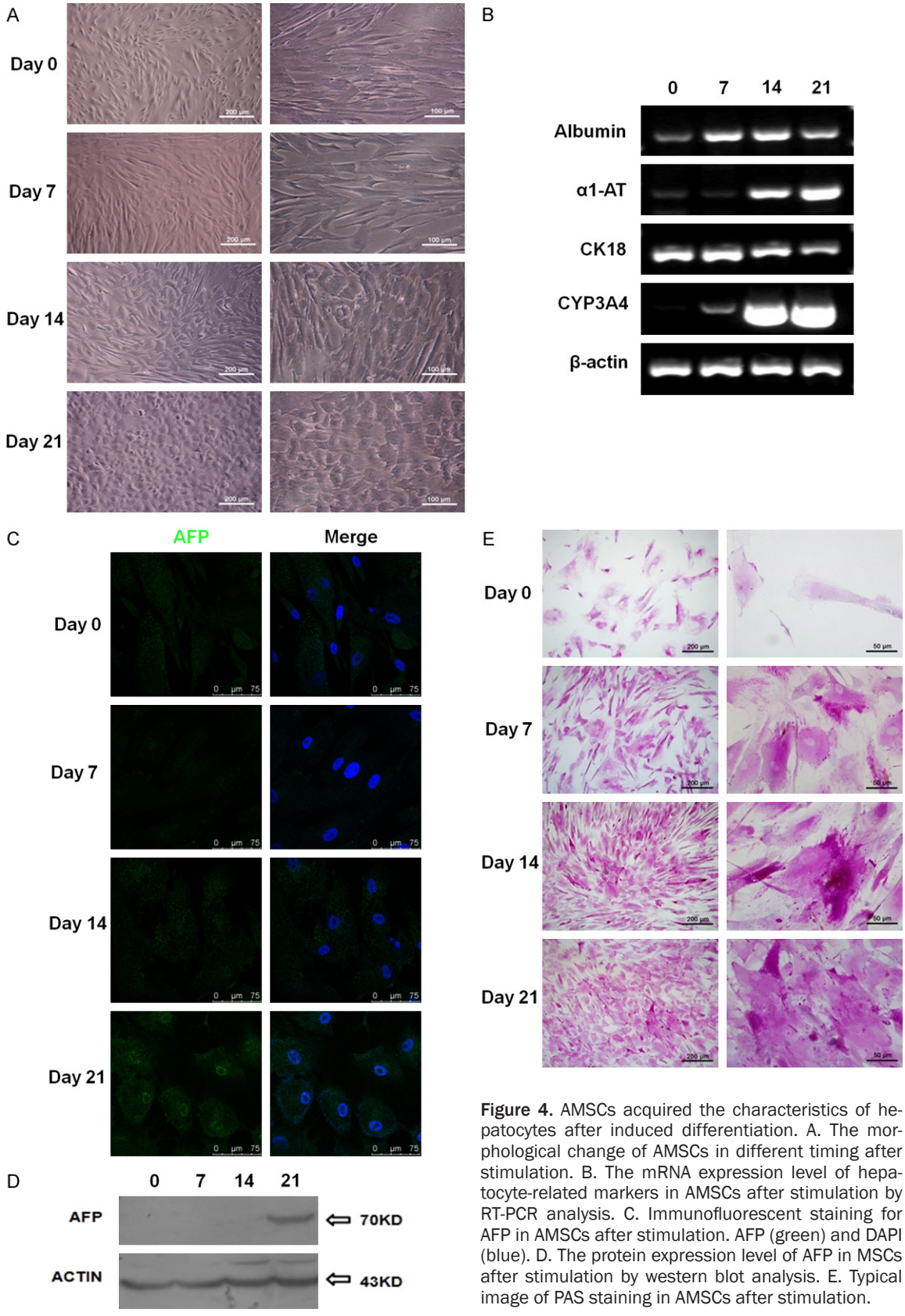

Figure 4. AMSCs acquired the characteristics of hepatocytes after induced differentiation. A. The morphological change of AMSCs in different timing after stimulation. B. The mRNA expression level of hepatocyte-related markers in AMSCs after stimulation by RT-PCR analysis. C. Immunofluorescent staining for AFP in AMSCs after stimulation. AFP (green) and DAPI (blue). D. The protein expression level of AFP in MSCs after stimulation by western blot analysis. E. Typical image of PAS staining in AMSCs after stimulation.

analyzer. We found that the levels of plasma AST and ALT were decreased in both HLCs-only transplantation group and mixed-cell transplantation group after transplantation (**Figure 7D**,

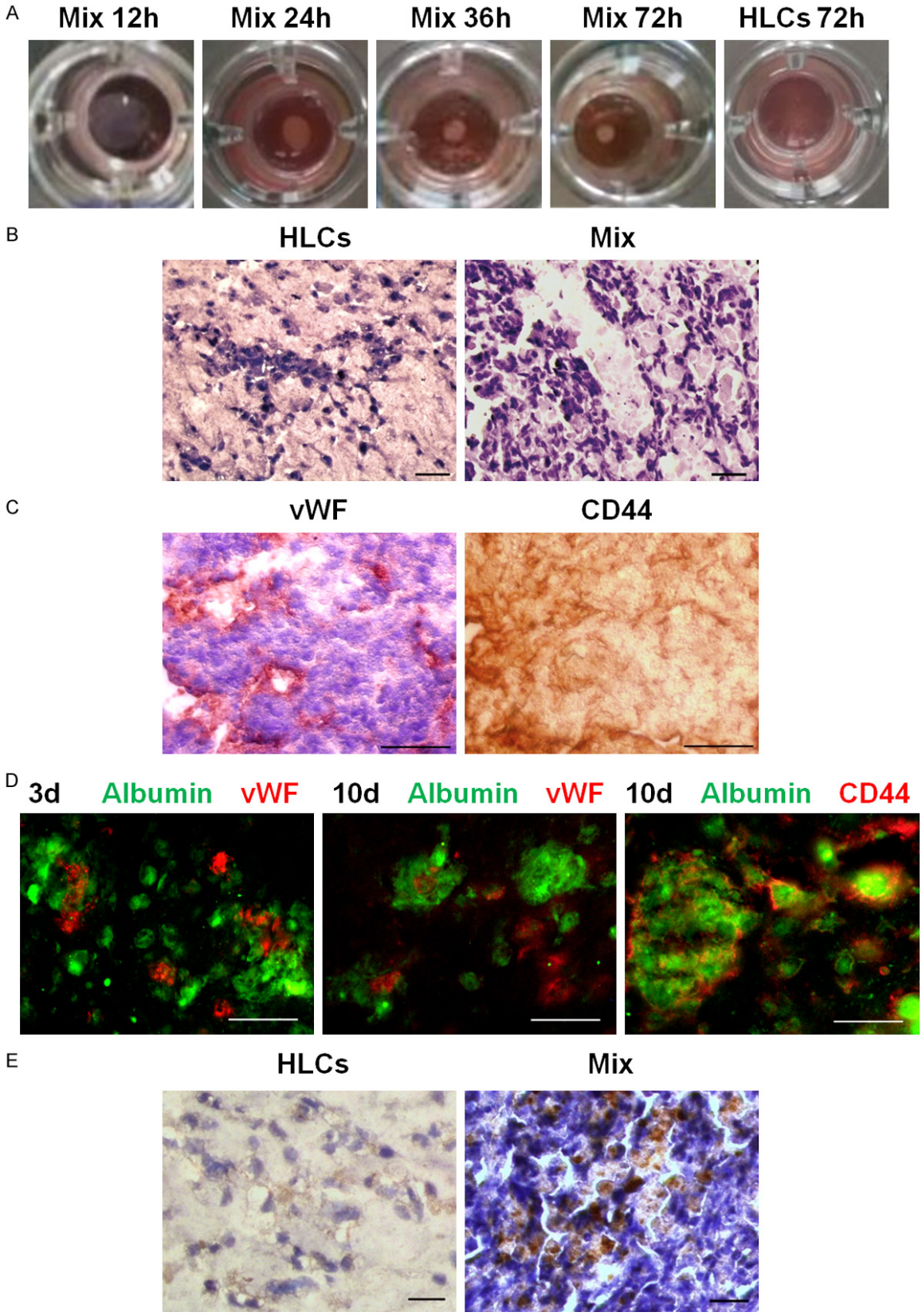

Figure 5. Generation of liver organoids in vitro. A. Time-dependent formation of liver organoids in 96-well plate. B. H&E staining for HLCs and mixed cells in 3D collagen hydrogel scaffold. Scale bars, 100 μm. C. Immunohistochem-

istry staining for vWF and CD44 expression in liver organoids. Scale bars, 100 μm. D. Immunofluorescent staining for cell-type specific markers in liver organoids. Scale bars, 100 μm. E. Immunohistochemistry staining for albumin expression in HLCs-only and mixed cells. Scale bars, 50 μm.

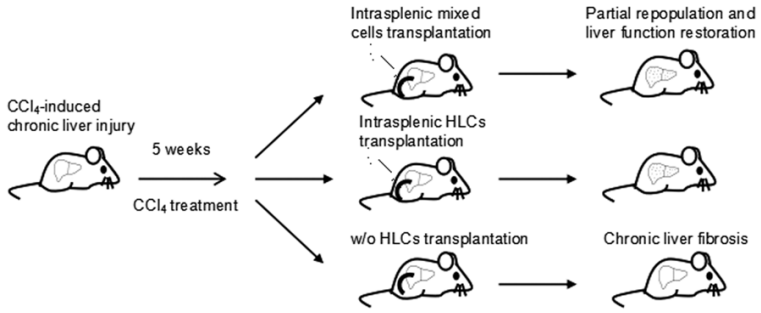

Figure 6. Schematic outline of the use of AMSCs-derived HLCs for treatment of chronic liver fibrosis mice. CD1 mice were intraperitoneally injected with CCl_4 to induce liver fibrosis. After CCl_4 treatment for 5 weeks, 2×10^6 HLCs or 1×10^6 HLCs mixed with 7×10^5 HUVECs and 3×10^5 AMSCs were transplanted to the spleen of mice with chronic liver fibrosis.

7E). Furthermore, the AST level in mice plasma was decreased markedly in mixed-cell transplantation group. From the above data, we suggest that AMSCs derived hepatocyte-like cells attenuated liver fibrosis and improved liver function more efficiently when transplanted along with HUVECs and AMSCs.

Engraftment of transplanted cells in recipient livers

To confirm the engraftment of transplanted cells in mice livers, we detected the human-specific Alu DNA sequences and albumin expression in recipient livers by genomic PCR and RT-PCR respectively. We found that HLCs were integrated in recipient livers in both HLCs-only transplantation group and mixed-cell transplantation group. Furthermore, the engrafted HLCs in mice liver was visibly higher in mixed-cell transplantation group 4 weeks post-transplantation, compared with HLCs-only transplantation group (**Figure 7F**, **7G**).

We also found that a few cells located around liver sinus for a half of transplanted mice after cell transplantation. Small portions of these cells were $CD83^+$ cells (Figure S1).

Discussion

In this study, based on the knowledge of liver development in embryo and other references from other lab's publications a population of functional effective HLCs from AMSCs were generated using a new two-step protocol. During the first stage, HGF, FGF2 and nicotinamide were utilized as induction factors. FGF2 is required for initiating early liver development [20, 21]. HGF is a pleiotropic growth factor and acts as a key factor for hepatogenesis [22, 23]. In addition, nicotinamide is involved in proliferation and colony formation of hepatocytes [24]. In the second stage of maturation, HGF, OSM and Dex as main factors. Pleiotropic cytokine OSM is essential for promoting maturation of fetal hepatocytes [25]. The combination of OSM and HGF not only promotes hepatocyte differentiation, but also triggers complete hepatic maturation with the addition of Dex, which is required for stimulating expression of liver specific genes [26-29]. Moreover, the cells were treated with 5'-AZ to inhibit the proliferation of AMSCs before the whole induction process, which could significantly improve the differentiation efficiency. This demonstrated a simple, efficient and easy handling protocol for the generation of functional HLCs from AMSCs.

Similar to primary human hepatocytes, the derived HLCs showed many typical hepatic features, including their polygonal and cobblestone-like morphology, expression of a panel of the mature hepatocyte markers including Albumin, α1-AT, CYP3A4 and CK18. These hepatocyte markers were selectively expressed during the differentiation of MSCs as described previously [9]. During the mid/late stage of MSC-to-hepatic differentiation, these cells expressed albumin and CK18, followed by the expression of mature hepatocytes markers, such as CYP3A4 and α1-AT during the late stage of MSC differentiation [30, 31]. The expression of AFP, which is a marker of immature hepatocytes, was also detected at late stage of differentiation. This observation suggested that the differentiated MSCs were heterogeneous and contained not only mature differenti-

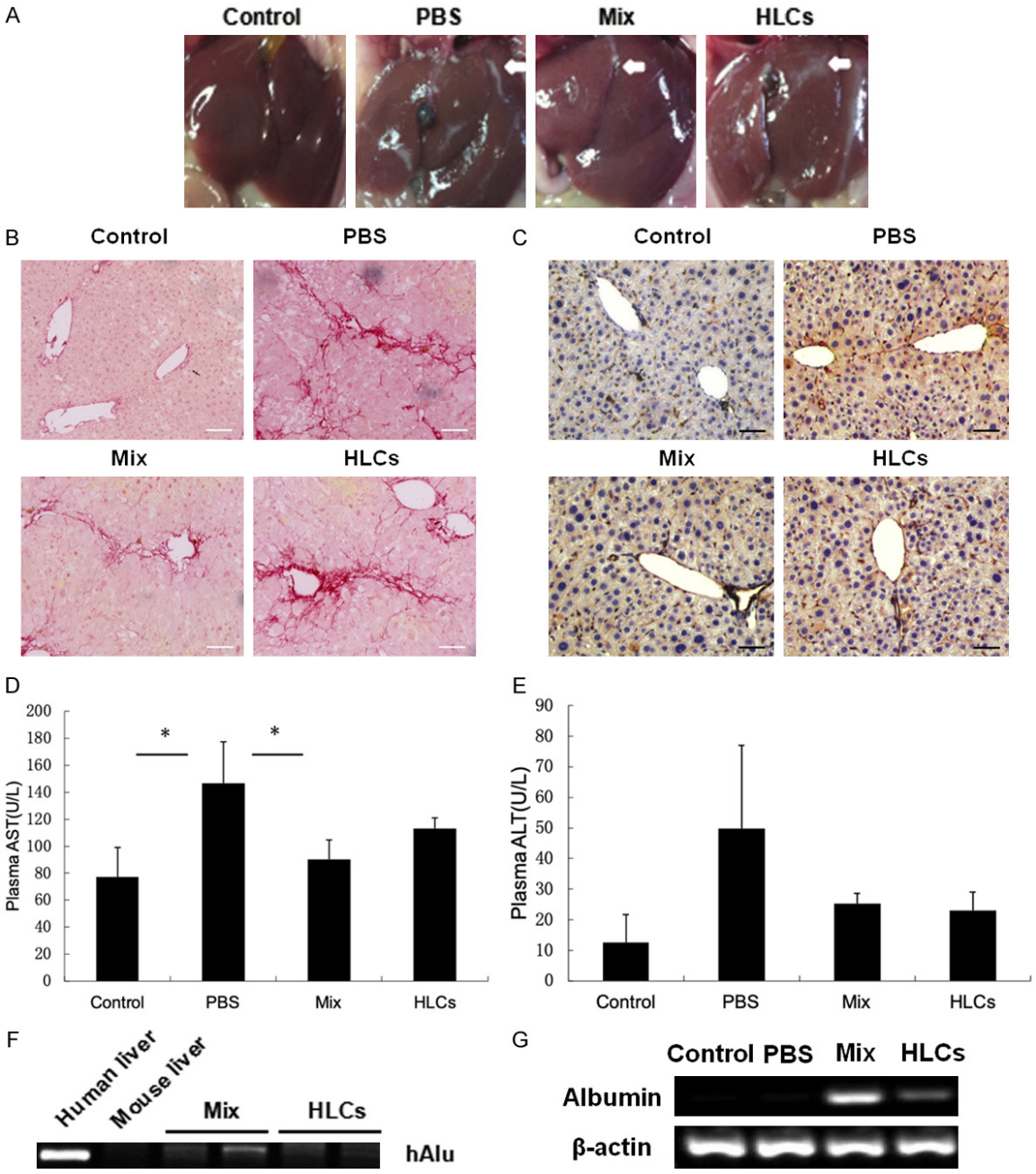

Figure 7. The effect of AMSCs-derived HLCs transplantation on liver fibrosis and liver function. A. Macroscopic images of freshly isolated livers 4 weeks post-transplantation. White arrows indicated the liver fibrotic areas. B. Sirius red staining of liver sections 4 weeks post-transplantation. Scale bars, 100 μm. C. Immunohistochemistry staining for α-SMA in mice liver 4 weeks post-transplantation. Scale bars, 100 μm. D. Plasma levels of AST in mice 4 weeks post-transplantation. E. Plasma levels of ALT in mice 4 weeks post-transplantation. *$P < 0.05$. F. Integrated cells in chronic liver injury mice. Human-specific Alu sequences were analyzed by PCR using genomic DNA extracted from mice livers. G. Human albumin expression in mice liver 4 weeks post-transplantation was analyzed by RT-PCR.

ated hepatocytes, but also some hepatoblasts or hepatic progenitors. MSCs isolated from primitive origin seem to have the immature phenotype even though they were driven toward specific lineage [23]. In addition, the HLCs acquired the characteristics of functional hepatocyte which was confirmed by PAS staining for glycogen storage. Collectively, our derived HLCs exhibited typical characteristics of primary human hepatocytes *in vitro*.

Conventional culture conditions of hepatocytes lack supportive cellular cross talk with stromal cells, thus long term culture *in vitro* leads to impaired phenotype and function of primary hepatocytes. Mice and human primary hepatocytes cultured in dish for 12 hour showed significant de-differentiation markers. It was demonstrated that AMSC-drived HLCs, together with HUVECs and undifferentiated MSCs, can generate liver organoids in a three-dimensional collagen scaffolds system. The HLCs within such organoids expressed albumin, which showed a more mature phenotype, compared to HLCs in the same culture system without HUVECs and MSCs. Moreover, the HLCs within the organoids were viable and functional for 10 days *in vitro*. These results imply that HUVECs and MSCs provide survival benefit for HLCs in the three-dimensional *in vitro* system.

The shortage of human donor livers and hepatocytes with good qualities is a major limitation of liver transplantation in clinical therapy. Taking advantages of the liver repopulation ability, a key characteristic of human hepatocyte *in vivo,* hepatocyte-like cells transplantation may be a promising alternative to orthotopic liver transplantation. However, previously reported stem cell-derived cell therapies in liver failure have failed mainly because of the poor engraftment efficiency of the transplanted cells and the temporal improvements in liver function [32-34]. In this study, it was observed that the HLCs mixed with HUVECs and undifferentiated AMSCs performed better in restoring the liver function, in contrast to previously failed MSC transplantation. It was found that compared to mock transplantation (PBS) or HLCs transplantation group, the liver fibrosis was attenuated more significantly in mixed-cell group four weeks after transplantation. Furthermore, the AST level in mice plasma was also decreased markedly in mixed-cell transplantation group. Thus, the therapeutic effects of HLCs transplantation on chronic liver fibrosis was improved with the presence of MSCs and HUVECs. One reasonable explanation for these differences may be that the HUVECs derived stromal cell populations provided a more suitable niche, which is generally required during normal organogenesis and liver repopulation, for AMSCs-derived HLCs.

It has been reported that solid liver buds, which were formed by iPSCs derived hepatic endoderm cells together with MSCs and HUVECs prior to transplantation, had successful engraftment and continued to mature and function for a long duration [11]. Restoration of injured liver requires the presence of hepatocytes, MSCs, and endothelial cells, and may involve in complex signaling processes and interactions [35, 36]. Previous studies also have shown vasculogenic endothelial cells and nascent vessels were critical for the earliest stages of organogenesis, prior to blood vessel function [12]. In addition, MSCs could inhibit hepatocellular apoptosis, secrete various bioactive molecules, or exert immune-modulatory effect to promote liver regeneration [9]. This study suggested that MSCs and endothelial cells may be beneficial to the functional differentiation, maturation and integration of HLCs into recipient livers. In this study, the engraftment of transplanted cells by detection of human specific Alu sequence and albumin expression was identified. Furthermore, the engraftment of HLCs in mice liver was better in the mixed-cell transplantation group 4 weeks post-transplantation, compared with the HLCs-only transplantation group.

In conclusion, we induced AMSCs differentiation into functional human hepatocyte-like cells using a new two-step protocol. The AMSCs derived HLCs exhibited hepatocyte-specific phenotype and glycogen storage function *in vitro*. It is very interesting to see if these functions may be applicable for diabetes patients. Furthermore, we demonstrated that the AMSCs-derived HLCs could attenuate liver fibrosis more efficiently when transplanted along with HUVECs and undifferentiated AMSCs, and this suggests that we could improve the efficiency of cell therapy by transplanting functional hepatocyte-like cells along with stromal cells with proper cells ratio. Although many aspects of hepatocyte-like cell transplantation remain to be further explored and to get more favorable conditions, our study highlights a therapeutic potential of the alternative approach by transplanting functional cells along with stromal cells for human liver disease treatment and other regenerative medicine.

Acknowledgements

We would like to acknowledge the department of pathology at 261st Hospital of Chinese PLA for the assistance with histological preparations.

Disclosure of conflict of interest

None.

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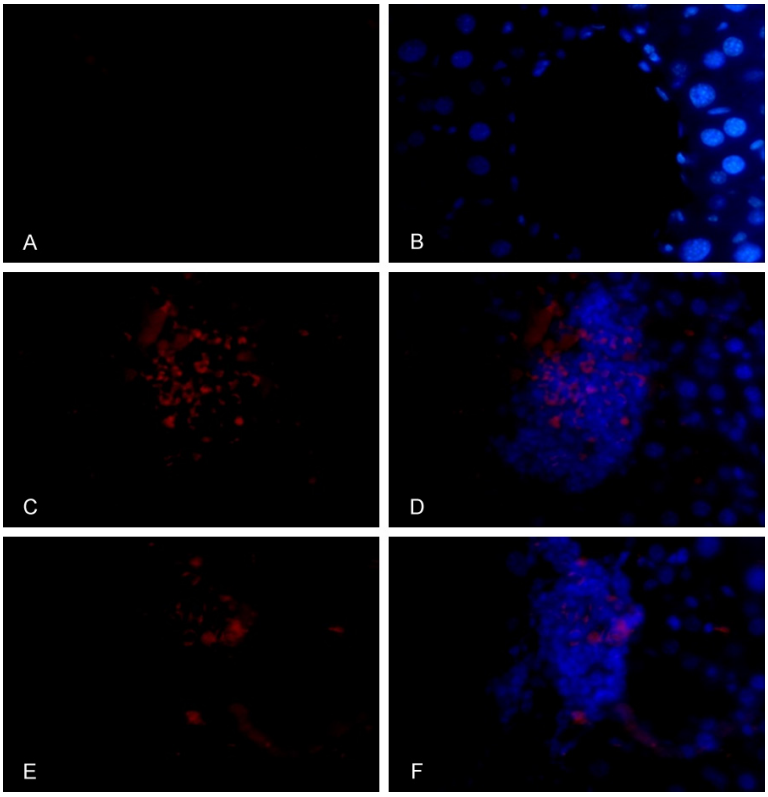

Figure S1. Immunofluorescent staining for $CD83^+$ cells around liver sinus in mock transplantation group (A), mixed-cell transplantation group (C) and HLCs transplantation group (E) 4 weeks post-transplantation. Overlay of CD83 and DAPI staining (B, D, F).