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Liver epithelioid progenitor cells derived from fetal Luxi bovine alleviate liver fibrosis

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Abstract Liver epithelioid progenitor cells (LEPCs) have important roles in liver therapy because of their hepatic differentiation potency in vitro and in vivo. Despite many researches on humans, mice, and rats, equivalent progenitor cells derived from bovine are relatively rare. The purpose of our current study is to characterize bovine LEPCs, and research on the cure potency of this heteroplastic progenitor cells on mice liver fibrosis. We have used collagenase IV digesting and differential adhesion method to isolate slabstone shape, EpCAM, LGR5, NCAM1 and SOX9 positive progenitor cells from fetal Luxi bovine liver. When cultured in hepatic differentiation media containing 20 ng/ml Oncostatin M, LEPCs can differentiate into hepatocytes in vitro. After 4 weeks of intravenous tail vein injection into CCl_4-injured mouse liver, LEPCs engrafted into liver parenchyma, differentiated into ALB positive hepatocytes, and could alleviate liver fibrosis through down regulating fibrosis genes-Tgfb1 and α-SMA as well as decreasing expression of collagen gene Col1a1, Col3a1, and Col4a1, and regain liver function by recovering ALT and AST. Our findings provided a useful tool for studying liver development in vitro, new cell resource for heterograft on mouse liver diseases, and a new platform for researches on immune rejection of heterogeneous cell transplantation.

Keywords Hepatocyte differentiation · Liver epithelioid progenitor cells · Stem cells therapy · Liver fibrosis · Heterogeneous cell transplantation

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Abbreviations

AFP	Alpha fetoprotein
CDM	Cholangiocytic differentiation medium
EPCAM	Epithelial cell adhesion molecule
G6PC	Glucose-6-phosphatase catalytic subunit
HDM	Hepatic differentiation medium
HepSCs	Hepatic stem cells
iHepSCs	Induced hepatic stem cells
Itgb4	Integrin subunit beta 4

LGR5	Leucine rich repeat containing G protein coupled receptor 5
LPCs	Liver progenitor cells
LEPCs	Liver epithelioid progenitor cells
NCAM1	Neural cell adhesion molecule 1
OSM	Oncostatin M
PAS	Periodic acid-Schiff
PFA	Paraformaldehyde
SOX9	SRY (sex determining region Y)-box 9

Introduction

Liver is an amazing organ. Its regenerative capacity remains in adulthood because it contains many types of stem cells—hepatic mesenchymal stem cells, liver stem cells (LSCs), liver epithelioid progenitor cells (LEPCs) and so on. Hepatic or liver mesenchymal stem cells possess elongated spindle shape with ovoid nuclei, and MSC properties (Wang et al. 2016). Hepatic or liver stem cells are pluripotent precursors of hepatoblasts, and EpCAM, NCAM, and claudin 3 positive, can differentiate into hepatoblasts in vitro and hepatocytes after transplantation (Schmelzer et al. 2007). LEPCs are bipotential cells,which can differentiate into hepatocytes and cholangiocytes, being the most extensive research stem cells (Arends et al. 2009). LEPCs can be cultured long-term whether isolated from a fetus or not (Kido et al. 2015). The bipotential nature and the high resistance to toxins make these cells of great (pre)clinical importance for currently untreatable liver diseases.

Leucine rich repeat containing G protein coupled receptor 5 (LGR5), which is a stem cell mark for self-renewal small intestine, colon, stomach and hair follicle tissues (Barker et al. 2007, 2008, 2010), is also expressed in damaged-induced liver stem cells (Huch et al. 2013a, b). Besides, other hepatic stem cell markers—epithelial cell adhesion molecule (EPCAM) (Okabe et al. 2009; Terris et al. 2010; Yoon et al. 2011), SRY (sex determining region Y)-box 9 (SOX9) (Liu et al. 2016; Paganelli et al. 2014), and neural cell adhesion molecule 1 (NCAM1) (Buzhor et al. 2013) are all expressed in liver stem cells as well.

Although rat and mouse liver-derived progenitor cells are intensively investigated, equivalent progenitor lines from the bovine are relatively rare. Domestic livestock stem cells (such as bovine) offer a unique opportunity to study developmental biology, serving as a resource to screen for harmful toxins or lifesaving drugs or even regenerative therapies for a number of diseases (Gao et al. 2015). Our previous study had characterized bovine liver mesenchymal stem cells (Lu et al. 2014). In our study, we isolated LEPCs from the bovine fetal livers and cultured them in HepSCs medium (Yu et al. 2013). Like the rodent hepatic stem cells, the clonally expanding bovine fetal liver epithelial cells express EPCAM, LGR5, SOX9 and NCAM1 hepatic stem cells markers, and show the capacity for hepatic differentiation under different conditions based on morphology, immunocytochemistry, and gene-expression profiles. Moreover, when transplanted into chronic injured liver induced by CCl_4, LEPCs engrafted into the liver parenchyma, differentiated into hepatocytes and remedied liver fibrosis.

Materials and methods

Ethics statement

All animal experiments were performed in accordance with national and international guidelines. The 6-week old ICR mice were bought from Beijing HFK Biotechnology Co. Ltd (Beijing, China). And the fetal Luxi bovine samples (5-month old) were obtained from the Chinese Academy of Agriculture Sciences' farm. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Peking University health science center (Permit number: SYXK (jing) 2011-0039).

Isolation and culture of bovine LEPCs

All fetal bovine samples had to be transported to laboratory within 4–8 h in ice tray. Under sterile conditions, we did caesarean section and collected fetal bovines. After gaining fetal bovine liver tissues, we directly plated them into sterile PBS, cut blood vessels and biliary ducts off with surgical scissors, and finally rinsed with PBS to remove blood cells more than 7 times. Then they were cut into approximately 1 mm^3 and digested with 0.1% collagenase IV (Sigma, St. Louis, MO, USA) at 37 °C for 20 min. The digested tissues were pipetting several times and passed through a 300-mesh filter, and then centrifuged at 1200 rpm for 7 min at room temperature. The supernatant was

removed, and the pellet was resuspended with basal medium. Then the resuspended cells were centrifuged twice at 500 rpm for 2 min to remove hemocytes. Finally the pellet was resuspended with growth medium (DMEM/F12 medium (Gibco, Grand Island, NY, USA), 10% FBS (Gibco), 1 × ITS (Gibco), 100 μM β-mercaptoethanol (Sigma), 10 ng/ml HGF (PeproTech, Rocky Hill, NJ, USA), 10 ng/ml EGF (PeproTech), and 10^{-7} M dexamethasone (Sigma-Aldrich, St. Louis, MO, USA)) to seed on 60 mm petri dishes 3 days for removing most no-epithelioid cells, and then transferred the supernatant to a fresh petri dish. Then 1 mL growth medium was added every 2 days. After 7 days, we picked epithelioid cells clone group for passage.

RNA extraction, RT-PCR, and real time PCR

RT-PCR: Total cellular RNA samples were extracted by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using a reverse transcription kit (TaKaRa, Dalian, China) and amplified by PCR using specific primers which are listed in supporting information Table 1. PCR products were visualized by 2% agarose gel electrophoresis.

Real time PCR: Total RNA was extracted from control, CCl_4 treated and LEPCs transplanted group by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was reversely transcribed using the RNA PCR kit (AMV) ver 3.0 (Takara). Real-time PCR was performed in 20 μl according to SYBR Premix Ex Taq II (Tli RNaseH Plus) kit (Takara). Each experiment was performed in duplicate in 96-well plates and repeated three times. Gene expression was detected on an ABI 7500 real-time PCR system (Foster City, CA, USA). The expression level was calculated using the 2-ΔΔCt method to compare relative expression. All primers used in this study are listed in Supplementary Table 2.

Flow cytometry

10^6 cells were harvested and fixed with 4% PFA (Sigma) for 30 min, and were then permeabilized in staining buffer (PBS with 10% FBS (Gibco) and 0.5% saponin (Sigma)) for 10 min. Cells were then incubated with primary antibody for 60 min in staining buffer, followed with secondary antibody incubation for 60 min. Cells were analysed by the FC500 flow cytometer (Beckman, Brea, CA, USA). Data were analysed with Windows FCS Express V3 Flow Cytometry.

Immunofluorescence

Cells were sub-cultured in 6-well plates at a density of 1×10^4 cells/well. The cells were fixed with 4% (w/v) paraformaldehyde at room temperature for 20 min, and then washed with phosphate buffer solution (PBS) three times. Cells were permeabilized with 0.1% (v/v) Triton X-100 (Sigma-Aldrich) for 15 min, and then washed with PBS three times. Cryostat sections were done as follows without being fixed and permeabilized. Next, cells and cryostat sections were incubated with 4% bovine serum albumin (BSA) (Sigma) for 60 min at room temperature. BSA was removed, and cells and sections were incubated with primary antibody at 4 °C overnight. Then the solution was decanted and the cells were washed with PBS three times. Cells and cryostat sections were incubated with the secondary antibody in 1% BSA for 60 min at room temperature. Afterwards, the secondary antibody solution was decanted and washed with PBS three times in the dark. Cells and sections were incubated with DAPI (Sigma-Aldrich) for 15 min and finally rinsed with PBS twice. Antibodies used for flow cytometry and immunofluorescence were listed in supporting information Table 3.

Hepatocyte differentiation in vitro

Hepatocyte differentiation was achieved by plating 5×10^4 cells/cm^2 LEPCs on 2% Matrigel-coated (BD Biosciences, San Jose, CA, USA) 6-well plates in hepatic differentiation medium (HDM) (DMEM/F12 medium supplemented with 10% fetal bovine serum (Gibco), 20 ng/ml Oncostatin M, 20 ng/ml EGF (both from PeproTech), 10 ng/ml nicotinamide, 0.1 mmol/l L-Ascorbic acid and 10^{-7} M dexamethasone (all from Sigma-Aldrich). During the differentiation course, medium was changed every 3 days. After 15 days, differentiation cultures were evaluated by RT-PCR for albumin, AFP and G6PC. Differentiated cells were evaluated by immunofluorescence for albumin protein expression. To assess the function of hepatocyte-like cells one assay was done (as described below).

PAS staining

Samples were oxidized for 5 min in 1% periodic acid-Schiff (PAS) (Sigma-Aldrich) and rinsed several times

with double-distilled H_2O (ddH_2O). Samples were incubated with Schiff's reagent for 15 min, rinsed several times with ddH_2O, immediately counter-stained with hematoxylin for 1 min, and washed several times with ddH_2O.

Hepatocyte differentiation of LEPCs in vivo and cure of liver fibrosis

To research the differentiation of LEPCs in vivo, DiI-labeled LEPCs (1×10^6) were suspended in DF12, and then injected into ICR mice (n = 6, 6 weeks old) through the caudal vein. Chronic liver damage was induced by intraperitoneal injection of 2.5 mg/kg carbon tetrachloride dissolved in olive oil twice a week for 4 weeks before transplantation. Mice were killed separately 1 or 6 months after cell implantation. Mouse sera were collected to detect the ALT and AST activity by using ALT and AST Activity Assay kits (Sigma). Some parts of recipient livers were fixed with 4% paraformaldehyde overnight at room temperature and embedded in OCT compound. Liver cryostat sections in 8 μm were observed under fluorescence microscope to detect transplanted cells. Paraffin sections were stained with FN and α-SMA antibodies to detect fibrosis. The rest of the liver tissues was stored in liquid nitrogen. Total RNA was extracted and real time PCR to quantify Tgfb1, collagen I, collagen II, and collagen IV expression was performed.

Fig. 2 Cell markers of LEPCs. **a** Detection of LEPCs cell marker by RT-PCR, the cells expressed EpCAM, LGR5, NCAM1, and Sox9, GAPDH was used as internal reference. M: DNA Marker I. **b** LEPCs are EpCAM-, LGR5-, and NCAM1-positive stem cells quantified by flow cytometry. **c**–**e** LEPCs can be stained by anti-EpCAM (**c**), anti-LGR5 (**d**), and anti-NCAM1 (**e**) antibodies. *Scale bar*, 50 μm

Statistical analysis

Data analysis was conducted using GraphPad Prism 6.0 software. Pictures were analyzed by Image J.

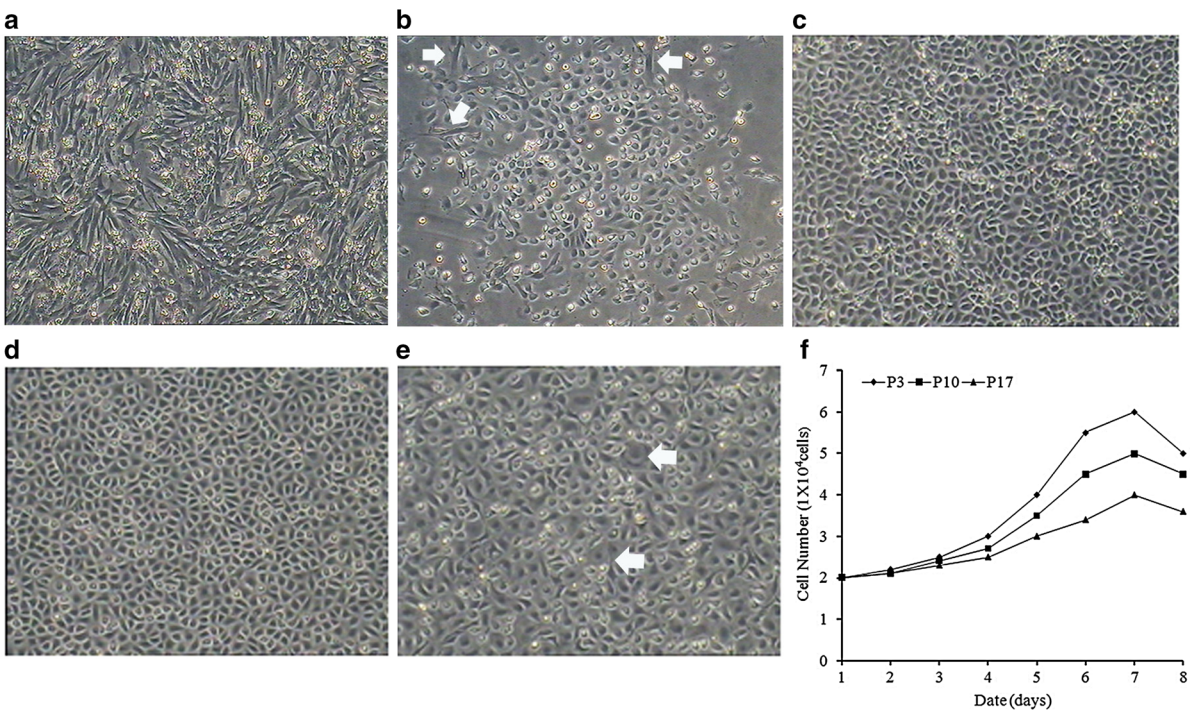
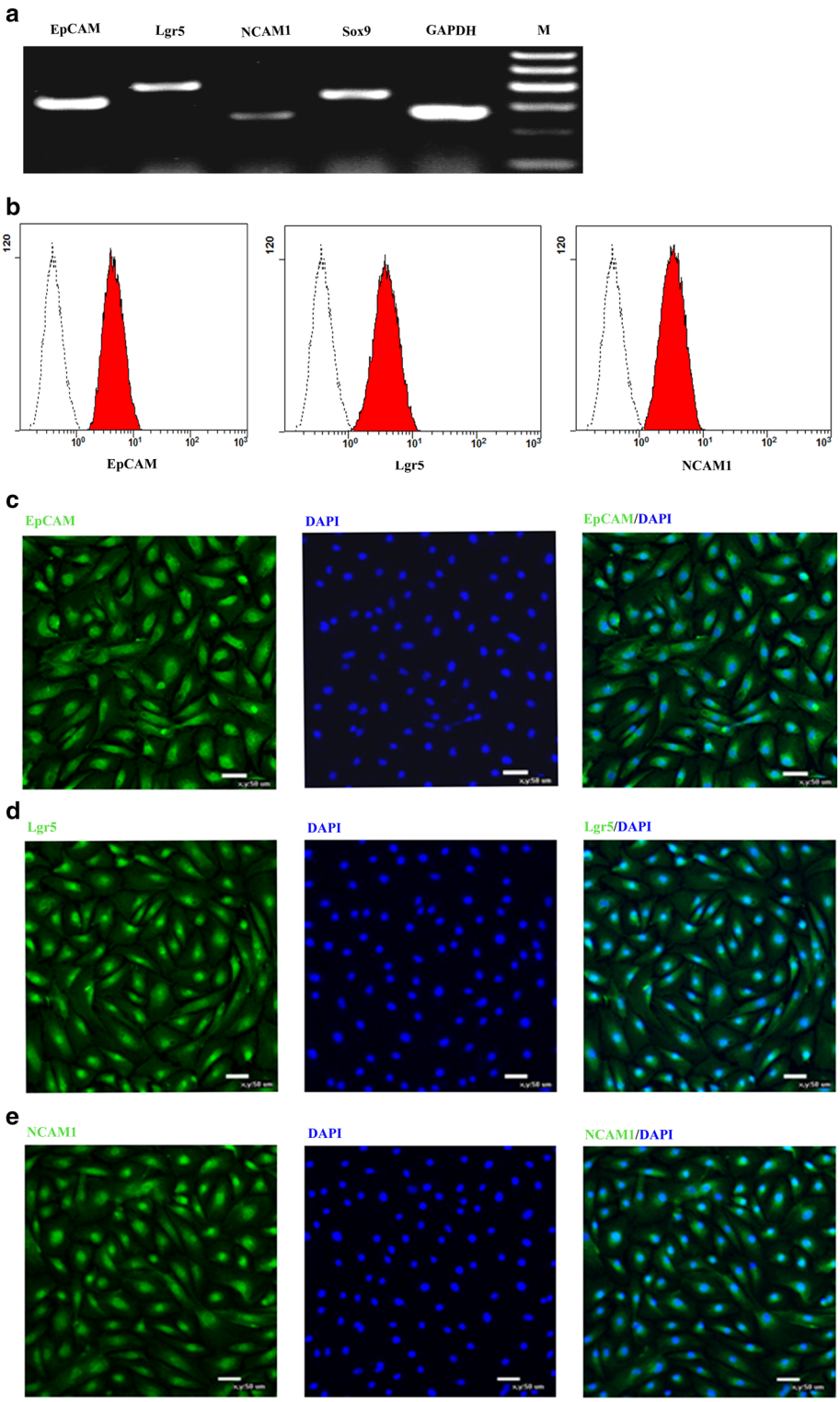


Fig. 1 Morphology of LSCs. **a** Cells attached at the first 3 days. **b** Epithelioid cells clones appeared at the second 3 days (*white arrows* indicated the spindle cells). **c** Passage 0 epithelioid cells cultured for about seven days. **d**–**e** Morphology of passage 13 and passage 23 epithelioid cells, *white arrows* indicated the flat and fired egg-like cells. **f** Growth curves of passage 3, 13, and 17 epithelioid cells. **a**–**e** ×40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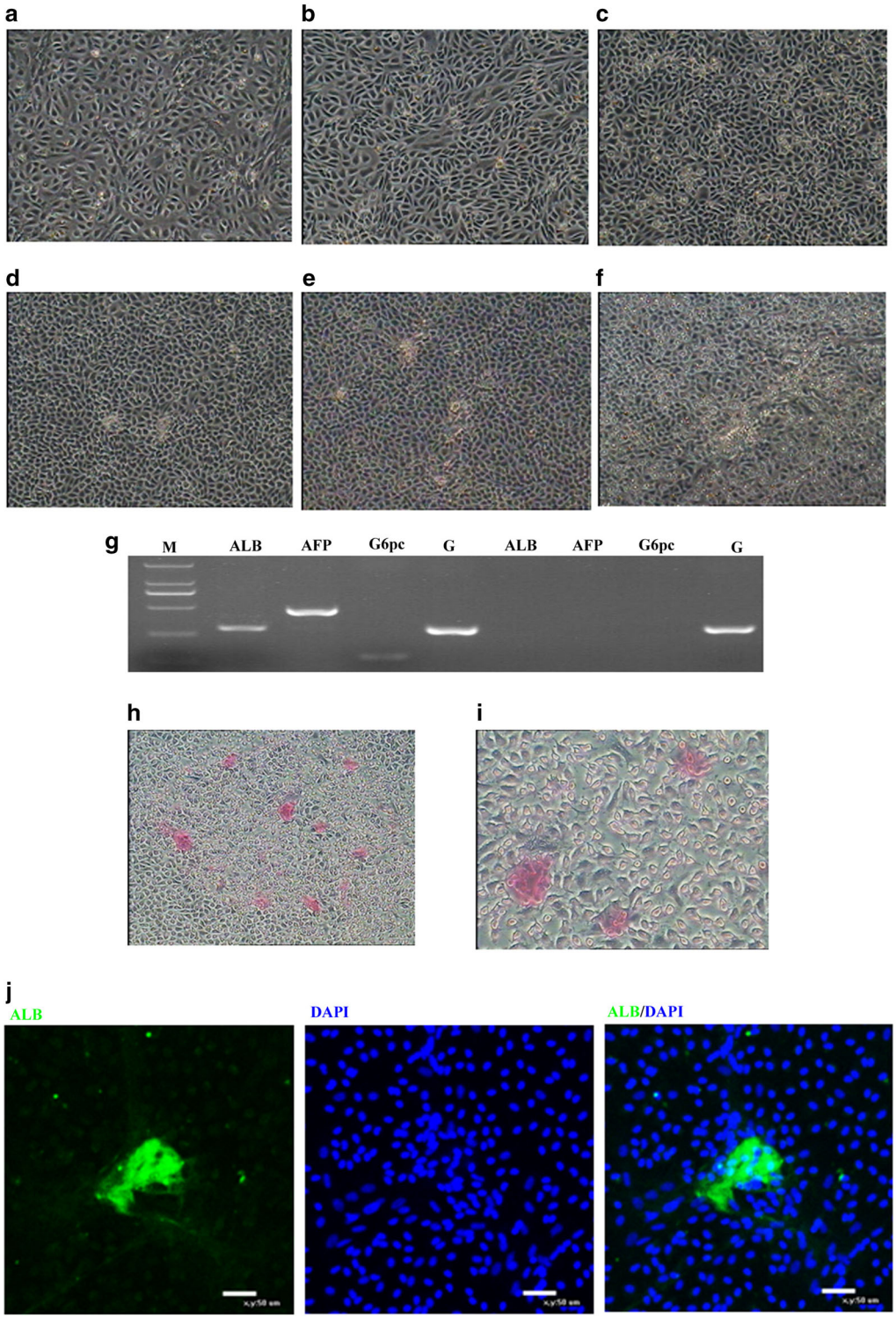
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◀**Fig. 3** LEPCs can differentiate into hepatocyte like cells. **a**–**f** Cell morphology changes during differentiation: a, induced 0 day; b, induced 3 days; c, induced 6 days; d, induced 9 days; e, induced 12 days; f, induced 15 days. **g** Hepatocyte specific markers (ALB, AFP and Gp6c, G: GAPDH was used as a internal reference) expressed between induced and un-induced groups. **h**, **i** PAS stained hepatocyte like cells after culture of LEPCs in hepatic differentiation medium 15 days. **j** The hepatocyte like cell mass expressed ALB protein detected by immunofluorescence. *Scale bar*, 50 μm. **a**–**f**, **h** ×40, **i** ×100

Results

Isolation and culture of fetal bovine LEPCs

In order to isolate epithelioid cells from digested liver cell mixtures, we attached the cell mixtures for different time periods. During the first 3 days, mostly long spindle cells (Fig. 1a) adhered to the surface of the dishes, and the epithelioid cells were still in supernatant. Then they were pipetted out the supernatant to a new dish. About another 3 days, epithelioid cells clones were formed (Fig. 1b). However, on the edge of these cells, there were also some spindle cells (white arrows). After another 4 days' culture, the epithelioid cell clones became a big clone, and occupied the domain position (Fig. 1c). When reaching up to 10 passages, the cells had a single morphology (Fig. 1d). At the end of the cells passage, cells expanded slowly and the cell morphology became irregular. They lost the tight junctions and became flat,

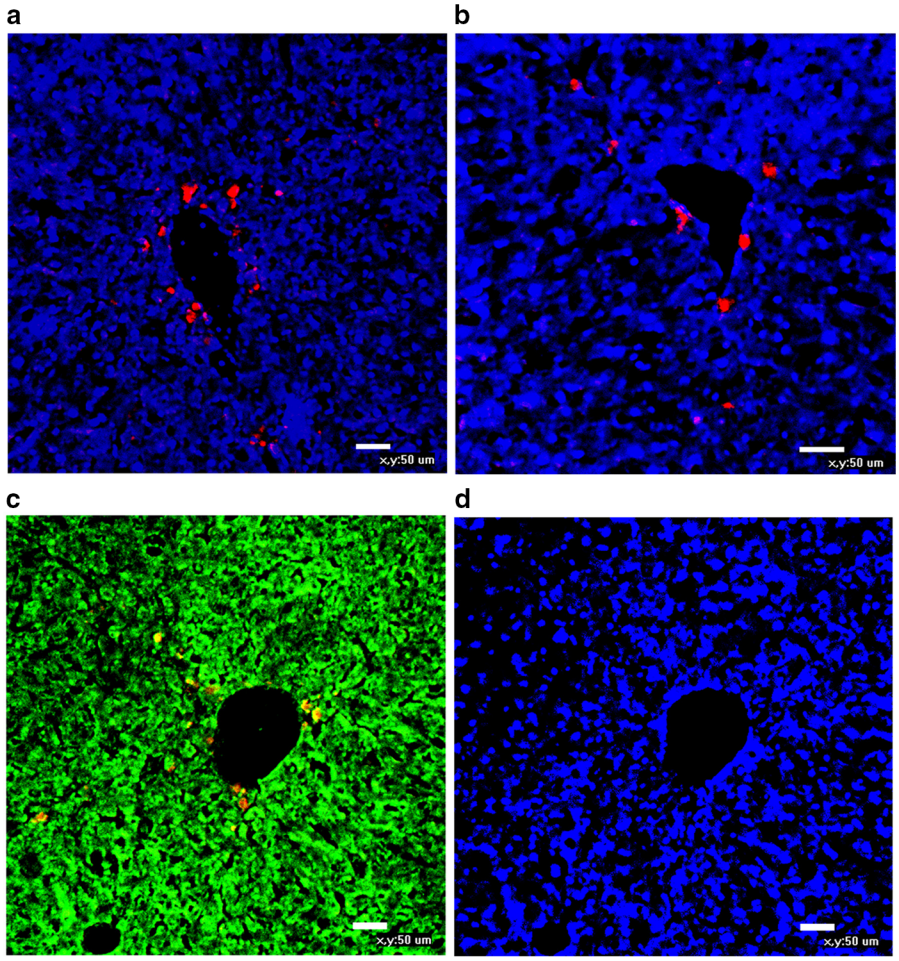
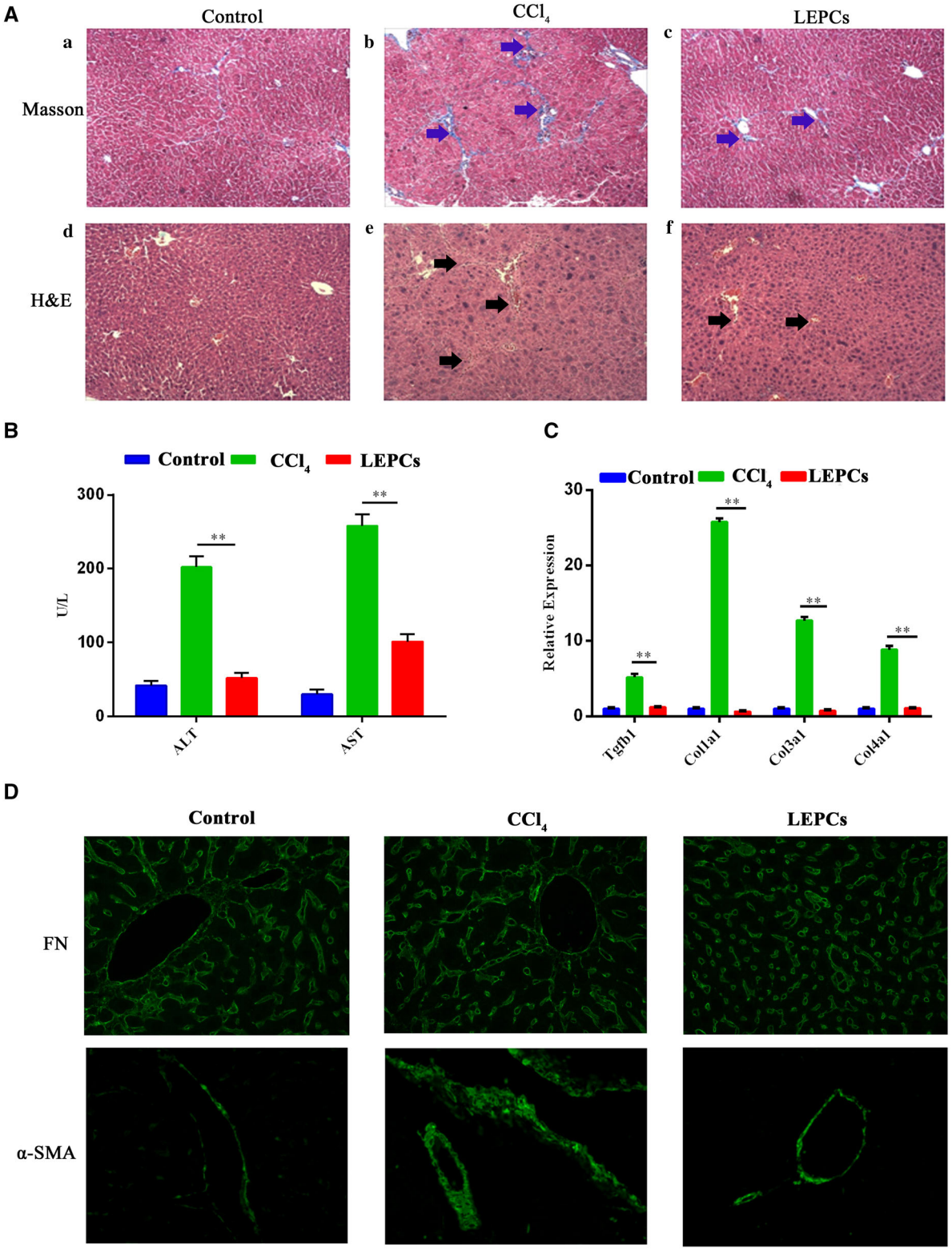


Fig. 4 Hepatic differentiation of LEPCs transplanted into CCl_4-treated recipient mice. **a**, **b** Dil-labeled LEPCs (*red*) were transplanted into mice livers by tail vein injection. Dil-positive cells were detected in experimental mice 4 weeks and 6 months after cell transplantation. **c** Overlay image of Dil (*red*) and section stained by anti-ALB antibody (*green*) shows that all Dil-positive cells were also ALB-positive hepatocytes (*yellow*) 6 months after cell transplantation. **d** DAPI staining of the same vision field of **c** to reveal nuclei. *Scale bar*, 50 μm. (Color figure online)


A
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Tgfb1
Col1a1
Col3a1
Col4a1
D
Control
CCl4
LEPCs
FN
α-SMA

◀**Fig. 5** LEPCs can reduce CCl_4 liver fibrosis. **A** Masson and H&E staining of mouse liver at 4 weeks after treatment. **a**, **d** Control group; **b**, **e** CCl_4 induced model group; **c**, **f** LEPCs transplanted group. **a**–**c** Masson staining (×200); **d**–**f** H&E staining (×200). *Black arrow* inflammation area, *blue arrow* collagen fibers. **B** Levels of mouse serum ALT and AST (liver injure markers) at 4 weeks after treatment. **C** Fibrosis associated gene expression of Tgfb1, Col1a1, Col3a1 and Col4a1 were analysised by real time PCR. **D** Immunofluorescence staining of FN and α-SMA at 4 weeks after treatment. **a**, **d** Control group; **b**, **e** CCl_4 induced model group; **c**, **f** LEPCs transplanted group. **a**–**c** FN staining (×400); **d**–**f** α-SMA staining (×400). Results are the means and SD; $p < 0.01$, Student's t test. (Color figure online)

fried eggs-like cells (white arrows) (Fig. 1e). During the first few passages, the cells were passaged every 2 days, and trypsinized about 1 min. With the passages increasing, the passage and trypsintion time were prolonged. After 20 passages, we needed 1 week for a passage, and digested 5 min by trypsin 0.25%-EDTA 0.02%. This was confirmed by the growth curve (Fig. 1f) at passages 3, 10, and 17. The cell growth curve was of "S" type and the expansion speed decreased with the increase of passages.

Detection of LEPCs cell markers

After isolation of the epithelioid cells, we did further researches to identify the cell type. First, we extracted the total RNA of the cells, and did reverse transcription PCR. We found the epithelioid cells expressed EpCAM, LGR5, NCAM1, and Sox9 (Fig. 2a). We further analyzed the percentage of the EpCAM, LGR5, and NCAM1 cells by flow cytometry. The results suggested that we have obtained highly purified Cells (Fig. 2b). In addition, we further detected that the cells were EpCAM, LGR5, and NCAM1 positive at the protein level by immunofluorescence (Fig. 2c–e). Therefore, we can confirm that we successfully isolated the liver epithelioid progenitor cells.

Hepatocyte differentiation of LEPCs in vitro

After identifying the isolated liver epithelioid progenitor cells, we detected the differentiation capacity of liver epithelioid progenitor cells. Therefore, we seeded the cells on 6-well plates with hepatic differentiation medium. During the first 3 days, the cells continued to expand (Fig. 3a, b). Some cells became bright round at the end of the sixth day (Fig. 3c). When it came to the ninth day, the bright round cells got together to form cell masses (Fig. 3d). With increasing culture days, masses were becoming larger (Fig. 3e, f). We isolated the total RNA and synthesized the cDNA. We detected by PCR that the differentiated cells expressed hepatocyte-specific genes ALB, AFP, and G6pc (Fig. 3g). Then we stained the hepatic differentiation cells with PAS on day 15. The cell masses were stained pink (Fig. 3h, i). This result indicated that the differentiated cell masses contain glycogen. We also confirmed by immunofluorescence that the cell masses expressed hepatocyte specific marker ALB protein (Fig. 3j).

Differentiation of LEPCs into hepatocytes in vivo after transplantation

After a 4-week treatment of CCl_4 for ICR male mice, we transplanted liver epithelioid progenitor cells through tail vein injection. Respectively after 4 weeks and after 6 months, we tracked that the red fluorescent cells were retained in the mouse liver (Fig. 4a, b). In addition, these cells could be stained with the hepatocyte specific marker ALB antibody (Fig. 4c, d).

Transplanted LEPCs recover liver damage

Because the LEPCs can be engrafted and settled in mouse liver, we further detected the anti-fibrotic effect of the LEPCs. We did H&E staining to detect the inflammation between CCl_4-treated and LEPCs-transplanted groups. In CCl_4-treated group, the inflammatory cells connected with line, but in LEPCs cure group, the inflammatory cells dispersed in single cell (Fig. 5A e, f). Then we detected the degree of liver fibrosis by Masson staining. The stained area of mouse liver of LEPCs transplanted group was obviously decreased relative to CCl_4-treated group (Fig. 5A b, c).

Then we studied the liver function by analyzing the ALT and AST activity of mouse serum. Compared with CCl_4-treated group (202.67 ± 1.76 U/L), the LEPCs group's ALT (50.33 ± 0.88 U/L) decreased ($p < 0.01$) and almost reached to the level of the control group (42.33 ± 1.45 U/L); compared with CCl_4 group's AST (245.67 ± 6.17 U/L), the LEPCs' AST (108.67 ± 4.09 U/L) just decreased ($p < 0.01$) and did not reach to the level of the control group (32.22 ± 1.45 U/L) (Fig. 5B).

In addition, we extracted the total RNA and did real time PCR detection of fibrosis associated genes Tgfb1 and collagens (I, III and IV) among the control, CCl_4-treated and LEPCs transplanted groups to confirm LEPCs' anti-fibrotic roles. Compared with CCl_4 group, the expression of all these genes was reduced significantly ($p < 0.01$) (Fig. 5C).

Finally, we detected that the expression of α-SMA protein got normal after transplantation of LEPCs (Fig. 5D e, f). The FN protein expression showed no significant differences among three groups (Fig. 5D a–c).

Discussion

In our study, we characterized Luxi bovine LEPCs and studied the capacity to alleviate liver fibrosis. At first, we aimed for isolation of hepatic stem cells from bovine fetal liver with HepSCs medium suitable for hepatic stem cells induction, maintenance, and expansion (Yu et al. 2013). Unfortunately, the isolated small round, high nucleo-cytoplasmic ratio cell clones (Wauthier et al. 2008) could not be passed more than three passages (data not shown). However, once in our experiments, epithelioid cells appeared 3 days after seeding. These epithelioid cells proliferated faster than the other cell types (Fig. 1). These cells are larger than hepatic stem cells in diameter and can be passaged more than 20 passages using HepSCs medium. These epithelioid cells have the similar morphology of iHepSCs (Yu et al. 2013) and LPCs (Li et al. 2006). With the increasing passages of culture of these epithelioid cells, the cell morphology became flat, and proliferated slowly (Fig. 1b, c). This is in accordance with the regularity of cell growth and passage.

Next, we detected the cell markers by RT-PCR, IF and flow cytometry. EPCAM, a cell marker shared with hepatic stem cells and hepatoblasts, being expressed in fetal liver epithelioid cells, was different from adult mouse liver progenitor cells (Li et al. 2006) and consistent with Hepatic stem cells (iHepSCs) and hepatoblasts of human fetal and adult liver (Huch et al. 2015; Wauthier et al. 2008; Yoon et al. 2011). LGR5, a stem cell marker, identified recently as a marker for selecting damage-induced liver stem cells by flow cytometry (Huch et al. 2013b), could also be detected in our purified liver epithelioid cells. SOX9, detected in mouse fetal liver and HepSCs, and adult human bipotent liver stem cells, was also expressed in bovine fetal liver epithelioid cells. All cell markers expressed in the bovine fetal cells suggested that we isolated a fetal bovine liver epithelioid progenitor cell line.

After that, we cultured the progenitor cells in hepatic differentiation medium containing OSM to detect the differentiation capacity. OSM is a member of the interleukin-6 cytokine family and pivotal in the differentiation of oval cells into mature hepatocytes. With the OSM in the hepatic differentiation conditions, the bovine epithelioid cells can form three-dimensional cell masses, expressing hepatic marker ALB and G6PC (Fig. 3g), as well as accumulating glycogen (Fig. 3h). The above results could be confirmed by PAS staining for glycogen and by IF for ALB staining (Fig. 3j). These results were consistent with differentiation of rat oval cells (Okaya et al. 2005) and iHepSCs (Yu et al. 2013). Without OSM, the cells could not form this functional mass. Therefore, the isolated progenitor cells also can differentiate into functional hepatocyte like cells in vitro.

Then we assessed the progenitor cells' plasticity in vivo in liver microenvironment. We treated 6-week old ICR mice with CCl_4 to damage endogenous hepatocytes, and then transplanted Dil-labeled fetal LEPCs into ICR mice through intravenous tail vein injection. 4 weeks after transplantation, we observed that the Dil-labeled cells were stained with ALB antibody (Fig. 3). This indicated that the transplanted progenitor cells have engrafted and differentiated into hepatocytes in mouse liver.

Finally, we explored the anti-fibrotic effect of this heteroplastic progenitor cells in mice. The LEPCs transplanted group down regulated both collagens (I, III and VI) and fibrogenesis genes (TGF-β1 and α-SMA), and recovered the liver function by decreasing ALT and AST secretion. These results are similar to the anti-fibrotic effects of transplanted rat fetal liver-derived epithelial stem/progenitor cells (Yovchev et al. 2014), CD34 + AMSPCs (CD34 + subpopulation of stem/progenitor cells derived from neonatal placental amnion membrane) (Lee et al. 2016), hAECs (Lin et al. 2015), SHED (Yamaza et al. 2015), and T-MSCs (Park et al. 2015). However, the ALB Dil-labeled cells appeared near portal vein, and the number was fewer. This may be contributed by heterogeneous immune rejection. Therefore, how to reduce or eliminate this action is a hinder for cell transplantation in the future.

In summary, we successfully isolated LEPCs from fetal Luxi bovine which expressed liver stem cells markers. The LEPCs can be easy purified, cultured for long time, and differentiated into hepatocytes in vitro and in vivo. After transplanting, the LEPCs can engraft into liver and update the hepatocytes, having anti-fibrotic effects. These results suggested that fetal bovine LEPCs will become a new resource for heteroplastic transplantation in liver disfunction in regenerative medicine and provide a platform for immune rejection research on heterogeneous cell transplantation.

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Author's contribution KW and HL participated in experimental designs, data acquisition and analysis, and data interpretation, as well as drafting of the manuscript. JY, CM and ZZ were involved in data acquisition and data interpretation, as well as drafting the manuscript. DZ and WG were also involved in data acquisition and data interpretation, as well as drafting the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interests.

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