

BASIC RESEARCH

Epithelial cells with hepatobiliary phenotype: Is it another stem cell candidate for healthy adult human liver?

Dung Ngoc Khuu, Mustapha Najimi, Etienne M Sokal

Dung Ngoc Khuu, Mustapha Najimi, Etienne M Sokal, Université Catholique de Louvain, Laboratory of Pediatric Hepatology & Cell therapy, Brussels, Belgium
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Correspondence to: Etienne M Sokal, Université Catholique de Louvain, Cliniques St Luc, 10 av. Hippocrate, B-1200 Brussels, Belgium. sokal@pedi.ucl.ac.be

Telephone: +32-2-7641387 Fax: +32-2-7648909

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Abstract

AIM: To investigate the presence and role of liver epithelial cells in the healthy human adult liver.

METHODS: Fifteen days after human hepatocyte primary culture, epithelial like cells emerged and started proliferating. Cell colonies were isolated and sub-cultured for more than 160 d under specific culture conditions. Cells were analyzed for each passage using immunofluorescence, flow cytometry and reverse transcription-polymerase chain reaction (RT-PCR).

RESULTS: Flow cytometry analysis demonstrated that liver epithelial cells expressed common markers for hepatic and stem cells such as CD90, CD44 and CD29 but were negative for CD34 and CD117. Using immunofluorescence we demonstrated that liver epithelial cells expressed not only immature (α -fetoprotein) but also differentiated hepatocyte (albumin and CK-18) and biliary markers (CK-7 and 19), whereas they were negative for OV-6. RT-PCR analysis confirmed immunofluorescence data and revealed that liver epithelial cells did not express mature hepatocyte markers such as CYP2B6, CYP3A4 and tyrosine amino-transferase. Purified liver epithelial cells were transplanted into SCID mice. One month after transplantation, albumin positive cell foci were detected in the recipient mouse parenchyma.

CONCLUSION: According to their immature and bipotential phenotype, liver epithelial cells might represent a pool of precursors in the healthy human adult liver other than oval cells.

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Key words: Epithelial-like cells; Liver stem cell;

INTRODUCTION

Mammalian adult liver shows a high capacity to regenerate under different physiopathological circumstances thanks to mature and progenitor cells with stem-like properties^[1-4]. Following cellular loss, fully differentiated hepatocytes and biliary epithelial cells can proliferate and completely repair the liver^[5-8]. When proliferation of these cells is impaired during chronic or extensive damage of the liver, non-parenchymal epithelial progenitor cells, expressing both biliary and hepatocytic phenotypes, are activated and participate in liver regeneration^[9-11].

Studies using hepato-toxins or chemical carcinogens treated animal models, revealed the existence of oval cells in the canals of Hering^[12-14]. These cells express both hepatic [albumin (Alb), alpha fetoprotein (AFP)] and biliary [cytokeratin (CK)-7, CK-19, OV-6, γ -glutamyltranspeptidase (GGT)] markers, and some antigens traditionally associated with hematopoietic cells, including c-kit, Thy-1 and CD34^[14,15] as well. During liver regeneration, stimulated oval cells express high levels of AFP, proliferate in the periportal area and migrate into liver parenchyma. Liver epithelial cells (LECs) differ from oval cells, and are as well candidate hepatic progenitor cells. Such liver epithelial cells were obtained from the livers of both normal^[16] and carcinogen treated rats^[17], normal pigs^[18], and human subacute hepatic failure patient^[10], but so far not from healthy human adult livers.

In the current study, we demonstrated the presence of LECs in healthy human liver and isolated these cells after primary culture. We successfully maintained their proliferation more than 160 d and analyzed their characteristics by flow cytometry, immunofluorescence, and RT-PCR. Furthermore, LECs were transplanted into SCID mice to evaluate their engraftment capacity.

MATERIALS AND METHODS

Cell isolation and culture

Liver parenchymal cell fraction was isolated from livers of

Table 1 Medical history of donors

Age (yr)	Sex	Cause of death
4	Male	Cranial trauma
9	Male	Cranial trauma
16	Female	Trauma
18	Male	Trauma
19	Male	Cranial trauma
36	Male	Cardiac arrest
43	Male	Euthanasia
43	Female	Cranial trauma + Cerebral hemorrhage
43	Female	Cerebral hemorrhage
44	Female	Cerebral hemorrhage
47	Female	Pulmonary embolism
51	Male	Suicide
61	Male	Cerebral hemorrhage

13 healthy cadaveric donors (Table 1) using the two-step perfusion technique^{19,20}. Ten to twenty million isolated cells were suspended in hepatocyte growth medium (HGM) [Williams' E medium (Invitrogen) containing 10% fetal bovine serum (FBS) (Perbio, Hyclone), 1% penicillin-streptomycin (Invitrogen), 100 nmol/L insulin (Lilly), 1 μ mol/L dexamethasone (Organon), and 25 ng/mL epidermal growth factor (Peprotech)] and plated on dishes coated with acid soluble type I collagen at a density of 10^5 cells/cm² in a fully humidified atmosphere containing 5% CO₂. Cells were allowed to attach and the first medium change was performed after 24 h and then every 3 d.

Fifteen days later, hepatocytes died and small colonies of LECs emerged from the culture dishes. The culture medium was then switched to DMEM containing 10% FBS and 1% penicillin/streptomycin in order to stimulate the growth of these cells. When colonies were evident, the other cell types were mechanically eliminated and LECs were lifted using trypsin (Invitrogen). Isolated LECs were replated on 6 well collagen I - coated plates and maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. For each passage, cells were analyzed using immunofluorescence, flow cytometry and RT-PCR.

Immunofluorescence

Isolated cells were cultured on collagen I-coated glass cover-slips and fixed with paraformaldehyde 4% (Merck). Then cells were permeabilised for 15 min using 1% Triton X-100 (Sigma) in TBS buffer (50 mmol/L Tris-HCl pH 7.4 and 150 mmol/L NaCl). Non-specific immunostaining was prevented by 1 h incubation in TBS buffer containing 3% non-fat dry milk at 37°C. The primary antibodies [monoclonal mouse anti-human Alb (Sigma), polyclonal rabbit anti-human AFP (Dakocytomation), monoclonal mouse anti-human CK-7 (Chemicon), monoclonal mouse anti-human CK-19 (Dakocytomation) and monoclonal mouse anti-human oval cell (OV)-6 (donation from professor Tania Roskams-Katholic Universteit van Leuven)] were incubated with cells for 1 h at room temperature. The secondary antibodies used [Cy3 conjugated anti-mouse IgG (Jackson immunoresearch laboratories) and FITC-conjugated anti-rabbit IgG (Sigma)] were incubated for 1 h at room temperature. The nuclei

were revealed by 4, 6-Diamidino-2-phénylindole (DAPI, Sigma) staining.

Periodic acid Schiff reaction

Cells were fixed with paraformaldehyde 4% (Merck) during 20 min at room temperature, incubated with 1% periodic acid solution for 10 min, rinsed in distilled water and placed in Schiff reagent for 15 min. After washing for 10 min with water, nuclei were revealed by hematoxylin staining.

Flow cytometry

Cells were harvested, suspended at a concentration of 500-1000/ μ L in PBS and incubated for 30 min at 4°C with the following antibodies: CD45-APC, CD34-APC (Becton Dickinson), CD90-PC5, CD73-PE, CD29-PE, HLA-DR-FITC (BD Pharmingen); CD44-FITC, CD117-PC5 (Immunotech); HLA-ABC-PE (Dako Systems), CD13-PE (Beckham Coulter) and CD105 Endoglin-FITC (Immunokontakt). The corresponding isotypes were used for evaluation of non-specific binding of monoclonal antibodies. Cells were then washed and suspended in Isoton[®] (Beckham Coulter) for reading with a Beckman Coulter Flow Cytometer.

Reverse transcription-polymerase chain reaction

Total RNA was prepared using Tripure isolation Reagent (Roche) and cDNA was generated using the ThermoScript[™] RT kit (Invitrogen). PCR amplification was performed for 28 cycles using specific primers (Table 2) and Elongase[™] Enzyme Mix (Invitrogen). PCR products were subject to 1% agarose gel electrophoresis and visualized after ethidium bromide staining.

Mouse transplantation and immunohistochemistry

One million LECs were suspended in 100 μ L PBS and injected into the spleen of 6-8 wk SCID mice, half of them being subject to 2/3 hepatectomy. Thirty days after transplantation, livers were harvested, formalin-fixed and embedded in paraffin. Five μ m liver sections were deparaffinized and hydrated to distilled water. Endogenous peroxidase activity was blocked by incubation for 15 min in a 3% hydrogen peroxide methanol solution. Slides were incubated overnight with polyclonal rabbit anti-human Alb antibody (Calbiochem) diluted at 1/2500 in PBS containing 0.1% bovine serum albumin. Staining detection was visualized by Envision Dako Anti-Rabbit (Dakocytomation) using diaminobenzidine (Sigma) as chromogenic substrate. The nuclei were revealed by hematoxylin staining.

RESULTS

Characterization of LECs

Isolated parenchymal cell fraction contained majority of hepatocytes with viability estimated at 90% using trypan blue exclusion technique. After 15 d culture in collagen type I - coated plates, hepatocytes died and small colonies of LECs emerged and proliferated (Figure 1A). The LECs appeared in polygonal shape with central nucleus, several

Table 2 Primers used for RT-PCR analysis of LECs

Gene name	Primers	PCR product size
AFP	5'-TGAAATGACTCCAGTAAACCC-3' 5'-GATGAAGCAAGAGTTTCTCATT-3'	199 pb
Oct-4	5'-CGA CCA TCT GCC GCT TTG AG-3' 5'-CCC CCT GTC CCC CAT TCC TA-3'	573 pb
Human hepatocyte nuclear factor 4 (HNF4)	5'-CCA AGT ACA TCC CAG CTT TC-3' 5'-TTG GCA TCT GGG TCA AAG-3'	295 pb
ALB	5'-CCTTGGTGTGATGCTTTGCTC-3' 5'-CAACACATCAACCTCGTCCACC-3'	308 pb
CK-8	5'-AAG GGC TGA CCG ACGAGA TC-3' 5'-GCT TCC TGT AGG TGG CGA TC-3'	537 pb
Glucose 6 phosphatase (G6P)	5'-TCA GCT CAG GTG GTC CTC TT-3' 5'-CCT CCT TAG GCA GCC TTC TT-3'	291 pb
α -antitrypsin (AAT)	5'-TCGCTACAGCCTTTGCAATG-3' 5'-GGAACCTCTCCGTACCCTCAA-3'	142 pb
Tyrosine aminotransferase (TAT)	5'-TGA GCA GTC TGT CCA CTG CCT-3' 5'-ATG TGA ATG AGG AGG ATC TGA G-3'	359 pb
Cytochrome P450 1B1 (CYP1B1)	5'-GAGAACGTACCGCCACTATCACT-3' 5'-GTTAGGCCACTTCACTGGGTCATGAT-3'	357 pb
CYP2B6	5'-CCT CTT CCA GTC CAT TAC CG-3' 5'-TGA CTG CCT CTG TGT ATG GC-3'	551 pb
CYP3A4	5'-TGC TGT CTC CAA CCT TCA CC-3' 5'-TAG CTT GGA ATC ATC ACC ACC-3'	802 pb
CK-19	5'-TTTGAGACGGAACAGGCTCT-3' 5'-CAGCTCAATCTCAAGACCCTG-3'	426 pb
GGT	5'-GAC GAC TTC AGC TCT CCC AG-3' 5'-CTT GTC CCT GGA TTG CTT GT-3'	489 pb
Multidrug resistance-associated protein 2 (MRP2)	5'-ACA CCA ACC AGA AAT GTG TC-3' 5'-CCA AGG CCT TCC AAA TCT C-3'	660 pb
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	5'-CGGAGTCAACGGATTGGTGTGAT-3' 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'	307 pb

nucleoli and a high cytoplasm/nucleus ratio (Figure 1B). LECs colonies grew up, increased in size and had contact inhibition after filling free spaces in the culture dishes. LECs were obtained both from freshly isolated and cryopreserved cell suspensions. Purified LECs resisted to trypsin application and maintained their proliferation potential of more than 5 passages in DMEM medium supplemented with 10% FBS (Figure 1C and D) that leads to purification of the cell population, indicating the self-renewing ability of LECs *in vitro*.

As shown in Figure 2, the flow cytometry analysis of LECs immuno-phenotype revealed that the cell population strongly expressed common markers of haematopoietic and mesenchymal stem cells. Indeed, LECs population was positive for CD90 (Thy-1) and CD44, moderately expressed CD73 and was negative for CD105 and CD13. LECs expressed CD29, an immature liver cell marker, but were negative for the hematopoietic markers CD34, and CD117 whereas expression of CD45 was weakly noted. Most of LECs expressed HLA-ABC but not HLA-DR.

As demonstrated by immunofluorescence, LECs were homogenously positive for AFP, Alb and CK-18 (Figure 3A-C). Biliary markers such as CK-19 and CK-7 were also positive in all the cell population (Figure 3D and E).

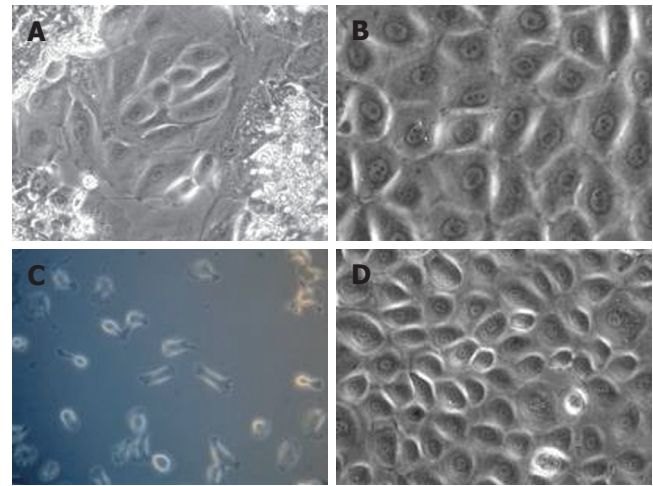


Figure 1 Morphology of LECs after liver cells primary culture ($\times 200$). A: After 15 d primary culture, small colonies of LECs emerged; B: organized in monolayers; C: When colonies were evident, the other cells types were mechanically eliminated and LECs were lifted and replated; D: LEC population reaching 70% confluence analyzed for the other passages.

Up to now, oval cells are not unambiguously defined as precursors of hepatocyte and biliary cells in adult liver diseases. But morphology of LECs was distinguishable from oval cells and did not express oval cell marker (OV-6) (Figure 3F). Metabolic function analysis of LECs also demonstrated their immature status. Thus LECs did not secrete Alb and urea in the culture medium (data not shown) and periodic acid-Schiff staining showed the absence of glycogen storage (Figure 3G).

The data were reproducible in 3 different liver cell suspensions and the bi-potential phenotype of LECs was confirmed using RT-PCR analysis. These cells strongly expressed Alb, CK-8, CK-19 and CYP1B1 (Figure 4). Furthermore, they expressed immature markers such as AFP and POU transcription factor octamer-binding protein 4 (Oct-4) but not HNF-4 (transcription factor expressed early in endoderm formation). Expression of mature hepatocyte markers such as G6P and AAT was weakly noted whereas hepatocyte markers of final maturation phase (CYP2B6, CYP3A4 and TAT) were not found. Expression of genes associated with biliary phenotype was also noted as for GGT and MRP2.

These parameters were maintained up to 5 passages. After that, LECs adopted a very large cytoplasm (Figure 5A and B). Whereas expression of Alb, CK-18 and CK-19 (Figure 5D-F) was maintained during all of cultured time, AFP (Figure 5C) and CK-7 (Figure 5G) staining was significantly decreased.

Intrasplenic transplantation of LECs

To investigate the *in vivo* outcome of LECs, transplantation of these cells was performed in the spleen of SCID mice, half of them were hepatectomized. One month after transplantation, mice were sacrificed and plasma analyzed for the detection of human Alb. No presence of this marker was noted after transplantation. In parallel, liver tissues were analyzed using immunohistochemistry. Foci or isolated cells positively stained for human Alb were

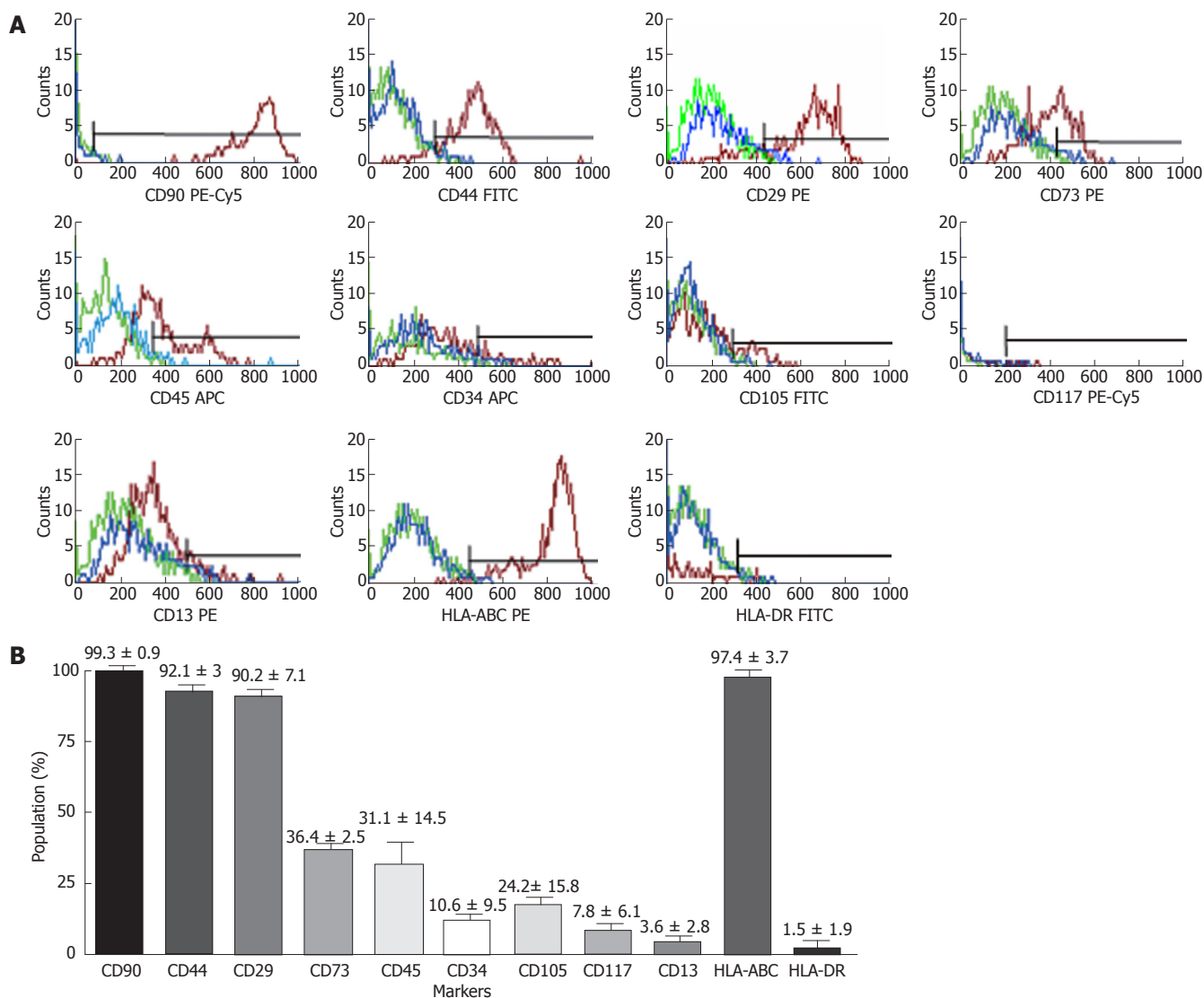


Figure 2 Flow cytometry analysis of purified LECs. **A:** Flow cytometry histograms showing three cell populations isolated from different livers. Antigens expression was detected with specific antibodies (red). Isotype-matched antibody controls (blue) and auto-fluorescence (green) showing the non-specific fluorescent labeling; **B:** Mean expression percentage of analyzed cell markers obtained from three different LEC populations after the third passage.

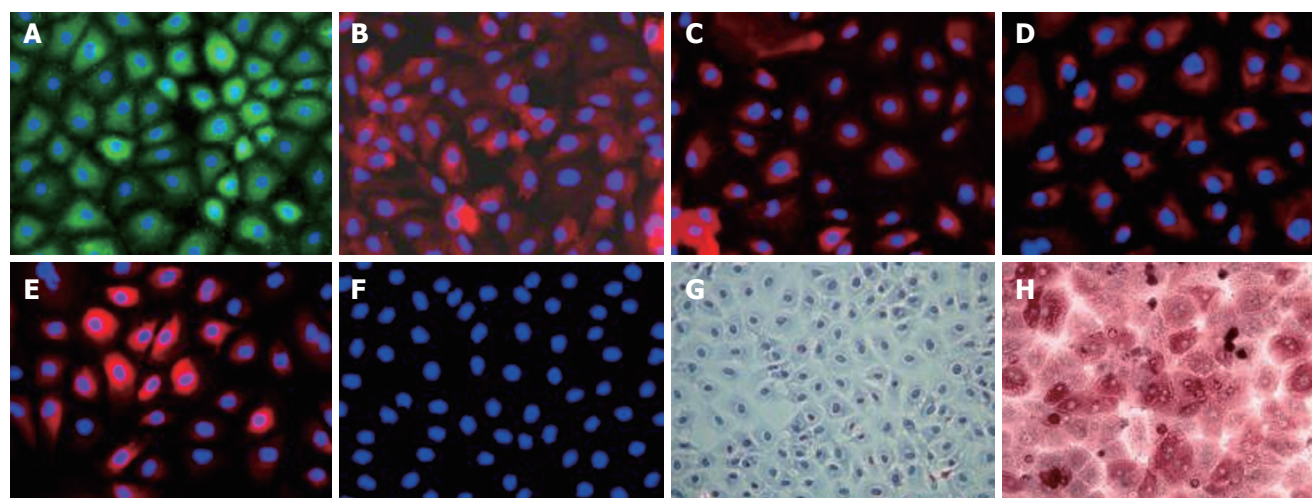


Figure 3 Immunofluorescence characterization of LECs in primary culture ($\times 200$). Immunofluorescence was visualized using anti-rabbit FITC (green) and anti-mouse Cy3 (rouge). The nuclei, stained by DAPI, appeared in blue. **A:** The expression of AFP, fetal liver marker, reflected their immature status; LECs expressed both hepatocytic (**B:** ALB; **C:** CK-18) and biliary markers (**D:** CK-19; **E:** CK-7); **F:** LECs did not express oval cell marker OV-6; **G, H:** The representative photographs at phase contrast microscopy after PAS staining. LECs in the primary culture were not able to store glycogen (**G**) as compared to human hepatocytes (**H**). The nuclei were stained with hematoxylin.

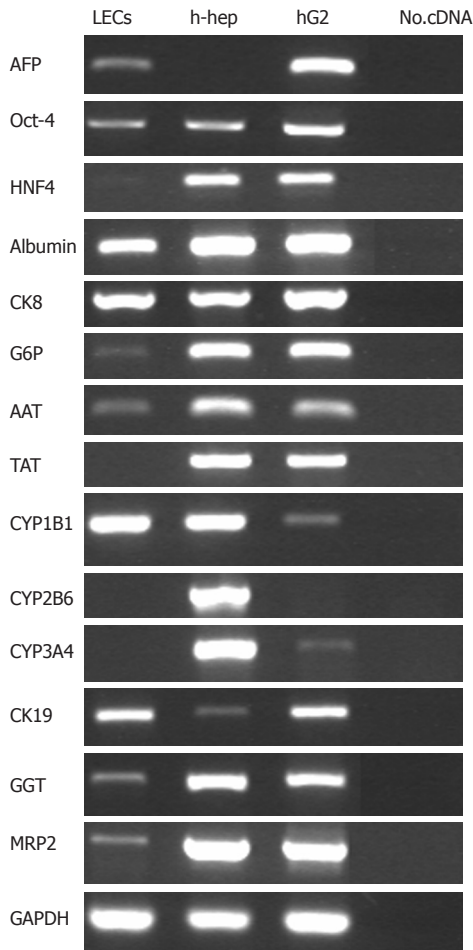


Figure 4 RT-PCR analysis of purified LECs. RT-PCR was assessed on mRNA extracted from purified LECs at the 3rd passage. Data shown are agarose gel electrophoresis of amplification products. Isolated human liver cells (h-hep) and HepG2 cells were used as positive controls. LECs expressed human fetal liver markers (α -fetoprotein (AFP) and transcription factor Oct-4) and adopted hepatocyte (positive for ALB, CK-8, G6P, AAT, and CYP1B1) and biliary (positive for CK-19, GGT and MRP2) phenotypes. These cells did not express HNF4, TAT, CYP2B6 and CYP3A4.

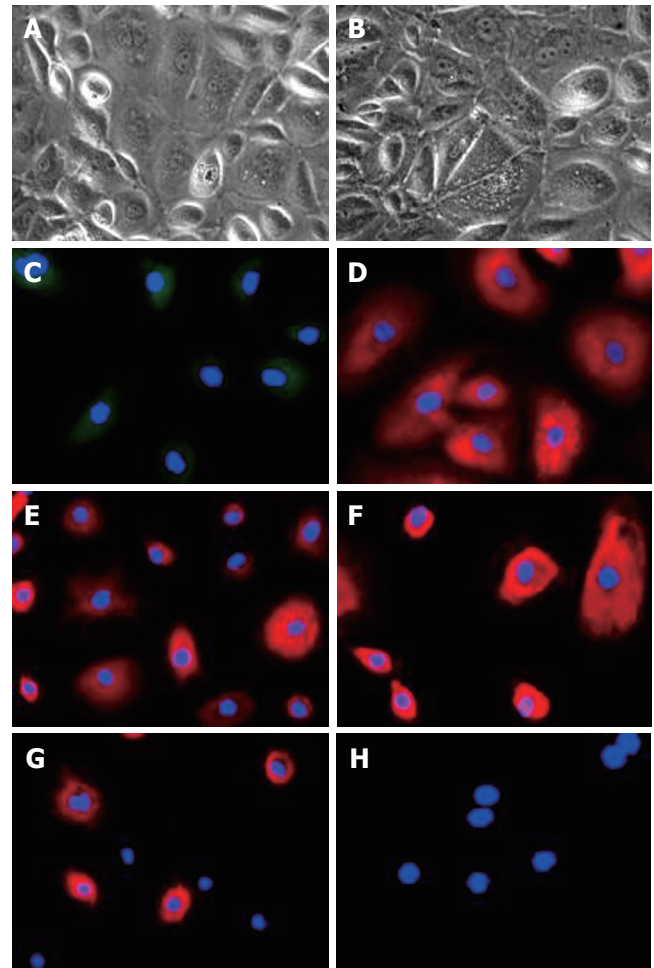


Figure 5 Characterization of LECs at the 5th passage ($\times 200$). The phase contrast microscopy photographs of LECs at the 5th passage showing an increased size (A and B). Using immunofluorescence, in parallel to morphological changes, LECs lost the expression of AFP (C) and CK-7 (G) but maintained the expression of ALB (D), CK-18 (E) and CK-19 (F). LECs remained mostly negative for oval cell marker OV-6 (H).

detected in the recipient liver tissue of 3 transplanted mice (Figure 6A and B) (2 of them were hepatectomized). The detected cells were mostly localized near vascular structures.

DISCUSSION

In the present study, we report the *in vitro* isolation of LECs from normal adult human liver. The culture of the purified cells was maintained *in vitro* for more than 160 d (seven passages) leading to their characterization at both the immuno-cytochemical and genetic levels. LECs injected into the spleen of SCID mice showed their ability to migrate and engraft into the recipient liver tissue.

Cell suspension obtained after collagenase liver disaggregation might contain all the cell types in the liver, the largest part being hepatocytes whereas the presence of minority of non-parenchymal cells is not excluded. When majority of hepatocytes undergoes cell death, LECs spontaneously emerge and proliferate. These cells were closely associated in a monolayer and formed several

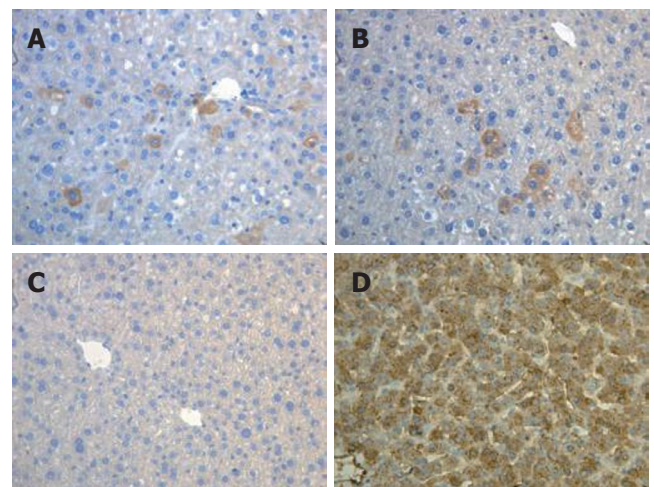


Figure 6 Immunohistochemical analysis of LECs in the liver of transplanted SCID mice. Foci or isolated cells stained for human Alb (brown) were detected around centrilobular vein (A) and portal area (B) of transplanted SCID mice (2 hepatectomized and 1 normal SCID mice; C: Human Alb cells were not detected in control mice; D: All of cells in human liver sections were stained for human Alb.

isolated colonies in the culture dishes. We hypothesized that these cells are resident in the normal adult liver and probably co-isolated with hepatocytes. Their detection in the intact liver may remain difficult because of absence of specific markers. Their proliferation may be stimulated due to chemical signals released in the culture medium after the death of mature hepatocytes (as proposed *in vivo* in liver injury animal models). Furthermore, LECs have been shown to survive *in vitro* after they were lifted in new culture dishes leading to the purification of cell population. LECs were evaluated both in the primary culture (heterogeneous environment) and after trypsin application, at the immunofluorescence level and showed that these cells homogeneously expressed hepatocytic (Alb, CK-18) and biliary (CK-7 and CK-19) markers. The hepatobiliary phenotype was confirmed using RT-PCR analysis up to the 5th passage. LECs were also positive for AFP and expressed Oct-4 but not the final maturation phase markers CYP2B6, CYP3A4 and TAT, indicating their immature state. The expression of these markers was maintained while cells continue proliferating. The bi-potential phenotype of LECs has already been described for the oval cells that were presumed to be precursors of hepatocyte and biliary cells in adult liver^[2,14,15,21]. LECs were morphologically different from oval cells (high cytoplasm/nuclear ratio) and did not express markers such as CD117, CD105 and OV-6^[14,15]. The bi-potential phenotype of LECs may propose these cells as originating from another resident progenitor/stem cells pool within the normal adult liver. LECs were positive for CD90, a marker of hematopoietic and fetal liver stem cells^[22-24]. They also expressed integrin β 1 (CD29), a marker of hepatoblasts, which persists after maturation process of both hepatocyte and biliary epithelial cells^[22,25].

The morphological and immunofluorescence analyses demonstrate that human LECs resemble those previously described in rats^[2]. In young adult Fisher 344 rats, LECs were isolated from the non-parenchymal fraction after an incomplete collagenase-digestion of the liver. In this study, hepatocytes were eliminated by differential centrifugation^[26]. In adult pig liver, epithelial cells have been described to emerge as clusters after the culture of parenchymal cell fraction. Nonetheless, a non-parenchymal cell fraction, rather than a parenchymal cell fraction, was recommended for obtaining such epithelial cells^[18,27]. The LECs reported in these studies even positive for Alb, AFP and the oval cell marker OV-6 did not express biliary cell markers such as CK-19 and GGT. In human, Selden and colleagues have isolated epithelial cell population from the liver of a 6 wk subacute liver failure patient^[10]. The authors hypothesized that this condition will enrich the recipient liver of progenitor liver cells. In our study, we demonstrate that LECs, although with low proportion, can be obtained from normal human liver tissue even when we plated a very limited initial cell suspension. Furthermore, we can observe that LECs isolated in our study express CK-8 in contrary to cells isolated from the subacute liver failure patient. The differences observed between these studies may be correlated to the use of liver isolation and cell culture techniques.

In our study, culture of purified LECs was maintained

after incubation in DMEM medium supplemented with 10% FBS. The immunofluorescence analysis revealed that after the 5th passage, some LECs had a very large cytoplasm and lose CK-7 although expression of Alb and CK-18 was maintained. A decrease in AFP expression was also noted. These data could suggest spontaneous *in vitro* differentiation of these cells. Such phenomenon has been documented for porcine LECs^[18,27]. Nevertheless, other studies demonstrated the ability of LECs to differentiate into hepatocytes and biliary cells after incubation with growth factors defined media^[28,29]. In the rat model, normal LECs have also been shown to engraft and to differentiate into hepatocytes or biliary cells^[30,31].

To evaluate this parameter, purified LECs were intrasplenically transplanted in 4 SCID mice, half of them being hepatectomized (70%). Evaluation of engraftment efficiency was analyzed 1 mo after transplantation. Although no human Alb was detected in serum, immunohistochemistry analysis demonstrated the presence of human cells within the recipient parenchyma mostly near vascular structures. These data suggest the ability of LECs to migrate and to engraft in the liver. In our study, hepatectomy did not stimulate the engraftment and proliferation potential of human LECs in SCID mouse liver suggesting a low ability of LECs to respond to murine differentiation signals. It is also possible that an inhibition of mouse liver cell regeneration induced by hepato-toxins is necessary for stimulating LECs proliferation.

In conclusion, we demonstrated the presence of LECs population within adult normal human liver. These cells expanded *in vitro*, presented bi-potential phenotype and were able to migrate and to engraft in the recipient parenchyma after intrasplenic transplantation. As being obtained after isolation of normal human liver, the pool of these progenitor/stem-like cells may reside in the liver, however, additional data regarding the progenitor's biology of this organ are needed.

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