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### Mice with humanized liver endothelium

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# Chapter

# 2

**Human liver endothelial cells, but not macrovascular  
or microvascular endothelial cells engraft in the  
mouse liver**

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## Abstract

Liver cell transplantation has had limited clinical success so far, partly due to poor engraftment of hepatocytes. Instead of hepatocytes other cell types, such as endothelial cells, could be used in *ex vivo* liver gene therapy. The goal of the present study was to compare the grafting and repopulation capacity of human endothelial cells derived from various tissues.

Human endothelial cells were isolated from adult and fetal livers using anti-human CD31 antibody conjugated magnetic beads. Human macrovascular endothelial cells were obtained from umbilical vein. Human microvascular endothelial cells were isolated from adipose tissue. Cells were characterized using flow cytometry. Liver engraftment and repopulation of endothelial cells was studied after intrasplenic transplantation in monocrotaline treated immunodeficient mice.

Following transplantation, human liver endothelial cells engrafted throughout the mouse liver. With immuno-scanning electron microscopy, fenestrae in engrafted human liver endothelial cells were identified, a characteristic feature of liver sinusoidal endothelial cells. In contrast, CD31 negative liver cells, human macrovascular and microvascular endothelial cells were not capable of repopulating mouse liver. Characterization of human liver-, macrovascular and microvascular endothelial cells demonstrated expression of CD31, CD34 and CD146 but not CD45.

Our study shows that only human liver endothelial cells, but not macro- and microvascular endothelial cells, have the unique capacity to engraft and repopulate the mouse liver. These results indicate that mature endothelial cells cannot transdifferentiate *in vivo* and thus do not exhibit phenotypic plasticity. Our results have set a basis for further research to the potential of human liver endothelial cells in liver-directed cell and gene therapy.

## Introduction

Whole liver transplantation remains the only curative treatment for many patients suffering from inherited and acquired liver disorders. Because donor livers are scarce, treatment options such as liver cell transplantation are an attractive alternative. Although clinical trials have shown that transplantation of hepatocytes is feasible, long-term outcome is disappointing (1). This is due to numerous factors such as poor engraftment of donor hepatocytes in the host liver and loss of hepatocytes after transplantation despite the use of immunosuppression (2-4). The use of *ex vivo* genetically modified autologous hepatocytes overcomes the problem of donor scarcity and immunosuppression. However, the problem with poor efficiency of hepatocyte engraftment and repopulation still remains (5).

Instead of hepatocytes, other cell types, such as endothelial cells, could potentially be used in *ex vivo* liver gene therapy. Cell transplantation studies performed in mice have shown that mouse liver sinusoidal endothelial cells can engraft and repopulate the liver endothelium of hemophilia A mice with subsequent correction of this bleeding disorder (6, 7). However, it is unknown whether human liver endothelial cells have the capacity to engraft and repopulate the liver and thus be used in cell transplantation therapy.

Unfortunately, obtaining sufficient liver endothelial cells for transplantation is difficult due to donor scarcity. Therefore, other sources of endothelial cells suitable for liver-directed cell therapy have to be examined if endothelial cell transplantation is going to be a viable alternative treatment option.

Endothelial cells form the inner lining of blood vessels throughout the human body. These cells exhibit phenotypic heterogeneity which is displayed by different morphology and functions depending on their environmental niche (8, 9). Liver sinusoidal endothelial cells for example are specialized endothelial cells, characterized by the presence of fenestrae and the lack of a basement membrane (10-12). Regeneration studies in rats have shown that bone marrow progenitor cells are capable of replacing liver sinusoidal endothelial cells after injury (13, 14). Thus far, it is unknown whether other types of human endothelial cells, such as microvascular endothelial cells, also exhibit this trait of phenotypic plasticity by functionally repopulating the mouse liver.

In the present study, we therefore compared the grafting and repopulation capacity of human liver endothelial cells with macrovascular and microvascular endothelial cells.

## Materials and Methods

### Animal experiments

Animal experiments were performed in accordance with the Animal Ethical committee guidelines at the Academic Medical Center of Amsterdam. Male and female *Rag2<sup>-/-</sup>yc<sup>-/-</sup>* (15) mice ages 3-8 weeks were used in all studies and fed *ad libitum* on standard laboratory chow.

Animals received an intraperitoneal injection of 200 mg/kg monocrotaline (16) (Sigma-Aldrich, St. Louis, MO, USA) in saline 7 days and 24 hours prior to the intrasplenic cell transplantation.

Mice were anaesthetized with an intraperitoneal injection of FFM mix (2.5 mg Fluanisone/0.105 mg Fentanyl citrate/1.25 mg Midazolam HCl/kg in H<sub>2</sub>O, 7 ml/kg). Under deep anesthesia, the spleen was exposed after a sub costal incision at the left flank. The cell suspension containing  $1 \times 10^6$  cells in 100  $\mu$ l PBS was injected into the tip of the spleen with a 30-gauge insulin needle. Control mice were injected with PBS. Homeostasis was secured by brief pressure for up to 5 minutes on the injection site. The animals were sutured and received the analgesic Temgesic (30-50  $\mu$ l, 0.03 mg/ml) subcutaneously following recovery from FFM.

The mice were sacrificed 14-28 days after the transplantation procedure by *in vivo* fixation. Under deep anesthesia, the peritoneal cavity was opened and the animals were perfused intracardially with 20 ml PBS followed by perfusion with 20 ml 2% paraformaldehyde in PBS. Prior to formaldehyde perfusion, part of the left liver lobe was cut off and snap frozen in liquid nitrogen for DNA analysis. After perfusion, the liver and spleen were removed and fixed in a 4% formaldehyde solution in PBS for 2-4 hours at 4°C. The tissues were transferred to a 30% sucrose solution and incubated overnight at 4°C. The following morning, the tissues were snap frozen in liquid nitrogen and stored at -80°C.

In order to determine the hepatotoxicity of monocrotaline, four animals received two intraperitoneal injections of 300 mg/kg monocrotaline 7 days apart. The

animals were sacrificed 24 hours after the second intraperitoneal injection by *in vivo* fixation under deep anesthesia as described above. Blood was collected before the first monocrotaline intraperitoneal injection by orbital puncture and prior to *in vivo* fixation by heart puncture.

Serum alanine aminotransferase (ALT) and aspartate transaminase (AST) were measured by routine clinical chemistry. Hyaluronic acid (HA) level was measured using the Kordia Life Sciences ELISA microplate kit.

### **Cell isolation**

The use of human fetal liver, human adult liver, human umbilical vein endothelial cells and human subcutaneous adipose tissue was obtained following informed consent.

*Human fetal liver* Human fetal liver was obtained from elective abortions. Gestational age ranged from 14-20 weeks. Fetal livers were digested in 0.05% collagenase IV (Sigma-Aldrich) in Hanks balanced salt solution (BioWhittaker) for 30 minutes at 37°C. Dissociated cells were pelleted at 1000 rpm for 5 minutes and washed once with Hanks balanced salt solution. The cells were seeded out on Primaria plates (BD Falcon) and cultured for 2-7 days in Williams' E medium (BioWhittaker) containing 10% heat-inactivated fetal bovine serum (HI-FBS, BioWhittaker), 2 mM glutamine (BioWhittaker), 10 µg/mL insulin, 5.5 µg/mL transferrin, 6.7 ng/ml selenium-X (ITS-mix, Life Technology), 100 U/ml penicillin, 100 µg/mL streptomycin (penicillin/streptomycin mix, BioWhittaker) and 1 µM Dexamethasone (Sigma). Medium was refreshed the first day following isolation to remove most of the non-adherent cells.

*Human adult liver* Mature primary human liver was obtained from non-tumor liver tissue from 3 female patients undergoing liver resection because of adenoma or secondary carcinoma with ages ranging from 36-67 years old. The liver tissue was used for hepatocyte isolation as described by Hoekstra *et al* (17). The endothelial cells were collected from the supernatant remaining after washing the hepatocytes. The endothelial cells were pelleted at 1000 rpm for 5 minutes and cultured on Primaria plates for 2-7 days in EGM2-basal medium plus EGM-2 MV bulletkit (Lonza, Walkersville, USA). Medium was refreshed the first day following isolation to remove most of the non-adherent cells.

*Human umbilical vein endothelial cells* were isolated as previously described (18) and kept in culture in M199 medium supplemented with 20% (v/v) fetal calf serum (FCS), glutamine, penicillin/streptomycin mix (Invitrogen), 0.05 mg/ml heparin and ECGS 12.5 µg/ml (Sigma Aldrich).

*Human subcutaneous adipose tissue* White adipose tissue was obtained from female patients undergoing reconstructive breast surgery using the deep inferior epigastric artery perforator (DIEP) flap procedure. Tissues from 6 different patients with ages ranging from 40-61 years old were included in this study. Microvascular endothelial cell isolation from white adipose tissue was performed by an adaptation of the protocol as described by Arts *et al* (19). In short, adipose tissue was mechanically minced and incubated in crude collagenase type 1 from *Clostridium histolyticum* (0.2% w/v, Sigma Aldrich) in a ratio of 1:3 (fat:tissue:collagenase solution) for 30-60 minutes at 37°C under continuous vigorous shaking. The adipose tissue was centrifuged for 12 minutes at 1200 rpm. The cell pellet containing microvascular endothelial cells was resuspended in Hanks balanced salt solution and centrifuged again for 5 minutes at 1200 rpm. The cell pellet was seeded out on Primaria plates and cultured in EGM2-basal medium plus EGM-2 MV bulletkit.

#### **Enrichment of endothelial cells from human fetal and adult liver**

The human liver endothelial cells were isolated from the human fetal and adult liver cell suspension, after 2-7 days in culture, via magnetic separation using anti-human CD31 antibody conjugated magnetic beads (Miltenyi Biotec, Auburn CA, USA) according to the protocol provided by the manufacturer. The human liver endothelial cells were seeded out in Primaria 6-wells plates in a density of  $0.5 \times 10^6$  cells per well overnight in EGM2-basal medium plus EGM-2 MV bulletkit.

#### **Lentiviral transduction**

To facilitate tracking the transplanted cells in the mouse liver, the cells were transduced with a GFP containing lentiviral vector driven by a PKG promoter 24 hours prior to transplantation with a multiplicity of infection of 3, as described earlier (20).

#### **Flow cytometry analysis**

The purity of the CD31 positive cell fraction from human adult and fetal liver was determined immediately after isolation by flow cytometry.  $1 \times 10^5$  CD31 positive

cells were resuspended in Williams' E medium containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 10 µg/mL insulin, 5.5 µg/mL transferrin, 6.7 ng/ml selenium-X (ITS-mix, Life Technology), 100 U/ml penicillin, 100 µg/mL streptomycin and 1 µM Dexamethasone. The cells were incubated for 10 minutes at 4°C with FITC-conjugated mouse anti-human CD31 (Miltenyi Biotec, Auburn CA, USA) according to the protocol provided by the manufacturer.

For further characterization of the isolated endothelial cells, the CD31 positive cell population from human adult (n=2) and fetal (n=3) liver, the macro- and microvascular endothelial cells (n= 5 and n=3 respectively) were simultaneously co-stained for CD45, CD146 and CD34 (Miltenyi Biotec, Auburn CA, USA). Cells were analyzed with a flow cytometer (FACS Calibur). Isotype matched nonspecific antibodies were used as controls.

### **Transmission electron microscopy**

To determine the effect of monocrotaline on sinusoidal endothelial cells, liver tissue was prepared for transmission electron microscopy (TEM) as previously described (21). Following *in vivo* fixation, small blocks of liver were put in 1% glutaraldehyde and 4% formaldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for subsequent immersion fixation and stored at 4°C. After fixation the liver was washed in distilled water, osmicated for 60 minutes in 1% OsO<sub>4</sub> in water and washed again in distilled water. For contrast enhancement the liver blocks were block stained overnight in 1.5% aqueous uranyl acetate. Dehydration was done through a series of ethanols and the tissue was embedded in epon LX-112 (Iadd). Ultrathin sections of 80 nm were stained with uranyl acetate and lead citrate and examined in a FEI Technai T-12.

### **Scanning electron microscopy**

The presence of fenestrae in human liver endothelial cells upon culturing, but prior to transplantation was determined by scanning electron microscopy (SEM). Liver endothelial cells were plated out on collagen coated cover slips following anti-human CD31 conjugated magnetic bead isolation as described above and kept in culture overnight in EGM2-basal medium plus EGM-2 MV bullet kit. The following day, the cells were fixed in 2.5% glutaraldehyde in 0.1 M PBS for 60 minutes and washed 3 times with 0.1 M PBS. Cells were fixed in 1% OsO<sub>4</sub> in 0.1 M PBS for 60 minutes, washed 3 times with PBS then dehydrated through a graded series of ethanol (30-100%) and finally chemically dried with hexamethyldisilazane. The

cells were visualized using a JEM-6335F field emission gun scanning electron microscope (JEOL, Peabody, MA, USA).

### **Immunohistochemistry**

Cryosections were made of the liver and spleen by embedding the tissue in Tissue-Tek OCT medium (Bayer). Sections of 5-6  $\mu\text{m}$  were cut, affixed to poly-L-lysine-coated glass slides and kept at  $-20^{\circ}\text{C}$  before use. Sections were washed in PBS for 15 minutes and subsequently incubated in specific primary monoclonal antibody against human CD31 (1:100, DakoCytomation) or human CD45 (1:100, eBioscience) for 1 hour at room temperature. After incubation, the sections were washed in PBS/Tween 0.05% followed by incubation with Texas red-conjugated goat anti-mouse (1:1000, Rockland Immunochemicals) for another hour at room temperature. The sections were washed for another 15 minutes and embedded in mounting medium containing DAPI.

### **Confocal imaging**

Livers were sectioned at 7  $\mu\text{m}$  and placed on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA). Immunostaining was performed essentially as described earlier (22). Livers were labeled for 1 hour with either rabbit anti-human LYVE-1 (Angiobio, Del Mar, CA) or rabbit anti-mouse LYVE-1 (Abcam, Cambridge, MA), both used 1:100 in PBG (PBS supplemented with 0.5% bovine serum albumin and 0.15% glycine). Goat anti-rabbit secondary antibodies conjugated to Cy3 (Jackson ImmunoLabs, West Grove, PA), were added at a 1:1000 dilution in PBG for 1 hour. Tissue was then rinsed 3 times with PBG, 3 times with PBS, counterstained with 0.001% Hoechst dye (bis Benzimide) in  $\text{ddH}_2\text{O}$  for 30 seconds. Samples were coverslipped using gelvatol (23 g poly (vinyl alcohol) 2000, 50 ml glycerol, 0.1% sodium azide to 100 ml PBS) and viewed on a Fluoview 1000 confocal microscope (Olympus, Central Valley, PA).

### **Immuno-Scanning electron microscopy**

The presence of fenestrae in the transplanted human endothelial cells in the mouse liver was determined by immuno-SEM. Liver from transplanted cell recipients and untreated control mice was removed after *in vivo* fixation with 2% paraformaldehyde in PBS. The tissue was incubated in fixative for another 2-4 hours and then transferred to PBS. One mm tissue slices were obtained and evaluated under a fluorescence microscope for the presence of GFP-positive human endothelial cells. The positive slices were washed 3 times with PBS then 3

times with PBG, blocked as above then stained with rabbit anti-human LYVE-1 (AngioBio 11-032) in PBG for 4 hrs. Following 6 washes with PBG, cells were labeled with goat anti-rabbit secondary antibody (1:25 in PBG) conjugated to 15 nm gold particles (Amersham) overnight at 4°C followed by 3 washes with PBG, then 3 washes with PBS. Cells were fixed in 2.5% glutaraldehyde in PBS for 1 hr. Cells were washed 3 times with PBS then dehydrated through a graded series of ethanol (30-100%), chemically dried with hexamethyldisilazane, then overcoated with vaporized carbon (Cressington 108 Carbon/A, Watford, England). The cells were visualized using a JEM-6335F field emission gun scanning electron microscope (JEOL, Peabody, MA, USA). Field emission backscattered electron and standard scanning electron digitized images were taken in tandem to identify areas of gold labeling on the cell surfaces.

### Quantification

We determined the repopulation success of the transplanted human endothelial cells in the mouse liver using the PCR approach involving the amplification of human repetitive sequences according to Becker *et al* (23). DNA was extracted from cryopreserved liver tissue of transplanted and control mice and normal human liver tissue using either the NucleoSpin Tissue DNA isolation kit (Bioke) or the QIAamp DNA FFPE Tissue (Qiagen) isolation kit for PFA fixed tissue. 100 ng of DNA was used to amplify the human DNA sequence by quantitative PCR in the Roche LightCycler480 with the fast-start SYBRgreen kit. Primers: *forward* GGG ATA ATT TCA GCT GAC TAA ACA G and TaqMan *reverse* AAA CGT CCA CTT GCA GAT TCT AG. The PCR cycles were as follows: 94°C for 10 minutes, 94°C for 10 seconds, 60°C for 10 seconds, and 72°C for 20 seconds (45x). Chromosomal DNA was isolated from human and mouse livers and dilutions of human DNA in mouse DNA were used to generate standard curves. The quantitative PCR data were analyzed using the LinRegPCR program (24).

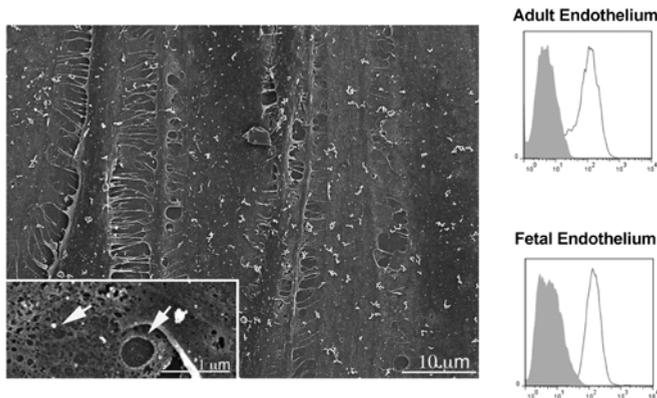
## Results

### Characterization and analysis of the endothelial cells

We isolated endothelial cells from human fetal and adult liver using magnetic separation with anti-human CD31 conjugated magnetic beads. As shown by flow cytometry, CD31 positive cells were efficiently purified from human adult and fetal liver (Fig. 1).

Human liver endothelial cells were examined for the presence of fenestrae *in vitro* using SEM. As shown in figure 1, the cells were largely defenestrated.

The CD31 positive cells were isolated from fetal liver as described and fixed on collagen coated cover slips. SEM imaging showed that liver endothelial cells were largely defenestrated, but occasional cells expressed fenestrations (arrows, inset).



**Figure 1. Purification and morphological characterization of human liver endothelial cells.**

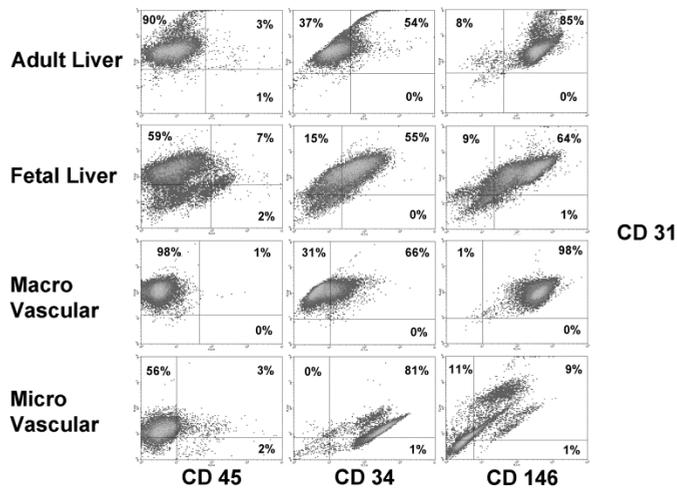
The purification of endothelial cells was analysed by flow cytometry using an antibody to CD31. CD31 positive cells were efficiently purified from human adult (top) and fetal liver (bottom). In each panel, the solid peaks represent staining with an isotype control antibody. The right peaks represent CD31 staining and show that the cells were efficiently purified.

The phenotype of isolated endothelial cells from human adult ( $n=2$ ) and fetal liver ( $n=3$ ) was characterized using flow cytometry. The CD31 positive cell fraction from both adult as well as fetal liver highly co-stained for CD146 ( $85\%$  and  $64\% \pm 14$  respectively). The endothelial cells isolated from fetal liver also highly co-stained with CD34 ( $55\% \pm 9$ ). In contrast, lower staining was observed of adult liver endothelial cells with CD34 ( $54\%$ ). There was a minimal amount of CD45 positive cells present in the CD31 positive cell suspension from both adult and fetal liver ( $3\%$  and  $7\% \pm 5$ ) that co-stained with CD31 (Fig. 2, upper two rows). This indicates that the majority of CD31 positive cells are not of hematopoietic origin.

The macrovascular endothelial cells were isolated from human umbilical veins and characterized after passage 0, 2 and 4. All macrovascular endothelial cells ( $n=5$ ) expressed CD31. They were positive for CD34 ( $66\% \pm 35$ ), CD146 ( $98\% \pm 1$ ) and CD45 negative ( $1\% \pm 1$ ) (Fig. 2, third row).

Microvascular endothelial cells were isolated from human subcutaneous adipose tissue. Using flow cytometry, we found that the microvascular endothelial cells (n=3) were positive for CD31 and highly positive for CD34 (81% ± 15). They were also CD146 positive (9% ± 3), although the signal was not as strong. They were negative for CD45 (3% ± 1) (Fig. 2, lower row).

All gates were corrected for background staining with isotype control matched antibodies (data not shown).

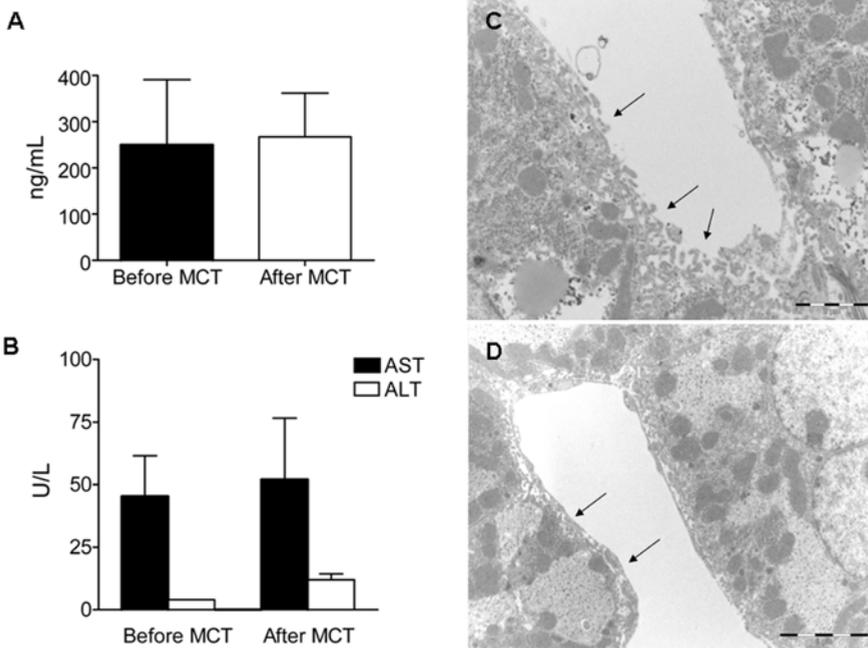


**Figure 2. Characterization of endothelial cells from different tissues.**

Endothelial cells isolated from different tissues were characterized using a panel of different antibodies. Endothelial cells isolated from adult liver and fetal liver are CD31 positive and CD45 negative. The CD31 positive cells from fetal and adult liver are highly CD146 positive. The endothelial cells from fetal liver are also highly CD34 positive. In contrast, endothelial cells isolated from adult liver have a low CD34 expression. All macrovascular (HUVECs) endothelial cells are CD31 positive and CD146 positive. There is no co-staining with CD45 and they are low positive for CD34. The microvascular (human white adipose tissue) endothelial cells are CD31 positive and few cells are CD45 positive. There is a high CD34 expression, but CD146 expression is low.

### Selective injury of mouse sinusoidal liver endothelium following monocrotaline treatment

The serum ALT and AST levels were not significantly increased following 2 intraperitoneal injections of 300 mg/kg monocrotaline 24 hours after the second injection (Fig 3B). Furthermore, the serum hyaluronic acid level, a marker for liver endothelial cell injury was also not significantly increased following monocrotaline treatment (Fig. 3A). However, sinusoidal endothelial cell injury could be observed by electron microscopy in monocrotaline treated mice (Fig. 3C) compared to the control mouse (Fig. 3D). The mouse endothelium was disrupted, but the morphology of the remaining liver was normal.



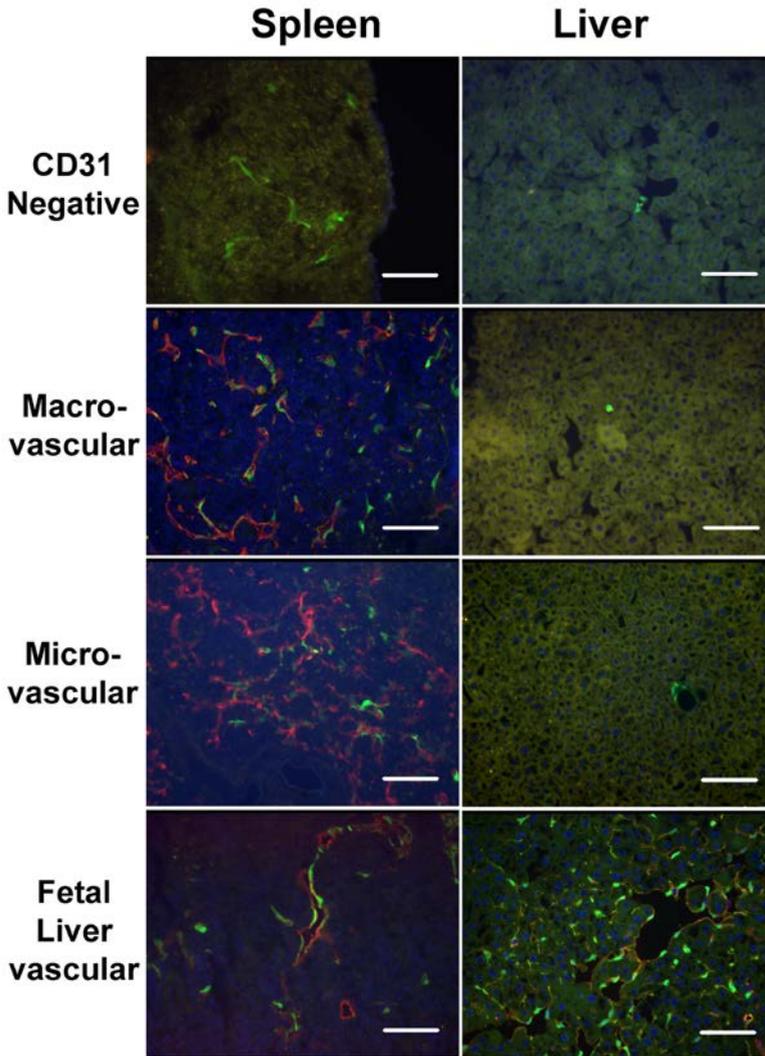
**Figure 3. Selective injury of liver sinusoidal endothelial cells following monocrotaline treatment.** Animals treated with monocrotaline were analyzed for liver injury. Following two intraperitoneal injections of monocrotaline no increase in serum liver hyaluronic acid was observed 24 hours after the second injection (A). Neither was there an increase in liver enzyme levels (B). However, electron microscopy shows disruption of the mouse liver endothelium (C) compared to the control mouse (D). Arrows point at the endothelium. The bar depicts 2  $\mu$ m and 5  $\mu$ m respectively.

**Human liver endothelial cells can engraft and repopulate the liver of *Rag2<sup>-/-</sup>γc<sup>-/-</sup>* mice**

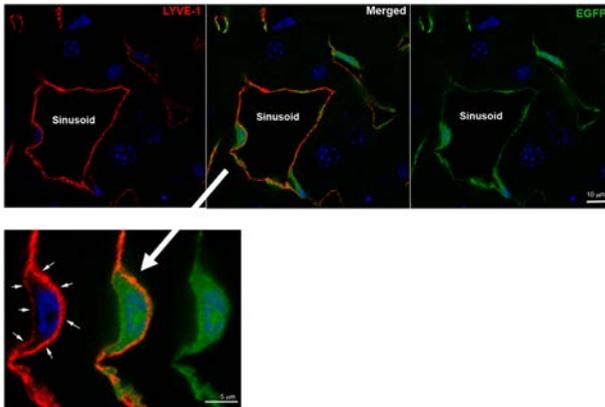
We determined whether human liver endothelial cells are suitable for transplantation and restoration of the liver endothelium by intrasplenically transplanting human liver endothelial cells in *Rag2<sup>-/-</sup>γc<sup>-/-</sup>* mice that had been treated with monocrotaline (16). To facilitate tracking, endothelial cells were transduced with a GFP-expressing lentiviral vector prior to transplantation. The mice did not show any signs of adverse effects after monocrotaline treatment. Intrasplenic transplantation of  $0.8-1.2 \times 10^6$  human fetal endothelial cells resulted in substantial engraftment and repopulation in mouse liver (n=6) (Fig. 4). Clusters of GFP-expressing cells were seen in the spleen and throughout the mouse liver. There was staining of the repopulated human liver endothelial cells with an anti-human CD31 antibody that does not cross react with mouse CD31. In sham-operated negative control mouse liver and spleen sections no GFP-expressing cells and no human CD31 staining was observed. There was no co-staining of GFP-expressing CD31 positive cells with specific anti-human CD45 (data not shown). Transplanted human liver endothelial cells were not observed in sections of the lungs and kidneys.

**Macrovascular and microvascular endothelial cells did not repopulate the mouse liver**

In order to compare grafting and repopulation capacity of different endothelial cell populations, we transplanted macrovascular and microvascular endothelial cells using the same transplantation protocol as with transplantation of human liver endothelial cells. Transplantation of  $1 \times 10^6$  macrovascular (n=9) or microvascular endothelial cells (n=9) showed GFP-expressing cells in the spleen, demonstrating successful transplantation. However, in contrast to human liver endothelial cells, macrovascular and microvascular cells did not engraft in the liver. Immunohistochemical anti-human CD31 staining confirmed that the macrovascular and microvascular cells did not repopulate the mouse liver (Fig. 4). Strikingly, in contrast to microvascular endothelial cells, the macrovascular endothelial cells did incorporate in the large vessel walls of the spleen (Fig. 4).

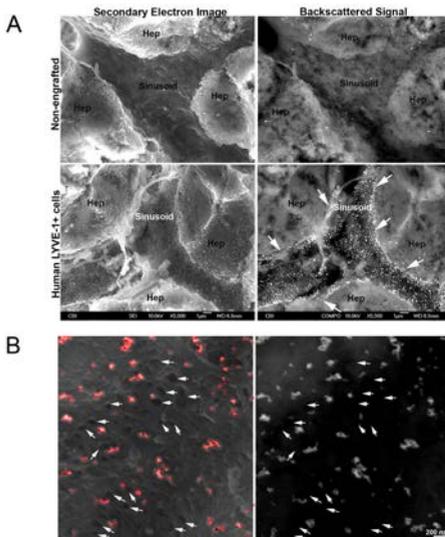


**Figure 4. Immunohistochemistry shows engraftment of human liver endothelial cells in mouse liver.** Cryosections of the spleen and liver of *Rag2<sup>-/-</sup>yc<sup>-/-</sup>* mice were stained with a specific anti-human CD31 antibody after intrasplenic transplantation with either CD31 negative cells from the human fetal liver, macrovascular (HUVECs) endothelial cells or microvascular (human white adipose tissue) or liver endothelial cells (CD31 positive) from the human fetal or adult liver. All spleen sections show the presence of the transplanted GFP positive cells, indicating the transplantations were successful. In the liver, clusters of GFP-expressing cells that exhibit specific anti-human CD31 red co-staining in mouse livers were only found after transplantation with human liver endothelial cells. Nuclei were stained blue with DAPI. Scale bar: 50  $\mu$ m.



**Figure 5. Confocal image showing GFP-positive cells with LYVE-1 membrane staining.**

Cryosections of the liver of *Rag2<sup>fl/yf</sup>* mice were stained with a specific anti-human LYVE-1 antibody after intrasplenic transplantation with endothelial cells from the human adult liver. The white arrows in the enlargement show LYVE-1 membrane staining of a transplanted GFP positive cell. Nuclei were stained blue with Hoechst's dye. Scale bar top panels: 10  $\mu\text{m}$ , bottom panel: 5  $\mu\text{m}$ .



**Figure 6. Immuno-scanning electron microscopy of LYVE-1 positive sinusoids.**

A) Bottom panels show a set of human LYVE-1 positive cells as determined by strong backscattered signal within the sinusoid (white punctata representing 20 nm gold-conjugated goat anti-rabbit LYVE-1). Top panels show no backscattered signal indicating there is no human LYVE-1 positive cells in this sinusoid. Hepatocytes also do not stain for LYVE-1. (B) High magnification of repopulated mouse liver shows human LYVE-1 positive sinusoidal endothelial cells form fenestrated endothelium. Gold particles are pseudo-colored pink in the left panel (backscattered signal merged on top of the scanning EM image) and are white in the right panel (backscattered signal only). Arrows identify some of the fenestrations, indicating the engrafted human cell has the specific characteristic of liver sinusoidal

endothelium. All LYVE-1 positive signals originates from the endothelium surface as none of the gold particles are found within the fenestrations which would suggest signal from the underlying hepatocyte or stellate cell.

### Quantification of repopulation

We determined the repopulation success of the transplanted human liver endothelial cells by quantifying the amount of human DNA in the repopulated mice livers using quantitative-PCR. After transplantation of human liver endothelial cells we found an average of  $0.1 \pm 0.3\%$  of total human DNA in repopulated mouse liver (table 1).

**Table 1. Quantitative PCR shows that only transplantation with human liver endothelial cells leads to detectable repopulation.**

| Cell type           | Microscopical detection of engraftment | Total percentage of human DNA in mouse liver | Repopulation percentage of mouse endothelium by human endothelial cells |
|---------------------|--|--|---|
| LSEC adult (n=2)    | Yes                                    | $0.1 \pm 0.1$                                | 1%  |
| LSEC fetal (n=5)    | Yes                                    | $0.3 \pm 0.4$                                | 3%  |
| CD31 negative (n=5) | No                                     | Below threshold                              | N/A   |
| macrovascular (n=4) | Sporadic single cells                  | Below threshold                              | N/A   |
| microvascular (n=4) | Sporadic single cells                  | Below threshold                              | N/A   |

We compared the repopulation percentage of different endothelial cell types by determining the amount of human DNA in transplanted mouse livers using quantitative PCR. The mice were transplanted with human liver endothelial cells (LSEC, adult or fetal), CD31 negative liver cells, macrovascular or microvascular endothelial cells. N/A: not applicable. Mean values are presented with  $\pm$  SD.

### Discussion

The present study is the first to show that transplanted human fetal and adult liver endothelial cells can effectively repopulate the liver of immunodeficient mice. The human fetal and adult liver endothelial cells were capable of incorporating in the mouse liver endothelium. We also show that the capacity to repopulate the liver endothelial niche is restricted to liver endothelial cells and that macro- and microvascular endothelial cells are incapable of repopulating the liver.

CD31 is a transmembrane glycoprotein also known as PECAM-1 (platelet endothelial cell adhesion molecule 1) and is present on the surface of most endothelial cells (25). In the present study we isolated endothelial cells from

human fetal and adult liver via magnetic separation using anti-human CD31 antibody conjugated magnetic beads. In addition to CD31, the human liver endothelial cells expressed both endothelial cell surface markers CD34 and CD146. After transplantation, the engrafted human liver endothelial cells still expressed CD31 and formed a fenestrated sinusoidal lining, a specific characteristic of liver sinusoidal endothelial cells, as shown by the immuno-SEM on repopulated mouse liver sections.

There are several studies that indicate that liver endothelial cells, in culture lose their fenestrae organized in sieve plates (26, 27). In our study, the liver endothelial cells dedifferentiated and lost their fenestrae *in vitro* as well. Regardless of their phenotype *in vitro*, we have shown that after transplantation, the CD31 positive cells were capable of engrafting and repopulating the mouse liver and furthermore exhibited fenestrae *in vivo*. This observation indicates that in the appropriate liver micro-environment, these cells resemble true liver sinusoidal endothelial cells *in vivo*.

In some of the transplantation experiments, endothelial cells isolated from human fetal liver were used. The human fetal liver is a prominent site of hematopoiesis and contains differentiated blood cells as well as hematopoietic stem cells (28, 29). Potentially, the human fetal liver can also contain endothelial progenitor cells. However, the CD45 positive hematopoietic cells present in the fetal liver were negative for CD31. Also, immunohistochemistry showed that CD31 positive transplanted human liver endothelial cells were negative for CD45. Furthermore, there are also CD45 positive hematopoietic cells present in the CD31 negative cell fraction and that fraction did not give rise to repopulation of the liver endothelium (data not shown). These experiments confirm that the repopulation can be entirely attributed to CD31 positive human liver endothelial cells.

Phenotypic plasticity is a trait most commonly attributed to stem cells. Recent *in vitro* studies however have suggested that endothelial cells may also exhibit phenotypic plasticity (30, 31). In order to examine whether mature endothelial cells can transdifferentiate *in vivo*, we compared the grafting and repopulation capacity of macro- and microvascular endothelial cells to human liver endothelial cells. *In vitro*, all three types of endothelial cells expressed CD31, CD146 and CD34 at different levels. However, in contrast to human liver endothelial cells, both

macrovascular and microvascular endothelial cells were incapable of repopulating the mouse liver after intrasplenic transplantation.

After transplantation of human liver endothelial cells we found a repopulation success of  $0.1 \pm 0.3\%$  of total DNA. Since human liver endothelial cells constitute 10-20% of total liver cells (9) an average of 1-3% of the mouse endothelium was replaced by human cells. In some cases, up to 10% replacement was detected.

In contrast, the livers of mice transplanted with either macrovascular or microvascular endothelial cells did not contain a detectable amount of human DNA. From the results of our *in vivo* transplantation experiments, it appears that repopulation of the mouse liver is a specific characteristic of human liver endothelial cells. Apparently, under our experimental conditions, mature endothelial cells do not have the capacity to transdifferentiate and thus do not exhibit phenotypic plasticity. Our results also indicate that macrovascular and microvascular endothelial cells are not suitable sources for cell transplantation for liver disorders.

In summary, human fetal and adult liver endothelial cells can incorporate in the mouse liver after transplantation. After transplantation, the engrafted human liver endothelial cells exhibited fenestrae. Compared to human macro- and microvascular endothelial cells, human liver endothelial cells can be transplanted with a high efficiency and are the only endothelial cell type that can incorporate in the mouse liver niche. These results show that mature endothelial cells do not exhibit phenotypic plasticity and the ability to repopulate the liver endothelium is a specific characteristic of human liver endothelial cells.

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*Potential conflict of interest:* nothing to report.

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