Mice with humanized liver endothelium

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Chapter 4

Human fetal liver cells for regulated *ex vivo* erythropoietin gene therapy

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Abstract

Possible risks and lack of donor livers limit application of liver transplantation. Liver cell transplantation is at this moment not a feasible alternative, because engraftment in the liver is poor. Furthermore, there is also shortage of cells suitable for transplantation. Fetal liver cells are able to proliferate in cell culture and could therefore present an alternative source of cells for transplantation. In this study we investigated the utility of human fetal liver cells for therapeutic protein delivery.

We transplanted human fetal liver cells in immunodeficient mice, but were not able to detect engraftment of human hepatocytes. In contrast, transplantation of human adult hepatocytes led to detectable engraftment of hepatocytes in murine liver.

Transplantation of fetal liver cells did lead to abundant reconstitution of murine liver with human endothelium, indicating that endothelial cells are the most promising cell type for *ex vivo* liver cell gene therapy. Human liver endothelial cells were subsequently transduced with a lentiviral autoregulatory erythropoietin expression vector. After transplantation in immunodeficient mice, these cells mediated long term regulation of murine hematocrits.

Our study shows the potential of human liver endothelial cells for long-term regulated gene therapy.

Introduction

Liver transplantation is the only available treatment for a variety of inherited deficiencies, but organ shortage and the risks associated with an invasive procedure limit the application of this technique. Because many inherited diseases would already be treated by partial restoration of the deficiency, complete organ replacement is often not necessary. Thus, hepatocyte transplantation seems an attractive alternative to whole liver transplantation. However, poor grafting of transplanted hepatocytes and shortage of donor organs limits the utility of this approach.

Fetal hepatocytes, or hepatoblasts, could represent an attractive source of liver cells for transplantation because they can be expanded in cell culture (1). Furthermore, studies in rats suggested that fetal hepatocytes might have better engraftment and repopulation properties than adult hepatocytes (2). In addition to hepatoblasts, fetal liver also contains large amounts of endothelial cells, forming the inner lining of the sinusoids of the liver.

We have shown previously that we are able to repopulate the liver of immunodeficient *Rag2*$^{-/-}$*γc*$^{-/-}$ mice with fully differentiated human liver endothelial cells (3). In this study we compare the grafting potential of liver endothelial cells and fetal hepatoblasts to identify the most suitable fetal liver cell type for therapeutic gene delivery. Our previous studies showed engraftment of cells derived from human fetal and adult liver in immunodeficient *Rag2*$^{-/-}$*γc*$^{-/-}$ mice (3, 4). These mice lack B and T lymphocytes and natural killer (NK) cells, but have residual macrophage function. Recent studies have shown that transplantation of human cells in *Rag2*$^{-/-}$*γc*$^{-/-}$ immunodeficient mice is improved by expressing murine CD47 in the transplanted human cells (5). CD47 is a membrane protein, also known as integrin-associated protein, which prevents phagocytosis through interaction with signal regulatory protein alpha (SIRPα) (6). In order to determine the full potential of human fetal liver cells in *ex vivo* gene therapy, we therefore used human fetal liver cells expressing murine CD47.

Lentiviral vectors have the ability to stably transduce dividing and non-dividing cells (7, 8) and lentivirus mediated *ex vivo* gene transfer is already clinically used to correct inherited hematopoietic disorders such as metachromatic leukodystrophy and Wiscott-Aldrich syndrome (9, 10). The safety record of lentiviral vectors appears to be better than that of older generation murine retroviral vectors and lentiviral vectors are now used in a number of clinical trials with promising results (9-11). The combination of *ex vivo* lentiviral gene transfer with fetal liver cell transplantation could thus represent an attractive treatment for metabolic disorders.

However, for many disorders, clinical implementation of *ex vivo gene* therapy will require the ability to regulate the expression of genes to maintain expression levels within a therapeutic window (12). Erythropoietin (Epo) is a glycoprotein with a critical role in erythropoiesis and is used for the treatment of patients suffering from anemia induced by a variety of causes (13). Overexpression of Epo can lead to serious adverse effects making regulated expression necessary. In previous experiments, we have shown that the tetracycline inducible system can be used to regulate the expression of Epo in rats following systemic administration (14, 15).
In this study we examined which fetal liver cell type can be most efficiently transplanted and used for regulated *ex vivo* gene therapy.

Results

Transplantation of fetal and adult liver cells

Unfractionated fetal liver cells were transduced with a mouse CD47-GFP expressing lentiviral vector to protect them from mouse phagocytic activity and increase transplantation efficiency (n=4). Adult hepatocytes were transduced with a GFP-expressing lentiviral vector for better visualization of engraftment (n=4). Intrasplenic transplantation of murine CD47 transduced human fetal liver cells resulted in substantial engraftment and repopulation of human liver endothelial cells throughout the mouse liver, as shown by positive human LYVE-1 staining (Figure 1). However, human fetal liver hepatoblasts were not able to engraft and differentiate into mature hepatocytes as shown by the absence of human albumin staining (Figure 1). In contrast to the human fetal liver hepatoblasts, transplanted mature hepatocytes did engraft in the mouse liver and expressed human albumin (Figure 1). These results show that fetal liver hepatocytes are not able to efficiently differentiate into adult hepatocytes and emphasize that human liver endothelial cells have a better potential for liver engraftment and repopulation than other fetal liver cell types.

Mice were also transplanted with purified fetal liver endothelial cells transduced with the murine CD47-GFP lentiviral vector (n=6). The repopulation success of the endothelial cells was determined by measuring the amount of human DNA in the repopulated mouse livers using quantitative-PCR. An average of 1.6 ± 1.2% of total human DNA in repopulated mouse livers was detected. Because the endothelium comprises only a small fraction of total liver cells, this represents an efficient reconstitution of murine livers with human endothelium.

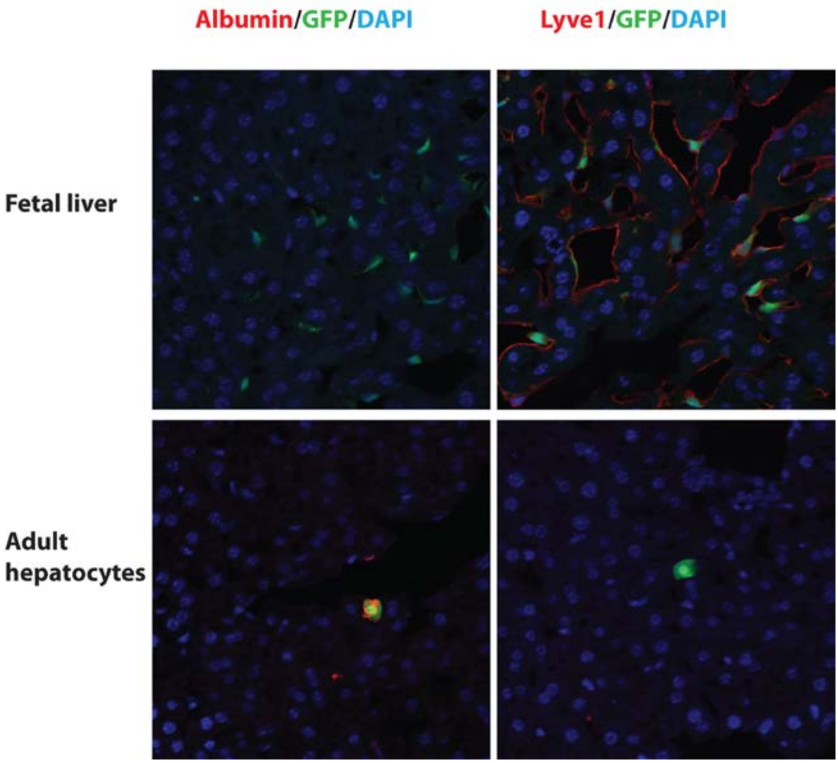

Figure 1. Fetal liver endothelial cells but not hepatoblasts engraft in murine liver.
Human adult hepatocytes were transduced with a GFP- lentiviral vector and human fetal liver cells were transduced with a mouse CD47-GFP lentiviral vector prior to transplantation. Human endothelium engrafted in the murine livers was identified by positive LYVE-1 staining and morphology one week after transplantation. Human hepatocytes engrafted in the murine livers were identified by positive staining for human albumin and morphology. After transplantation of adult hepatocytes, occasional engrafted hepatocytes, positive for albumin, were observed (bottom left). No endothelial cells positive for LYVE-1 were observed (bottom right). Transplantation of mCD47-expressing human fetal liver cells did not lead to engraftment of hepatocytes, as shown by negative human albumin staining, (top left), but did result in abundant engraftment and repopulation of human liver endothelial cells throughout the mouse liver as shown by positive LYVE-1 staining (top right). Sections of 240 µM squared are shown.

Human liver endothelial cells can be used for long-term regulated gene therapy

We have shown previously in rats that *in vivo* intramuscular administration of the doxycycline regulated erythropoietin expressing lentiviral vector TREAutoR4rEPO resulted in a doxycycline dependent Epo expression and subsequent regulation of hematocrit *in vivo*. However, an immune response rapidly cleared the transduced muscle cells. In the present study we examined the use of the TREAutoR4rEPO for *ex vivo* gene therapy by transducing human liver endothelial cells with this vector followed by intrasplenic transplantation in *Rag2*$^{-/-}$*γc*$^{-/-}$ mice (n=8). Blood was collected every two weeks for hematocrit determination. The hematocrit level of the mice was on average 0.5 ± 0 PCV (packed cell volume) before transplantation. Two weeks after transplantation, but before starting doxycycline administration, the hematocrit remained at the same level showing tight regulation of background expression of the lentiviral vector. Two weeks after transplantation, the mice received doxycycline in their drinking water for two weeks. The hematocrit level increased significantly to 0.7 ± 0 PCV and 0.7 ± 0.1 PCV two and four weeks after starting the doxycycline water respectively (Figure 2). After withdrawal of doxycycline, the hematocrit returned back to baseline. Second and third rounds of doxycycline administration resulted in similar responses showing robust and long term (7 months) regulation by this vector system. These results not only show that human liver endothelial cells are capable of engraftment and long-term repopulation following transplantation, but also that they can be used for successful regulated gene expression.

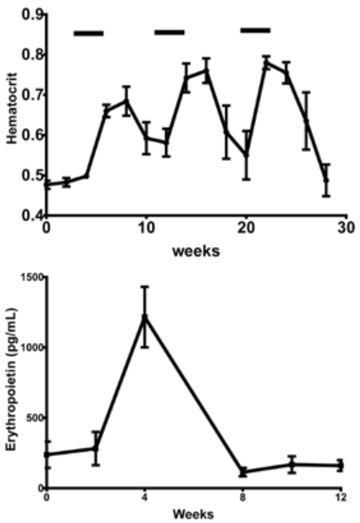

Figure 2. Long-term doxycycline regulation of hematocrit.

Mice were transplanted with 50.000 human liver endothelial cells transduced with the autoregulatory Epo expression vector at day 0 (n=8). Blood was sampled every two weeks. Doxycycline was administered in the drinking water at 2, 12 and 20 weeks after cell transplantation and continued for two weeks as indicated by the solid bars. Top panel: average hematocrit levels are shown on the y-axis in PVC (packed cell volume). Up to 16 weeks n=8, up to 28 weeks, n=4. Bottom panel: the amount of rat erythropoietin in transplanted mouse serum was determined by ELISA. Following induction by doxycycline administration in the drinking water, the concentration of erythropoietin increased significantly, P< 0.0001. Mean values are shown ± SD (n=6).

The regulated hematocrits were mirrored by Epo concentrations in the serum of transplanted mice. Epo concentration was on average 234 ± 61 pg/ml (n=6) before starting administration of doxycycline water and increased significantly (P>0.0001) to an average of 1216 ± 452 pg/ml (n=8) after 14 days of administration of doxycycline water. After stopping administration of doxycycline water the concentration decreased again to baseline levels (Figure 2).

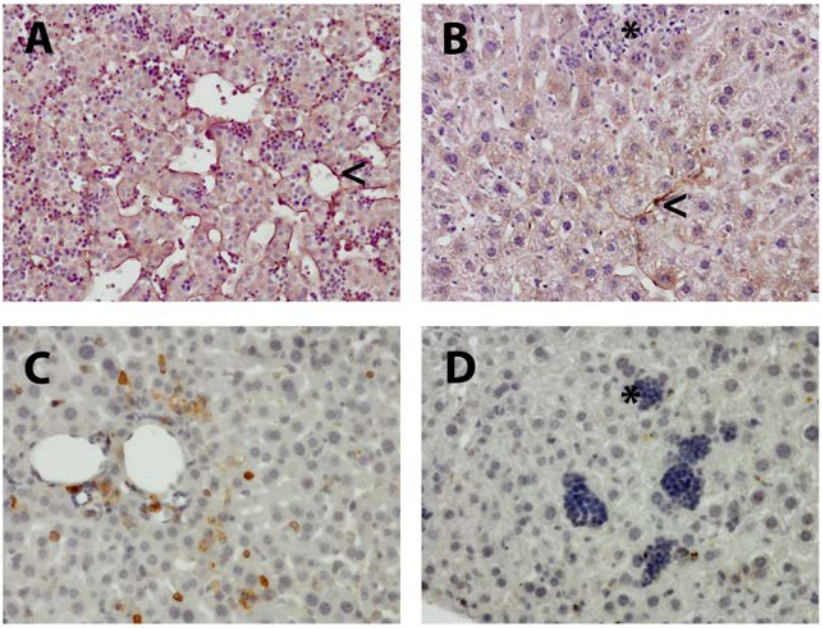

Figure 3 Histological staining for hematopoietic and endothelial markers in murine liver transplanted with endothelial cells expressing Epo.

Sections of paraplast embedded human fetal livers (3A) or murine livers transplanted with endothelial cells transduced with an autoregulatory Epo expression vector were stained with a specific anti-human LYVE-1 antibody (3B). The murine liver was harvested 3 months after transplantation in a period the mouse did not receive doxycycline water. In the human fetal liver (gestation 14-18 weeks) that is shown as a positive control, LYVE-1 positive sinusoidal endothelium lines islands of hematopoietic cells and hepatoblasts (arrow). In the transplanted murine liver, clusters of hematopoietic cells can be seen showing extramedullary hematopoiesis (asterisk). Occasionally, LYVE-1 human liver endothelial cells were also detected (arrow). Liver tissue from mice with a humanized immune system that is shown as a positive control (3C) or mice transplanted with endothelial cells transduced with an autoregulatory Epo expression vector (3D) were stained for human CD45. The mouse transplanted with endothelial cells was harvested 4 months after transplantation in a period the mouse received doxycycline water. Human CD45 positive hematopoietic cells were detected in mice with humanized immune systems but not in mice transplanted with endothelial cells.

Four months after transplantation, the mice were sacrificed and sections of spleen and liver were embedded in paraffin and stained for human LYVE-1 (Figure 3B). Interestingly, the transplanted mice showed signs of extramedullary hematopoiesis in liver en spleen (not shown). For comparison, in Figure 3A a section of human fetal liver with LYVE-1 positive endothelium surrounding islands of hepatoblasts and hematopoiesis is shown. To determine whether the hematopoietic cells in the liver were of human or murine origin, we used an antibody specific for the human hematopoietic marker CD45. No CD45 positive cells were detected in our transplanted mice (Figure 3 D). As a control, positive CD45 staining in a mouse with a humanized immune system is shown (Figure 3 C). No other gross abnormalities were observed.

Discussion

Because *ex vivo* lentiviral gene therapy has been successful in the treatment of hematological disorders (9-11), this approach could also be promising for inherited disorders affecting the liver. In order to determine the full potential of human liver cells in *ex vivo* gene therapy, it is important to know their capacity to integrate and repopulate the liver under conditions that resemble transplantation in human hosts as closely as possible.

In the present study we therefore transduced human fetal liver cells with a lentiviral vector conferring murine CD47-expression. Following transplantation, multiple clusters of human liver endothelial cells were present. Although the murine CD47-expressing transplanted human fetal liver cell suspension also contained a large proportion of hepatoblasts, no human hepatocytes were detected in the transplanted livers. Using the same transplantation strategy, sporadic engraftment of human adult hepatocytes was observed, showing that our approach is suitable for engraftment and detection of mature hepatocytes. These results emphasize the high potential of human liver endothelial cells for cell transplantation therapy and show that the potential of human fetal liver hepatoblast for the replacement of liver parenchyma is poor. Because we also show that undifferentiated human hepatocytes poorly engraft in the liver, our results parallel recent studies using human cells in a model with severe liver damage (16) and a milder model using murine fetal liver cells (17) and thus further question the utility of undifferentiated cell transplantation in the treatment of liver disorders.

Because CD31 is also present on several types of hematopoietic lineages (18) it would be possible that the engrafted endothelial cells were derived from a hematopoietic precursor or through cell fusion with a hematopoietic cell type. However, we did not detect cells positive for the human pan hematopoietic marker CD45 in mice transplanted with CD31 positive endothelium.
We have also transplanted purified hematopoietic stem cells in our model and treated mice with humanized immune systems with monocrotaline. In neither of these experiments engraftment of human endothelial cells was detected (El Filali *et al*, unpublished, submitted). Thus, the human endothelium in our transplanted animals is derived from endothelial cells and not, directly or indirectly through cell fusion, from a hematopoietic precursor.

After transplantation of purified murine CD47-expressing human liver endothelial cells, 1.6 ± 1.2% of total liver DNA was human. Since human liver endothelial cells constitute 10-20% of total liver cells (19, 20) an average of 16-32% of the mouse endothelium was replaced by human cells. Since 1 gram rat liver contains approximately 217 million cells (21) and our mice had average liver weights of 1.3 gram, the total number of human endothelial cells in our fully repopulated mice would be approximately 4.5 million. Previously, we transplanted an enriched suspension of GFP-expressing human liver endothelial cells, that did not express mCD47 and found a repopulation success of 0.3 ± 0.4% of total DNA (3). Thus, in the absence of macrophage activity against xenogenic cells, such as will be the case in human/human transplantation, grafting of transplanted endothelial cells is likely going to be very efficient.

We also examined whether human liver endothelial cells can be used for *ex vivo* regulated gene therapy by transplanting human liver endothelial cells transduced with an autoregulatory lentiviral vector that mediates erythropoietin expression controlled by doxycycline. Mouse hematocrits could successfully be regulated by doxycycline following transplantation of human liver endothelial cells transduced with the erythropoietin auto-regulatory lentiviral vector. Comparable results were found in immunodeficient mice that had received a subcutaneous implant of human erythropoietin expressing endothelial colony forming cells (22). Yet, these experiments were continued for a maximum of four weeks. In our present study, multiple rounds of doxycycline stimulation for a total duration up to 7 months were possible, indicating that long-term functional engraftment of human liver endothelial cells is feasible.

Transplantation of as little as 50.000 human liver endothelial cells transduced *in vitro* with an erythropoietin expressing autoregulatory lentiviral vector, was enough to result in robust therapeutic and regulated Epo expression. At the end of our experiment, our mice had an average weight of 29 grams. To regulate Epo expression in a human with a body weight of 75 kg, we would thus need 129 million cells. Since the hematocrits we obtained in mice were supraphysiological, a lower relative dosing of cells would likely suffice for human use. Because the amount of transplanted cells was low, few LYVE-1 positive human endothelial cells were detected by histology. Epo is a protein hormone, low expression levels already give a robust therapeutic effect. However, for correction of inherited disorders such as hemophilia, a larger amount of cells is likely necessary. Together, our results emphasize the potential of liver endothelial cells for therapeutic gene delivery. We observed extramedullary hematopoiesis in the mice transplanted with Epo expressing endothelial cells. The absence of human CD45 positive cells showed that this process was of murine origin. The tetracycline inducible system is characterised by low basal expression of rtTA and erythropoietin in the absence of doxycycline stimulation. Previously, *in vivo* administration of this vector in Wistar rats led to an immune response to rtTA regardless of the low basal level of rtTA (15). In the present study, we examined whether human liver endothelial cells can be used for *ex vivo* regulated gene therapy using the same tetracycline inducible system in immune deficient mice. It is likely that transduction of antigen presenting cells by lentiviral vectors administered *in vivo* is responsible for the strong immune response to transduced cells (23, 24). However, transplantation in immune competent animals is necessary to investigate whether *ex vivo* transduction of transplanted cells prevents the induction of a cytotoxic immune response.

Our animals were pretreated with monocrotaline which caused a mild disruption of endothelium in mice. Because of potential side effects, the use of monocrotaline is not clinically acceptable. In the absence of endothelial damage, engraftment of endothelium is very low. For human use a mild pretreatment with antineoplastic drugs that disturb liver endothelium such as the tyrosine kinase inhibitor sorafenib (25) or the alkylating agent cyclophosphamide(26) might be required.

In conclusion, the results of our transplantation experiments show that human liver endothelial cells can be used for long-term regulated gene therapy and might be an excellent platform for clinical implementation of *ex vivo* gene therapy.

Materials and Methods

Cell isolation

Human fetal liver The use of human fetal liver was obtained following informed consent. Human fetal liver was obtained from elective abortions. Gestational age ranged from 14-20 weeks. Fetal livers were processed and cells cultured as described earlier (3, 27).

Human adult liver Mature primary cells were obtained from non-tumor liver tissue following informed consent from a patient undergoing liver resection because of adenoma aged 55 years. The liver tissue was used for hepatocyte (28) and endothelial cell isolation as described earlier (3).

Enrichment of endothelial cells from human fetal 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) 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 x 10^6 cells per well overnight in EGM2-basal medium plus EGM-2 MV bulletkit (Lonza).

Lentiviral vector production and transduction

The autoregulatory lentiviral vector (TREAutoR4rEPO) expressing rat Epo (15) was produced as previously described (29). Human fetal liver endothelial cells used for studying regulated *ex vivo* gene therapy were transduced overnight with the TREAutoR4rEPO lentiviral vector. Human fetal liver cells and human fetal liver endothelial cells were transduced overnight with a combination of a codon-optimized mCD47-expressing pHEF (30) lentiviral vector and a green fluorescent protein (GFP) containing lentiviral vector driven by a phosphoglycerate kinase (PGK) promoter 24 hours prior to transplantation as described earlier (29). Adult human hepatocytes were transduced with a green fluorescent protein (GFP) containing lentiviral vector driven by a phosphoglycerate kinase (PGK) promoter 24 hours prior to transplantation as described earlier (29). Transduction efficiency of fetal liver cells was routinely approaching 100%, whereas primary hepatocytes were transduced with maximal 68% efficiency (29).

Animals

Animal experiments were performed in accordance with the Animal Ethical committee guidelines at the Academic Medical Center of Amsterdam. Male and female *Rag2*$^{-/-}$*γc*$^{-/-}$ (31) mice ages 3 weeks to 6 months were used in all studies and fed *ad libitum* on standard laboratory chow.

Animal experiments

Mice were treated with monocrotaline (Sigma-Aldrich) by intraperitoneal injection of 200 mg/kg monocrotaline (32) in saline 7 days and 24 hours prior to the intrasplenic cell transplantation. Transplantation experiments were performed as described earlier(3). In short, under deep anesthesia, the spleen was exposed after a sub costal incision at the left flank. The cell suspension, in 100 µl PBS (Frensius), was injected into the tip of the spleen with a 30-gauge insulin needle (Terumo). For experiments with primary adult hepatocytes (n=4), fetal liver cells (n=4) and purified fetal liver endothelium (n=6), 1 x 10^6 cells were transplanted. For experiments with fetal liver endothelial cells transduced with the autoregulatory Epo expression vector (n=8), 5 x 10^4 cells were transplanted.

Mice with humanized immune systems were generated as described (33).

Control mice were injected with PBS. Transplanted mice were sacrificed by *in vivo* fixation for tissue sampling as described earlier (3).

Doxycycline administration, blood collection and analysis

Drinking water was prepared containing 200 ug/ml doxycycline, 1% sucrose pH 6.0 and administered *ad libitum* for induction of Epo expression in periods of two weeks. Blood was collected every two weeks by cheek puncture. Hematocrit levels were determined by centrifugation using a heparin coated 75 mm long glass capillary (Hirschmann). Plasma was frozen at -20°C for determining erythropoietin level using the Quantikine Epo Elisa (R&D Systems) according to the protocol provided by the manufacturer.

Immunohistochemistry

Cryosections were made of the liver and spleen by embedding the tissue in Tissue-Tek OCT medium (Bayer). Sections of 5-6 µm were cut, affixed to poly-L-lysinecoated glass slides and kept at -20°C before use. Sections were stained for human LYVE-1 (Dilution 1/100, DakoCytomation) as described earlier (3) or human albumin (dilution 1/100 Bethyl laboratories). Sections were embedded in mounting medium containing DAPI for nuclear staining. Images were taken using Leica SP8 confocal microscope.

For histology, tissues were processed and embedded in paraplast as described previously. Tissues were stained for human LYVE-1 (1:100, DakoCytomation), CD45 (dilution 1/250, clone HI30, eBioscience) processed and counterstained with hematoxylin and eosin, for the LYVE-1 staining only, as described (34). Pictures were taken using an Olympus BX51 microscope.

Quantification

We determined the repopulation success of transplanted human endothelial cells in the mouse liver using a PCR approach involving the amplification of human repetitive sequences according to Becker *et al* (35). 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 as described earlier (3).

Statistics

Statistical analysis was performed using the Mann-Whitney U test with SPSS software. Values were indicated as significantly different with $p<0.05$. Mean values are presented with ± SD.

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