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 \textit{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.

\textbf{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 sinuses of the liver.

We have shown previously that we are able to repopulate the liver of immunodeficient \textit{Rag2} \textsuperscript{-/-}\textit{yc} \textsuperscript{-/-} 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 \textit{Rag2} \textsuperscript{-/-}\textit{yc} \textsuperscript{-/-} mice.\(^4\) These mice lack B and T lymphocytes and natural killer cells, but have residual macrophage function. Recent studies have shown that transplantation of human cells in \textit{Rag2} \textsuperscript{-/-}\textit{yc} \textsuperscript{-/-} 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 \(\alpha\) (SIRP\(\alpha\)).\(^6\) In order to determine the full potential of human fetal liver cells in \textit{ex vivo} gene therapy, we therefore used human fetal liver cells expressing murine CD47.

Lentiviral vectors have the ability to stably transduce dividing and nondividing cells\(^7\) and lentivirus mediated \textit{ex vivo} gene transfer is already clinically used to correct inherited hematopoietic disorders such as metachromatic leukodystrophy and Wiscott–Aldrich syndrome.\(^8,9\) 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 \textit{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 \textit{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
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inducible system can be used to regulate the expression of Epo in rats following systemic administration. 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 green fluorescent protein (GFP)-expressing lentiviral vector for better visualization of engraftment (n = 4).

Intraspinal 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 Lyve1 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 polymerase chain reaction. 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.

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 this study, we examined the use of the TREAutoR4rEPO for ex vivo gene therapy by transducing human liver endothelial cells with this vector followed by intraspinal transplantation in Rag 2−/−γc−/− mice (n = 8). Blood was collected every 2 weeks for hematocrit determination. The hematocrit level of the mice was on average 0.5 ± 0 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 2 weeks. The hematocrit level increased significantly to 0.7 ± 0 packed cell volume and 0.7 ± 0.1 packed cell volume 2 and 4 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 show not only 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.

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 1,216 ± 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).

Four months after transplantation, the mice were sacrificed and sections of spleen and liver were embedded in paraffin and stained for human Lyve1 (Figure 3b). Interestingly, the transplanted mice showed signs of extramedullary hematopoeisis in liver and spleen (data not shown). For comparison, in Figure 3a, a section of human fetal liver with Lyve1-positive endothelium surrounding islands of hepatoblasts and hematopoeisis 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 3d). As a control, positive CD45 staining in a mouse with a humanized immune system is shown (Figure 3c). 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
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 (E.E.F. et al., unpublished data). 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 cells18,20, an average of 16–32% of the mouse endothelium was replaced by human cells. Since 1 g of rat liver contains ~217 million cells21 and our mice had average liver weights of 1.3 g, the total number of human endothelial cells in our fully repopulated mice would be ~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 autoregulatory 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 4 weeks. In this 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 g. 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 Ly6e-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 hematopoeisis 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 characterized 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.13 In this 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 to 20 weeks. Fetal livers were processed and cells cultured as described earlier.\(^{3,27}\)

*Human adult liver.* Mature primary cells were obtained from nontumor 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, Bergisch Gladbach, Germany) according to the protocol provided by the manufacturer. The human liver endothelial cells were seeded out in Primaria six-wells plates in a density of 0.5\(\times 10^6\) cells per well overnight in EGM2-basal medium plus EGM-2 MV bulletkit (Lonza, Wijchen, The Netherlands).

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 GFP-containing lentiviral vector driven by a phosphoglycerate kinase promoter 24 hours prior to transplantation as described earlier.\(^{29}\) Adult human hepatocytes were transduced with a GFP-containing lentiviral vector driven by a phosphoglycerate kinase 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\(^{−/−}\)Ly5.2\(^{−/−}\) mice ages 3 weeks to 6 months were used in all studies and fed ad libitum on standard laboratory chow.

**Figure 3**  
Histological staining for hematopoietic and endothelial markers in murine liver transplanted with endothelial cells expressing Epo. (a) Sections of paraplast embedded human fetal livers or (b) murine livers transplanted with endothelial cells transduced with an autoregulatory Epo expression vector were stained with a specific antihuman Lyve 1 antibody. 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, Lyve1-positive sinusoidal endothelium lines islets 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). (c) Liver tissue from mice with a humanized immune system that is shown as a positive control or (d) mice transplanted with endothelial cells transduced with an autoregulatory Epo expression vector 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.
Animal experiments
Mice were treated with monocrotaline (Sigma-Aldrich, Zwijndrecht, The Netherlands) by intraperitoneal injection of 200 mg/kg monocrotaline[2] in saline 7 days and 24 hours prior to the intrasplenic cell transplantation. Transplantation experiments were performed as described earlier.[1] In short, under deep anesthesia, the spleen was exposed after a subcostal incision at the left flank. The cell suspension, in 100 µl phosphate-buffered saline (Frenesius, Zeist, The Netherlands), 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 10⁴ cells were transplanted. For experiments with fetal liver endothelial cells transduced with the autoregulatory Epo expression vector (n = 8), 5 x 10⁴ cells were transplanted. Mice with humanized immune systems were generated as described.[3] Control mice were injected with phosphate-buffered saline. Transplanted mice were sacrificed by in vivo fixation for tissue sampling as described earlier.[1]

Doxycycline administration, blood collection, and analysis
Drinking water was prepared containing 200 µg/ml doxycycline, 1% sucrose pH 6.0 and administered ad libitum for induction of Epo expression in periods of 2 weeks. Blood was collected every 2 weeks by cheek puncture. Hematocrit levels were determined by centrifugation using a heparin-coated, 75 mm long glass capillary (Hirschmann, Elberfeld, Germany). Plasma was frozen at −20 °C for determining erythropoietin (Epo) levels. Values were indicated as significantly different with a P-value < 0.05. Mean ± SEM.

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-lysine–coated glass slides and kept at −20 °C before use. Sections were stained for human Lyve1 (Dilution 1/100; DakoCytomation, Herlev, Belgium) as described earlier[6] or human albumin (dilution 1/100 Beryl Laboratories, Montgomery, TX). Sections were embedded in mounting medium containing DAPI for nuclear staining. Images were taken using Leica SP8 confocal microscope.

Quantification
We determined the repopulation success of transplanted human endothelial cells in the mouse liver using a polymerase chain reaction 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, Leiden, The Netherlands) or the QiAamp DNA FFPE Tissue (Qiagen, Venlo, The Netherlands) isolation kit for PFA fixed tissue as described earlier.[1]

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.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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