Towards efficient perfusion recellularization of porcine liver grafts with liver-derived organoids

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Abstract

Currently, the only effective treatment for end-stage liver diseases is liver transplantation. However, the waiting list with patients who are in need of a new liver is long, because too few good-quality donor livers are available. New techniques in the field of tissue engineering are being investigated to create a reconstructed liver, which might offer a solution for the shortage of transplantable livers. In this study, after decellularization had been performed, the potential of recellularization of porcine liver grafts with liver-derived organoids was researched. In advance of organoid recellularization, the reseeding protocol was optimized by studying different flow rates and different numbers of cell injections in recellularization experiments with HepG2 cells. A flow rate of either 5 ml/min or 11 ml/min was used. The experiments were performed by multi-step perfusion of either 4 or 10 cell injections inside the perfusion system. In addition to optimization experiments and experiments with organoids, reendothelialization of vascular structures inside a porcine liver graft was tested by HUVEC reseeding. The results of these experiments have shown that regarding cellular spreading, cell position and cells viability, the optimal parameters are a flow rate of 5 ml/min and 10 injections. Furthermore, this study has shown that it is possible to culture HepG2 cells, HUVECs as well as organoids inside a porcine liver graft, and that it is possible to keep the cells viable for up to one week. Organoid reseeding was successful and the organoids have shown their organoid-like behaviour. Although more experiments should be done, organoid reseeding has shown to be a promising cell type for recellularization of liver grafts.
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1 Introduction

Currently, the only effective treatment for patients with end-stage liver diseases, for example caused by cirrhosis, chronic viral hepatitis, hepatocellular carcinoma or liver injuries due to alcohol abuse, is liver transplantation [1, 2]. Due to a rising need for donor-livers by patients with end-stage liver diseases as well as too few good-quality donor livers for transplantation, organ shortage is a remaining problem [3]. The worldwide prevalence of liver cirrhosis is approximately 100 patients in 100,000 people [4]. According to the World Health Organization (WHO), each year 170,000 patients in Europe die from liver cirrhosis alone [2, 5]. According to The Dutch Transplant Foundation (NTS), each year over 110 people are still on the waiting list without receiving a new liver in the Netherlands, of which about one in four patients dies while waiting for a donor-liver [6]. More good-quality donor livers could strongly decrease this number.

Research into the field of liver transplantation, in order to increase the amount of transplantable donor livers, is important because the liver has some crucial functions in the human body. Among these important functions are: filtration and storage of blood; metabolism of carbohydrates, proteins, fats, hormones and foreign chemicals; formation of bile; storage of vitamins and iron and formation of coagulation factors [7]. Figure 1 shows the liver anatomy on both macroscopic and microscopical level. As can be seen, on microscopical level, the liver consists of many individual compartments, called lobules [7]. Each lobule has a hexagonal structure, which contains a portal triad at each corner [8]. These portal triads consist of a hepatic artery which carries oxygen-rich blood; a hepatic portal vein, carrying nutrient-rich venous blood from the digestive system for filtration; and a bile duct for bile transport [8]. The artery, vein and bile duct are all connected by sinusoids, which are surrounded by plates of liver cells, called hepatocytes [8]. Hepatocytes are the main component of the liver; they account for about 60%-80% of the total mass of the liver [9, 10]. Hepatocytes are situated in the parenchymal part of the liver, and they can secrete bile, process nutrients, store fat-soluble vitamins and they play an important role in detoxification [7]. The non-parenchymal cells of the liver include sinusoidal endothelial cells, which coat the vessel walls; cholangiocytes, for integrity of the bile ducts and modulation carrying of bile and Kupffer cells and stellate cells, which are important for immune responses to damage and infection [10, 11]. Blood filtration is done by hepatic or stellate macrophages inside the sinusoids [8].

![Liver anatomy](image)

Figure 1: Liver anatomy [12]

Because of this crucial role of the liver for the well-being of patients, researchers in various fields of regenerative medicine and tissue engineering are searching for ways to create reconstructed tissues and organs. This research can help to overcome the donor-shortage problem [3, 10]. Examples of these techniques are hepatocyte transplantation, 3D printing of liver tissue and xenotransplantation [10, 13, 14, 15, 16]. However, there are certain disadvantages to these techniques. For example, xenotransplantation has led to organ rejection and death [10]. Whereas
hepatocyte transplantation has mostly shown only short-term efficiency and the cell engraftment seems to be low [10, 17]. Regarding 3D engineering solutions, the main difficulty seems to create artificial liver tissue with functional vascular and biliary trees [18]. To overcome this problem with reconstructed liver matrices, decellularization and recellularization, might be a solution. In this way, the natural lay-out, including a intact vascular network and the biliary tree, of the liver is maintained. Retaining the vasculature and biliary networks ensures that they do not have to be reconstructed. Another advantage of decellularization and recellularization compared to matrix reconstruction, is that the native extracellular matrix (ECM) is maintained. It is hypothesized that the structures inside this natural matrix, such as laminin, collagen and glycosaminoglycans (GAGs) improve proliferation and differentiation of cells inside the matrix [19, 20, 21]. This is caused by the fact that the ECM is highly tissue specific. By maintaining the original matrix, the niches needed for optimal cell proliferation and differentiation of different cell types are already present.

The idea behind decellularization and recellularization is to use a donor liver that is not suitable for transplantation, for example due to macroscopic evaluation of the transplant surgeon or because of ischemia criteria. During decellularization, all cells are removed from the liver. Decellularization is performed by flushing detergents, for example Triton-X-100 or Sodium Dodecyl Sulfate (SDS), through the native vessels of the liver [21]. These detergents interact with the bilipid layers of the cells, causing the cells to rupture and spill their contents. Constant perfusion removes cellular remnant from the ECM, for example by shear stress. [3].

Decellularization is followed by recellularization of the ECM; cells are reseeded into the liver scaffold via its native vessels to create a functional unit again. Cellular reseeding is mostly performed by using a bioreactor perfusion system. The perfusion systems contain mechanical chambers providing the appropriate native environmental conditions for cells to develop and repopulate the decellularized liver matrix [22]. Bioreactors need to support the decellularized liver and the cell cultures in a sterile environment for long periods of time [2, 22, 23]. Although most perfusion systems are custom-designed and custom-made, nearly all bioreactors consist of the same components. Most systems use normothermic machine perfusion, which means that the cells are perfused inside the liver matrix at normal body temperature (37 °C). The ideal perfusion temperature seems to be equal to the physiological, or near physiological temperature to ensure that the cells are able to attach to the decellularized matrix and are able to self-assemble and differentiate [2, 23, 24]. The temperature in the bioreactor is maintained either by means of keeping the cell culture inside an incubator or by using a water-jacketed medium reservoir [23, 25]. Furthermore, nearly all perfusion systems for liver graft recellularization contain the following components: a (peristaltic) pump; an oxygenator; a bubble trap, preventing bubbles inside the system and tubing; a medium reservoir for the cell culture; and a (glass) chamber for the liver that has several adjustable inflows and outflows that can be connected to the perfusion tubing [21, 24, 25, 26, 27, 28, 29, 30, 31].

Cells can be injected into the liver via the perfusion or bioreactor system in several ways. The three most commonly used methods are direct injection, continuous perfusion and multi-step perfusion. For the direct injection approach, cells are suspended in medium, and this cell suspension is being injected into the liver ECM scaffold through injections into various liver lobes. The cells are injected directly into the scaffold by means of a syringe [27]. This method is significantly different from continuous perfusion and multi-step perfusion, because for these methods, the liver is perfused with culture medium while the cells are injected into the perfusion circulation [27]. The difference between continuous perfusion and multi-step perfusion is that for continuous perfusion, the total number of cells is injected into the circulation at the same time and the cells are recirculated through the liver. For multi-step perfusion, the cells are injected through a port near the liver in various steps with a time-interval of 10-15 minutes. When comparing these three methods, hepatocytes have shown significantly better engraftment in the liver ECM scaffold via multi-step perfusion compared to the other methods [27]. The multi-step approach resulted in approximately 86% grafting efficiency, while continuous perfusion resulted in about 69% cell engraftment and the five direct injections into the liver lobes resulted in only 13% engraftment [27]. Additionally, the liver perfused by multiple steps, showed more albumin production after seven days as well as more viability, secretion abilities, metabolic activity and fewer complications such as obstruction, thrombosis and embolism [27, 32].

Besides an efficient reseeding method, also the optimal cell type should be selected in order to establish effective recellularization. When comparing cell types for recellularization, the ultimate cell types have still to be discovered due to differentiation considerations and because of the diffi-
ulty to obtain a sufficient number of cells. However, in order to achieve effective recellularization, three important goals have been identified [33]:

- The scaffold should be reseeded with an appropriate number of cells to meet metabolic needs for the recipient;
- Re-endothelialization of the vascular tree is necessary to avoid thrombosis, and to ensure that the liver cells will be provided with a sufficient amount of oxygen and nutrients;
- The biliary tree should be recellularized with functional cholangiocytes to prevent biliary complications after transplantation.

To achieve efficient recellularization of the entire liver, it is important to reseed the parenchymal part of the liver with hepatocytes as well as the vascular tree and the biliary tree with endothelial cells and cholangiocytes, respectively. Although it is important that an appropriate number of cells is available for the liver recellularization process, the exact number of cells that is necessary remains unclear, because the number of cells is highly dependent on the cell type and the volume of the liver scaffold [34]. Using hepatocytes can be beneficial for curating of liver diseases because they are fully functional, and therefore, they are able to quickly replace damaged hepatocytes. However, these cells are less suitable for repopulation of decellularized liver scaffolds, due to the fact that primary hepatocytes only achieve short-term survival. Also, because of limited donor livers, limited proliferation capacities of the primary hepatocytes and possible changes in physiological and functional characteristics, using these cell types for recellularization has limited clinical success [11, 23, 27, 35, 36]. Other cell types such as HepG2 cells and C3A have also been commonly used for research to recellularization of livers [37]. These are tumor derived cells and they are able to proliferate rapidly because of its tumorigenic properties. Which is also the reason that these cells cannot be used in the clinic. Therefore, adult stem cells are potentially ideal cells to recellularize the liver scaffold. They proliferate quickly and in addition, they have the ability to differentiate into hepatocytes and cholangiocytes. However, for example induced pluripotent stem cells (iPSCs) also have the tendency to differentiate into multiple different cells instead of differentiating only into hepatocytes. Teratoma formation is a serious risk when using iPSCs. Adult hepatic stem cells, on the other hand, are not prone to teratoma formation and are still able to proliferate and differentiate towards hepatocytes and cholangiocytes. Recently, Huch et al. established a culture platform for these adult stem cells, that retain liver-like features [38]. These liver derived organoids are a potential source of hepatocytes and biliary epithelial cells and originate from Lgr5+ adult stem cells isolated from the human liver. Liver organoids are spheric structures of stem cells that can be isolated from liver biopsies. The cells are cultured in a hydrogel, which enables self-organization in a 3D structure [11]. Organoids have the potential to proliferate rapidly: a relatively small number of stem cells would be enough to generate the necessary number of cells for liver recellularization [11, 18, 38]. Furthermore, organoids have shown to be genetically stable [38]. After months of in vitro culturing, the genetic integrity is maintained; according to Huch et al., 10-fold fewer base substitutions are observed when culturing organoids compared to culturing other types of stem cells [38]. Another advantage of using organoids for recellularization, is that they are bipotent, which means that they are able to differentiate into both hepatocytes and cholangiocytes [11, 18]. Research has shown that organoids have the potential to differentiate into functional hepatocytes with elevated levels of hepatocytic markers [38]. Although organoids have great potential for tissue engineering research, organoids have not been used for this purpose before.

The aim of this project was to find an optimal method to recellularize a decellularized liver ECM using a HepG2 cell line and to test the potential of organoid reseeding. In order to study the effectiveness of the recellularization protocol, mostly cellular engraftment, a homogenous cell distribution, and the quality of the engrafted cells inside the liver scaffold have been studied. In this research, firstly the recellularization method was optimized by measuring various levels of flow rate and various numbers of injections of cells by multi-step perfusion. Optimization was achieved with HepG2 cells, due to the fact that these cells are easy to culture and because of their large proliferation capacity. Secondly, after the protocol was optimized, reseeding with organoids has been performed to test the organoid reseeding potential in liver grafts and in an effort to recellularize the parenchymal part. Lastly, reseeding with Human Umbilical Vein Endothelial Cells (HUVECs) is performed to recellularize the vascular tree of the liver.
2 Methods

2.1 Porcine livers

The porcine livers, used for decellularization and recellularization were obtained from healthy pigs from the bio-industry. The pigs were averagely eight to ten weeks old and their mean weight was 40 kilograms. The livers were obtained after the pigs were sacrificed for the food industry. The weight of the livers was between 900 and 1500 grams. After the livers were obtained, the hepatic artery and portal vein were cannulated and the livers were flushed with a saline solution and Heparin via the cannula to remove the blood from the liver. The livers were stored in the freezer at -20 °C in advance of the decellularization process.

2.2 Decellularization

Prior to decellularization, the porcine livers were thawed and connected to decellularization set-up. The set-up was custom-made and contained a perfusion chamber in which the liver was placed and the perfusate could be stored. During the washing-steps with detergents, the perfusate could recirculate in this chamber via tubing connected to one of the vessels (e.g. the hepatic artery) into the liver. The perfusate left the liver via tubing connected to the other vessel (e.g. the portal vein) and flows into the perfusion chamber again for recirculation. The washing-steps with saline solution and water, the fluids did not recirculate. In these steps, the fluids were collected in a tank and discharged immediately. Percussion of the liver was pressure controlled and the arterial pressure was set to an upper level of 120 mm Hg.

The first step of decellularization was flushing the liver with saline solution, where after it was flushed with demineralized water (dH$_2$O). The liver was reperfused with 10 liters of 4% Triton-X-100 (Brunschwig Chemie) with 1% ammonium hydroxide (Sigma) for five times 120 minutes. After decellularization with Triton-X-100, the liver was flushed with 100 liters dH$_2$O to remove the detergents. The liver was placed in a storage box with dH$_2$O in a cold-room at 4 °C, the water was changed every 24 to 72 hours for up to 10 days.

In advance of the last step of the decellularization procedure, in which the remaining DNA fragments are removed by DNase I, the liver was washed by 10 liters of 1x Phosphate Buffered saline (PBS) (Lonza). Then, the liver was flushed with 2 liters of 400 µg/l DNase I (Sigma). Where after the DNase was washed out of the liver by 10 liter of 1x PBS. Biopsies were taken from the liver before and after the procedure to test the efficiency of decellularization. After a biopsy was taken from the decellularized porcine liver, the liver was cold-stored until further use. Figure 2a and b show the decellularized process during and after decellularization and figure 2c and d show histological analysis of a normal versus a decellularized porcine liver by Hematoxylin and Eosin (H&E) staining. In figure 2 can be seen that the decellularization process was effective; the liver has a white appearance after decellularization and the H&E staining shows that the ECM does not contain cells anymore. Other analysis such as DNA quantification also proved that the ECM was deprived of cells. This implies that the livers can be used for recellularization procedures.

2.3 Liver grafts

Decellularized porcine livers were thawed and cut in small segments of 4 to 10 cm diameter and cannulated through its native vessels (mostly portal veins) by the surgeon in a sterile environment. Recellularization within liver segments has been performed because this reduces the required number of porcine livers and the number of cells for recellularization. Also, reseeded cells could be tracked more easily under the microscope in smaller segments.

Figure 3 shows a schematic overview of the human liver divided into 8 segments according to the Couinaud classification of functional anatomy. Although the anatomy of a porcine liver is different from the anatomy of a human liver, figure 3 gives an idea of how the segmentation is performed by the surgeon. Segmentation allows for recellularization by perfusion through the native vessels with minimal leakage, which is needed to reduce the loss of cells into the culture medium and to increase cellular engraftment into the liver ECM.

In figure 4, one of the liver segments for recellularization is shown. Before recellularization, the wet weight of the segments was measured. The average mass of the segments (including the cannula shown in figure 4) was 24.36 ± 13.86 grams.
2.4 Experimental design

Firstly, the protocol was optimized by studying flow rate and the number of injections for recellularization with HepG2 cells for $t = 1$ day, $t = 2$ days and $t = 3$ days. Reseeding at a flow rate of 11 ml/min was compared to reseeding with a flow rate of 5 ml/min. Furthermore, 4 injections with an intermittent period of 15 minutes was compared to 10 injections with an intermittent period of 15 minutes. An evaluation was performed after each experiment and the results of previous exper-
ments determined the new experimental parameters. After optimization of the protocol regarding flow rate and the number of injections, HepG2 recellularization was performed for longer periods of time ($t = 5$ days and $t = 7$ days), to study whether it was possible to recellularize porcine livers without the set-up being contaminated. For experiments with an incubation time of five and seven days, the culture medium was checked for infections every other day and after the experiment was finished. Lastly, recellularization with HUVECs and organoids was done to test revascularization and to test the potential of organoid reseeding. An overview of the experiments can be found in table 1.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cell-type</th>
<th>Number of cells</th>
<th>Flow rate</th>
<th>Number of injections</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HepG2</td>
<td>$70 \times 10^6$</td>
<td>11 ml/min</td>
<td>4</td>
<td>1 day</td>
</tr>
<tr>
<td>2</td>
<td>HepG2</td>
<td>$40 \times 10^6$</td>
<td>11 ml/min</td>
<td>10</td>
<td>3 days</td>
</tr>
<tr>
<td>3</td>
<td>HepG2</td>
<td>$40 \times 10^6$</td>
<td>5 ml/min</td>
<td>4</td>
<td>2 days</td>
</tr>
<tr>
<td>4</td>
<td>HepG2</td>
<td>$40 \times 10^6$</td>
<td>5 ml/min</td>
<td>10</td>
<td>2 days</td>
</tr>
<tr>
<td>5</td>
<td>HepG2</td>
<td>$40 \times 10^6$</td>
<td>5 ml/min</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>HepG2</td>
<td>$30 \times 10^6$</td>
<td>5 ml/min</td>
<td>10</td>
<td>7 days</td>
</tr>
<tr>
<td>7</td>
<td>HepG2</td>
<td>$40 \times 10^6$</td>
<td>5 ml/min</td>
<td>10</td>
<td>5 days</td>
</tr>
<tr>
<td>8</td>
<td>HepG2</td>
<td>$80 \times 10^6$</td>
<td>5 ml/min</td>
<td>10</td>
<td>7 days</td>
</tr>
<tr>
<td>9</td>
<td>Organoids</td>
<td>$10 \times 10^6$</td>
<td>11 ml/min</td>
<td>10</td>
<td>2 days</td>
</tr>
<tr>
<td>10</td>
<td>Organoids</td>
<td>$16 \times 10^6$</td>
<td>5 ml/min</td>
<td>10</td>
<td>7 days</td>
</tr>
<tr>
<td>11</td>
<td>HUVEC (uncoated)</td>
<td>$40 \times 10^6$</td>
<td>5 ml/min</td>
<td>10</td>
<td>5 days</td>
</tr>
<tr>
<td>12</td>
<td>HUVEC (coated with CD31)</td>
<td>$40 \times 10^6$</td>
<td>5 ml/min</td>
<td>10</td>
<td>5 days</td>
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</tbody>
</table>

2.5 Cell culturing

HepG2 cells, HUVECs and liver derived-organoids have been cultured for recellularization. All cells were stable transduced using a lentiviral vector expressing a green Green Fluorescence Protein
(GFP) label, shown in figure 5. The same method was used as performed by Huston et al. [40]. The transduction of the HUVEC was almost 100% effective. Almost all cells visible with bright field microscope were also visible with fluorescent microscope using green fluorescent light. The organoids were GFP transduced as well. The efficiency was approximately 50% (shown in figure 5). The grown GFP-labeled organoids were imaged by a fluorescence microscope, and one of the organoids with an transduction efficiency of about 100% was handpicked and expanded in a new plate, to allow for clonal growth and 100% GFP expression. The overall efficiency of these transduced organoids used for this project, was thus 100%.

HepG2 is an immortalized tumor cell line consisting of human liver carcinoma cells and they can be grown successfully in large scale. HepG2 cells and HUVECs are cultured in a T175 culture flask (Greiner) in standard culture medium: 1x Dulbecco’s Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% Fetal Calf Serum (FCS), 1% UltraGlutamine (Lonza) and 1% Penicillin and Streptomycin (Pen/Strep). The cells were cultured in culture medium and incubated in 95% O2 and 5% CO2 at 37 °C. The cells were split at 80% to 90% confluency by using a solution of Trypsin (Lonza). Culture medium was changed once every two to three days.

Organoids are cultured inside a 24 well/plate in Matrigel (BD Biosciences) , which allows for 3D growth of the cells (figure 5). Organoids were split with Advanced DMEM F12 (Gibco) supplemented with 1% HEPES-buffered Saline solution (Lonza), 1% UltraGlutamine, 1% Pen/Strep and 0.1% Primocin (ADV F12 ++++). Organoids were expanded with Filtered Expansion Medium (EM). All components of filtered EM can be found in table 4 in appendix 2. Organoids were cultured and incubated in 95% O2 and 5% CO2 at 37 °C. Medium was changed once every 2 to 3 days.

**Figure 5:** (a) GFP-labeled HepG2 cells. (b) Organoids in 3D culture. (c) GFP-labeled organoids.

### 2.6 Recellularization set-up

To recellularize porcine liver grafts, the set-up shown in figure 6 was used. The set-up was placed inside a flow cabinet during cell infusion for a sterile environment. After cell injection, the set-up was moved to an incubator to control temperature and O2 (95%)/CO2 (5%) levels. The temperature of the experiment was 37 °C, to maintain a physiological temperature for the cells. The set-up consisted of a medium reservoir, which contained culture medium (standard culture medium for HepG2 cells and HUVECs and Expansion Medium for organoids) and was pumped towards the liver segments. The culture medium was pumped from the medium reservoir towards the segment by a peristaltic pump, which controlled the flow of culture medium. During cell injection, the porcine liver segment was kept inside a culture disc and during incubation, the liver segment was placed inside the medium reservoir. During cell injection, the temperature was controlled by a water bath and during incubation, the temperature was controlled by the incubator.

### 2.7 Porcine liver graft reseeding

The day before cell injection, the liver segment was thawed and washed via the cannula by injection of 1x PBS (Lonza) and culture medium. The washing-steps are presented in table 2. Firstly, the segment was washed three times with 50 ml 1x PBS. Secondly, it was washed three times with 50 ml standard culture medium (when the liver was recellularized with HepG2 cells or HUVECs) with 10% FCS, 1% Pen/Strep and Primocin or Advanced medium (when the liver graft was recellularized with organoids) with 1% HEPES-buffered saline solution, 1% Pen/Strep and Primocin. Thirdly, the liver segment was washed with either standard culture medium or ADV F12 ++++ with 10%
Pen/Strep and the segment was incubated for one hour. Lastly, the liver segment was again washed three times with 50 ml standard culture medium or ADV F12 ++++ with 1% Pen/Strep and was incubated for 24 hours. During the washing-steps, the segment was inspected to see whether there were any perfusion defects inside the liver graft. In addition, the washing protocol was done in order to perfuse all vessels with medium and to let the segment swell to its original size. After 24 hours, at the day of cell injection, the set-up shown in figure 6 was built inside the flow cabinet to maintain a sterile environment during the experiment.

2.7.1 HepG2 cell reseeding

For HepG2 cell reseeding, the segment was connected to the tubing by the three-way port attached to the cannula to start perfusion one hour before cell injection. The cells were harvested by Trypsin EDTA (0.25%). The number of cells used for each experiment depended on the mass of the segment; for HepG2 reseeding, approximately two million cells per gram were used. The cells were diluted into standard culture medium (2 ml per injection). The cells were dynamically infused inside the segment by multi-step perfusion with intermittent periods of 15 minutes. The cells were injected via a syringe connected to the three-way port shown in figure 7. The cells were diluted in 2 ml medium per injection. The flow rate during perfusion was either 5 ml/min or 11 ml/min to test the effect of flow rate on the efficiency of recellularization.

2.7.2 HUVEC reseeding

The same washing protocol used for HepG2 recellularization applied for recellularization with HUVECs. The protocol for HUVEC reseeding was slightly different. The cells were statically infused inside the segment in order to give the cells the opportunity to adhere to the vascular wall without experiencing shear stress. The effect of vascular coating was researched by comparing CD31 coated vessels to uncoated vessels. For experiments with coated vessels, two hours before reseeding, the segment was coated with 6 ml of 50 µg CD31 (PECAM-1) (Santa Cruz) per 1 ml 1x PBS. Two hours after coating, the segments were washed with 50 ml of culture medium. The cells were harvested by trypsinization and the number of cells used for recellularization, was 2 million per 1 gram of wet liver weight. The cells were diluted in culture medium (2 ml per injection) and the cells were injected inside the liver via the cannula, shown in figure 7. For HUVECs reseeding, the cells were statically injected, which means that the segment was not perfused by culture medium during cellular injection. After injection, the segment was placed inside the incubator, for static
incubation for 2 hours. After 2 hours, the segment was perfused with standard culture medium for 24 hours where after the flow rate was raised to 5 ml/min. The segments were incubated for 5 days.

### 2.7.3 Organoid reseeding

Two recellularization experiments with human liver-derived organoids were performed. The protocol of organoid reseeding was similar to the HepG2 cells protocol. The washing steps were performed with ADV F12 ++++. Recellularization was done by dynamic multi-step perfusion with intermittent periods of 15 minutes. Again, the number of cells depended on the wet weight of the segment. For organoid reseeding about one million cells per gram was used. Single cell organoids, harvested by trypsinization, were infused into the segment. The organoids were diluted into 2 ml medium per injection. The culture medium used for organoid reseeding was filtered EM (as described in table 4 in appendix 2. The cells were injected via the cannula (figure 7) similar to the HepG2 cells and HUVECs protocols. The flow rate was either 5 ml/min or 11 ml/min and the number of injections per experiment was 10. The segments were incubated for 2 and 7 days.

### 2.8 Analysis

#### 2.8.1 Fluorescence microscopy imaging, sample preparation and H&E staining

Directly after perfusion in the incubator at 37 °C and 5% CO$_2$ and prior to fixation, the recellularized porcine livers were screened under a fluorescence microscope (EVOS FL Cell Imaging System, Invitrogen) to check cellular spreading by means of the GFP signals of the cells. Biopsies of the normal liver, decellularized porcine liver and recellularized porcine liver grafts were taken and were either snap-frozen or fixed with 4% formaldehyde for 24 hours. Biopsies of the recellularized liver are taken from the parts with most GFP signal. After fixation, the samples were embedded in paraffin. Two to three biopsies were sectioned (4 µm) with a microtome for histological examination. The histological slides processed for H&E staining and analyzed using a bright field microscope to test the efficiency of the recellularization procedure by analyzing the general morphology of the cells the cellular spreading throughout the liver matrix.

#### 2.8.2 IHC staining

Immunohistochemistry (IHC) staining was performed for experiments 7 (HepG2 cells), 10 (organoids), 11 and 12 (both HUVECs) in order to detect and localize specific antigens for liver cells by means of antibody markers. The markers used for IHC staining of experiments 7 and 10 were Albumin, whose presence indicates hepatocyte viability and function; Cytokeratin (CK)19 (Agilent Dako, Clone ECK 108), a cholangiocyte marker, which is also organoid-specific; CK7 (Agilent Dako, clone OV-TO 12/30) and Mucin 1 (Muc1) (FISCHER Scientific, clone E29), two

<table>
<thead>
<tr>
<th>Washing-step</th>
<th>Amount of fluid per step</th>
<th>Incubation time</th>
</tr>
</thead>
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<td>1x PBS</td>
<td>50 ml</td>
<td></td>
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<tr>
<td>1x PBS</td>
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<tr>
<td>1x PBS</td>
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<td>DMEM/Advanced DMEM F12 (10% Pen/Strep)</td>
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markers specific for cholangiocytes. The samples were incubated with either mouse monoclonal anti-albumin, mouse monoclonal anti-CK7, mouse monoclonal anti-CK19 or mouse monoclonal anti-Muc1. The secondary anti-body used was rabbit anti-mouse-HRP. The tissue sections were stained with 3,3′-diaminobenzidine (DAB) and counter staining was performed with H&E. Samples were imaged using a bright field microscope.

2.8.3 qPCR analysis

Quantitative polymerase chain reaction (qPCR) was performed to analyze the expression of certain genes of the cells inside the recellularized ECM of experiment 6 (HepG2 cells), experiment 10 (organoids), experiment 11 (uncoated segment with HUVECs) and experiment 12 (coated segment with HUVECs). qPCR was performed using the StepOnePlus™ Real-Time PCR System (Applied Bioscience). Firstly, RNA was purified using the miRNeasy Mini Kit (cat. no 217007, QIAGEN). QIAzol Lysis Reagent (QIAGEN) was used for lysis of the sample. The sample was disrupted and homogenized and treated following the QIAGEN Quick-Start Protocol of the miRNeasy Mini Kit for RNA purification. cDNA was made from purified RNA and was used for qPCR analysis. Primers used for qPCR analysis were Ki67 (cell proliferation), NOTCH (cellular proliferation, specification, and differentiation), CK19 (cholangiocyte marker and organoid-specific), CK7 (cholangiocyte marker), Albumin (hepatocyte marker), LGR5 (organoid marker), HNF4α (hepatocyte gene expression control) and CYP3A4 (drug metabolism in the liver). qPCR of these primers was performed in duplicates for 4 experiments mentioned earlier in this section. In addition a water control was used as reference. The qPCR results were analyzed using dC_T values of all genes, which were corrected for GAPDH, the housekeeping gene. Mean C_T values higher than 35 and standard deviations (SD) of dC_T higher than 1, were treated as undetermined and were left out.
3 Results

3.1 Optimization of the recellularization procedure with HepG2 cells

The first three experiments were performed to analyze the effect of the number of injections on the recellularization efficiency. Experiments 4 and 5 were performed in order to analyze the effect of flow rate. The first two experiments are presented in the next section (3.1.1), the results of the third experiment can be found in appendix 3 because most cells flowed directly out of the segment after injection. The results of experiments 4 and 5 are presented in section 3.1.2. After optimization of the number of injections and the flow rate, recellularization was performed with a prolonged incubation time (experiments 6, 7 and 8). Experiments 7 and 8 are presented in section 3.1.3 and the Fluorescence microscopy imaging results and H&E staining results of experiment 6 can be found in appendix 4, because no healthy cells were found inside the matrix by H&E staining.

3.1.1 Effect of the number of injections

The results of the first experiment can be seen in figure 8. The culture medium did not show any signs of contamination during or after the experiment. The medium had a clear appearance and the color of the medium did not change during the experiment, indicating a healthy balance between O$_2$ and CO$_2$. No cells were seen in the culture medium after the experiment. Figure 8a and b show images made by the fluorescence microscope at t = 24h. During recellularization, cells spread through the matrix via the vasculature of the liver. The cells flowed through the vessels and attached to the vascular walls, as can be seen in the figure. Analyzing the spreading of the cells with the fluorescence microscope, it can be seen that most cells stayed within the vasculature structures of the liver. However, also some clumps of cells can be seen. One of those clumps is indicated by the arrow in figure 8a and shown in the close up in figure 8b. These clumps of cells imply that the cells did not only stay in the vascular structures of the matrix, but also reseeded the parenchymal part of the matrix. In addition, it can be seen that the cells were engrafted inside the matrix, which can be distinguished because of the difficulty of focusing on the cells with the microscope. This implicates that the cells were not engrafted inside of the matrix in stead of on top of the matrix, which would have made it more easy to focus on the cells. It is also shown that not all the areas of the ECM were reached by the cells. These results are confirmed by the H&E staining, shown in figure 8c and d. Figure 8c and d, show that some parts of the matrix were effectively recellularized and that the cells are engrafted inside the parenchymal parts of the liver. However, the figures also show that other parts of the ECM are not recellularized at all. This is shown in figure 8a, where healthy cells are engrafted in the left part of matrix, whereas no cells can be found in the right part of the matrix. The condition of the cells looks good: the cells inside the matrix seem to be alive.

The second experiment was performed with more injections and a longer incubation time. The culture medium did not show any signs of infection and the color of the medium did not change. Some cells were found inside the culture medium after the injection phase and after 3 days of incubation. The results are shown in figure 9.

The GFP-labeled HepG2 cells show less clumping and they seem to have stayed within the vasculature of the liver graft. Comparing this cellular spreading to the cellular spreading of the previous experiment, it seems that the cells were more evenly distributed throughout the vessels of the ECM (figure 9a). In addition, more parts of the matrix of this experiment seem to be recellularized compared to the previous experiment.

Also, figure 9b shows a better distribution of cells within the graft. However, most cells stayed within the vascular structures (indicated by the arrows in figure 9), supporting the results of the images made by the fluorescence microscope. Subsequently, in experiment 2, the condition of the HepG2 cells was worse than the condition of the cells of experiment 1. When analyzing the H&E staining results, the close ups shown in figure 9c and d smaller recellularized areas as well as more cell death. However, some singular cells could be found in between the dead cells, indicated by the arrow in figure 9d. However, also these cells do not look healthy.
3.1.2 The effect of flow rate

Experiments 4 and 5 were performed to test the effect of flow rate on the effectiveness of recellularization. Both experiments were performed with the same parameters. For both experiments, the culture medium did not show any signs of infections after 2 days of incubation, also the color did not change during the experiments and no cells were found in the medium reservoir. The results of these experiments were similar and they are shown in figure 10.

More GFP-labeled cells were found in the matrix (figure 10a) compared to previous experiments. However, the distribution does not seem different from the previous experiment. The cells seem to have migrated deeper inside the matrix and the HepG2 cells seem to appear in clumps to a larger extent compared to previous experiments with a flow rate of 11 ml/min. In figure 10a can be seen that mostly the right part of the figure is recellularized and the cells seem to appear in large clumps, indicating that the cells recellularized both vascular structures and the parenchymal parts of the matrix.

Figure 10b shows H&E staining of experiment 4. Figure 10c, d, e and f depict the H&E staining of experiment 5. Both experiments show good engraftment inside the parenchymal parts of the liver, as large areas of the liver graft are recellularized. Furthermore, the distribution of cells seem better compared to previous experiments. Also, the recellularized areas are much larger compared to all previous experiments, especially the recellularized parts in experiment 5 (figure 10e). The condition of the HepG2 cells of both experiment 4 and 5 was good, the cells were alive and they look healthy. Even though the liver was recellularized with a tumor cell line, the cells seem to arrange themselves inside the matrix, as can be seen in figure 10f. Moreover, in the H&E staining of both experiments, small sinusoid-like structures can be distinguished.
Figure 9: Experiment 2, recellularized liver matrix after 3 days of incubation. Recellularization was performed by multi-step perfusion with 10 injections of 4 million cells per injection. The flow rate was 11 ml/min. (a) Captured by a fluorescence microscope, scale bar: 2000 µm. Most cells have stayed within the vascular structures of the liver matrix. The GFP-signal shows an even distribution of cells throughout the vessels of the liver. (b) H&E staining of a recellularized liver graft, scale bar: 100 µm. Most of the HepG2 cells have stayed within the vasculature of the liver, indicated by the arrows in the figure. Moreover, most cells seem dead. (c) Close up of liver vessels, scale bar: 50 µm. A lot of dead debris can be found within the vascular structures, some cells seem semi-healthy. (d) Close up of a vascular structure of the porcine liver, scale bar: 25 µm. Mostly dead debris is seen inside the matrix. Some singular cells can be distinguished, indicated by the arrows. Also these cells do not look healthy.

In table 3, an overview is given of the results of all optimization experiments. Column five describes the effectiveness of cellular distribution throughout the segment: + for desired distribution pattern, the cells did evenly spread throughout the matrix, and - for bad cellular spreading. Column six describes the cell position: + for cells engrafted inside the parenchymal part of the liver and - when no cells were present in the parenchymal part of the liver. Lastly, the cell condition is described in column 7: + for good cell condition (alive and healthy cells) and - for bad cell condition (e.g. a lot of cell death).

Table 3: Overview of the results of experiments 1-5. The flow rate, the number of injections and the incubation time is given. Column 5, 6 and 7 sum the cell distribution, the position of the cells and the cell condition.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Flow rate</th>
<th>Number of injections</th>
<th>Incubation time</th>
<th>Cell distribution</th>
<th>Cell position</th>
<th>Cell condition</th>
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<tr>
<td>1</td>
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<td>11 ml/min</td>
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<td>3 days</td>
<td>+</td>
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<td>3</td>
<td>5 ml/min</td>
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<td>2 days</td>
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<td>+</td>
</tr>
<tr>
<td>4</td>
<td>5 ml/min</td>
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<td>2 days</td>
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<td>+</td>
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<tr>
<td>5</td>
<td>5 ml/min</td>
<td>10</td>
<td>2 days</td>
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Figure 10: Experiments 4 and 5, recellularized liver matrix after 2 days of incubation. Recellularization was performed by multi-step perfusion with 10 injections of 4 million cells per injection. The flow rate was 5 ml/min. (a) Experiment 4, captured by a fluorescence microscope, scale bar: 2000 µm. Mostly the right part of the figure is effectively recellularized, as large clumps of HepG2 cells are shown, implicating that both vascular structures as well as parenchymal parts being recellularized. (b) H&E staining of experiment 4, scale bar: 100 µm. The cells show a good distribution throughout the matrix. In some areas, large parts of the parenchyme of the liver are recellularized. (c, d, e) H&E staining of experiment 5, scale bar: 100 µm. Large areas of the porcine liver matrix are recellularized by HepG2 cells. The cells seem alive and healthy. (f) Close-up of H&E staining of experiment 5, scale bar: 50 µm. A large part of the matrix is recellularized and the cells seem to be arranged. The cells are healthy.

3.1.3 HepG2 cell recellularization with a prolonged incubation time

To test whether the recellularized HepG2 cells were able to survive within the ECM for a longer period of time, three experiments were performed. These recellularization experiments all have the same flow rate (5 ml/min) and number of injections (10 injections). Experiment 6 and 8 were had an incubation time of 7 days and experiment 7 had an incubation time of 5 days.

In figure 11, the results of experiment 7 are shown. Analyzing the results of this experiment, it can be seen that the majority of the ECM of experiment 7 was not recellularized, as depicted in figure 11a and b. In addition, the cells in the parts of the matrix that were
recellularized did not evenly spread throughout the matrix. In figure 11a, a couple of cell-clumps can be distinguished and in figure 11b, it is shown that inside a certain part of the liver graft, no cells were engrafted at all.

Analyzing the H&E staining results (figure 11c, d, e and f), it can be seen that some parts of the porcine liver matrix contain large recellularized areas. In figure 11c, one of those large parts is shown. The cells seem to be healthy. In figure 11d, it seems that the matrix is not recellularized at all. However, in this part some areas with dead cell debris can be distinguished (one of these areas is indicated by the arrow). In addition, figure 11e and f show recellularized areas with dead cell debris too. When comparing histological slides of various parts processed for H&E staining, most slides show large areas of dead HepG2 cells, comparable to the results shown in figure 11.

The conditions for experiment 8 were the same as for experiment 7, except for the incubation time, which was 7 days in stead of 5 days. The cellular spreading within the matrix, analyzed by a fluorescence microscope shown in figure 12a and b, was better compared to the previous experiment. The cells spread throughout the matrix more evenly, although it seems that many cells stayed within the vessels of the liver graft (shown in figure 12). In figure 12b can be seen that the HepG2 cells also formed clumps within the matrix.

H&E staining shows recellularization of large areas of the porcine liver matrix with healthy-looking cells. In addition, some smaller areas are reseeded by HepG2 cells, it seems that in these areas, the cells stayed within the vascular structures and did not reach the parenchymal parts of the liver (shown by the arrows in figure 12d). In figure 12e and f, close-ups of one of the recellularized areas are shown. As can be appreciated from the image, the cells look healthy.

Figure 13 shows the results for the IHC staining of experiment 7, recellularization of a porcine liver graft with HepG2 cells, compared to a normal liver graft before decellularization. As can be seen in the figure, the cells stain positive for Albumin and CK19, whereas the HepG2 cells stained negative for CK7 and Muc1.

The qPCR results of the first experiment with a prolonged incubation time (7 days), are shown in figure 14. As can be appreciated from figure 14, CK7, CK19, Albumin, HNFα and CYP3A4 were expressed. Ki67, NOTCH and LGR5 were not determinable (indicated by ND). Albumin was highly expressed, compared to the housekeeping gene GAPDH, while the other genes showed low expression.
Figure 11: Experiment 7, recellularized liver matrix after 5 days of incubation. Recellularization was performed by multi-step perfusion with 10 injections of 4 million cells per injection. The flow rate was 5 ml/min. (a, b) Captured by a fluorescence microscope, scale bar: 2000 µm. (c) H&E staining of experiment 7, scale bar: 100 µm. A large part of the matrix is recellularized by HepG2 cells. The appearance of the cells looks healthy. (d, e) H&E staining of experiment 7, scale bar: 100 µm. Some areas of the matrix are reseeded by HepG2 cells. Only dead cellular debris can be distinguished, indicated by arrows. (f) H&E staining of experiment 7, scale bar: 25 µm. Close up of an recellularized area with HepG2 cells, only dead debris is shown.
Figure 12: Experiment 8, recellularized liver matrix after 7 days of incubation. Recellularization was performed by multi-step perfusion with 10 injections of 4 million cells per injection. The flow rate was 5 ml/min. (a, b) Captured by a fluorescence microscope, scale bar: 2000 µm. Cells can be distinguished inside the vascular structures inside the ECM by GFP signal. (c, d) H&E staining of experiment 8, scale bar: 100 µm. Large areas inside the ECM are recellularized by HepG2 cells, they are engrafted in the parenchymal part of the liver. The cells have also stayed within the vascular structures, indicated by the arrows. (e, f) H&E staining of experiment 8, scale bars: 50 and 25 µm. Close-up of the cells reseeded in the parenchyme, the cells look healthy.
Figure 13: Recellularization with HepG2 cells. First two columns: IHC staining of recellularized grafts after 5 days of incubation, scale bar: 50 and 25 μm. Third column: IHC of normal liver sections as control, scale bar: 50 and 25 μm. Staining for Albumin, CK7, CK19 and Muc1. HepG2 cells stained positive for Albumin and CK19.
Figure 14: qPCR analysis of experiment 6 with HepG2 cells. ND indicates that the genes could not be determined by the qPCR procedure. CK7, CK19, Albumin, HNFα and CYP3A4 were expressed. Whereas Ki67, NOTCH and LGR5 were not determinable.
3.2 Recellularization with organoids

Two recellularization experiments with organoids were performed. For the first experiment (experiment 9), the flow rate was 11 ml/min. The results are shown in figure 15. When analyzing the images captured by the fluorescence microscope, only sparse amounts of cells are found within the ECM. Some green fluorescent signal could be distinguished in the matrix, however, it seems that most cells adhered to the external part of the graft, because little fluorescent signal comes from within the matrix. This result was also confirmed by the images obtained by H&E staining. A small number of cells was found in the matrix. Some of the cells did show their typical organoid-like shape, as presented in figure 15b and c: the organoids seem to grow in a circular shape. According to H&E staining in figure 15b, some of the cells have died during the experiment; dead debris can be distinguished from this figure inside the vascular structures. In figure 15b and d can be seen however, that most cells look healthy. The major part of the cells in figure 15d, as well as a few cells in figure 15c appear in a cuboidal shape (indicated by the arrows in the figure). Whereas other cells, mostly in figure 15b have a more elongated shape.

When comparing the results of experiment 9 to the results of experiment 10, a stronger fluorescent signal as well as more healthy cells were found within the matrix of experiment 10. The organoids of this experiment show different kinds of behaviour; again, the organoid-like structures were present in this experiment (figure 16b). In figure 16b can be seen that multiple organoid-like structure with a circular shape were found inside the matrix, although it seems that these shapes have not engrafted to the matrix, as they lay loosely in between two parts of the matrix. One of these organoid-structures is indicated by an arrow. In other areas, the cells did engraft inside the matrix, which is shown in figure 16c, d and e. The arrow is indicating the area where the cells engrafted inside the matrix, as also part of the organoids grew in between the matrix. Furthermore, the organoids seem to align around the vascular walls of the matrix, as shown in figure 16f. The arrow indicates the engongated cells, aligning the vascular wall.

Figure 17 shows the results for the IHC staining of experiment 10, recellularization of a porcine liver graft with organoids, compared to a normal liver graft before decellularization. As can be seen in the figure, the cells stain positive for Albumin, CK7, CK19 as well as for Muc1.

The results of the qPCR analysis of experiment 10 are presented in figure 18. According to the qPCR data, Ki67, CK7, CK19, Albumin, HNFα and CYP3A4 were expressed, whereas NOTCH and LGR5 could not be determined. Mostly, CK19 showed high expression compared to the housekeeping gene GAPDH.
Figure 15: Experiment 9, recellularization with organoids after 2 days of incubation. Recellularization was performed by multi-step perfusion with 10 injections of 1 million cells per injection. The flow rate was 11 ml/min. (a) captured by a fluorescence microscope, scale bar: 2000 µm. Only a few cells can be distinguished inside the liver matrix. Most cells seen to be engrafted on the external part of the liver. (b) H&E staining of experiment 9, scale: 100 µm. A few areas of the matrix are recellularized. Some cells appear in their typical organoid-like structure, as they have a circular-shape. (c) H&E staining of experiment 9, scale bar: 50 µm. Some healthy cells can be distinguished inside the liver graft and some of the cells are arranged in a circular organoid-like shape. Most cells in the circular shape have an elongated appearance, although also a cuboidal shaped cell is found and indicated by the arrow. (d) H&E staining of experiment 9, scale: 25 µm. An organoid structure is shown. Most cells have an cuboidal shape. One of the cuboidal cells is indicated by an arrow.
Figure 16: Experiment 10, recellularization with organoids after 7 days of incubation. Recellularization was performed by multi-step perfusion with 10 injections of 1.6 million cells per injection. The flow rate was 5 ml/min. (a) captured by a fluorescence microscope, scale bar: 2000 µm. Only a small part of the matrix shows GFP-signal coming from inside the liver matrix. In addition, some organoids seem to have engrafted on the external part of the porcine liver matrix. (b) H&E staining of experiment 10, scale bar: 100 µm. Organoid-like structures are found in between the matrix. In some areas, the cells did not engraft to the matrix and one of these areas is shown by the arrow. (c) H&E staining of experiment 10, scale bar: 100 µm. Cells are engrafted to inside the matrix, indicated by the arrow. (d, e) H&E staining of experiment 10, scale bar: 50 µm. Close-up of the cellular engraftment inside the porcine liver matrix, indicated by the arrow. (f) Organoids have aligned the vascular structures of the porcine liver. The cells have a elongated shape, indicated by the arrows.
Figure 17: Recellularization with organoids. IHC staining of recellularized grafts after 7 days of incubation and IHC of normal liver sections as control. Staining for Albumin, CK7, CK19 and Muc1. Organoids stained positive for Albumin, CK7, CK19 as well as Muc1.
Figure 18: qPCR for experiment 10 with organoids. Ki67, CK7, CK19, Albumin, HNFα and CYP3A4 were expressed, whereas NOTCH and LGR5 could not be determined.
3.3 Recellularization with HUVECs

Two experiments were performed with HUVECs in order to recellularize the vasculature of the porcine liver grafts. Comparing images made with a fluorescent microscope (figure 19a and b) of the uncoated and the coated experiment with HUVECs, a big difference was seen. In figure 19a, many HUVECs were seen inside the ECM, whereas in figure 19b, fewer cells were found inside the matrix. The spreading of the cells of the uncoated experiment, was comparable to the previous experiments with HepG2 cells. It can be seen in figure 19a that many cells entered the vascular structures of the liver graft and that the cells formed clumps inside the matrix. In figure 19b, the cells seem to appear in singular cells in a spot-like structure, as well as they appear in smaller numbers.

When comparing the H&E staining (figure 19c-h), similar results were shown. Larger areas of the uncoated porcine liver matrix (figure 19c and e) were recellularized compared to the coated liver matrix (figure 19d and f). Also, when looking at the vascular structures of the liver graft, it can be appreciated that more cells adhered to and nestled into the vascular walls, indicated by the arrows in figure 19c and e and as presented by figure 19g. As can be seen in figure 19h, nearly any cell did not attach to the vascular wall. Moreover, the cells in the uncoated graft look healthier than the cells in the coated liver graft. In figure 19d and f, dead debris can be distinguished inside the vascular structures and in figure 19h, the cells do not have a healthy appearance.

The results of the qPCR analysis of the recellularization experiments of the uncoated liver segment are shown in figure 20. Since the expression of non of the genes of the coated segment could be determined, these results are not presented. As can be seen in figure 20, Ki67, CK7, CK19, Albumin, HNFα and CYP3A4 were expressed, whereas NOTCH and LGR5 could not be determined. Mostly, Albumin showed high expression compared to the housekeeping gene GAPDH.
Figure 19: Experiments 11 and 12, recellularized liver matrix after 5 days of incubation. Recellularization was performed by multi-step perfusion with 10 injections of 4 million cells per injection. The flow rate was 5 ml/min. The segment used for experiment 12 was coated with CD31 in advance of the recellularization steps. Experiment 11 was performed with an uncoated segment. (a) The uncoated experiment, captured by a fluorescence microscope, scale bar: 2000 µm. Many cells have engrafted inside the porcine liver graft. The cells have engrafted inside the vascular structured and some cell clumps can be distinguished. (b) Image of the coated experiment, captured by a fluorescence microscope, scale bar: 2000 µm. Hardly any cell has engrafted inside the porcine liver matrix. (c, e) H&E staining of the uncoated experiment, scale bar: 100 µm. Some areas of the liver are recellularized. The cells did not only adhere to the vascular walls, as they were also engrafted inside the parenchymal part of the liver. Some cells did engraft to the inside the vasculature of the liver, indicated by the arrows. (d, f) H&E staining of the coated experiment, scale bar: 100 µm. Fewer cells can be distinguished inside the liver graft and most cells did not adhere to the vascular structures. In addition, the cells look less healthy compared to the cells in the uncoated segment. (g) H&E staining of the uncoated experiment, scale bar: 25 µm. Healthy cells adhered to the vascular structure of the liver graft. The cells have an elongated appearance. (h) H&E staining of the coated experiment, scale bar: 50 µm. Most cells did not attach to the vascular walls. The cells do not look as healthy as the cells in the uncoated experiment.
Figure 20: qPCR for experiment 11 with HUVECs. Ki67, CK7, CK19, Albumin, HNFα and CYP3A4 were expressed, whereas NOTCH and LGR5 could not be determined (indicated by ND).
4 Discussion

In this research, the potential of porcine liver graft recellularization with liver-derived organoids was studied. Using rejected donor-livers for decellularization and recellularization could be a solution to the donor-liver shortage. This research mainly focused on organoid reseeding and optimization of reseeding techniques. Organoids are potentially an ideal cell type because they have the potential to proliferate rapidly, they are genetically stable and they are bipotent: organoids are able to differentiate into both hepatocytes and cholangiocytes [11, 18, 38]. In advance of organoid recellularization experiments, the reseeding protocol was optimized by using HepG2 cells. During the optimization experiments, flow rate and the number of injections were analyzed. In addition to HepG2 cell and organoid recellularization, reseeding of endothelial layers of the vascular structures of the liver graft was studied by using HUVECs. Endothelial coverage of the vasculature is essential to prevent thrombosis and to provide proper vascular functions [41]. The level of recellularization efficiency was determined by: analyzing cellular spreading throughout the liver segment; the position of the cells and the viability of the cells.

To optimize the recellularization procedure, either a flow rate of 5 ml/min or 11 ml/min was used. In addition, the effect of 4 injections was compared to 10 injections. Analyzing the effect of the amount of injections, the spreading of cells in the ECM seems better using a high number of injections. To my best knowledge, this effect has not been analyzed before. However, the reason for this could be that injection causes random infusion and division of cells through the matrix. This implies that when 4 injections are performed, only a small part of the liver will be recellularized compared to a situation where more cell injections are used. In addition, some cells flowed out of the liver segment directly after injection. When using only a few injections, the risk of losing a significant number of cells during the injection phase is higher. Therefore, using a high number of injections improves the recellularization procedure, because the amount of engrafted cells will probably be higher and the cellular spreading is improved.

A low flow rate is superior to a high flow rate. Using a flow rate of 5 ml/min has shown a good cell viability as well as favorable cell position and cellular spreading. In contrast, a flow rate of 11 ml/min resulted in considerable more cell death; especially when the experiment was performed with a prolonged incubation time. This is also confirmed in literature: it has been argued that a high flow rate causes shear stress to vascular walls [27, 42]. Shear stress causes rupture of the cells and results in cell death [27, 42]. In this research, two experiments at 11 ml/min were performed, the first was incubated for one day and the second was incubated for three days. Since the incubation time was extended, the cells were exposed to shear stress for a longer period of time and this probably resulted in more cell death.

The optimized protocol was used for experiments with a prolonged incubation time. Analyzing these experiments, it can be seen that the cells have migrated to the parenchyme and some parts of the segment show healthy cells inside the liver graft. However, some parts of the segments show healthy cells, a lot of cell death is encountered. Therefore it seems difficult maintain cell viability for a prolonged period of time. Most certainly, there are many different parameters affecting the efficiency of recellularization. It is of utmost importance that the experiments are performed in the same manner, with constant parameters such as volume, pressure, flow rate, culture time and mechanical stimuli [26]. For example, during the recellularization, the porosity of the matrix will decrease due to rising numbers of cells. This causes an increased pressure inside the matrix [43]. Monitoring pressure, and adjusting flow rate to this pressure might be critical to prevent shear stress and mechanical damage during the reseeding process.

In the experiments performed during this research, a relatively simple experimental set-up was used. This made it difficult to keep the experiment at constant conditions. In order to make the recellularization process more efficient, a number of improvements could be made regarding the experimental set-up. For example, an oxygenator and a bubble trap were not available for the recellularization experiments of this research, although they could have beneficial effects by analyzing media turnover. It has been argued that due to the absence of capillaries, it may be necessary to supply the liver with higher levels of O$_2$ than the atmospheric O$_2$ saturation levels. Mostly a level of 95% O$_2$ and 5% CO$_2$ is used to support survival or reseeded cells [23]. To keep these levels constant, adding a pH sensor and a pO$_2$ sensor to monitor media conditions could improve the experimental conditions. Also a mixing gas apparatus with a flow controller allowing for fully automated conditioning of the media with O$_2$ and CO$_2$ in response to pH and pO$_2$ could
enhance the outcomes of the experiments [26]. Caralt et al. measured glucose and lactate in the culture medium via a valve for media sampling [44]. Via glucose and lactate sampling, oxygen in the culture medium can be determined. Oxygen and nutrient measurement inside the medium can be correlated to cell metabolism and cell number in the matrix, which gives information about adequacy of the perfusion [44]. During the experiments in this research, the appearance of the culture medium was checked daily in order to determine whether the culture medium was in a proper condition. The color of the culture medium did not change during the experiments, implying that the CO$_2$ buffer was working properly.

A method to improve cell proliferation and cellular migration towards the parenchymal area, could be the use of multiple cell types or growth factors. Kadota et al. and Barakat et al., used stellate cells to improve hepatocyte attachment and viability inside a liver scaffold [28, 45]. Co-culturing of cells might replicate part of the normal physiology and may help induce migration in vitro as well as the growth of complex biological tissue structures [46]. Additionally, the incorporation of certain growth factors or bio-active molecules into the decellularized scaffolds could result in enhanced functionality of the recellularized liver constructs [47]. Factors such as VEGF165, GRGDSPC peptides and hepatocyte growth factors have been used to improve proliferation and functionality [21, 48, 49].

After optimization and reseeding with a prolonged incubation time, recellularization of porcine liver grafts with liver-derived organoids was performed in two experiments. The behaviour of the cells seem to be organoid-like as they grew in both the parenchymal part of the liver and they have shown a cuboidal shape, typical for cholangiocytes surrounding the bile duct. In addition, organoids have shown a elongated shape lining liver structures. These various shapes of the cells are caused by the fact that organoids can differentiate into both hepatocytes as well as cholangiocytes [38].

The organoids cultured and reseeded in these experiments, were treated with EM with Y which normally keeps the cells in an undifferentiated state. Differentiation of cells in vitro requires change of culture medium [11]. Organoid differentiation into hepatocytes has shown potential: the organoids seem to acquire both morphological characteristics of hepatocytes as well as an up-regulation of hepatocyte markers and hepatocyte-like functions, such as production of albumin and bile acid salts production [38]. Differentiation of liver-derived organoids into cholangiocytes is less established. However, Sampaziotis et al. showed a protocol in which cholangiocytes can be derived from human iPSCs and the cells showed biliary specification markers [50].

Organoids normally have to be cultured in differentiation medium in order to differentiate, however, it seems that the cells did differentiate inside the porcine liver ECM. The IHC slides stained positive for all markers, including the cholangiociptic markers. This confirms previous research, which argued that the liver ECM is able to support the differentiation of cells into biliary and hepatocytic lineages. The liver scaffold provides not only a three-dimensional vascularized scaffold for nutrient delivery, it also retains the environmental cues necessary for progenitor hepatic and endothelial cells to grow, differentiate, and maintain functionality [44]. Looking at the CK7 and Muc1 positive cells, it is difficult to see whether these cells are positioned at locations particular for cholangiocytes. Therefore, it is hard to conclude whether the cells might have differentiated towards cholangiocytes. Comparing IHC results of organoids to the IHC staining of HepG2 cells, it can be seen that HepG2 cells stain positive for Albumin. This implies that Albumin antigens are present on both HepG2 cells and organoids. It is striking that the HepG2 cells stain positive for CK19 cells. Healthy hepatocyte-like cells should stain positive for Albumin and negative for the other markers tested in this research. A possible explanation could be that the HepG2 cells were not viable, which could have made the cells adhesive, causing DAB to attach to the HepG2 cells. Similar results were encountered in IHC staining of histological slides of organoids. In the lower left corner of figure 24 in appendix 5, a large area is stained with DAB, although no viable cells can be distinguished in this area. Another explanation could be that HepG2 cells are CK19 positive. Several researches have shown that some hepatocellular carcinoma cell lines produce CK19 [51] and HepG2 cells have shown that they are able to stain positive for CK19, performing IHC [51, 52, 53]. This could be a reason why in this research, the HepG2 cells stain positive for CK19. The HepG2 cells did not stain positive for CK7 and Muc1, whereas IHC staining of organoids did show positive results for these markers. This supports the findings that the ECM contributes to organoid differentiation in recellularization procedures.

Moreover, qPCR analysis showed expression of all genes except for NOTCH and LGR5.
LGR5 is specific for organoids, which may implicate that the organoids were in a differentiated state. This also might explain why the gene expression CK19 of the organoid experiment was high. NOTCH was not expressed for all experiments, which is a marker for cell proliferation, specification, and differentiation. It is difficult to explain these results and in order to draw any conclusions, more qPCR experiments should be done.

Endothelialization of the vascular structures was analyzed by reseeding of HUVECs inside the liver graft by static infusion. The effect of vascular coating was studied. Comparing the segment coated with CD31 to the uncoated segment, it was seen that the uncoated segment showed more viable cells aligning the vascular walls, which is contradicting to previous research [54]. According to Ko et al., "conjugation of CD31 antibodies to the vascular matrix improved endothelial cell retention on the vasculatures. Endothelial cell seeding combined with antibody conjugation improves endothelial cell attachment and retention leading to vascular patency of tissue-engineered kidneys" [55]. The vasculature was coated in the same way as described by Ko et al. [54].

An explanation for these results could be that the coating protocol might have failed. Ko et al. confirmed CD31 antibody conjugation on the vasculature within the sections by treatment of the segment with Alexa588 goat anti-rat antibody and imaging the segments by a fluorescent microscope [54]. This procedure was not performed in this research, and the effectiveness of the coating procedure was not confirmed. A reason why the procedure might have failed can be because in this research, a smaller amount of CD31 was used: 6 ml in this research compared to 20 ml by Ko et al. [54], or because the CD31 was not effective. Research by Ko et al. describes different seeding methods within CD31 coated livers [55]. Those results show different outcomes, comparing small vasculature structures with large-sized blood vessels. CD31 antibody conjugation significantly maintained endothelialization of small-sized vasculatures, however, these techniques resulted in limited or no cell attachment in the intermediate and large-sized blood vessels [55]. Whereas reseeding of small capillaries resulted in cell clogging within the renal cortex [55]. This suggests that there is need for improvement of cell seeding and coating methodology, in order to obtain better revascularization results.

A drawback of the analysis protocol of this research, was that only a few biopsies were processed for analysis. It is therefore difficult to say whether these biopsies were representative for the entire liver graft. During fluorescence microscopy analysis, most representative parts of the liver were selected for taking biopsies for staining, this protocol was used for all experiments. However, it might be better to process the entire liver for either staining analysis or RNA and DNA analysis. Moreover, recellularization efficiency was not measured during the experiments in this research. Taking a biopsy during the reseeding process would most probably have resulted in a perfusion defect, effecting the perfusion efficiency of the liver graft. Therefore, developing non-invasive techniques to assess liver reseeding during the experiments is required. Multiple imaging techniques have already been incorporated into bioreactors and could be used for reseeding efficiency analysis [26, 56, 57, 44].

As mentioned before, in future experiments, segments could be used completely for either qPCR and DNA or RNA quantification methods or staining methods. To improve the qPCR experiments even further, a positive control should be included. In this way, the experimental data can be compared to gene expression of original liver tissue and the fold-change can be determined. This will quantify the up-regulation of certain genes compared to normal liver tissue and this will eventually give an indication about the reseeding efficiency. Lastly, in addition to normal IHC-staining, whole mount staining could be performed. This gives the possibility of detecting and localizing specific antigens for liver cells by means of antibody markers throughout a larger part of the liver.

Besides that more replications of the the qPCR experiments in this research should be performed, also additional quantification techniques could be used. Some research groups collected their culture medium after cell injection in order to determine cellular engraftment efficiency [27]. The number of cells that was not retained in the liver after the cell injection phase could be determined by calculating the difference between the number of cells seeded and the number cells present in the medium [27]. Also other types of DNA and RNA quantification analyses can be performed to test the amount of cells inside the matrix. For example by spectrophotometry or by DNA quantification assays such as the PicoGreen DNA assay (Invitrogen) [58].
In order to study cell viability, a TUNEL-staining or other cell viability assays could be performed. TUNEL-staining is a method to determine apoptotic DNA fragmentation. Thus, the TUNEL-positive percentage gives the percentage of apoptotic cells compared to the total number of cells. This can give an indication of whether the reseeding procedure was performed by the right conditions. Additionally, analyzing the liver graft by a confocal microscope gives a 3D overview of the reseeded segment. Since the confocal microscope is able to detect GFP signal, the cells can be tracked inside the liver graft. This might give a more reliable indication of the cellular spreading throughout the segment, compared to fluorescence microscopy imaging, used in this research. Lastly, the hepatic function of the reseeded liver grafts have been studied by other research groups [32, 27]. For example albumin and urea production can give an indication of the condition of the reseeded liver segment and thus the efficiency of the recellularization procedure.

To obtain a sufficient number of organoids for liver graft recellularization, a long culturing period is necessary. Due to the long culturing time needed for one experiment, it was not possible within the scope of this research to execute more organoid recellularization experiments. Yet, the potential and prove of concept of organoid recellularization is shown in this research. In order to be able to use recellularization with organoids in clinical settings in the future, several problems should be solved. Although organoids can be expanded to large amounts in a relative short time, it still seems to be difficult to obtain a sufficient number of cells in order to reseed an entire liver matrix. Moreover, culturing organoids requires Matrigel, which currently cannot be used in clinical settings because it is not FDA approved. In future directions, recellularization experiments with a larger number of organoids should be performed, and the problems regarding organoid reseeding should be solved to be able to use organoid recellularized livers in the clinic.

5 Conclusion

In conclusion, liver-derived organoids have proven to be a promising source of cells to recellularize a-cellular liver scaffolds for tissue engineering purposes. Although more experiments on efficient recellularization should be done, some careful preliminary conclusions can be made. In fact, this optimization study has shown that a high flow rate gives more cell death. Therefore, a low flow rate of for example 5 ml/min is preferred. Furthermore, a low number of injections raises the risk of losing cells during the injection phase, which results in a lower number of engrafted cells inside the liver matrix. Additionally, a low number of injections showed worse cellular spreading compared to a high number of injections. Therefore, a using 10 injections is preferred over 4 injections. After the protocol was optimized, organoids reseeding was performed. Organoids have shown to engraft inside the liver matrix and they have various kinds of behaviour inside the ECM. They appeared in a cuboidal shape, they were found in the parenchyme and they aligned in an elongated shape. This shows the organoids might be able to differentiate, probably facilitated by cues of the ECM. Differentiation capacity has also been supported by IHC staining and qPCR results. Although, more long-term studies are required to evaluate the efficacy of organoid recellularization and to assess the functionality of organoids inside a liver graft, this study showed proof of concept and has shown the potential of liver graft recellularization with organoids.
6 Appendices

6.1 Appendix 1: Abbreviations

CK: Cytokeratin
DAB: 3,3’-diaminobenzidine
dH₂O: demineralized water
FCS: fetal calf serum
ECM: extracellular matrix
EM: expansion medium
GAGs: glycosaminoglycans
GFP: green fluorescent protein
H&E: hematoxylin & eosin
HUVECs: Human Umbilical Vein Endothelial Cells
iPSCs: induced pluripotent stem cells
IHC: immunohistochemistry
LGR5: Leucine-rich repeat-containing G-protein coupled receptor 5
Muc: Mucin
ND: not determined
NTS: the Dutch Transplant Foundation
PBS: phosphate buffered saline
Pen/Strep: Penicillin and Streptomycin
qPCR: quantitative polymerase chain reaction
SD: standard deviation
SDS: sodium dodecyl sulfate
WHO: World Health Organization

6.2 Appendix 2: Components of Expansion Medium

Table 4: Expansion Medium was based on ADV ++++ and supplemented with the components listed in the table.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>1%</td>
</tr>
<tr>
<td>B27</td>
<td>1%</td>
</tr>
<tr>
<td>Acetylcysteine</td>
<td>1.25 mM</td>
</tr>
<tr>
<td>Gastrin I</td>
<td>10 nM</td>
</tr>
<tr>
<td>EGF</td>
<td>50 ng/ml</td>
</tr>
<tr>
<td>R-Spondin</td>
<td>10%</td>
</tr>
<tr>
<td>FGF10</td>
<td>100 ng/ml</td>
</tr>
<tr>
<td>HGF</td>
<td>25 ng/ml</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>10 mM</td>
</tr>
<tr>
<td>A8301</td>
<td>5 µM</td>
</tr>
<tr>
<td>Forskolin</td>
<td>10 µM</td>
</tr>
<tr>
<td>Y-27632 dihydrochloride</td>
<td>1 µ/μl</td>
</tr>
</tbody>
</table>

6.3 Appendix 3: Experiment 3

Figure 21 shows images made by the fluorescence microscope and H&E staining of experiment 3. Experiment 3 was performed with 4 injections of 10 million cells per injection. The flow rate was decreased to 5 ml/min and the incubation time was 2 days. As can be appreciated from figure 21a and b, GFP-labeled HepG2 cells were found in only a small part of the segment. Figure 21b shows that in large parts of the porcine liver, no cells were engrafted inside the matrix. A few singular cells can be distinguished in this figure, however, these cells lie most probably on top of the ECM in stead of being engrafted inside the matrix. The green area that can be seen in the figure 21b (white arrow) is presumably back-ground fluorescence. In the recellularized parts, the cells were clumped together (figure 21a).
Figure 21c and d also show that most of the ECM was not recellularized as only a small number of cells was found inside the porcine liver matrix. The HepG2 cells that were found inside the matrix, were in relatively good condition compared to experiment 2.

Figure 21: Experiment 3, recellularized liver matrix after 2 days of incubation. Recellularization was performed by multi-step perfusion with 4 injections of 10 million cells per injection. The flow rate was 5 ml/min (a, b) Captured by a fluorescence microscope, scale bar: 2000 µm. (a) Shows small recellularized areas, mostly with cells that are clumped together, whereas (b) Shows a large part that was not recellularized. (c, d) H&E staining of a recellularized liver graft, magnification: 10x normal size.

Figure 22: Experiment 3: recellularized liver matrix after 2 days of incubation. Recellularization was performed by multi-step perfusion with 4 injections of 10 million cells per injection. The flow rate was 5 ml/min. As can be appreciated from the picture, most cells engrafted on the outside of the matrix. This could be an explanation for the low amount of cells engrafted inside the liver matrix. Scale bar: 2000 µm
6.4 Appendix 4: Experiment 6

The images taken with the fluorescence microscope, shown in figure 23, can be seen that the fluorescent signals show an even spreading of the HepG2 cells throughout the ECM. It can be seen that the cells have migrated inside the matrix. However, the H&E staining shows different results compared to the fluorescence microscope. Figure 23b shows the H&E staining of experiment 6 and it can be seen that the HepG2 cells have died during the experiment. A lot of dead debris was present in the matrix and no healthy cells were found in the samples processed for H&E staining. During and after the experiments, no contamination was found in the culture medium.

Figure 23: Experiment 6, recellularized liver matrix after 7 days of incubation. Recellularization was performed by multi-step perfusion with 10 injections of 4 million cells per injection. The flow rate was 5 ml/min. (a) Captured by a fluorescence microscope, scale bar: 2000 µm. (b) H&E staining of experiment 6, magnification: 10x normal size.

6.5 Appendix 5: IHC staining of organoids

Figure 24: IHC staining of the experiment with organoids. DAB attached to both healthy cells as well as to dead debris, scale bar: 50 µm.
References


