Repopulating a decellularized liver scaffold with liver-derived organoids in a perfusion-based bioreactor

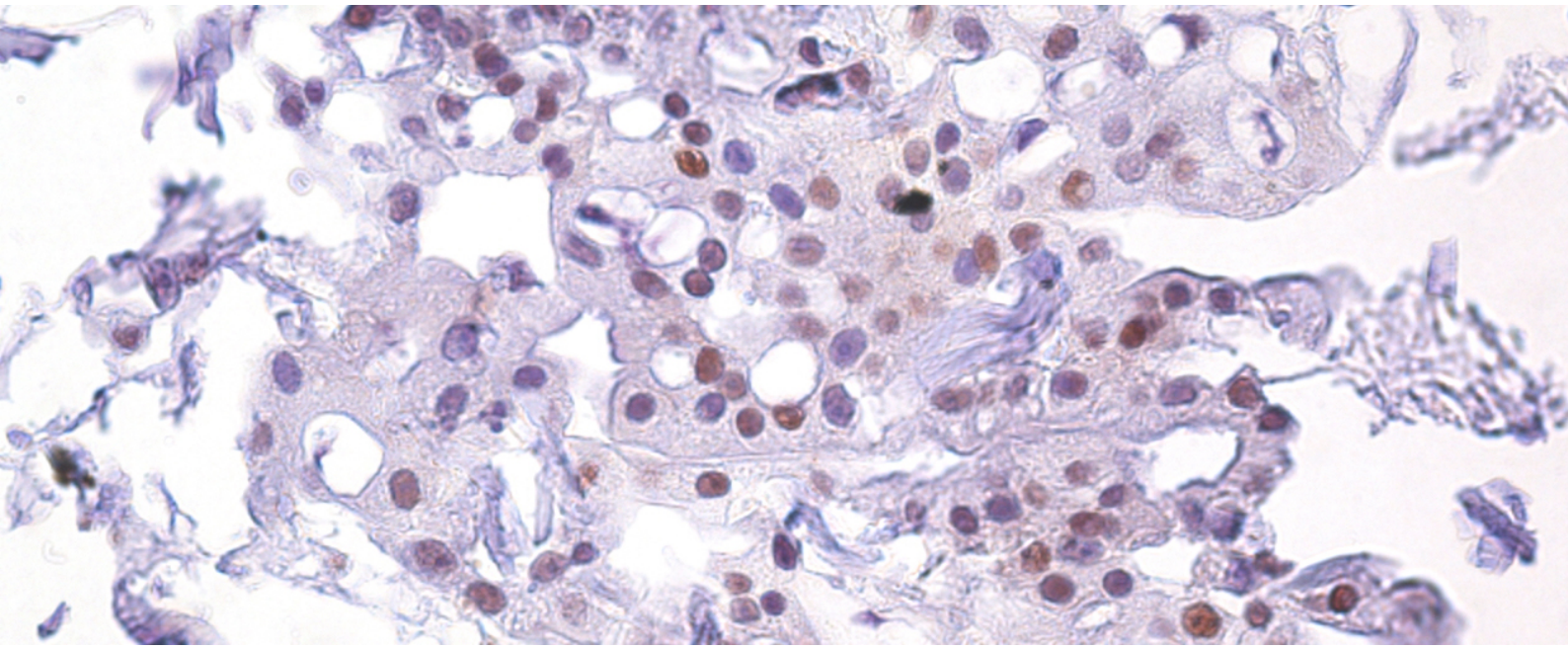
by

A. Vermeulen

in partial fulfillment of the requirements for the degree of

Master of Science
In Biomedical Engineering,
With the specialization in Biomaterials and Tissue Biomechanics,

at the Delft University of Technology,
to be defended publicly on Tuesday September 24, 2019.



Student number:	4281861	
Supervisors:	Prof. dr. A. A. Zadpoor,	TU Delft
	Dr. ir. E. L. Fratila-Apachitei,	TU Delft
	Dr. M. Verstegen,	Erasmus MC
	MSc. J. Willemse,	Erasmus MC

TU Delft

Abstract

Over the last decade new methods are explored in the field of tissue engineering to minimize the donor organ shortage [1]. Engineering organs with a complex structure and large vascular network, such as the liver, remain a challenge. Luckily, the decellularization of an organ creates a scaffold that consists of the extracellular matrix (ECM) with important growth factors, bifunctional molecules such a fibronectin and multiple collagen types [2]. This ECM provides the biophysical and biochemical cues needed for cells to adhere, proliferate and differentiate [3]. However, there is no optimal method yet to recellularize such a decellularized liver scaffold. This project shows that it is possible to use a perfusion-based bioreactor for repopulating a porcine liver scaffold with liver-derived organoids. In the bioreactor, the Harvard Apparatus (Hugo Sachs Elektronik), decellularized porcine liver segments were infused with a HepG2 cell line and liver-derived organoids in seperate experiments. A setback in the project was the proneness to infections in the Harvard Apparatus (HA), which shortened the duration of experiments and influenced the results. The experiments were analyzed by histological and immunochemical staining and by qPCR. The HepG2 cell line validated the set up and recellularization with the HA, the cells engrafted throughout the scaffold and showed viability and signs of proliferation. The liver-derived organoids were successfully cultured and expanded in spinnerflasks, and were found engrafted and alive after 10 days in the scaffold. The qPCR data showed variability between the different organoid lines and between the different phases of the organoid culture. The results combined of this project are promising for future research, especially regarding the use of liver-derived organoids for recellularization.

List of abbreviations

CK	Cytokeratin
DMEM	Dulbecco's Modified Eagle Medium
EM	Expansion Medium
EpCAM	Epithelial cell adhesion molecule
FCS	Fetal Calf Serum
GFP	Green Fluorescent Protein
H&E	Hematoxylin & Eosin
HA	Harvard Apparatus
HNF4α	Hepatocyte Nuclear Factor 4 alpha
HNF1β	Hepatocyte Nuclear Factor 1 beta
IHC	Immunohistochemistry
Lgr5	Leucine-rich repeat-containing G-protein coupled receptor 5
PBS	Phosphate Buffered Saline
Pen/Strep	Penicillin and Streptomycin
qPCR	quantitative Polymerase Chain Reaction

Contents

1 Introduction

1.1. Background

Over the last decade new methods are explored in the field of tissue engineering to minimize the donor organ shortage [1]. Tissue engineering up until now, was mostly done for thin layer tissues like vessels and skin making use of 3D printing or tissue layering. However, engineering organs with a complex structure and a large vascular network still remain a challenge.
An organ that is therefore particularly interesting in the field of whole organ engineering, is the liver. The liver plays an important role in all metabolic processes of the human body. The structural and functional units in the liver are called 'lobules' and can be seen in part B of figure 1.1. At each corner of this lobule, the portal triad can be found which consists of a bile ductule, hepatic artery and portal vein. Sinusoids are the vessels that run from the portal triad to the central vein. This creates a well vascularized tissue for uptake of nutrients and waste removal. The most abundant cells that are present in the liver lobules are hepatocytes which can secrete bile, store glucose and vitamins and help with the detoxification [4].

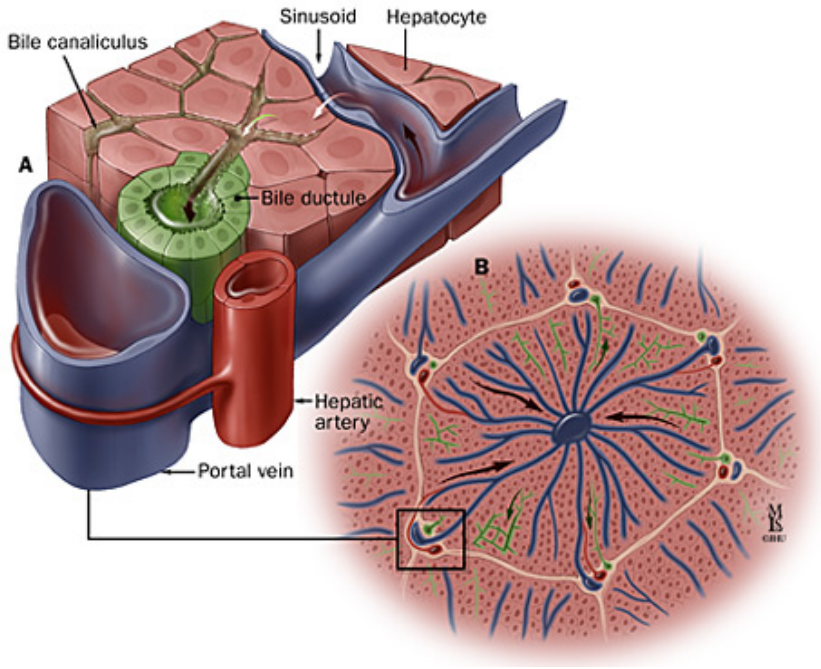


Figure 1.1: Schematic drawing of the liver lobule. Part A shows the portal triad which consists of the bile ductule, hepatic artery and portal vein. Part B shows the typical structure of the lobule [5].

An ideal scaffold for tissue engineering, needs to be biocompatible, biodegradable, has good porosity, and is able to offer the needed structural support [6]. One of the methods to create such a scaffold has been proven to be successful. It involves the decellularization of a whole organ tissue by flushing out all the cells and cellular debris [3]. This decellularized scaffold, which after the decellularization process mainly exists of the extracellular matrix (ECM), can provide biological cues of the physical and chemical components. These cues are necessary in the process of proliferation, adherence of the cells and differentiation to match the original organ [3]. What provides these biophysical and biochemical cues, are the richness in growth factors, bifunctional molecules such a fibronectin and multiple collagen types present in the ECM [2]. Focusing on the liver, the ECM can have a direct effect on the fate of the native cells, because of its structure and behaviour [2]. Figure 1.2 shows the histological staining of porcine liver tissues, image A shows the tissue before decellularization and image B shows it after the decellularization process. The outlined section in image A is a portal triad from the tissue, the portal vein is clearly visible. In image B, part 1 is again the portal triad and part 2 that is outlined shows the typical structure of the parenchyma in a decellularized porcine liver.

The next step is the recellularization of the decellularized organ with an appropriate mixture and number of cells and the correct distribution of these cells within the organ to populate the parenchymal space [2]. Studies that carried out a recellularization of organ scaffolds used direct cell injection as main method, though the downside to this method is damage to the scaffolds microarchitecture and it is responsible for heterogeneous scaffold seeding [7]. Focusing on recellularizing the liver, the success of parenchymal repopulation depends on the route where the new cells are going to be injected [8]. Several studies state that cell seeding can also be done using a bioreactor [3][9], which makes use of dynamic cell seeding by injecting a suspension with a high number of cells into the perfusion vessel, where cells can infiltrate to the organs parenchyma by perfusion to enhance efficiency and uniformity of the injected cells [3][9]. Figure 1.3 shows a schematic drawing of the different steps needed in the recellularization process. As menthioned earlier, the first step is to decellularize the scaffold to prepare it for the infusion of new cells and after it will be perfused and cultured in a bioreactor-like system. Bioreactors seem fit for this process because it can deliver a suf-

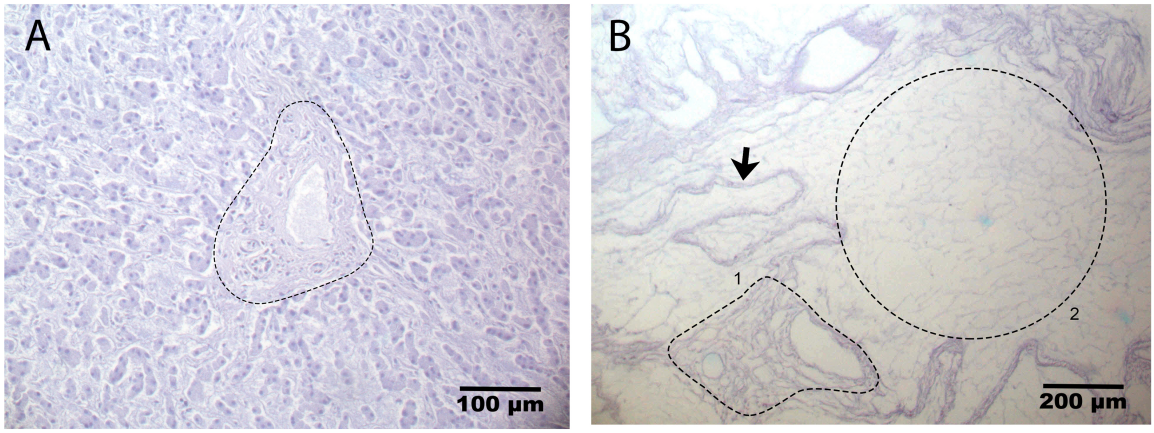


Figure 1.2: H&E images taken with a microscope of a porcine liver. Image A shows the tissue before decellularization, the selection shows the portal triad. Image B shows the tissue after decellularization, selection (1) shows the portal triad, selection (2) shows the parenchymal space and the arrow shows connective tissue.

ficient supply of nutrients and oxygen into the scaffold and simultaneously remove waste through the perfusate [9].

At this moment multiple studies have tried to repopulate a liver scaffold in different ways. Methods tested and compared by Soto-Gutierrez et al. (2011)[11] include direct parenchymal injection, multistep infusion and continuous perfusion. Infusion and perfusion are both a type of dynamic cell seeding. Studies that use a perfusion system have had the best delivery rates for scaffolds which are dense and have a complex 3D structure, like the liver [9]. However, reason for a lower engraftment efficiency of the injected liver cells can be the lack of a cell lining in the vasculatur network of a decellularized liver scaffold. The vasculature can in this case be coated with endothelial cells for the reconstruction of a functional organ [12]. These vessels can express growth factors to attract endothelial cells, but if the endothelial layer on the wall indeed is absent, the cells could directly migrate into the parenchyma instead of attaching to the wall[13]. Therefore it is of importance that the vascular network is re-endothelized before repopulating the scaffold with liver cells.

Besides analyzing the engraftment efficiency, a common method to test the functionality of the cells in the engineered scaffold is to measure the albumin and urea levels. The variations in the results of the studies can be the result of a number of factors, like the decellularization and recellularization techniques which include different cell types and seeding methods [1].

Although multiple studies have shown promising results in liver tissue engineering, there are still many and important challenges to overcome. These challenges include finding the best species for the decellularized scaffold which brings graft acceptance in the human body, reconstruct the vascular network of the scaffold, finding the best cell source for repopulating and improving the bioreactor perfusion [12].

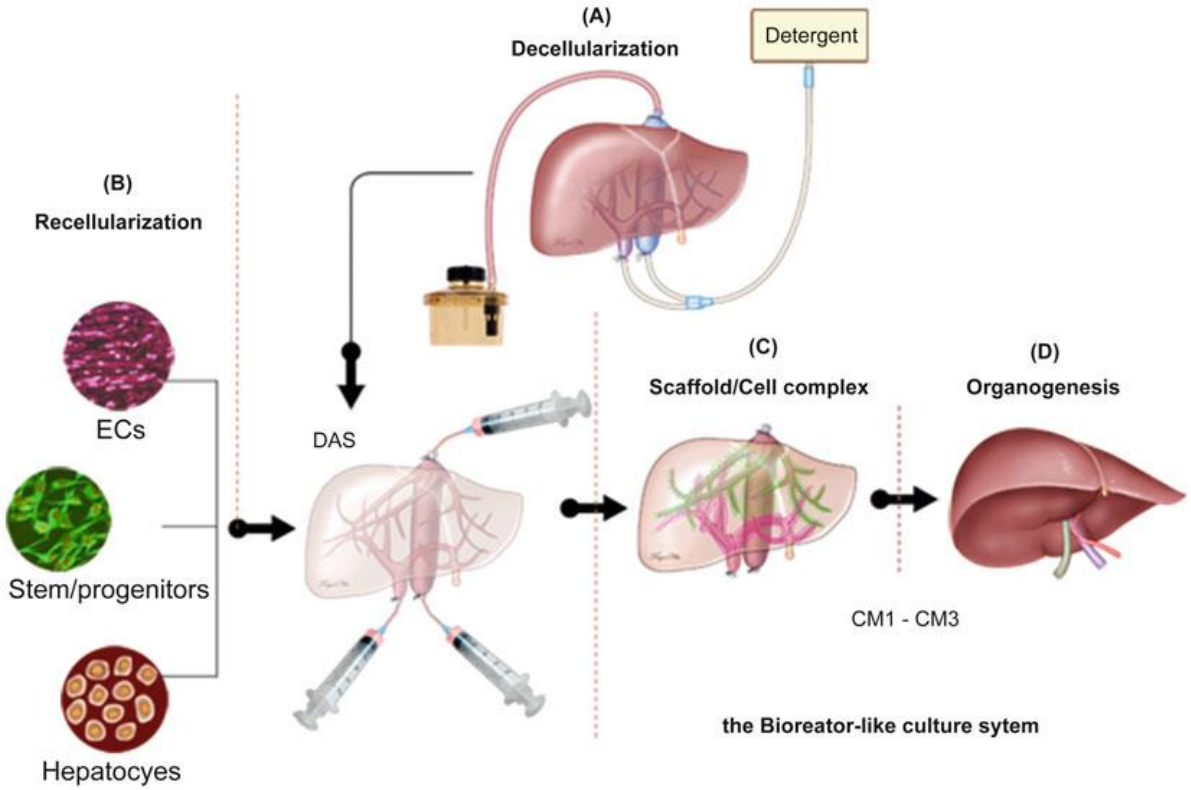


Figure 1.3: Schematic drawing of the recellularization process of a decellularized liver scaffold [10]

1.2. The research

The aim of this project is to find the optimal methods to repopulate a decellularized porcine liver scaffold which is infused with a cancer cell line , and a novel cell group, in a perfusion-based bioreactor. For this research a new bioreactor was obtained, the Harvard Apparatus (Hugo Sachs Elektronik). This machine allows for the control of, and insight into: the flow rate, pressure, oxygen and carbon dioxide levels, pH value and temperature. This creates the preferred circumstances for cells to survive and proliferate. The novelty of this research compared to previous published papers is the use of liver-derived organoids to repopulate the scaffold.
The cancer cell line that is used for this research is the HepG2 cell line. It belongs to the Hepatoma cell line, which stands for hepatocellular carcinoma (HCC). Characteristics that belong to this cell line are: unlimited life span, stable phenotype, high availability and are easy to culture. The HepG2s in particular, are non-tumorigenic and have a high proliferation rate [14]. These attributes make the HepG2 cell line suitable for the validation of the Harvard Apparatus perfusion for liver segment recellularization.
An organoid is a single cell or a group of cells that is self-organizing in a 3D structure and has organ-like functionality. They can form a near-physiological model system for studying adult stem cells and tissues in different environments and can be cultured for a long period of time without altering its genomes [15]. Organoids can be derived from the primary tissue of the donor, for instance from a liver biopt. They are cultured in BME which is a substitute for the ECM in a living tissue. In the culture well plates Expansion Medium (EM) is added to promote growth of the organoids. The application of organoids in a decellularized scaffold could give groundbreaking results. Taking the different factors of this research into account, the following research objectives were established:

- To validate the new set up with the Harvard Apparatus for recellularization of a decellularized liver segment with a HepG2 cell line
- To determine if it is possible to use liver-derived organoids for perfusion-based recellularization and culturing/expanding them to big numbers
- To determine the engraftment and functionality of both cell groups in the new derived constructs

The experiment itself consists of culturing the different cell lines, the preparation of the liver scaffold, injecting the cell suspension and perfuse the scaffold for about 10 days. Analysis of the scaffold will be done using a fluorescence microscope, by doing histological and immunochemical staining and qPCR. From the results a discussion and conclusion will arise with a recommendation for future research.

2

Materials and Methods

2.1. Harvard Apparatus

The whole set up of the Harvard Apparatus (HA) consists of the following components which can be seen in figure 2.1:

- Moist chamber (1)
- Medium reservoir (2)
- Oxygenator (3)
- 2 roller pumps (4)
- Thermostatic circulator (Grant) (5)
- Mounting plate for O_2, CO_2 and pH sensors (6)
- Pressure unit (7)
- Plugsys basic system case for the different modules (figure 2.2):
    - TAM-D (pressure)
    - SCP (flow)
    - OPPM (O_2)
    - PHMM (pH)
    - EMM (CO_2)
- SuperFish Airflow 2
- Self-fabricated bubble counter
- Laptop with logging software (BDAS v2.0)

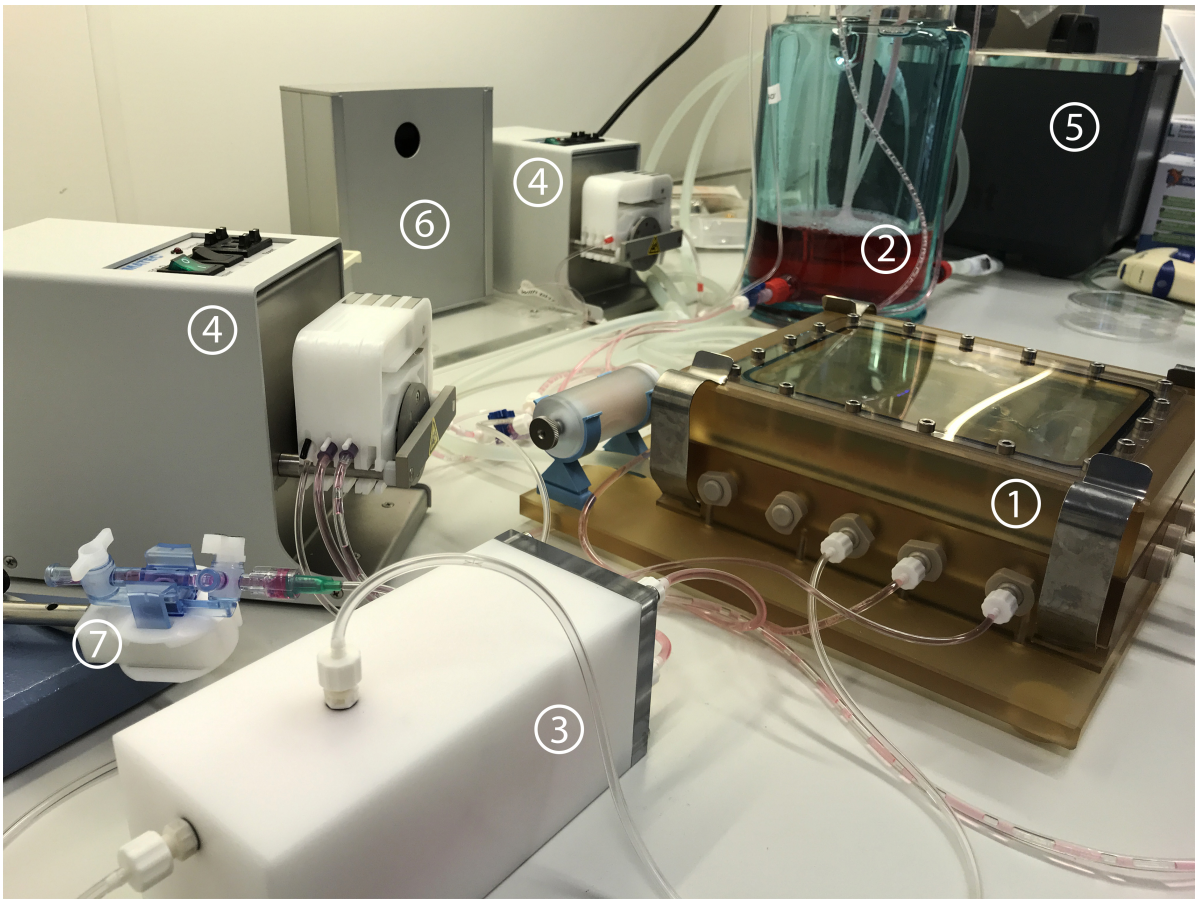


Figure 2.1: Set up of the Harvard Apparatus. Image shows: moist chamber (1), medium reservoir (2), oxygenator (3), roller pump (4), thermostatic circulator (5), mounting plate with sensors (6) and the pressure unit (7).

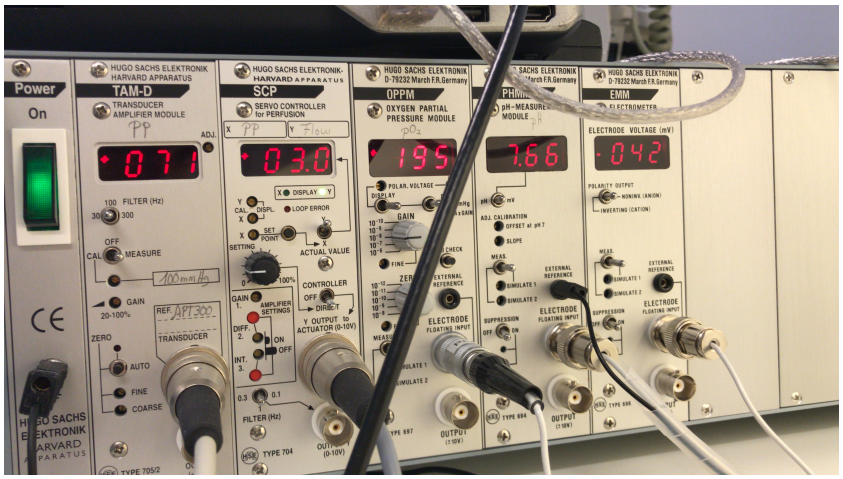


Figure 2.2: The 5 different modules of the Plugsys sysemt case

2.1.1. Preparation

Before starting the experiments all the modules in the system case (Plugsys) have to be calibrated. The pressure module is calibrated to a minimum of zero and a control value of 100mmHg, the flow rate unit is only used with the turn wheel and does not need calibration. All the sensors in the mounting plate (figure 2.1) (O_2, CO_2 and pH) do need frequent calibration, preferably before every experiment. The O_2 sensor came with pO_2 zero solution packages for calibration. The CO_2 sensor has to be calibrated with a 0% (± room air) solution and a 5% (incubator air), this was accomplished by placing the culture medium used for the coming experiment in petri dishes in the room and one in the incubator for at least 30 minutes. The pH sensor was calibrated with a 7.00pH and 4.01pH solution. After the calibration the whole set up was cleaned according to the protocol (figure 2.3, Cleaning A), to rule out possible infections caused by the calibration process. After cleaning the HA, the system was almost dry from the demineralized (demi) water used for flushing and from this moment the medium reservoir and the moist chamber were only opened when necessary. To the reservoir 300mL DMEM+ (which consisted of DMEM (Gibco) with 10% FCS, 1% Pen/Strep and 1mL Primocin) or Advanced DMEM+ (which consisted of Advanced (no serum) DMEM (Gibco) with 1% Pen/Strep and 1mL Primocin) was added by injecting the medium via the lid through a (0,45 μm poresize) air filter and ran through the system for at least 30 minutes to remove detergent and water remnants. The thermostatic circulator was set to 38° Celsius (body temperature and buffer for heat loss) and the air supply was installed. For the O_2 filtered room air was used and supplied by the SuperFish Airflow 2, for the CO_2 there was a tube coming from a CO_2 tab in the lab with 100% CO_2. The ratio of the air was set to be around 95% O_2 and 5% CO_2. The HA system was drained from medium and 450mL fresh DMEM+ or EM was added. All the air filters (0,45 μm poresize, Sarstedt) present in the circulation were replaced by fresh ones. When the temperature and pH stabilized, the scaffold was retrieved from the incubator. The moist chamber was swiftly opened and with sterile gloves and tweezers the 3-way stopcock of the scaffold (figure 2.5(2)) was connected to the cannula of the moist chamber to start the continuous perfusion through the scaffold.

2.1.2. Operation

During perfusion the HA was monitored. All levels were checked daily, where the pH should range between 7 and 8. Every day or every other day the air filters were replaced because they get clogged due to the warm moist present in the system. The ratio of the air components was checked, the room air and CO_2 should be stable and air should be bubbling in the medium reservoir. Several times during an experiment a supernatant was taken from the tubing (see figure 2.5 (3)) used for the injections. Only a small syringe was needed and mounted on the outlet in the wall and medium was extracted from the cannula going to the scaffold. This medium was inserted into a cell counting chamber and analyzed under a microscope. When the tubing used in the roller pumps was worn out, it was replaced before cleaning the HA after an experiment.

2.1.3. Cleaning

In figure 2.3 two protocols are explained to clean the HA at the end of an experiment. After an infection-free experiment protocol 'Cleaning A' could be used, in case of an infection 'Cleaning B' was used. Protocol B was done after experiment 5 and involved cleaning of all the separate parts of the HA (shown in 2.4). This method was followed by 2 infection-free experiments. The system was cleaned by perfusion with a detergent, two recommended detergents are Mucasol (Schulke+) and Cidex OPA (Johnson & Johnson). Mucasol was added to demiwater (at room temperature or 37°C) and the Cidex was a ready to use solution (at 20°C to 30°C). If the system runs with the detergent, make sure the moist chamber is filled by putting the medium outlet (figure 2.5 (5)) upwards. The cells that had grown onto the surface of the moist chamber were removed using a pipetboy or syringe with demiwater.

Cleaning A	Cleaning B	Injection A	Injection B
Drain HA system of medium into waste bottle Flush system with fresh demiwater Remove cell remnants from moist chamber Detach and drain reservoir (Figure 2.1 (2)). Clean with demiwater and Ethanol --- Fill reservoir with 1L demiwater and add 20mL Mucasol (or only 0,5L Cidex) and let HA run (overnight) Drain whole system and flush thoroughly with demiwater Start first run with culture medium and keep system closed	Follow part 1 of Cleaning A --- Take the HA apart and clean every part with warm water, Mucasol solution with brush and demiwater Small parts are bathed overnight in Ethanol See Figure 2.4 Let all parts dry and put the HA together again --- Follow part 2 of Cleaning A	Cell suspension in 10 syringes of 5mL The moist chamber is swiftely opened (Figure 2.1 (1)) The syringe is attached to the 3-way stopcock of to the scaffold (Figure 2.5 (2)) Cell suspension is infused during continuous perfusion The syringe is detached and the moist chamber closed Interval of 5 minutes before next injection	Cell suspension in 10 syringes of 5mL The syringe is attached to the moist chamber outlet with a 3-way stopcock and a large syringe containing medium (Figure 2.8) Cell suspension is infused during continuous perfusion through port (Figure 2.5 (3)) The medium syringe flushes the remaining cells through the tube. Syringes keep attached to the port for the next injections Intervals of 5 minutes

Figure 2.3: Overview of the different protocols used for HA cleaning (Cleaning A, Cleaning B) and infusion of cell suspensions (Injection A, Injection B).

Figure 2.4: Overview of the HA during Cleaning B protocol, in case of an infection.

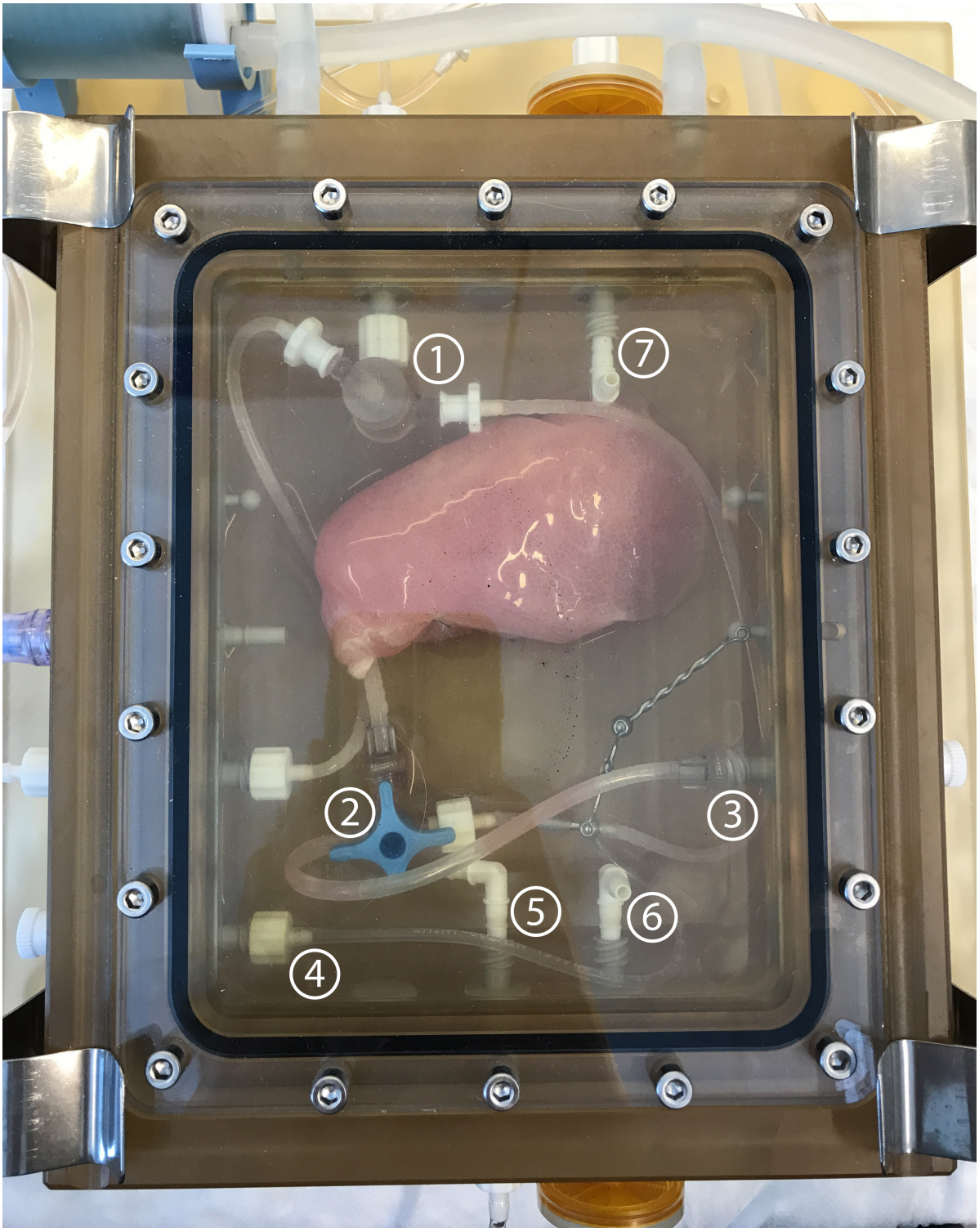


Figure 2.5: Recellularized scaffold from experiment 7 in the HA on day 13. Numbered parts are: bubble trap (1), 3-way stopcock (2), inlet port of injections (3), tube going from cannula to the pressure gauge (4), medium outlet (5), air outlet (6), air inlet (7)

Table 2.1: Summary of the performed HA experiments.

Experiment	Cell type	Cell number (approx.)	Injections, interval	Flow (ml/min)	Duration	Remarks
1	HepG2	300 x 10^6	10x 2ml, 5 min	2.5 (seeding), 3	5 days	Try out
2	HepG2	125 x 10^6	10x 1ml, 5 min	3	11 days	Infection (fungi)
3	HepG2	2 x 10^9	10x 4ml, 5 min	5 (seeding), 8	4 days	Infection (fungi) on segment
4	Organoid	100 x 10^6	10x 3ml, 5 min	3 (seeding), 5	8 days	Infection (bacteria)
5	HepG2	1,1 x 10^9	10x 3ml, 5 min	5	7 days	Double infection
6	Organoid	20 x 10^6	10x 3ml, 5 min	5	10 days	Infection free
7	HepG2	300 x 10^6	10x 3ml, 5 min	5	8 days	Infection free
8	HepG2	550 x 10^6	10x 3ml, 5 min	5 (seeding), 8	19 days	Infection (fungi) in segment
9	Organoid	5 x 10^6	10x 2ml, 5 min	3 (seeding), 5	5 days	Infection (fungi) on segment

2.2. Recellularization

2.2.1. HepG2 cell culture

The culture medium used for the HepG2 cell line consisted of: DMEM+ which is made from DMEM (Gibco) with 10% FCS, 1% Pen/Strep and 1mL Primocin. For the first experiment the GFP labeled HepG2s (transduced using a lentiviral vector) were prepared and cultured according to normal procedure. A confluent (80%-100%) culture flask (Thermo Scientific) was split and placed in larger or multiple culture flasks. Before splitting, the cells were washed with PBS (HyClone, GE) and after Trypsin (Lonza) was added. The cells were washed with medium and centrifuged in a centrifuge (Eppendorf) for 5 minutes at 1500 rpm. The cell pellet created was diluted and divided to fill new culture flasks. Medium was replaced once between splitting and the start of the experiment. The HepG2 line was kept in culture for the next experiments.

2.2.2. Organoid culture in spinner flasks

The culture medium used for the expansion of organoids consisted of: filtered Expansion Medium (EM) (see the table in appendix A.2 for materials). Liver-derived organoids were initaited from liver biopsies according to Huch et al. (2015) [16] and were cultured in a 24-well plate in BME (Cultrex) with supplemented EM in the first step. In confluent organoid well(s), the organoids were fragmented into smaller pieces and placed in a tube, supplemented with Advanced (all serum excluded) DMEM+ and centrifuged. The created cell pellet was washed twice with cold Advanced DMEM+ in order to remove BME. To these cells 5mL EM supplemented with 10% BME was added in a 50mL tube. The first tube started with 5mL and the spinner was added (see figure 2.6) which spins at 30 rpm and creates a bigger volume where organoids can expand. When confluent, the volume of the spinner is increased to 10mL. The volume was doubled each time, at a volume of 40mL it is increased to 80mL and divided over the 4 spinners available which resulted in 4 suspensions of 20mL each. This was doubled one more time to 40mL and at the moment that the 4 spinners were confluent, the organoids were ready to be harvested for the injections.
After usage, the spinner flasks were rinsed with demiwater and 70% ethanol and were stored in a closed clean tube. The lids of the spinners were cleaned with an ethanol soaked tissue and the filters (0.2 μm poresize, Whatman) were replaced.

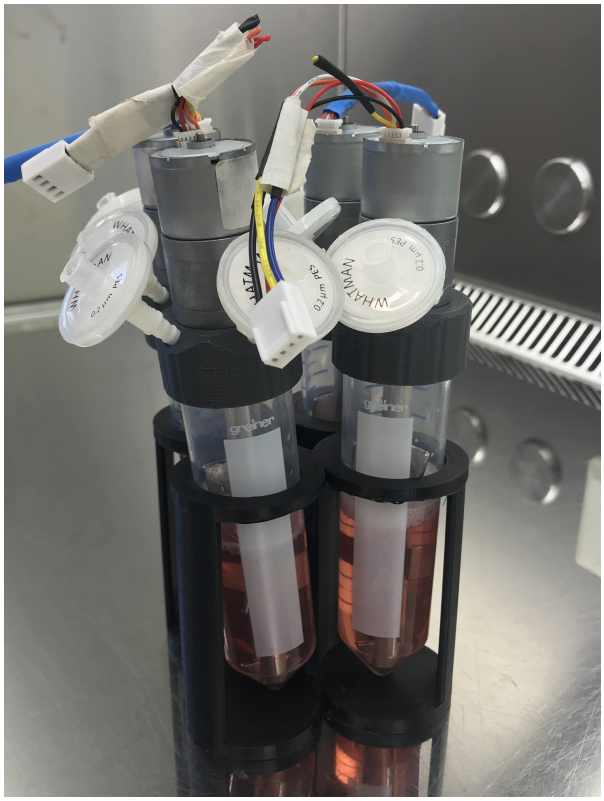


Figure 2.6: The spinner flasks disconnected and just splitted to 4 tubes with 20mL

Figure 2.7: Washing protocol of the liver scaffold. The scaffold was flushed multiple times with DMEM+ medium and after incubated in the container

50mL syringe perfusion	Solution composition
1	DMEM +
2	DMEM +
3	DMEM + 30mL, 20mL Pen/Strep, 1mL Primocin
Incubate (overnight)	
4	DMEM +
5	DMEM +
6	DMEM + 30mL, 20mL Pen/Strep, 1mL Primocin
Incubate	

Table 2.2: This table shows the composition of the filled syringes to flush the scaffold and prepare it for the experiment

2.2.3. The scaffold

The scaffolds used in this research were created by the decellularization of a porcine liver. Willemse et al. (2019) [17] established a method to successfully decellularize a whole liver of a pig and human in a shortened time compared to previous studies. This method consists of using only Tx100 and ammonia in the solution, in contrast to the other studies who used SDS next to the Tx100 [18][19]. The obtained porcine livers were first flushed with H_2O to remove all the blood and residue. Which was followed by 5 cycles of 120 minutes flushing the scaffold with a 4% Tx100 + 1% NH_3 solution in a 50L dH_2O continuous perfusion set up. When the liver is pale enough it is again perfused with H_2O to remove the detergent from the scaffold. The last step of the decellularization process is the addition of DNase to remove the remaining DNA. After thorough analysis it could be concluded that the ECM components of the liver scaffold were retained and remained intact. Segments of these porcine livers were surgically partitioned and cannulated for use in the Harvard Apparatus.
The created segments were stored in a -20° Celsius freezer and were thawed before they could be used in the HA. A 50mL syringe was filled with DMEM+ for the HepG2 experiments and Adv. DMEM+ for organoid experiments and was attached to the 3-way stopcock that was inserted and fixed with a cannula into the scaffold before freezing. The medium was gently flushed through the scaffold, see figure 2.7 for the set up. This is repeated three times, with in the last syringe a 40% Pen/Strep dose of the solution and 1mL extra dose of Primocin. The scaffold was bathed in the solution overnight. On the following day the washing steps were repeated and the scaffold was placed in the incubator until it could be attached to the HA at the beginning of an experiment. In appendix A.1 images of the scaffolds placed in the HA for every experiment can be found. After finishing the perfusion experiment the scaffold was disconnected from the system, placed in a petri dish and analyzed with a fluorescence microscope (EVOS, Invitrogen) in case of a HepG2 experiment. Parts of interest were partitioned in biopsies due to a high GFP signal for the HepG2 experiments, and the whole scaffold was partitioned in case of an organoid experiment. Followed by fixation with formaldehyde and embedding in paraffin. From the scaffold small pieces of tissue were snapfrozen for RNA isolation and later qPCR (quantitative Polymerase Chain Reaction).

2.2.4. HepG2 suspension

To the confluent t175 flasks Trypsin was added and the flasks were incubated. After the incubation, DMEM+ was used to wash the cells and the solution was divided over 50mL tubes and centrifuged. The created pellets were combined and supplemented with the needed amount of DMEM+, dependent on the mL injected at every step during recellularization. From this suspension the total number of cells was estimated.

2.2.5. Organoid suspension

The spinners are turned off and removed from the tubes. The suspension was divided over multiple tubes and Adv. DMEM+ was added dilute. The tubes were centrifuged for 5 minutes at 1500 rpm. Supernatant containing BME gel was removed and again Adv. DMEM+ was added to further wash the cells. The cell pellet created was trypsinized and washed again. The pellet was the basis of the cell suspension.

2.2.6. Injection

The suspension will be distributed along 10 15mL tubes and be set on ice. For every injection the 15mL tube is agitated and inserted through a needle into a 5mL syringe. In figure 2.3 two infusion methods are explained, where method A proved to be prone to infection. Method B was created for a more sterile and more easy way to infuse the cell suspension into the scaffold in a closed moist chamber. The set up of method B can be found in figure 2.8.

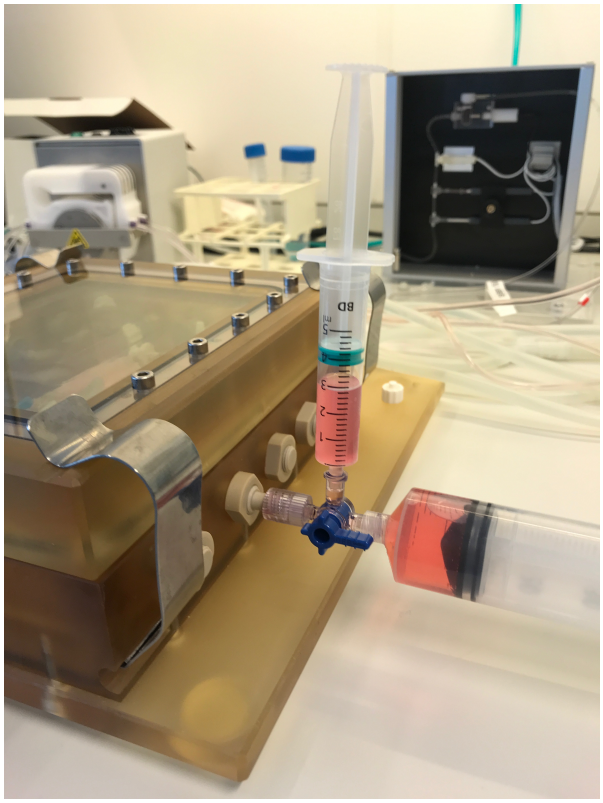


Figure 2.8: Image of the new injection protocol from experiment 4 and onward

2.3. The analysis

2.3.1. GFP signal

As described previously the scaffold repopulated with lentiviral GFP transduced HepG2 cells was placed in a petri dish and under the Fluorescence Microscope (EVOS) to analyze the GFP signal from the top and bottom. Areas with a strong signal contained most likely a large number of living cells and were therefore interesting to analyze.

2.3.2. Histological and DAPI staining

The slides were deparaffinized in a cycle of xylene and ethanol baths and after were placed from water into the Hematoxylin for 1.5 minutes, followed by multiple rinses of water. The slides were dipped for 2 seconds in acidic ethanol and again rinsed with water. The next step was the Eosin staining, this was done by placing the slides in a container filled with Eosin for 2 minutes and thoroughly rinsing the slides with water afterwards. The last step was to dehydrate the slides in a reversed cycle of ethanol and xylene. The slides were removed one by one from the xylene, a drop of Pertex was placed and the slide covered with a coverslip. The slides were analyzed under a microscope. For DAPI, the slides were taken from the last ethanol bath and placed on tinfoil. Onto each slide a drop of DAPI stain was dropped and a coverslip was placed. The slides were analyzed in the EVOS with ultraviolet light, which should be done on time because the DAPI signal fades after a while.

2.3.3. Immunohistochemical staining

Immunohistochemistry (IHC) was used to stain specific antigens with help of antibodies in biological tissues. After the deparaffinization of the slides they were washed and in different steps the primary and secondary antigens were added (see appendix A.3 for antibodies and dilutions). In this protocol Shandon covers were used for keeping the slides wet and for adding the solutions evenly. Both HepG2 and organoid coupes were stained. The staining was done with DAB (3, 3 –diaminobenzidine) and gave a brown color to the targeted antigens. Antigens that were stained for are: albumin (hepatocyte function), Ki67 (proliferation, cells in metastase phase), HFN4α (hepatocyte nuclei stain), Ck7 (cholangiocyte), Ck19 (organoid/cholangiocyte) and Collagen type I (connective tissue). A light Hematoxylin stain was used to color the adjacent tissues like the ECM for reference.

2.3.4. qPCR

Snapfrozen samples from experiments 7 (HepG2s), 6 (organoids) and 9 (organoids) were used to isolate RNA. To the experiment scaffold samples and cell samples (HepG2s and the different organoid lines) QIAzol was added to break down the proteins. The miRNeasy Kit was used for the purification of the RNA. The isolated RNA was measured for the nucleic acid ratio in a nanodrop, to test the RNA for pollution. cDNA is made from the isolated RNA and mixed with the primers of the chosen genes to run a qPCR. The genes chosen are: GAPDH (housekeeping gene), ALBorg (albumin/hepatocyte marker), Ki67 (cell proliferation), CK19 (organoid/cholangiocyte marker), CK7 (cholangiocyte), Lgr5 (organoid marker), HNF4α (hepatocyte marker), HNF1β (early cholangiocyte marker) and EpCAM (organoid/cholangiocyte marker). The forward and reversed primers can be found in appendix A.3.

3

Results

3.1. Harvard Apparatus

3.1.1. Observations

Included in the results section is the HA itself, which gave insight into the logged parameters and its participation in the multiple infections. In figure 3.1 a screenshot is taken from the log file during the 10 injections of experiment 2 with time in minutes on the x-axis. The most interesting parameter that was logged in the program (BDAS v2.0) is the pressure. Clear differences in pressure are seen during the multiple injections in figure 3.1. At the start of every injection there was a decrease in pressure because of initiated back-flow to trap air bubbles from going into the scaffold with the cell suspension. The high peak (left arrow) was an effect of the dense cell suspension entering the scaffold. All ten time points of the injections were marked in the file with a time stamp. There was a slight buildup in pressure visible after each injection step. The other parameters (pH, flow rate, pO_2) kept stable.

In the last experiments (6 to 9) the mounting plate was excluded from the circuit. In this case the pH was monitored by the color of the medium and checked once in a while with a pH meter with a supernatant of the perfusate. If the air filters were clogged, the pressure in the system rose and changed the supply ratio of the room air and pure CO_2. In this case there is more CO_2 present than favorable, which lowers the pH beneath the value of 7 and makes the medium yellow instead of red (figure 3.2).

During this study, multiple parts of the HA broke down. The pO_2, pCO_2 and pressure sensor quit working, hence the log files are not extensively included in the results or taken into account during an experiment. In experiment 8, the duration of an experiment was pushed to a total of 19 days. The main problem at this point was the roller pump tubing. It was worn out and flattened to a level where the system would create vacuum cause a reversed flow. This flow emptied the scaffold of perfusate instead of filling it, the experiment had to be ended at this point.

Figure 3.1: Image of the log file of experiment 2 during the 10 cell suspension injections into the scaffold. The arrows indicate the pressure peak at the beginning of every injection and the overall pressure build up after multiple injections.

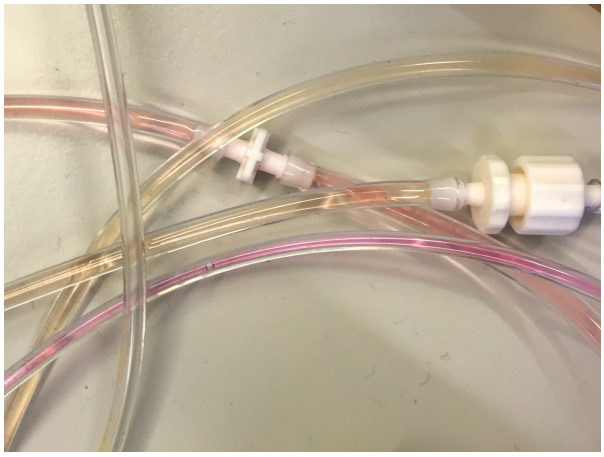


Figure 3.2: Image of the tubing of the HA during an experiment. The different hues of the medium can be seen: purple is high pH (>8), red is favorable pH (7-8) and yellow is low pH (<7).

3.1.2. Infections

Experiments 2 until 5 and 8 plus 9 have known to be infected by either fungi or bacteria. The infections had as result changing protocols for washing the scaffold, injecting the cells and cleaning the HA. In experiments 3 and 9 fungi was growing on the scaffold, which asked for a more thorough washing protocol. More medium with higher dosage of Pen/Strep was flushed through the scaffold and incubated in between and before usage in the HA. During the first three experiments at every cell injection the HA was opened and the syringe with the cell suspension was manually put in the 3-way stopcock (figure 2.5(2)) to inject the cells. After multiple infections this step was changed. In experiment 4 a new method was used for the injections, the 3-way stopcock attached to the scaffold was connected to a port in the wall of the moist chamber (figure 2.5(3)) to make it possible to inject from the outside of the chamber and only open it once when the scaffold is connected and placed in the chamber. This method made the injections easier and less susceptible for mistakes. Still, after

the new protocols for the scaffold washing and cell suspension injections, there was an infection in the next experiment. This called for a drastic cleaning job of the whole HA. What was done is described in figure 2.3 in Cleaning B. This cleaning method proved successful, without having to use the autoclave for the moist chamber. Because of this, experiment 6 and 7 were infection free. First signs of an infection were a reason to immediately stop the experiments. In this way, the cells in the scaffold could be still alive when fixated and analyzed afterwards. After most experiments that experienced an infection, a test run was done with only culture medium in the system to see if the infection was still present. These test runs lasted around 4 days, which proved to be insufficient to test if the infection was resolved after the cleaning steps.
The first infection detected was a fungal infection in experiment 2 that ran for 11 days. White clouds of fungi could be found in the small tubing of the HA. Especially parts with a low flow like the tubing and small reservoir present on the mounting plate were heavily infected. In experiments 3 and 9 a fungal infection was detected on the scaffold after a mere 4 or 5 days of running (see figure 3.3). The first organoid experiment, experiment 4, luckily reached 8 days before the infection was signalized. The bacterial infections were discovered in the supernatants taken of the perfusate during the experiment and the medium had to stay clear in the reservoir stayed clear and not cloudy. Under the microscope with a 40x magnification lens small moving bars could be detected in the supernatant in case of a bacterial infection. In images A and B from figure 3.4 it is clearly visible that the cells have not left the vessels yet. In experiment 3 the difference to the previous experiments was a higher number of cells injected, around the $1x10^9$ HepG2s, in a larger segment. This made us chose to increase the flow rate at the start and during the experiment. The cells that are present in the vessels (see images A and B of 3.4) are from what can be seen still alive.

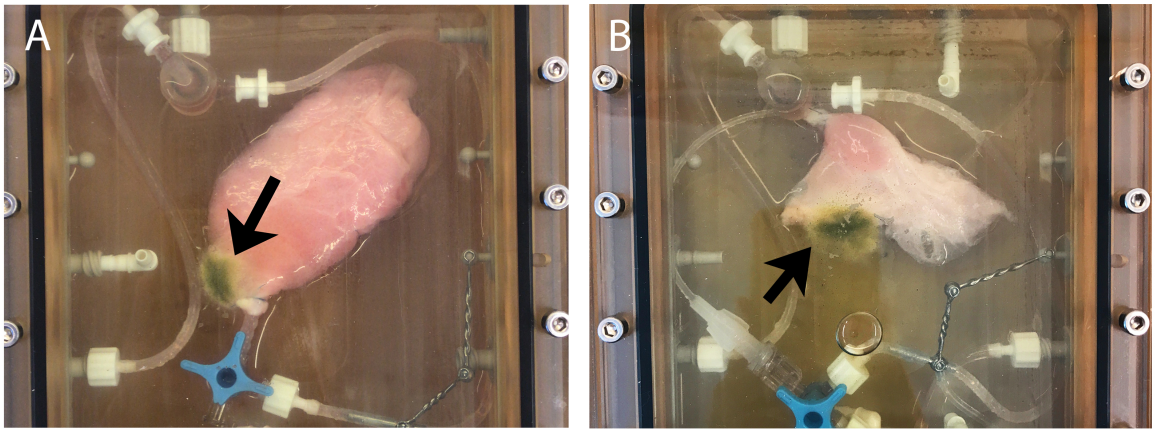


Figure 3.3: Image of the fungus that was growing on the scaffold of experiment 3 and experiment 9, indicated by the arrow.

Unfortunately experiment 3 was stopped after only 4 days caused by a fungal infection on the scaffold (figure 3.3(A)). This infection most likely originated from the scaffold, that should have been washed more properly. But, experiment 9 which also gave rise to a mold on the scaffold, was washed more thoroughly according to the second protocol (see table 2.2). This suggests that the fungus was already present in the scaffold before the start of the experiments and this particular infection could not have been prevented. From the segment a biopt was taken at the infection site for fixation and afterwards histological staining. Results of this H&E staining can be seen in images C and D from figure 3.4. The infection spreads with small thread like stripes and dots, with quite striking bodies (C) of the fungi. In D a difference can be seen between the ECM that is covered with fungus on the right and on the left the ECM that is not yet collonized.

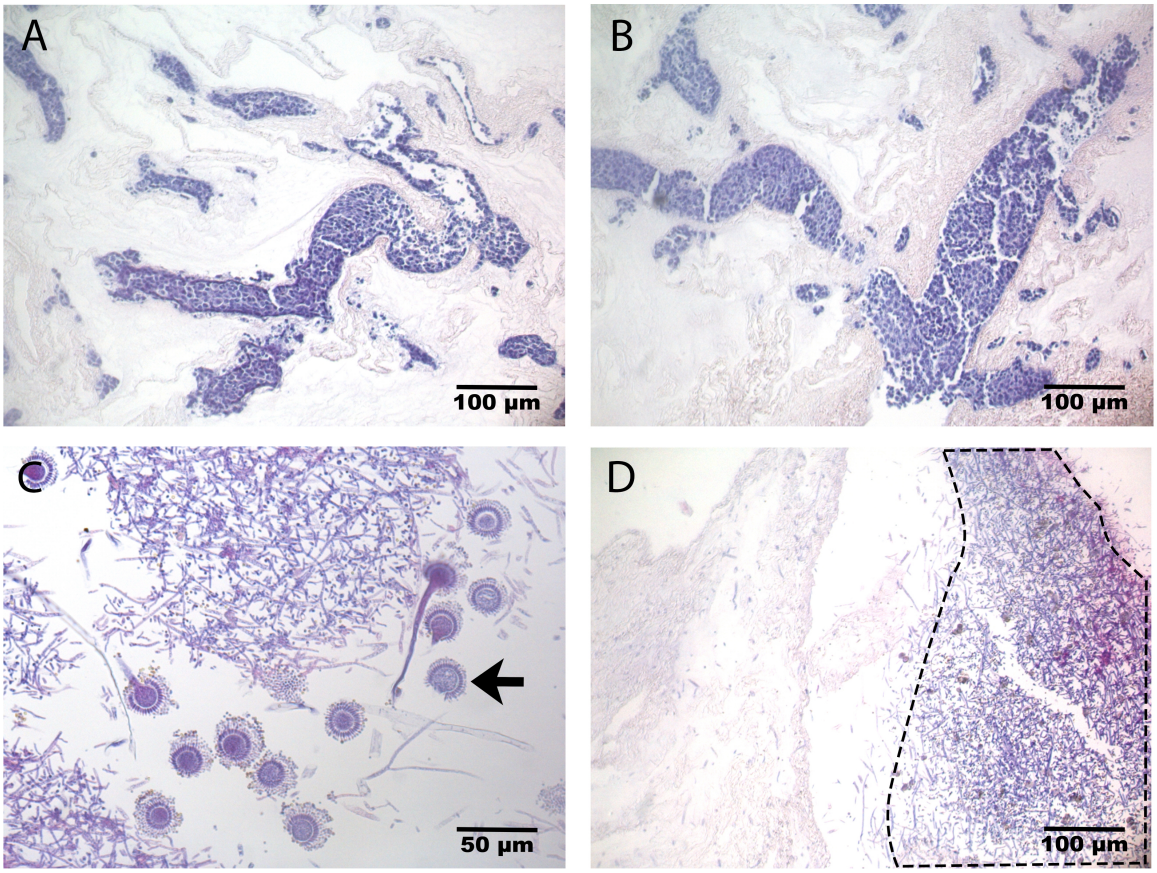


Figure 3.4: Images taken with a microscope from H&E stained slides from experiment 3 with HepG2s. (A)(B) Images of HepG2 cells that have grown and settled only in the vessels of the structures and almost none in the parenchyma. (C)(D) Images of the fungi that was growing on the liver segment after 4 days.

3.2. HepG2 recellularization

The purpose of recellularizing the scaffold with HepG2 cells was to validate the application of the HA. The HepG2 cells were a favorable choice because of its proliferative character, which means that they can be cultured to large numbers in a small period of time. All HepG2 experiments (1, 2, 3, 5, 7 and 8) were done with the same batch of HepG2 cells that were cultured and continued to be cultured during and after every experiment (up until passage 13 which was used for the qPCR data). The results of the HepG2 experiments consist of: GFP signal, histological staining, immunohistochemical staining and qPCR data.

3.2.1. Localizing HepG2s

Fluorescence images were taken with the EVOS from every HepG2 injected segment just after detachment from the HA. Figure 3.5 shows two images that illustrate different outcomes. Image A is the GFP signal from the HepG2s that were only 4 days in the scaffold, this shows the vessels and its branches because the cells are still in the vasculature and have not moved to the parenchyma yet. This is in accordance with the histological stained images from figure 3.4. Image B in contrary, shows a blurry but strong GFP signal through the scaffold wall. The honeycomb structure of the liver scaffold lays in front of the green signal. This means that in the scaffold grouped cells could be present and this makes this area suitable for further analyses and discovery of possible engrafted cells.

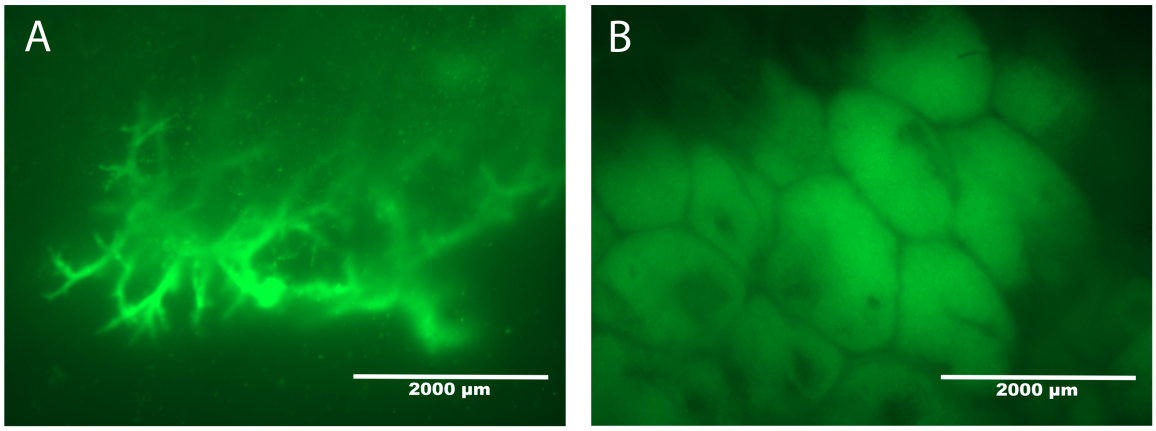


Figure 3.5: Images taken with the EVOS to evaluate the GFP signal that is caused by the GFP labeled HepG2s injected into the liver scaffolds. (A) Image taken at the end of experiment 3 which shows presence of the HepG2s in the vessels of the scaffold. (B) Image taken at the end of experiment 7 which shows a spread signal from inside the scaffold.

3.2.2. Cell distribution and engraftment

The first two experiments were a trial-and-error case using HepG2s as cell source. For these experiments the injections were done by opening up the HA for each injection. Both experiments had a flow rate of 3 mL/min for the entire experiment. Experiment 1 ran for 5 days for the first try and to see if cells could be found back in the scaffold. In figure 3.6, images A and C correspond with the results of experiment 1. These images are from a biopt that was located close to the cannula where all the cells were injected. In image A the HepG2s have lined a vessel wall in bulk and in the top left corner at the arrow cells have distributed inside the parenchyma. For a first experiment these were promising results. Image C and D are DAPI stain images of neighboring slides of image A and B, the same structure and distribution of cells can be found. DAPI stains the nuclei of the cells.
Experiment 2 involved a leaky segment, it did not swell on flushing and during perfusion. This may have caused minor cell engraftment in the scaffold. However, some groups of cells were found in the

scaffold as can be seen in images B and D of figure 3.6. These cells were again close to the main vessel where the cannula was placed. For experiment 2 the goal was to try let the perfusion run longer. Because of this the cells had 11 days to grow and proliferate, but the end of the experiment was marked by a fungal infection found in the tubing of the HA. In the H&E image (B) the cells still look alive after this longer period of time and have left the vessels to populate the parenchymal space.

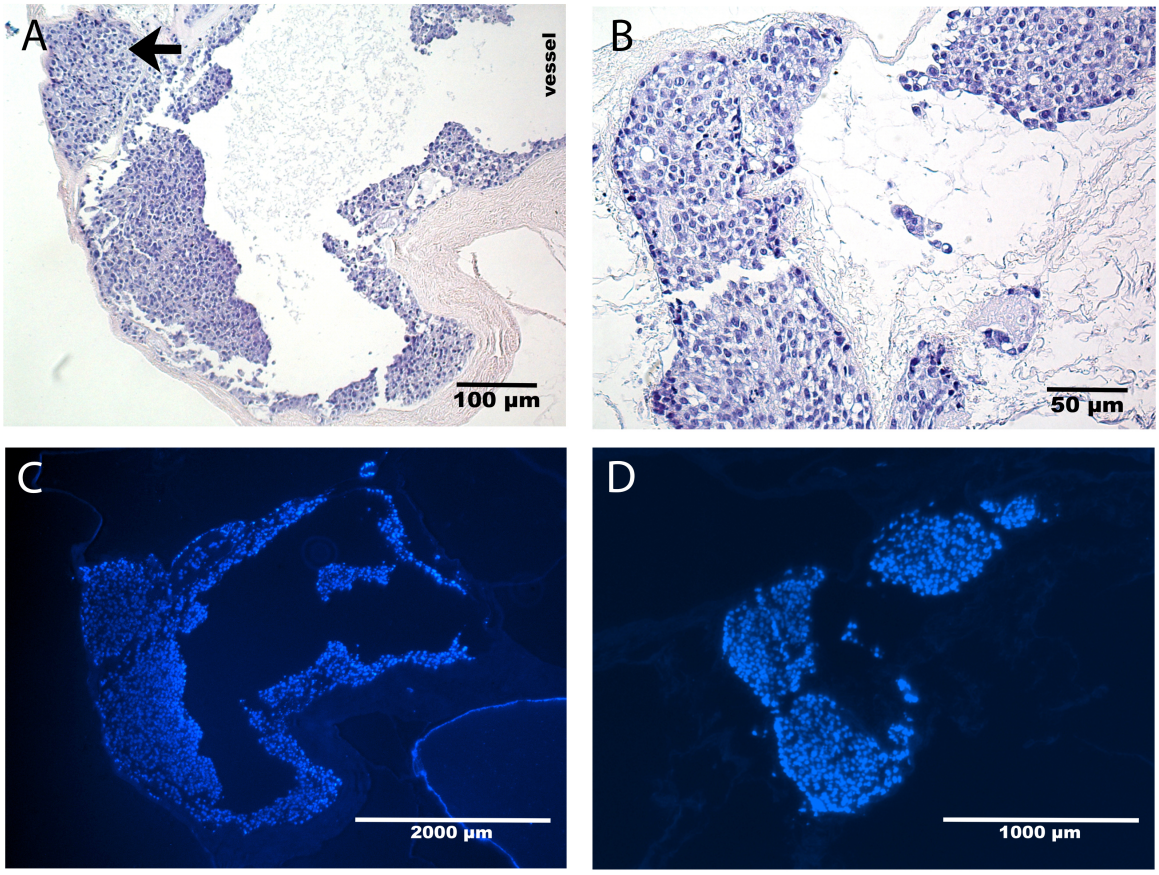


Figure 3.6: Images taken with the microscope of H&E stained and DAPI stained slides. (A) Image of engrafted HepG2s from experiment 1 and (B) experiment 2. (C) Image taken with ultraviolet light of DAPI stained slide from experiment 1 and (D) experiment 2.

Experiment 7 was the first HepG2 experiment again that was infection free. This made that it lasted for the planned 8 days in total. What was striking to see in this experiment for the scaffold, was the part where the cannula went into the scaffold, here the scaffold began to turn yellowish as can be seen in figure 3.7. This could have been a sign of abundant HepG2 cell growth into the scaffold. The H&E stained images A, B and C confirm this assumption (see figure 3.8). Around the vessel the cells went through the cell wall and engrafted into the parenchymal space. Image B shows the vessel wall and the neighboring parenchyma, only a single line of cells is still present inside the vessel walls because HepG2s tend to grow 2D along a surface. The bulk of the cells is distributed throughout the parenchyma. In this bulk, parts are found where no cells have grown (arrow in image A). This could be patches of proteins or remnants of dead cells. Image C shows the outer wall of the scaffold and the well repopulated inside. Striking in this image is the difference in cell shape, with no abundant cell death. Most cells are full and round, but as can be spotted in the outlined section are also smaller dots present. This could be a sign of apoptotic cells caused by the lack of nutrients reaching the cells, or a variation in cell shape because of characteristics a cancer cell line has. Image D is a slide from one biopt further than A-C. More cells are lining the vessel wall

and less have entered the parenchyma. A reason for this decrease in parenchymal engraftment can be a consequence of decreasing flow and pressure. The further away from the cannula, the lower the pressure may be in the vessel and the lower the number of cells go through the vessel wall.

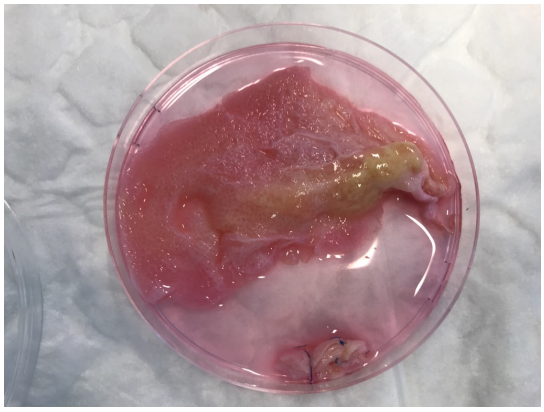


Figure 3.7: Photo of the scaffold just removed from the HA at the end of experiment 7. The cannula was attached at the yellowish part of the scaffold.

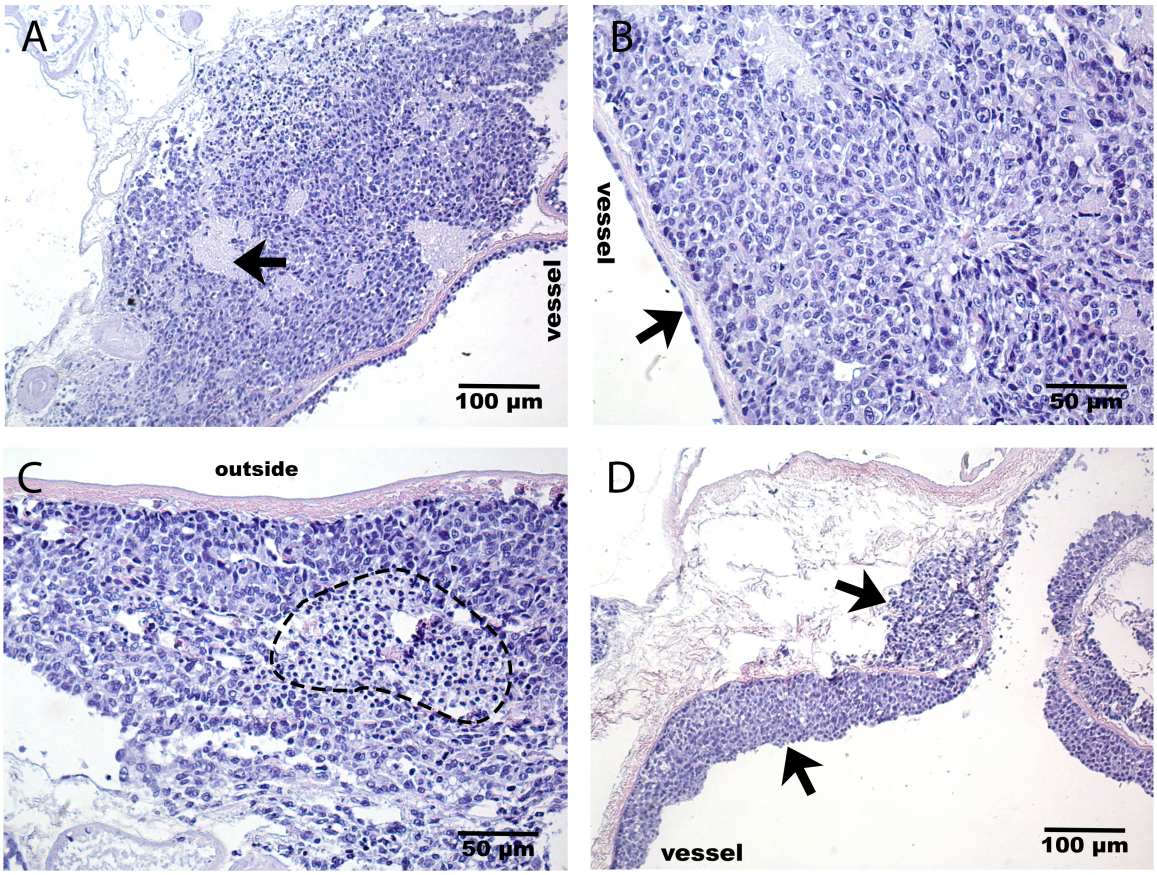


Figure 3.8: Images taken with a microscope of H&E stained slides. (A)(B)(C) Fully filled parenchyma with HepG2 cells next to main vessel of the inserted cannula. (D) Slide of one biopt further down the vessel. The white spaces that are marked with 'vessel' illustrate where in the tissue the cells have engrafted.

The last HepG2 experiment that was done, experiment 8, lasted for 19 days. The histological staining with H&E can be seen in figure 3.9. This experiment was a partial success, compared to the previous HepG2 experiments the cells had distributed and engrafted through the whole scaffold (image A). Unfortunately, in this longer period of incubation, a fungus found its way into the scaffold (image D). At the end of the experiment, the roller pump tubing had worn out and created vacuum within the system and back-flow going from the liver segment. These multiple factors caused the cells to look less full and dense compared to the previous experiment (figure 3.8). In image B it can clearly be seen that the center of the bulk of cells most probably have had less nutrients from the perfusate and are therefore in apoptosis, the nuclei lost their staining. Image C shows the engraftment of the cells through the whole parenchyma.

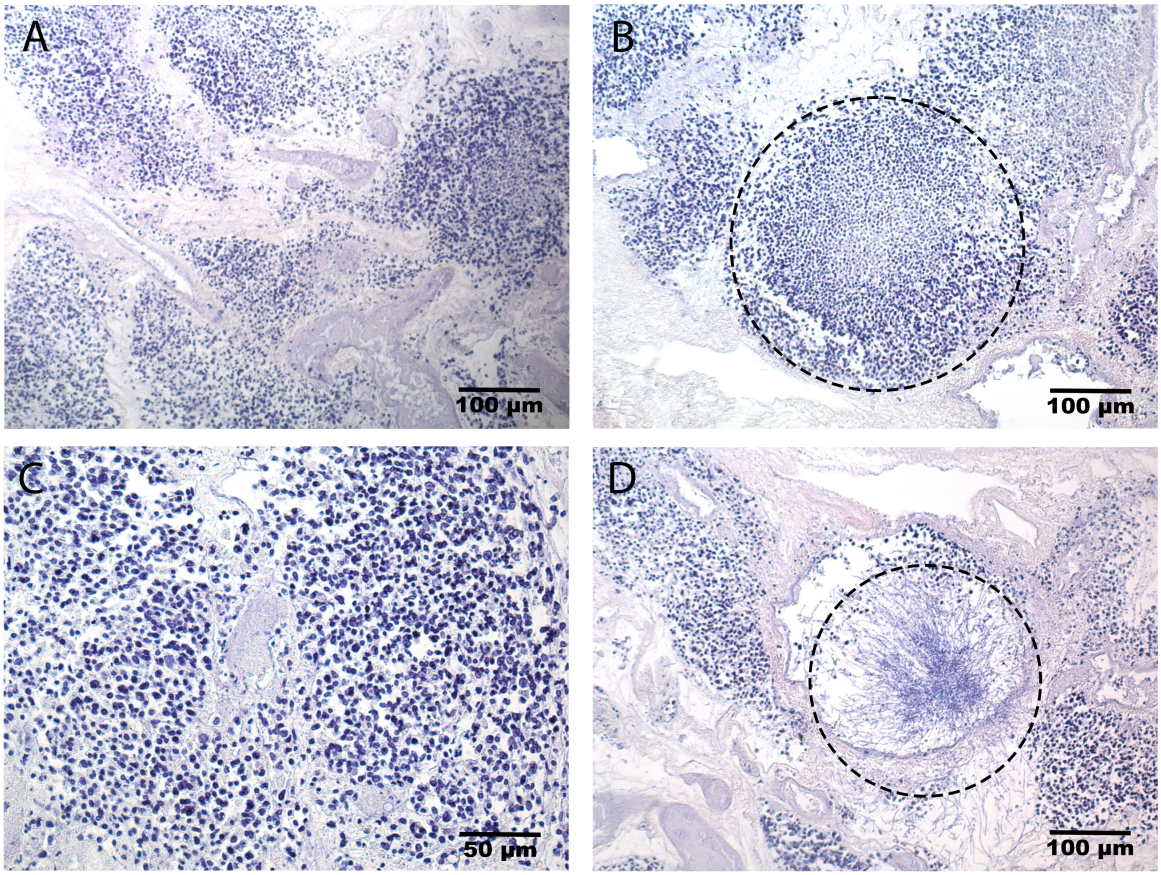


Figure 3.9: Images taken with microscope of H&E stained slides. (A)(B)(C) Fully filled parenchyma with HepG2 cells after 19 days of perfusion. (D) Image showing the infiltration of fungi into the scaffold.

3.2.3. Immunohistochemistry

Slides of HepG2 experiment 7 (infection-free) were used for the IHC staining analysis. The slides were stained for the antigens: Collagen type I, Ki67 and HNF4α. Results can be found in figures 3.10 and 3.11. The HepG2 cells in the scaffold stained positive for all antigens.

Figure 3.10 shows two images in which Collagen type I was colored with the DAB stain. Collagen type I is an indication of the presence of ECM, because collagen is one of the main components of the scaffolds ECM. The earlier hypothesis that the patches in figure 3.8(A) at the arrow could be proteins or dead cells, is highly probable because these patches are not part of the ECM (negative for Collagen type I). The collagen can be found in the vessel wall of both image A and B. Subtle lines

of collagen are present in between the bulk of the HepG2 cells (arrow in B), which confirms that the cells have engrafted into the ECM and therefore the parenchyma of the scaffold.
Both images of figure 3.11 show a nuclei staining, HNF4α is a hepatocyte nucleus marker and Ki67 is a marker of cell proliferation also found in the nucleus of the cell. In image A in the upper half of the image, a dense structure of engrafted HepG2s can be found where the majority of the nuclei have a positive staining. Going deeper into the scaffold, less full and less dense structure is found with a decrease in cells that are positively stained for HNF4α. A reason for this can be that the cells are less content in terms of lacking nutrients, still busy finding their final place or being in apoptosis. Image B shows a few positive stained cells for the Ki67 protein. This protein is less abundant because cells are only stained when they are in the G1, S, G2 and M phases of the cell cycle [20].

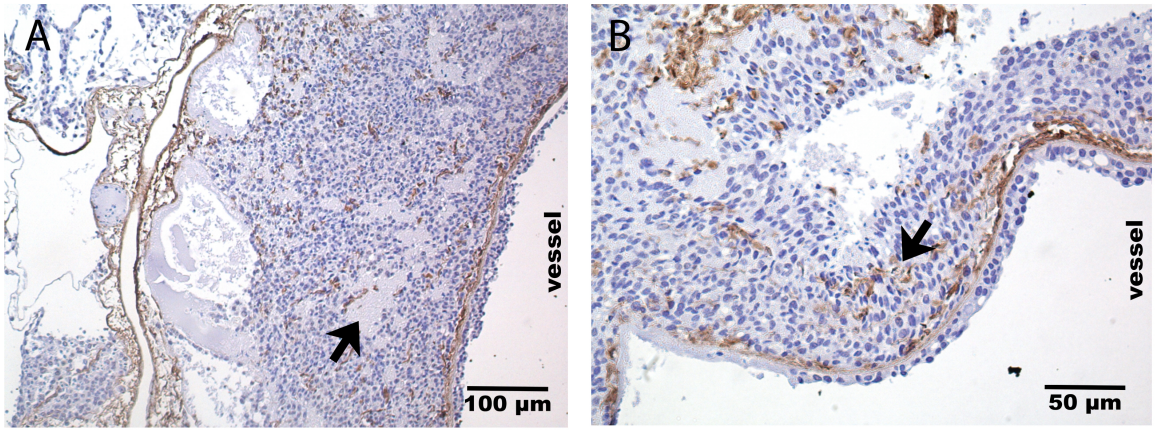


Figure 3.10: Images taken with a microscope of IHC stained slides. Image A and B are stained for Collagen I.

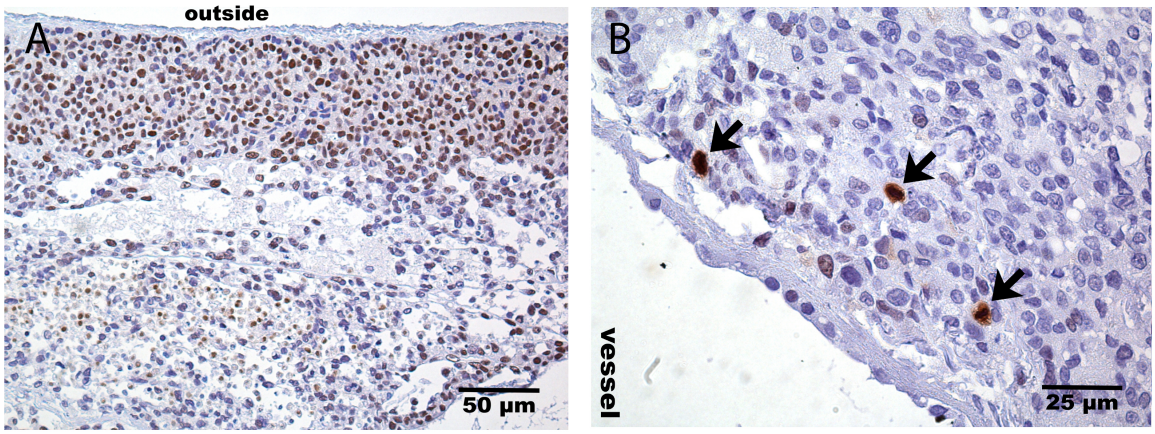


Figure 3.11: Images taken with a microscope of IHC stained slides. Image A is stained for HNF4α and image B is stained for Ki67, both are nuclei stainings.

3.2.4. Gene expression

Snapfrozen samples were taken from the scaffolds of experiments 1, 2, 3 and 7. Unfortunately evaluation of the RNA isolation of these samples showed inconsistencies in the samples from experiments 1, 2 and 3. This was seen in the Ct values of the samples too, the housekeeping gene (GAPDH) gave Ct values higher than 25. For the housekeeping gene a Ct value of below 20 is preferred because the data for the other genes will be more reliable. Therefore, the qPCR data of only experiment 7 is used and as control the HepG2 cells from the culture flasks went through the RNA isolation and cDNA cycle as well. Those results can be seen in figure 3.12, the two graphs are a $2\wedge - \Delta\Delta Ct$ representation of the data. This shows in which 'fold' RNA is expressed in the injected HepG2s compared to the control HepG2s from the culture flask. A high increase of Ki67 is visible in graph A and a slight decrease of albumin is seen in the graph B.

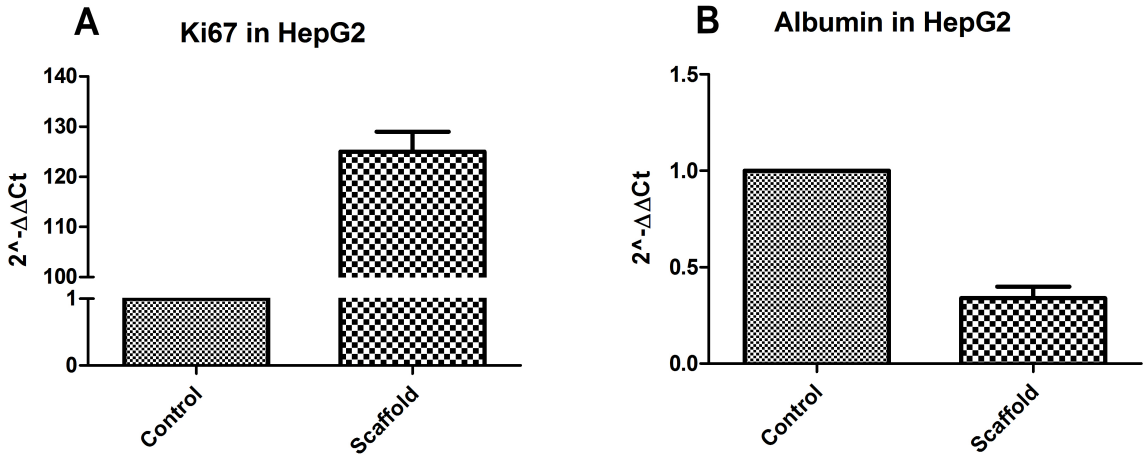


Figure 3.12: Graphs representing the RNA expression of albumin and Ki67 in HepG2s. HepG2 samples taken from the culture flask function as control, the other samples originate from experiment 7 and were taken after injection into the scaffold and incubation of 8-10 days.

3.3. Organoid recellularization

In total three of the experiments were done with liver-derived organoids. This is due to the long culture time needed to get a large number of cells, in contrast to the HepG2 cell line. On average, it took about three weeks to culture the organoids from the start in the spinner flasks till the end. The organoid culture knows 3 stages, the culture in the well plate, the culture in the spinner flasks and the culture after injection in the scaffold. These different stages are taken into account with the qPCR data. Every organoid experiment is done with another cell line of organoids, from different donor livers. The results of the organoid experiments consist of: histological staining, immunohistochemical staining and qPCR data.

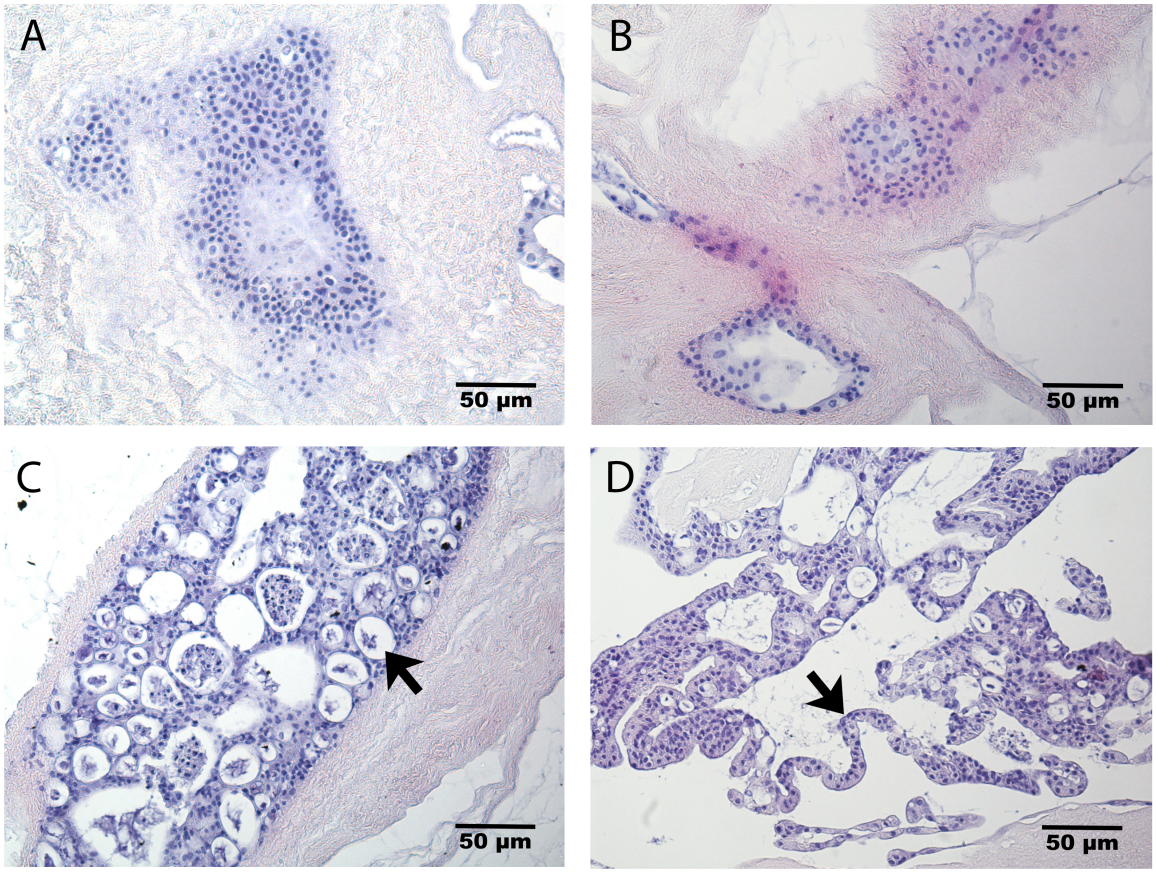


Figure 3.13: Images taken with microscope from H&E stained slides of experiment 4, scaffold was repopulated with organoids and perfused for 8 days. (A)(B) These images show cells that have successfully engrafted into dense parenchymal structure. (C)(D) Images of organoids that have settled in open space parenchyma and formed cyst-like structures and strands.

3.3.1. Cell distribution and engraftment

The first experiment done with organoids as cell group was experiment 4. The difficulty of the organoid experiments was the absence of GFP labelling, the scaffold as a whole had to be cut in biopsies for analysis. There were fewer organoids at the end of the spinner flask culture compared to the HepG2s in the culture flasks. The parameters were comparable to those of the previous experiments. Except for the duration of the experiment, it was favorable to have the scaffold perfused for more than 10 days which is the time needed for organoids to settle down and start to proliferate. Despite a bacterial infection that was early on detected in the supernatant, the experiment had already run for 8 days. In the scaffold live and engrafted cells can be found as seen in images

A-D of figure 3.13. Images A and B show cells that were engrafted into the denser structures of the parenchyma and with no cyst-like formations. In images C and D on the other hand the cyst-like structures can be found (arrow in C). This is a characteristic of how organoids like to grow which are cultured primarily with EM that contains Forskolin. Likewise, there was strand formation which is visible at the arrow in image D. It seems that the organoids only tend to form cyst-like structures if they have the space, not in the denser parts of the ECM. In general, the engraftment results are quite good for the first try with organoids as cell group.

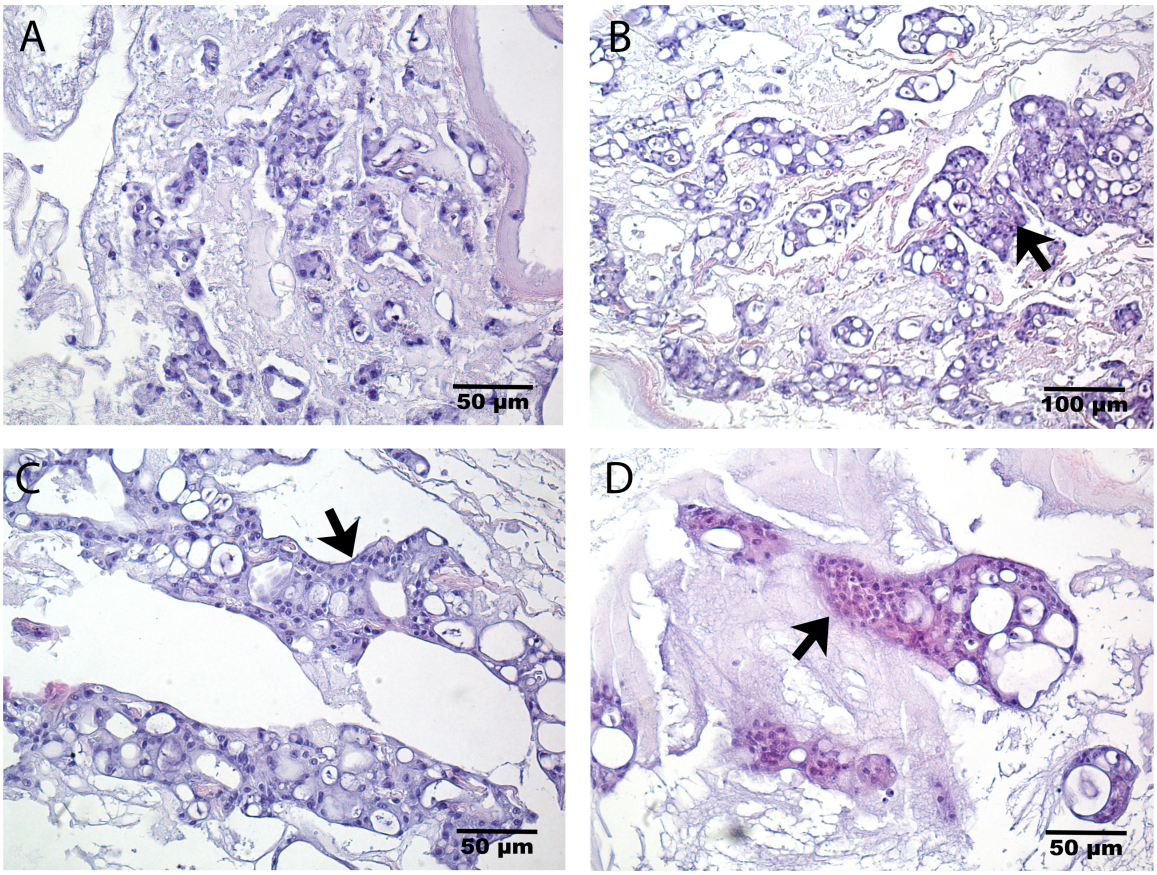


Figure 3.14: Images taken with microscope from H&E stained slides of experiment 6, scaffold was repopulated with organoids and perfused for 10 days. In all images the tendency of the organoids to forms cysts is clearly visible.

The next experiment that used organoids as infused cell group and had no sign of any infection after 10 days was experiment 6. In the images of figure 3.14 a lot of engrafted organoids can be seen. In the case of experiment 6, many live cells were found and were distributed throughout the scaffold biopsies instead of concentrated in a few spots. The cells were engrafted in the parenchymal space, yet again they tend to form many cysts. This tendency can differ between different organoid lines from different donors. The organoids are encouraged to form 3D bubbles and take in the surrounding liquids in the BME in the well plates and after a while they change their structure. In the scaffold the organoids had no time to make this structure change and they hold on to the cyst formation. In image B and C the arrows point at areas that contain many cells that were alligned close to each other with no cyst formation in between. There is a difference between the cysts, some have a small wall and are intact and for some this wall lacks and looks like it has collapsed. Image D shows again that the organoids grew more controlled in the dense structure of the parenchyma.

The last experiment done with organoids was experiment 9. The culture of the organoids in the spinner flasks took longer than the usual three weeks and a smaller number of cells was infused into the scaffold, in total only $5x100^6$. Unfortunately the experiment only lasted a total of 5 days because a fungal infection was found on the scaffold (see figure 3.3). In this experiment a parameter was changed, the Forskolin was left out from the Expansion Medium (appendix A.2) in the HA set up. In this way, the assumption that Forskolin was responsible for the cyst formation in the previous experiments could be investigated. In figure 3.15 two images are shown of the scaffold after histological staining with H&E. The cells were harder to find because of the smaller number of infused cells and the shortened incubation time. The cells that were found on the other hand, did show less cyst formation than the previous organoid experiments.

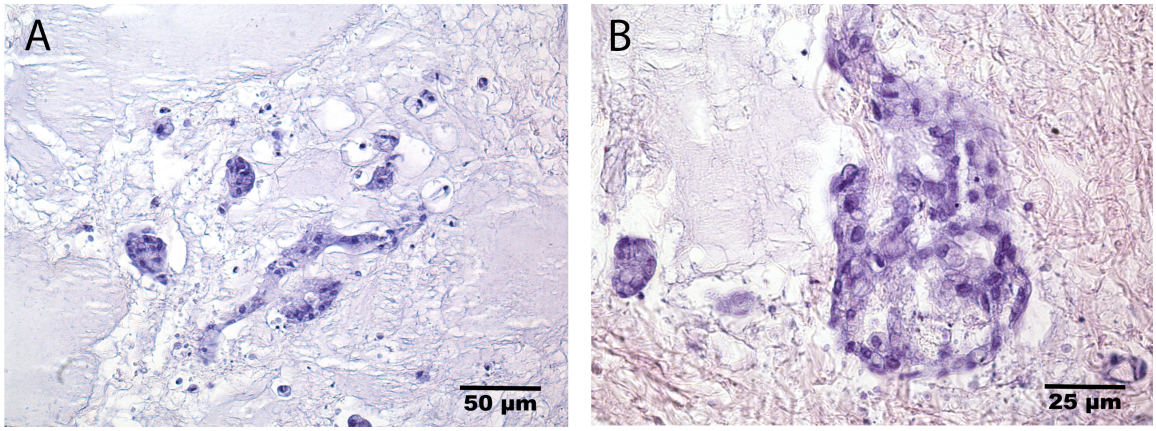


Figure 3.15: Images taken with microscope from H&E stained slides of experiment 9, scaffold was repopulated with organoids and perfused for 5 days. In both images the the organoids did not have the tendency to forms cysts like the previous experiments.

3.3.2. Immunohistochemistry

Slides of organoid experiment 6 (infection-free) were used for the IHC staining analysis. The slides were stained for the antigens: CK7, CK19, Ki67 and HNF4α. Results can be found in figures 3.16 and 3.17. Figure 3.16 shows the results of the DAB staining for the CK7 and CK19 proteins, which are known to be cholangiocyte markers. What is striking about image A, is the intensity of the staining. The intensity could be explained by a too long incubation of the slides in DAB. All cells shown in this image, seem positive for this protein, but still the nucleus is visible because of the hematoxylin staining. Above that, in the bottom of image A it is of notice that the ECM looks damaged. This damage could have been caused by the 'cooking' step of the IHC protocol. Image B on the other hand, shows a more mild signal for the CK19 compared to that of CK7. The slides had the same DAB incubation time, it may be found that there is more CK7 present than CK19.
Figure 3.17 shows two images that were stained for the HNFα and Ki67, as previously seen in figure 3.11 for the HepG2 experiment. Comparable results are reached in these nuclei specific stains; the HNF4α shows positivity in most cell nuclei and Ki67 shows positivity in a few nuclei. Image B is another proof of an extended DAB incubation time, the Ki67 should preferably be only stained in the nucleus and not fade out to the surrounding tissue. The images resulting from the IHC staining, are again proof that the organoids engrafted into the ECM in big numbers and seem to have settled.

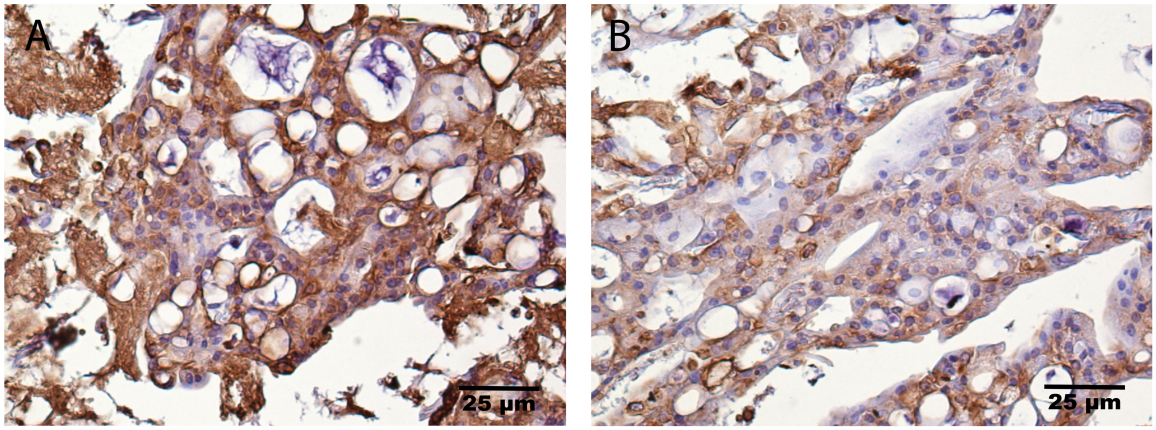


Figure 3.16: Images taken with microscope of IHC stained slides. Image A is stained for CK7 and B is stained for CK19, both are cholangiocyte specific.

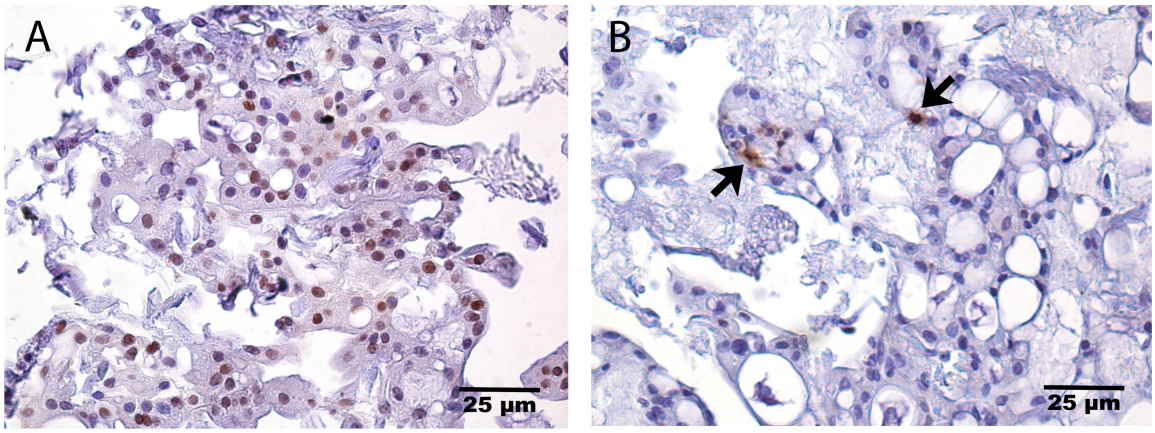


Figure 3.17: Images taken with microscope of IHC stained slides. Image A is stained for HNF4α and image B is stained for Ki67, both are nucleus proteins.

3.3.3. Gene expression

Snapfrozen samples were taken from the scaffolds of experiment 6 and 9, because experiment 4 had the presence of a bacterial infection which could alter the data of the qPCR. For these experiments, samples were taken of the organoids in the stages of the wells plate, the spinner flasks and after injection into the scaffold with an incubation time of 10 (Exp6) or 5 (Exp9) days. The $2\wedge -\Delta Ct$ representation of the qPCR data is shown in the four graphs of figure 3.18. In figure 3.19 expressions of other genes are shown in the $2\wedge -\Delta\Delta Ct$ representation where the organoids from the wells plate functioned as the control. A small side note before analyzing these data, the RNA isolation and the GAPDH Ct value of the organoid samples taken from the scaffold imply that there is some kind of pollution in the samples. This could be caused by the tissue that was used, that alters the ratio of the nucleotides. Still, we will take the values of the scaffold samples into account when comparing the data of the different samples. The data of both experiment 6 and experiment 9 are placed in each graph, although the comparison is hard to make because of the different variables, trends could be found.

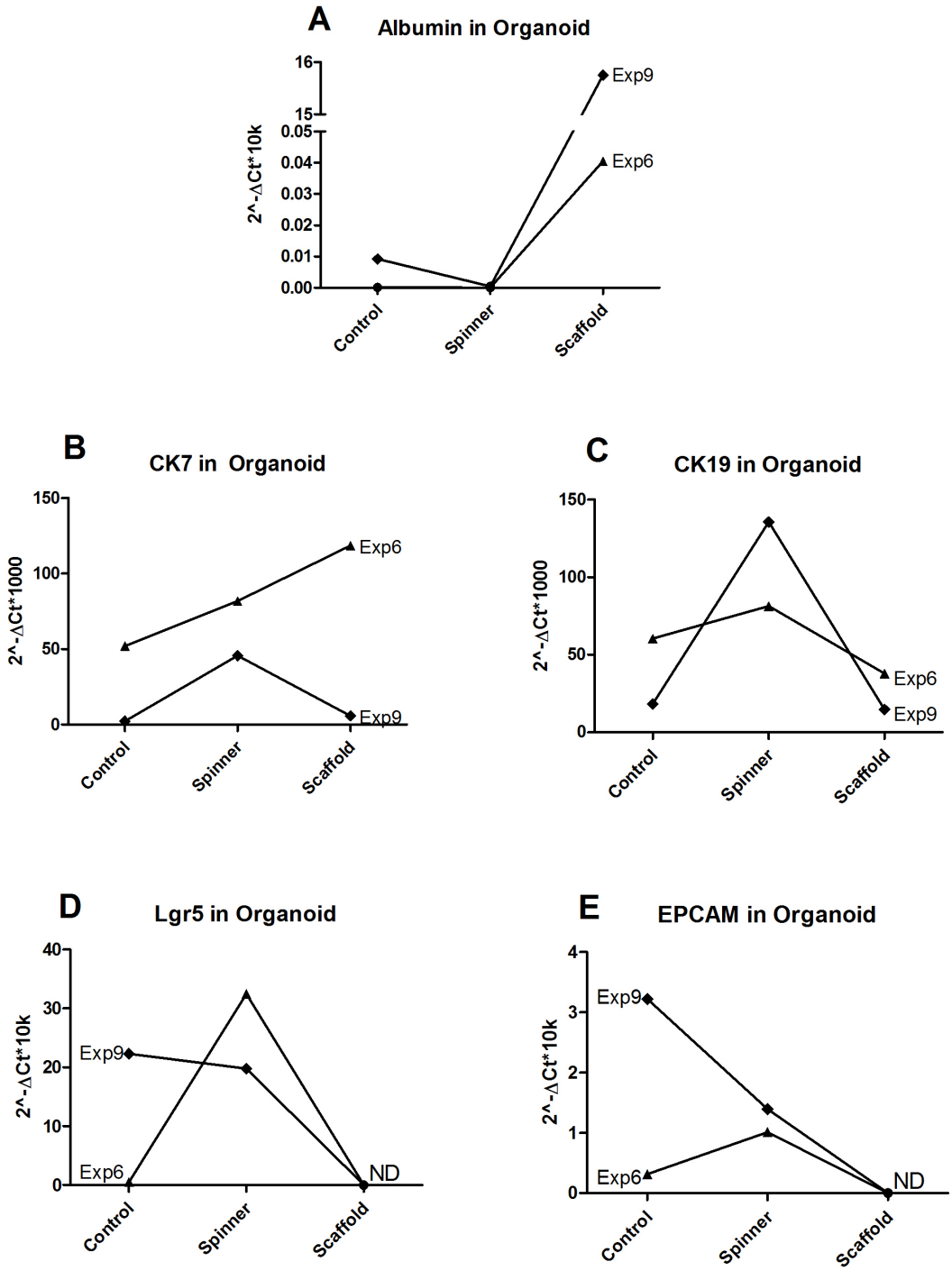


Figure 3.18: Graphs representing the RNA expression of albumin, CK7, CK19, Lgr5 and EpCAM in organoids. Organoid samples taken from the wells plate function as control, the other samples were taken during culture in the spinner flasks and after injection of the cells into the scaffold after 10 (Exp6) or 5 (Exp9) days of incubation.

In figure 3.18 the $2\wedge-\Delta Ct$ is shown for albumin, CK7, CK19, Lgr5 and EpCAM. The values shown, are the difference between the GAPDH (house keeping gene) Ct value and the Ct value of the targeted gene in that specific sample. The first image, image A, gives insight into the albumin expression levels in both organoid experiments. What is most striking in this graph, is the large difference

of the albumin levels in the scaffold culture phase. This is a clear example of the effect of different donor material that is used for the primary organoid culture. Also, after transferring the organoids from the spinner flask into the scaffold, the expression levels go from almost non-detectable to detectable. It is likely that this increase is caused by the environment consisting of the ECM and the circumstances created by the perfusion flow.
In images B and C, the expression levels of CK7 and CK19, which are both cholangiocyte markers, are shown. The first difference that stands out, is the starting level of both experiments. The levels of the organoids in the plate that function as control, are undeniably higher in experiment 6 than experiment 9. In these images it can also be seen that both CK markers increase in expression when they are cultured in the spinner flasks. Except for CK7 in experiment 6, all the other samples of the CK markers go down when cultured in the scaffold. Image D shows the expression levels of Lgr5, a stem cell marker, of both experiments. Experiment 6 experiences an increase towards the spinner flask culture, where experiment 9 starts with a higher level and slightly decreases. Both experiments have undetectable expression levels in the scaffold culture. The last image, image E, shows the expression levels of EpCAM. EpCAM, such as CK19, is a cholangiocyte and organoid marker. It peaks in experiment 9 in the control phase and decreases to an undetectable value. Experiment 6 has the same trend as the CK19 marker, only with lower values.
In figure 3.19 the $2\wedge-\Delta\Delta$Ct is shown for Ki67, HNF1 and HNF4. The values shown, are the difference between the GAPDH (house keeping gene) Ct value and the Ct value of the targeted gene in that specific sample. Image A shows the expression of Ki67 in all culture phases, it marks the proliferation of the cells. For both experiments there is almost no proliferation in the spinner flask culture and a large increase towards the scaffold culture. This indicates that the cells proliferate when infused and engrafted into the decellularized liver scaffold, where experiment 6 shows almost a two-fold of experiment 9. HNF1β is an indicator for early cholangiocytes, it is a phase between a stem cell maturing into an adult cell. In image B, with each step of the organoid culture the HNF1β expression increases for both experiments, surprisingly the expression of experiment 9 is higher than that of experiment 6. The last image, image C, shows the expression of HNFα. This is a hepatocyte marker with a high increase for experiment 6 from the spinner flask to the scaffold culture. The expression of HNF1β is not determined for experiment 9 in the scaffold. What could be derived from this result, is that after a longer period of time (10 days in the scaffold for experiment 6), the cells tend to mature into hepatocytes more likely than into cholangiocytes. The early cholangiocytes are seen in experiment 9 in image B, which had a scaffold culture that lasted only 5 days.

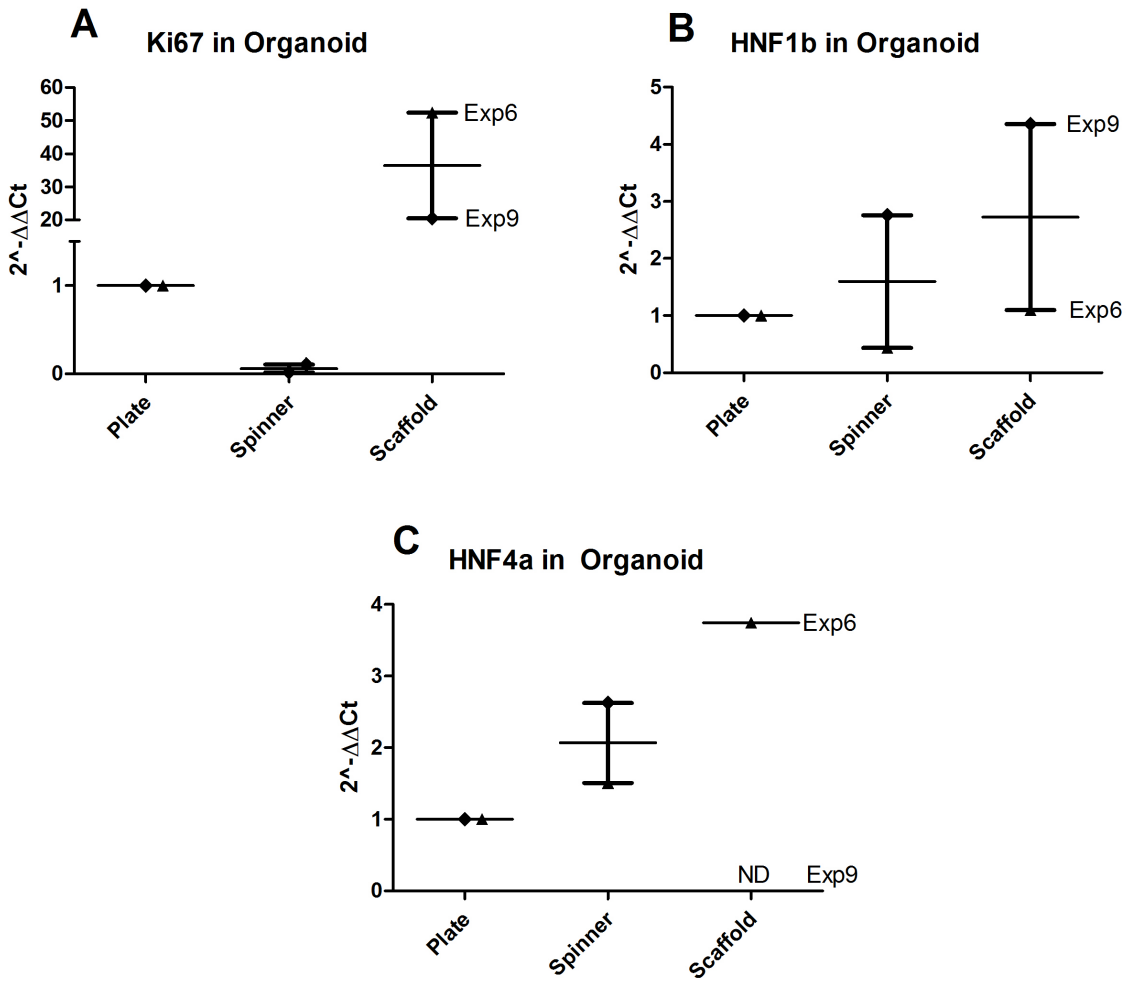


Figure 3.19: Graphs representing the RNA expression of Ki67, HNF1β and HNF4α in organoids. Organoid samples taken from the wells plate function as control, the other samples were taken during culture in the spinner flasks and after injection of the cells into the scaffold after 10 (Exp6) or 5 (Exp9) days of incubation.

3.4. Albumin levels

Figure 3.20 shows two images of cells that are stained for the production of the albumin protein. Image A represents the cells from the HepG2 experiment and image B those of the organoid experiment. A clear difference can be seen, overall the HepG2 cells are stained positive and the organoids seem to be stained non-specific. The DAB mostly stained the ECM and not the cells. It is known, that HepG2 cells secrete more albumin than the liver-derived organoids. The graph in image C shows a comparison of the $2\wedge -\Delta Cts$ of albumin levels of the HepG2 experiment 7 and organoid experiment 6 (both infection-free). This value is a representation of the difference between the Ct values of the housekeeping gene (GAPDH) and the measured albumin levels. A noticeable difference is present between the HepG2 and organoid data. Going back to the IHC staining results, a trend can be seen between higher albumin secretion in HepG2s compared to the organoids.

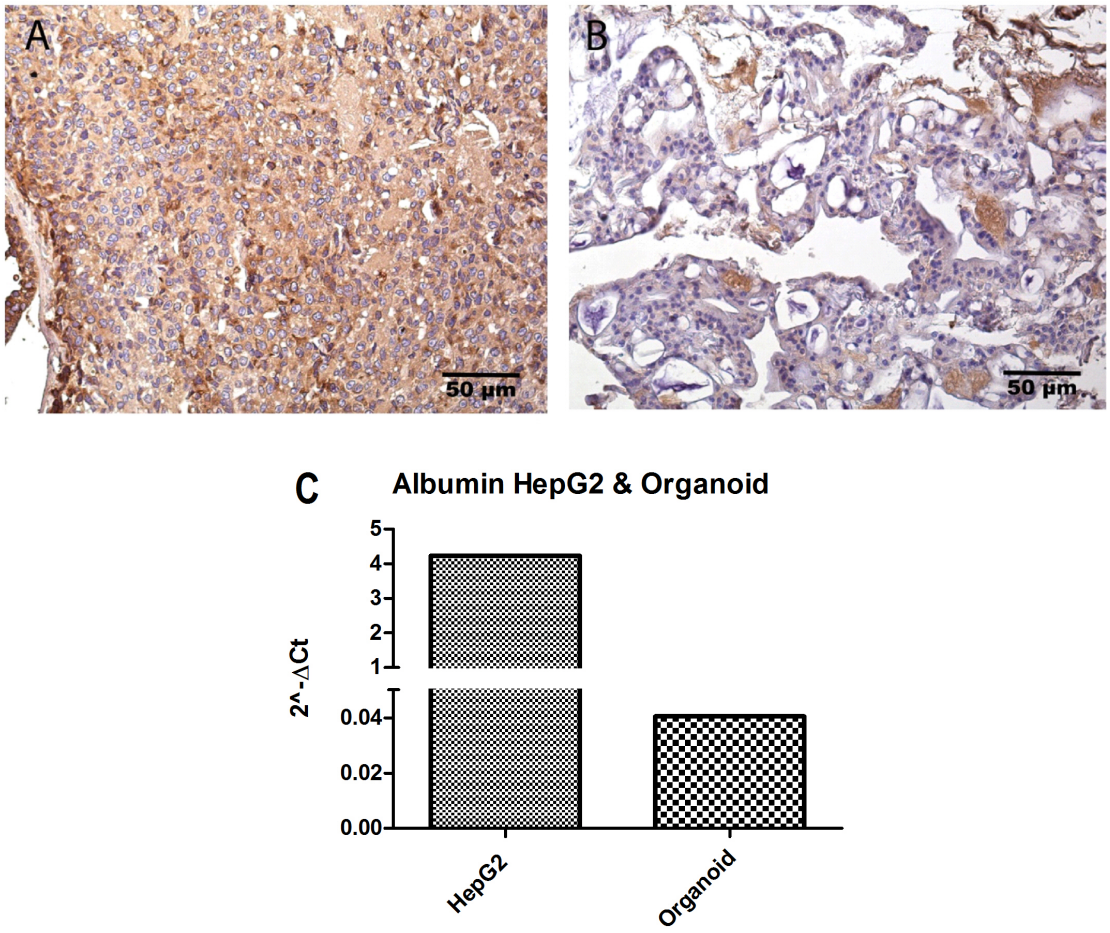


Figure 3.20: Images taken with microscope of IHC stained slides. Image A is from the HepG2 experiment and image B is from the organoid experiment. The graph in image C represents the δCt of albumin levels against the housekeeping gene after qPCR analysis.

4

Discussion

The aim of this project was to validate the use of a perfusion-based bioreactor to recellularize a decellularized porcine liver scaffold with different cell lines. This research is a step towards engineering complex organs from a decellularized scaffold and thereby minimizing the current donor organ shortage. In this case, it will target the liver and the cell groups of interest, which are HepG2s and organoids. The focus lies on the engraftment and viability of the injected cells influenced by variables in the experiment. If the set up and method of this research will prove successful, next steps can be taken towards whole organ engineering.
An important result of this research is the amount of living cells that engrafted into the scaffold during the incubation. The longer the experiment, the more cells moved from the vessels to the parenchyma and proliferated. Especially the organoid experiments showed new and promising results. It is a pity that there were only three experiments done with organoids, with two of them marked with an infection. This low number was due to the time needed for culturing and expanding the organoids. Luckily, the spinners flasks have proven to be useful to shorten the culture time of organoids to a high number. Previous research done on the recellularization of porcine liver scaffolds with HepG2s and organoids with the same research group was the thesis of A. M. de Vries (2018)[21]. In this thesis, the recellularization set up consisted of a self-built bioreactor like system in an incubator with a constant temperature and air composition (5% CO_2 and 95% O_2). The set up contained roller pumps, a culture medium reservoir and the scaffolds were placed in a petri dish. All components were connected by tubing that was replaced each experiment. This research had no occurrence of any infection, due to the infusion step taken in a flow cabinet and the perfusion set up was placed in an incubator which was less prone to infection. Multiple parameters in our research originated from both the results retrieved from de Vries (2018)[21] and from literature, for instance the multiple injections and the flow rate. However, less cells and especially living cells were found in the recellularized liver grafts of de Vries (2018)[21]. Our research went a step further in the infusion of a higher cell number, longer incubation time and the use of the HA set up.
The infections were a challenge to overcome, being that the sources of the infections were hard to locate. Once an infection manifested in the HA, there was a high probability it would still be there in the next experiment. Flushing the system with demiwater and Mucasol proved insufficient in fighting an infection. The HA has the option to autoclave the moist chamber, yet the infection could carry on in any other part of the system. The perfusate is pumped around the entire system continuously and thus an infection can immediately spread to every surface or corner in the system. Luckily, a thorough cleaning protocol of every component and single parts of the set up , plus the exclusion of the mounting plate with multiple sensors, kept the system infection free during experiment 6 and 7. Still, in experiments 8 and 9 new infections were located, one was probably caused by the prolonged incubation time of 19 days and the other by an infection on the scaffold as seen pre-

viously in experiment 3. These infections that most likely originated from the scaffold in less than 4 days of incubation, were already present in the decellularization phase or during the cannulation of the scaffold. It was unavoidable that these infections took place during the recellularization process. A solution to the other infections that occurred, could be to place the whole set up in a sterile environment. Still, the cell culture before recellularization and the multiple injections could give rise to other infections. It will stay an infection prone process.
Difficulties concerning the organoid recellularization are the differences in how the cells behave in the scaffold. Every organoid culture originates from an other donor or expand liver and influences the way in how fast or slow they settle down and start proliferating and/or differentiating. As stated in Uygun et al. (2015)[22], even primary hepatocytes need 7 to 10 days of culture to stabilize the gene transcription levels and their metabolic fuctions. Striking to see in the H&E stained images of the organoid experiments is the cyst-like structures that formed. This is due to the Expansion Medium (EM) used, EM is intended for organoid cultures in a wells plate. They are encouraged to form 3D structures that tend to take in surrounding fluids. It is not certain that the cyst structures have a negative influence on the performance and engraftment of the organoids. A way in which this tendency could be reduced is to leave out the Forskolin in the EM, which was done in experiment 9 and proved to be successful.
Experiment 7, which had a high number of HepG2s infused into the scaffold and was incubated infection-free for 8 days, showed that parts of the scaffold turned an opaque yellow. This is in line with obtained results in the study of Baptista et al. (2011) [7]. The histological staining of these cells showed the distribution of the cells in the matrix comparable to that of Zhou et al. (2015) [23] which also resembles the structure and distribution of a normal liver. The difference between shorter and longer incubation times showed after histological staining that the cells that were first located in the vessels, moved to the matrix and parenchyma of the scaffold after more days. Similar results were reached in the study of Uygun et al. (2010) [22] that compared the H&E stainings of the recellularized scaffold at 4 hours, 1 day, 2 days and 5 days of perfusion culture. The results originating from the IHC in our study prove that the HepG2 cells are alive, engrafted in bulk and have a high albumin expression. To further prove the albumin production of the cells, Baptista et al. (2011) [7] took and analyzed the culture medium for albumin concentrations. This would be a great next analysis for this research. Cells were found to be proliferating by staining it for Ki67, showing an overlap with results of previous studies [7] [11].
During the experiments of this study, the HA has functioned for a period of time of almost 3 weeks. A previous study done by Robertson et al. (2018) [24] with the recellularization of a rat liver, even lasted up to 28 days which proves it should be possible to incubate liver cells in the scaffold for this period of time. In this project the extended periods of continuous usage of the HA caused multiple broken-down parts. Still, proof for the validation of the HA set up in a non-sterile environment are experiments 6 and 7 that lasted up to 10 days without any sign of infection and high cell engraftment in the histological staining. These results show great promise for further research with the Harvard Apparatus, which had only known experiments that lasted less than a day.
A previous search in literature gave recommendations (see the table in appendix A.4) for the parameters applied to the perfusion system and injection techniques. A multi-step infusion method, which is the use of multiple injections during the continuous perfusion of the scaffold, resulted in the best outcomes in terms of engraftment and cell viability [11]. Those studies were mostly done with rat liver scaffolds, which gave a starting point for this project. The majority of the successful applied flow rates were taken into account for the starting flow rate of 3mL/min in the first experiments. Experience learned that the scaffolds and cells could withstand higher flow rates and it could even promote the distribution into the parenchyma of the scaffold. During the experiments almost no changes were made to the parameters used for the perfusion, which had a lower priority in this research. For further research using the HA, more in-depth analysis could be done concerning dif-

ferences in flow rate, pressure, pO_2, pCO_2 and pH to optimize the results.
The biggest shortcoming of this research is that it is not yet statistically significant. A high N number could not be reached because of the changing parameters and other factors. Every liver segment differed in size, cannulation and swelling when perfused. The results were also highly dependent on the cell types and cell number. This also indicates a low reliability of the values obtained from the organoid scaffold samples. Between experiment 6 and 9 the circumstances were too different which made a full comparison impossible. Only trends could be obtained from the scarce data. The number of experiments that were done in this study were highly dependent on the time the cell cultures took, for the organoids meaning more than 3 weeks. Above that, the infections made the results in a way unusable and caused delays when the HA had to be cleaned repeatedly. This research can be classified as a trial-and-error case, still with promising results for further research.

5

Conclusion and Recommendations for future work

5.1. Conclusion

In conclusion, the new set up with the Harvard Apparatus for recellularizing a decellularized porcine liver scaffold with a HepG2 cell line and liver-derived organoids proved partly successful. The HA itself and the perfusion, created an ideal environment for the cells to engraft into the scaffold and proliferate/differentiate. The downside of the recellularization experiments using the HA, were the multiple infections that occurred and components of the system that broke down.

In this project, the most promising results were obtained during the recellularization with liver-derived organoids, next to the validation of the HA set up with the HepG2 cell line. The organoids could be expanded to high numbers with use of the spinner flasks in about three weeks. Histological staining of the repopulated scaffolds, showed cells that were viable and had engrafted into the parenchymal space adjacent to the infused vessels. In the infection free experiments, immunohistochemical staining showed that the HepG2 cells had engrafted in a dense formation of live cells, showed albumin secretion and the presence of profilerating cells. These results were supported by the values obtained from the qPCR. For the organoid experiments, immunohistochemical staining proved viability, proliferation and showed positive stained cells for cholangiocyte markers. The qPCR data gave varying results, which could be explained by the difference in organoid lines and the characteristics of each experiment. Trends that could be seen in the organoid experiments were an increase in the cholangiocyte and organoid expression during the spinner flask phase and an increase in proliferation, differentiation and albumin expression in the scaffold phase. It could be stated, that the presence of the ECM and the influence of the perfusion have a positive effect on both cell types infused into the decellularized scaffold.

This project is only a small part of the journey towards whole organ engineering and the experiments should be repeated for more reliable results, nevertheless this project has proven the benefits of using a perfusion-based bioreactor for repopulating a decellularized porcine liver scaffold and it has shown that liver-derived organoids can be used for liver scaffold recellularization.

5.2. Further research

This project could only include certain aspects of the liver recellularization using the HA. Mentioned in the Discussion section (section 4), more infection free experiments should be done using the liver-derived organoids as cell source for the recellularization of a decellularized porcine liver scaffold. These would give a broader spectrum of data and make the results more reliable, especially the qPCR data. In these additional experiments, focus should lie on keeping the parameters and duration the same for at least 3 experiments before changing any factors.

There are some interesting parameters that could be changed and above that could be monitored, which was impossible during this project due to malfunction of the HA. The activity and metabolism of the cells could be tracked by measuring the change in the air composition. More cells and live cells, use more oxygen and therefore produce more carbon dioxide. A way to measure the metabolism of the cells, is the albumin production and secretion. If a supernatant is taken to calculate the concentration of albumin in the culture medium, it is important to know the total volume of culture medium in the HA. During the experiment, part of the culture medium evaporates and new medium is added for a boost in growth factors. The concentration of for instance added Inulin can tell the total volume present compared to the starting volume with the corresponding concentration in the system. Inulin is a material that has no influence on the performance of the cells in the liver scaffold. A second value that can be obtained from the culture medium, is the engraftment efficiency. If the medium that exits the scaffold just after infusion is collected, the number of cells present in the medium can be estimated.

The efficiency of the engraftment of the cells could be further improved in two ways. In this project most liver segments were cannulated through a portal vein. The influence of infusing the scaffold through the bile duct instead, or multiple vessels simultaneously can give new insights and possibly improve the engraftment. Regarding the cell type, this project did not include injecting cell types other than liver cells (HepG2s and organoids). In previous studies (see table A.4) multiple cell types were injected into the liver scaffolds, mostly a combination of endothelial cells and liver cells. These different cells, were injected through different vessels or at multiple time points for better distribution and engraftment. The moist chamber of the HA is customizable which will allow for more than 2 inlet ports to be used during infusion of multiple cell types through different vessels.

In the end, each experiment done in this project and the next steps taken will contribute to the end goal of whole organ engineering of the liver.

Bibliography

[1] F. W. Meng, A. Assiri, D. Dhar, and D. Broering. Whole liver engineering: A promising approach to develop functional liver surrogates. *Liver International*, 37(12):1759–1772, 2017.

[2] S. F. Badylak, D. Taylor, and K. Uygun. Whole-organ tissue engineering: decellularization and recellularization of three-dimensional matrix scaffolds. *Annu Rev Biomed Eng*, 13:27–53, 2011.

[3] D. Rana, H. Zreiqat, N. Benkirane-Jessel, S. Ramakrishna, and M. Ramalingam. Development of decellularized scaffolds for stem cell-driven tissue engineering. *Journal of Tissue Engineering and Regenerative Medicine*, 11(4):942–965, 2017.

[4] E. N. Marieb and K. Hoehn. *Human anatomy & physiology*. Pearson Education, 10th edition, 2015.

[5] Arora K. Normal histology. `http://www.pathologyoutlines.com/topic/livernormalhistology.html`. Accessed: 2019-07-23.

[6] M. Caralt. Present and future of regenerative medicine: Liver transplantation. *Transplant Proc*, 47(8):2377–2379, 2015.

[7] Pedro M. Baptista, Mohummad M. Siddiqui, Genevieve Lozier, Sergio R. Rodriguez, Anthony Atala, and Shay Soker. The use of whole organ decellularization for the generation of a vascularized liver organoid. *Hepatology*, 53(2):604–617

[8] M. Cesaretti, A. Zarzavajian Le Bian, S. Moccia, A. Iannelli, L. Schiavo, and A. Diaspro. From deceased to bioengineered graft: New frontiers in liver transplantation. *Transplant Rev (Orlando)*, 2018.

[9] R. A. Junka, L. E. Daly, and X. Yu. Bioreactors for evaluating cell infiltration and tissue formation in biomaterials. In M. Jaffe, W. Hammond, P. Tolias, and T. Arinzeh, editors, *Characterization of Biomaterials*, volume 64 of *Woodhead Publishing Series in Biomaterials*, pages 138–181. 2013.

[10] W. Yang, R. Xia, Y. Zhang, H. Zhang, and L. Bai. Decellularized liver scaffold for liver regeneration. *Methods Mol Biol*, 1577:11–23, 2018.

[11] A. Soto-Gutierrez, L. Zhang, C. Medberry, K. Fukumitsu, D. Faulk, H. Jiang, J. Reing, R. Gramignoli, J. Komori, M. Ross, M. Nagaya, E. Lagasse, D. Stolz, S. C. Strom, I. J. Fox, and S. F. Badylak. A whole-organ regenerative medicine approach for liver replacement. *Tissue Eng Part C Methods*, 17(6):677–686, 2011.

[12] E. C. Moran, A. Dhal, D. Vyas, A. Lanas, S. Soker, and P. M. Baptista. Whole-organ bioengineering: current tales of modern alchemy. *Translational Research*, 163(4):259–267, 2014.

[13] Kamal Hany Hussein, Kyung-Mee Park, Kyung-Sun Kang, and Heung-Myong Woo. Heparin-gelatin mixture improves vascular reconstruction efficiency and hepatic function in bioengineered livers. *Acta biomaterialia*, 38:82–93

[14] María Teresa Donato, Laia Tolosa, and María José Gómez-Lechón. Culture and functional characterization of human hepatoma hepg2 cells. In *Protocols in In Vitro Hepatocyte Research*, pages 77–93. Springer, 2015.

[15] Aliya Fatehullah, Si Hui Tan, and Nick Barker. Organoids as an in vitro model of human development and disease. *Nature cell biology*, 18(3):246, 2016.

[16] Meritxell Huch, Helmuth Gehart, Ruben van Boxtel, Karien Hamer, Francis Blokzijl, Monique M. A. Verstegen, Ewa Ellis, Martien van Wenum, Sabine A. Fuchs, and Joep de Ligt. Long-term culture of genome-stable bipotent stem cells from adult human liver. *Cell*, 160(1-2):299–312

[17] J. Willemse, M. Verstegen, A. Vermeulen, I. Schurink, H. Roest, L. van der Laan, and J. de Jonge. Fast, robust and effective decellularization of whole human livers using mild detergents and pressure controlled perfusion. *manuscript in submission*, 2019.

[18] S. Sabetkish, A. M. Kajbafzadeh, N. Sabetkish, R. Khorramirouz, A. Akbarzadeh, S. L. Seyedian, P. Pasalar, S. Orangian, R. S. Beigi, Z. Aryan, H. Akbari, and S. M. Tavangar. Whole-organ tissue engineering: decellularization and recellularization of three-dimensional matrix liver scaffolds. *J Biomed Mater Res A*, 103(4):1498–1508, 2015.

[19] V. Navarro-Tableros, M. B. Herrera Sanchez, F. Figliolini, R. Romagnoli, C. Tetta, and G. Camussi. Recellularization of rat liver scaffolds by human liver stem cells. *Tissue Eng Part A*, 21(11-12):1929–1939, 2015.

[20] Thomas Scholzen and Johannes Gerdes. The ki-67 protein: from the known and the unknown. *Journal of cellular physiology*, 182(3):311–322, 2000.

[21] A. M. de Vries. Towards efficient perfusion recellularization of porcine liver grafts with liver-derived organoids. *Thesis report*, 2018.

[22] B. E. Uygun, A. Soto-Gutierrez, H. Yagi, M. L. Izamis, M. A. Guzzardi, C. Shulman, J. Milwid, N. Kobayashi, A. Tilles, F. Berthiaume, M. Hertl, Y. Nahmias, M. L. Yarmush, and K. Uygun. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. *Nat Med*, 16(7):814–820, 2010.

[23] Q. Zhou, L. Li, and J. Li. Stem cells with decellularized liver scaffolds in liver regeneration and their potential clinical applications. *Liver Int*, 35(3):687–694, 2015.

[24] M. J. Robertson, B. Soibam, J. G. O'Leary, L. C. Sampaio, and D. A. Taylor. Recellularization of rat liver: An in vitro model for assessing human drug metabolism and liver biology. *PLoS One*, 13(1):e0191892, 2018.

[25] Ji Bao, Yujun Shi, Huaiqiang Sun, Xiangli Yin, Ruina Yang, Li Li, Xi Chen, and Hong Bu. Construction of a portal implantable functional tissue-engineered liver using perfusion-decellularized matrix and hepatocytes in rats. *Cell transplantation*, 20(5):753–766

[26] O. Barakat, S. Abbasi, G. Rodriguez, J. Rios, R. P. Wood, C. Ozaki, L. S. Holley, and P. K. Gauthier. Use of decellularized porcine liver for engineering humanized liver organ. *J Surg Res*, 173(1):e11–25, 2012.

[27] Y. Chen, S. Geerts, M. Jaramillo, and B. E. Uygun. Preparation of decellularized liver scaffolds and recellularized liver grafts. *Methods Mol Biol*, 1577:255–270, 2018.

[28] W. Hassanein, M. C. Uluer, J. Langford, J. D. Woodall, A. Cimeno, U. Dhru, A. Werdesheim, J. Harrison, C. Rivera-Pratt, S. Klepfer, A. Khalifeh, B. Buckingham, P. S. Brazio, D. Parsell, C. Klassen, C. Drachenberg, R. N. Barth, and J. C. LaMattina. Recellularization via the bile duct supports functional allogenic and xenogenic cell growth on a decellularized rat liver scaffold. *Organogenesis*, 13(1):16–27, 2017.

[29] Ru Ji, Ning Zhang, Nan You, Qiang Li, Weihui Liu, Nan Jiang, Jie Liu, Hongtao Zhang, Desheng Wang, and Kaishan Tao. The differentiation of mscs into functional hepatocyte-like cells in a liver biomatrix scaffold and their transplantation into liver-fibrotic mice. *Biomaterials*, 33(35):8995–9008

[30] Wei-Cheng Jiang, Yu-Hao Cheng, Meng-Hua Yen, Yin Chang, Vincent W. Yang, and Oscar K. Lee. Cryo-chemical decellularization of the whole liver for mesenchymal stem cells-based functional hepatic tissue engineering. *Biomaterials*, 35(11):3607–3617

[31] In Kap Ko, Li Peng, Andrea Peloso, Charesa J. Smith, Abritee Dhal, Daniel B. Deegan, Cindy Zimmerman, Cara Clouse, Weixin Zhao, and Thomas D. Shupe. Bioengineered transplantable porcine livers with re-endothelialized vasculature. *Biomaterials*, 40:72–79

[32] H. Kojima, K. Yasuchika, K. Fukumitsu, T. Ishii, S. Ogiso, Y. Miyauchi, R. Yamaoka, T. Kawai, H. Katayama, E. Y. Yoshitoshi-Uebayashi, S. Kita, K. Yasuda, N. Sasaki, J. Komori, and S. Uemoto. Establishment of practical recellularized liver graft for blood perfusion using primary rat hepatocytes and liver sinusoidal endothelial cells. *Am J Transplant*, 18(6):1351–1359, 2018.

[33] K. M. Park, K. H. Hussein, S. H. Hong, C. Ahn, S. R. Yang, S. M. Park, O. K. Kweon, B. M. Kim, and H. M. Woo. Decellularized liver extracellular matrix as promising tools for transplantable bioengineered liver promotes hepatic lineage commitments of induced pluripotent stem cells. *Tissue Eng Part A*, 22(5-6):449–460, 2016.

[34] I. Pla-Palacin, P. Sainz-Arnal, S. Morini, M. Almeida, and P. M. Baptista. Liver bioengineering using decellularized whole-liver scaffolds. *Methods Mol Biol*, 1577:293–305, 2018.

[35] M. Watanabe, K. Yano, K. Okawa, T. Yamashita, K. Tajima, K. Sawada, H. Yagi, Y. Kitagawa, K. Tanishita, and R. Sudo. Construction of sinusoid-scale microvessels in perfusion culture of a decellularized liver. *Acta Biomater*, 2018.

[36] Hiroshi Yagi, Ken Fukumitsu, Kazumasa Fukuda, Minoru Kitago, Masahiro Shinoda, Hideaki Obara, Osamu Itano, Shigeyuki Kawachi, Minoru Tanabe, and Gina M. Coudriet. Human-scale whole-organ bioengineering for liver transplantation: a regenerative medicine approach. *Cell transplantation*, 22(2):231–242

A

Appendix

A.1. Overview of experiments

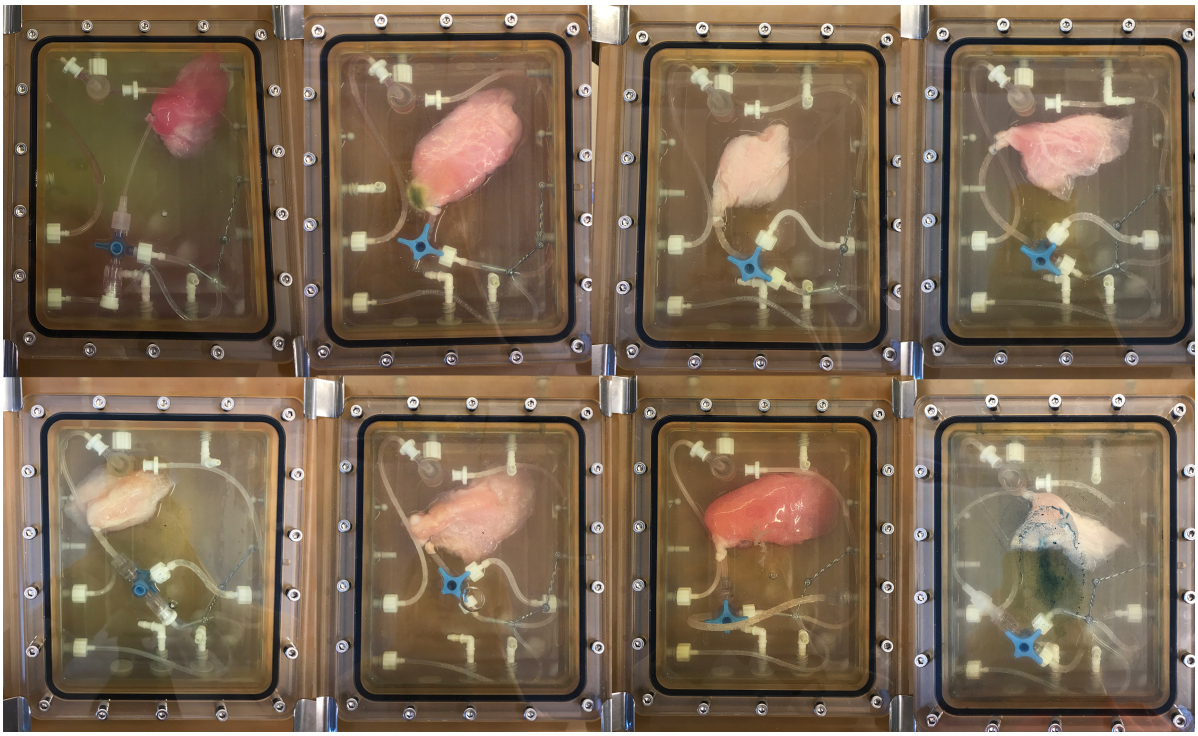


Figure A.1: From left to right and top to bottom, an overview of the porcine liver scaffolds perfused in the HA during the experiment. Except for experiment 2, this picture was taken just after the end of the experiment.

A.2. Table EM

Table A.1: Composition of the Expansion Medium used for the organoid culture.

Component	Amount (per 1 mL)
Advanced DMEM +	852,5 µL
N2	10 µL
B27	20 µL
Gastrin I	1 µL
EGF	1 µL
FGF10	1 µL
HGF	0,5 µL
A8301 (Ti)	1 µL
Nicotinamide	10 µL
Forskolin	1 µL
Acetylcysteine	2 µL
R-spondin CM	100 µL

A.3. Table primers & antibodies

Table A.2: Primers used in the qPCR analysis.

Primer	Species	Forward	Reversed
Albumin (o)	Human	CTGCCTGCCTGTTGCCAAAGC	GGCAAGGTCCGCCCTGTCATC
GAPDH	Human	AAGGTCGGAGTCAACGGATTT	ACCAGAGTTAAAAGCAGCCCTG
Ki-67	Human	TTGTTTGGAAGGTGAGCCCC	GCACCAAGGAAAAGTGACGG
LGR5	Human	TGCTGGCTGGTGTGGATGCG	GCCAGCAGGGCACAGAGCAA
CK7	Human	GGGGACGACCTCCGGAATAC	CTTGGCACGCTGGTTCTTGA
CK19	Human	GCACTACAGCCACTACTACACGA	CTCATGCGCAGAGCCTGTT
HNF1β	Human	TCACAGATACCAGCAGCATCAGT	GGGCATCACCAGGCTTGTA
HNF4α	Human	GTACTCCTGCAGATTTAGCC	CTGTCCTCATAGCTTGACCT

Table A.3: Antibodies used in the immunohistochemistry analysis.

Antibody	Dilution	Species	Company
Primary			
Ki67	1:200	Rabbit	Novus Biologicals
HNF4α	1:200	Rabbit	Thermofisher Scientific
Albumin	1:400	Mouse	Sigma
CK7	1:200	Mouse	Biogenex
CK19	1:200	Mouse	Dako
Collagen I	1:60	Mouse	Novus Bio
Secondary			
Anti-mouse	Ready to use	Rabbit	Dako
Anti-rabbit	Ready to use	Goat	Dako

A.4. Table literature study

Table A.4: Summary of perfusion based recellularization parameters.(between brackets are the 'before seeding' and 'after seeding' flow rates, no brackets means during seeding, 'static' means that during the seeding process the liver was not continuously perfused.

Reference	Species	Perfusion vessel	Cell type	Cell number	Injections (interval)	perfusion flow* (ml/min)
Bao et al. (2011)[25]	Rats	Portal vein	Hepatocytes	1 x 10^8	1x	-2 static -1
Baptista et al. (2011)[7]	Mice, rats, ferrets, rabbits & pigs	Vena cava & portal vein	ECs, hFLCs & hUVECs	100 x10^6, 70 x 10^6 & 30 x 10^6	Continuous	-5 3 (0.5)
Barakat et al. (2012)[26]	Pigs	Portal vein & suprahepatic inferior vena cava	hFH & hFSC	3.5 x 10^6 & 1 x 10^9	1x each	(1.5) static -1
Chen et al. (2017)[27]	Rats	Portal vein	Adult primary rat hepatocytes	40-120 x 10^6	4x	-5 static (10) -5
Hassanein et al. (2016) [28]	Rats	Bile duct, portal vein	HepG2s, hUVECs	100-200 x 10^6	4x (15min)	static -25
Hussein et al. (2016) [13]	Pigs	Portal vein, PV+HA	HepG2s, ECs	450 x 10^6	3x (15min)	2
Ji et al. (2012)[29]	Rats	-	Murine MSCs	1 x 10^6	1x	static (0.5/1/2/4 /6)
Jiang et al. (2014)[30]	Mice	Portal vein	MSCs	50 x 10^6	5x (10min)	1
Ko et al. (2015)[31]	Pigs	PV, HA, SH-IVC, IH-IVC & PV	Endothelial (MS1)	70, 30, 50, 50 x 10^6	1x	static and (PV) 3 (5 – 10)
Kojima et al. (2018)[32]	Rats	Bile duct, portal vein	Hepatocytes, LSECs or hUVECs	3 × 10^7, 1.5 × 10^7	1x	Static (1) (0.5)
Navarro 2015	Rats	PV, IVC, SVC, CD	hLSCs (and extra)	?	1x each	?
Park et al. (2016)[33]	Pigs	Portal vein	iPSCs	20x10^6	4x (30min)	static -3
Pla-Palacin et al. (2018)[34]	Pigs	Portal vein & hepatic artery	Hepatoblasts, pUVECs, pMSCs	60, 45, 12 x 10^6	2x (4h)	25 mmHg & 60 mmHg
Robertson et al. (2018)[24]	Rats	Portal vein	Rat or human liver cells	20 x 10^6	1x	static -1
Sabetkish 2014	Rats/ sheep	Portal vein	GFP-positive cells	18 x 10^6	1x	5
Soto-Gutierrez et al. (2011)[11]	Rats	Portal vein	Hepatocytes	10 – 50 x 10^6	multiple (10-15min)	2
Uygun et al. (2010)[22]	Rats	Portal vein	Rat hepatocytes	50 x 10^6	4x (10min)	-5 static -10
Watanabe 2018 [35]	Rats	Portal vein	GFP-HUVECs	10 x 10^6	2x	4.7
Yagi et al. (2013)[36]	Pigs	PV (?)	Hepatocytes	1 x 10^9	multiple (5-10min)	4
Yang et al. (2018)[10]	Mice	PV & IVC	Hepatocytes or stem/progenitor	1–5 x 10^6	2x (4h)	needle
Zhou et al. (2015)[23]	Rats	Portal vein	Hepatocytes & EPCs	2 x 10^7	4x (10-15min)	static (1-2)