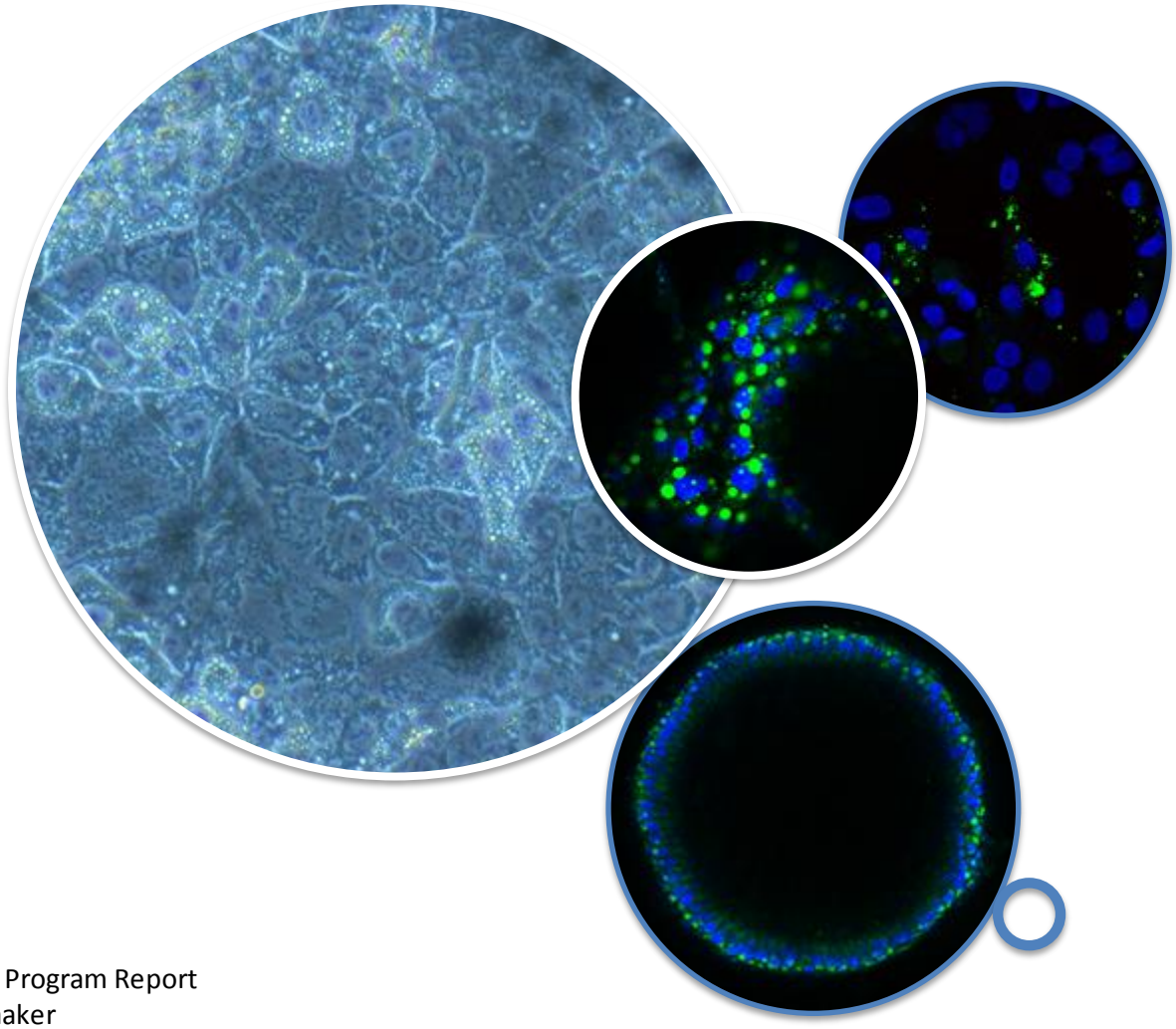
Effects of lipid overload on Hepatic Progenitor Cells



Honours Program Report
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September 2013 - August 2014
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Contents

Research at the University of Utrecht

The University of Utrecht has divided their research into four strategic themes:

- Sustainability
- Institutions
- Dynamic of Youth
- Life Sciences

The Life Sciences theme has tree sub-themes:

- Cancer
- Public Health
- Regenerative Medicine & Stem cells

The faculty of veterinary medicine has six research programmes.

- Biology of Reproductive Cells
- Tissue Repair
- Emotion & Cognition
- Risk Assessment
- Strategic Infection Biology
- Advances in Veterinary Medicine

Research on Tissue Repair is performed at the department of Equine Sciences, Pathobiology, Clinical Sciences of Companion Animals and Biochemistry and Cell Biology. It is focused on bone/cartilage, liver and cancer.
My honours program fits in the sub-theme Regenerative Medicine & Stem cells, Tissue Repair of the liver at Clinical Sciences of Companion Animals and Biochemistry and Cell Biology.

Abstract

One of the causes for damage to the liver is lipid overload, like in obesity, leading to fatty liver disease. As with other causes of liver damage, lipid overload can cause activation of hepatic progenitor cells(HPCs). HPCs can differentiate towards hepatocytes. Both the topics of HPC activation and fatty liver disease are subject to many studies, but nothing is known about the effect of lipid overload on HPCs.
In this study HepaRG cells and organoïd culture were used as models for HPCs. The lipid overload was simulated by supplementing the culture medium with 0.2mM oleate and 0.1mM palmitate. During differentiation (as measured with qRT-PCR samples) measurements were taken to assess the viability with a MTT assay, the lipid droplet formation with fluorescent staining and the lipid content with mass spectrometry.
Both the cells with and without extra fatty acids showed an increase in hepatocyte markers like HNF4a and albumin. Cyp3a4 increased gradually in the control without extra fatty acids, in contrast 24 hours after the addition of fatty acids the mRNA levels of CYP3a4 were reduced. The HepaRGs cultured with extra fatty acids for a long time changed in morphology. It looked like a mesenchymal transition, but no significant difference in the expression of mesenchymal genes was found.
HPC models (both HepaRG and organoids) had more lipid droplets and triacylglycerol in differentiated than in undifferentiated state. When extra fatty acids were added the amount of TAG and lipid droplets increased further in the next 24 hours. After a week the HepaRG cells and their media showed almost no trace of the extra fatty acids. After adding etomoxir (inhibitor of the carnitine shuttle) the amounts of triacylglycerol increased even more, where as orlistat (lipase inhibitor) had almost no effect. This might indicate that the HPCs use fatty acids for energy.
Our results suggest that HPCs are protected from lipid overload by rapid lipid metabolism and less uptake than hepatocytes. Unravelling the molecular mechanisms of these protective pathways might give new ways of protecting hepatocytes against lipid overload.

Keywords:
Hepatic progenitor cells, fatty liver disease, non-alcoholic fatty liver disease, CYP3a4, etomoxir, orlistat, lipid metabolism, differentiation

Introduction

Liver damage is a severe problem for the health of people and animals. In 2011 2456 people in the USA died while waiting for a new liver. The amount of people removed from the liver transplant list because they were too sick to undergo the transplantation doubled that year (Department of Health and Human Services 2011). Animals also develop a variety of liver diseases, but the treatment is mostly symptomatic (Bonagura & Twedt 2013).

When the liver is damaged, hepatocytes and cholangiocytes can re-enter the cell cycle and proliferate (reviewed by (Fausto 2000) (Michalopoulos 1997)). The normal hepatocytes are quiescent most of the time, but when stimulated for example by liver damage, they can divide for a number of cell doublings, thereby recovering the liver function (reviewed by (Fausto 2000) (Duncan et al. 2009).
One of the possible causes for liver damage is too much accumulation of lipids in the hepatocytes. This is one of the cases in which the regeneration of the liver by normal hepatocyte and cholangiocyte proliferation is impaired. In these cases the hepatic progenitor cell compartment is activated. Hepatic progenitor cells (HPC) are precursor cells that can differentiate into hepatocytes and cholangiocytes (reviewed by (Fausto 2000)). They are believed to be situated in the canal of Hering, around the smallest bile caniculi. After activation they start to proliferate and migrate, after which they can differentiate (reviewed by (Duncan et al. 2009)(Erker & Grompe 2007)).
Not all the cases of steatosis (fatty liver) give rise to clinical symptoms, but fatty liver can lead to or be present during hepatitis and cirrhosis, and can be predisposing for hepatocellular carcinoma. Fat accumulation is often seen in alcohol abuse. For non-alcoholic fatty liver disease (NAFLD) associations with obesity, insulin resistance and hepatitis C virus infection have been found (reviewed by: Kopec & Burns 2011) (Brunt 2004). Around 30% of the donor livers has steatosis. There is still a lot of discussion if these livers should be discarded as donor livers. Rescue strategies have been proposed, while some articles describe no negative effect of these graft on the patients, depending on the health status of the donor and the degree of steatosis (reviewed by(Liu et al. 2013; McCormack et al. 2011)). In animals liver transplants are not a therapeutic option. However research is done on the possibilities to transplant HPCs, to serve as a cure for liver disease (Kruitwagen et al. 2014; Schotanus et al. 2013).

Because of the relevance of the problems of obesity in the Western world a substantial amount of research has been done on NAFLD. Possible ways of regeneration of the liver are the topic of several researches as well, because of the clear clinical possibilities. However there is very little known about the effect of fatty liver disease on hepatic progenitor cells. The amount of damage to hepatocytes caused by the lipid overload (causing oxidative stress), the severity of NAFLD and the amount of HPC activation correspond with each other. A logical and histologically confirmed consequence is an expansion of the intermediate hepatocyte pool (T. Roskams et al. 2003; Nobili et al. 2012). Therefore it is known that the hepatic progenitor cells are activated with NAFLD. Pilot studies had shown us that HPCs did not accumulate lipids. It is unknown whether lipids in itself play a role in activating the HPCs, and how the HPCs are capable of being proliferative while the normal hepatocytes are not.

In this study we looked at the effects of adding extra fatty acids to the media of HPC cell lines. Palmitic and oleic acid are the fatty acids most commonly found in the liver of patients with and without NAFLD (Araya et al. 2004). That's why supplementing the media of hepatic cell lines with these fatty acids can be used as a model of steatosis (Gómez-Lechón et al. 2007).
There are several *in vitro* models for HPCs. The cell line HepaRG was isolated from a female patient suffering from a hepatitis C virus infection and from hepatocarcinoma (Gripon et al. 2002). HepaRGs

are capable of differentiation towards both hepatocytes and cholangiocytes (Parent et al. 2004). There are two ways to differentiate the HepaRG cells. One is by long time culture without dimethylsulfoxide (DMSO), the other is by long-time culture with 2% DMSO. Both have advantages and disadvantages (Hoekstra et al. 2011). We choose the option without DMSO to have a more constant timeline, and no possible interference of DMSO.
The THLE-5b cell line is a SV40 T antigen-immortalized, non-tumorigenic CK18 positive liver cell line (Tokiwa et al. 2006). THLE-5b cells have been reported to have the characteristics of immature liver cells, but when cultured under the right conditions they can obtain mature hepatocyte and cholangiocyte properties (Tokiwa et al. 1998; Tokiwa et al. 2006).
Organoids are a 3D model for HPCs. They consist of primary cells from biliary duct fragments cultured in matrigel with several growth factors. In this culture system they grow into round structures, expressing progenitor characteristics (Huch et al. 2013).
It is difficult to find the hepatic progenitor cells, since there are almost no HPCs in the healthy liver. Only in some cases of liver damage the HPC compartment is activated. Also, a HPC specific marker is still lacking. HPCs express both hepatic and biliary markers (reviewed by Libbrecht & Roskams 2002) (see figure 1). KRT8 and KRT18 stain both liver parenchymal cells and cholangiocytes, KRT7 and KRT19 are markers of cholangiocytes and not of hepatocytes (van Eyken et al. 1987). Some of the markers found on hepatic progenitor cells are KRT7, KRT19, Oct4, Lgr5 and NCAM. Markers found on hepatocytes (and not on progenitor cells) are cytochrome P450 3a4, HNF4a, AFP and albumin (reviewed by Hengstler et al. 2005) (Roskams et al. 1998; Schmelzer et al. 2006; Huch et al. 2013; Strick-Marchand & Weiss 2002). Some of these markers were used to determine if our cells were differentiating.

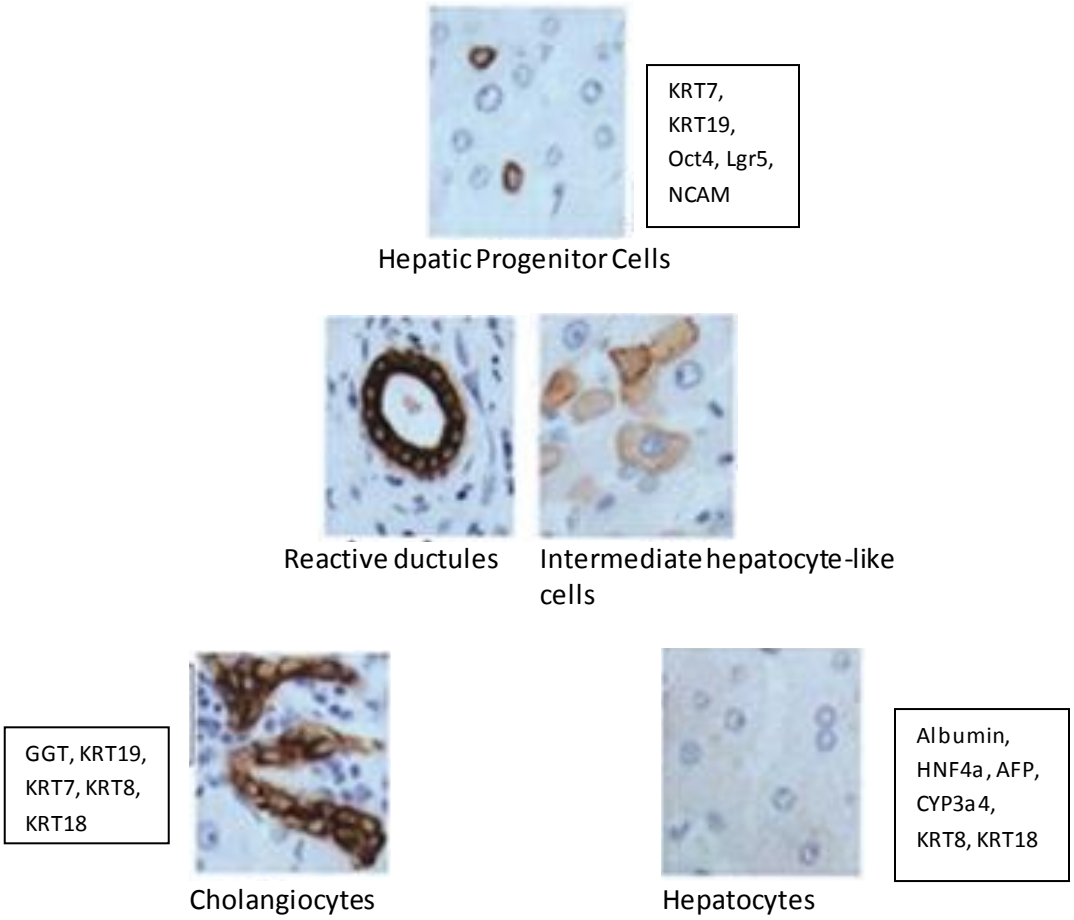


Figure 1: the pictures show staining for KRT7. The scheme shows the changing from a hepatic progenitor cell to either hepatocytes or cholangiocytes, through their intermediate cell types. The markers tested positive are listed next to the pictures.

KRT7; keratin 7, KRT19; keratin 19, Oct4; octamer-binding transcription factor 4, Lgr5; leucine-rich-repeat-containing Gprotein-coupled receptor 5, NCAM; neural cell adhesion molecule, GGT; gamma-glutamyltransferase, KRT8; keratin 8, KRT18; keratin 18, HNF4a; Hepatocyte Nuclear Factor 4 alpha, AFP; Alpha-fetoprotein, CYP3a4; Cytochroom P450 3A4. Pictures from: (T. a Roskams et al. 2003)

In our study we investigated effects of artificial lipid overload (supplementing the culture medium with palmitate and oleic acid) on proliferation, lipid droplet formation and lipid content during differentiation of HPCs. Our results show a difference in response to extra fatty acids between undifferentiated and differentiated HPCs.

Materials and methods

Cell culture:

HepaRG

HepaRG cells, obtained from BioPredic International (Rennes, France), were cultured in Williams Medium E with glutamax, with 10% FCS, 1% penicillin/streptomycin, 5ug/ml insulin (Sigma-Aldrich) and 25uM hydrocortisone hemisuccinate (Sigma-Aldrich). Differentiation was accomplished by confluent cell growth for extended periods of time (Hoekstra et al. 2011). See figure 2 and table 1. Experiments 1, 2 and 3 were experiments consisting of samples for qPCR, MTT, fluorescent staining and mass spectrometry. The others are sub-experiments based on findings from these large experiments.

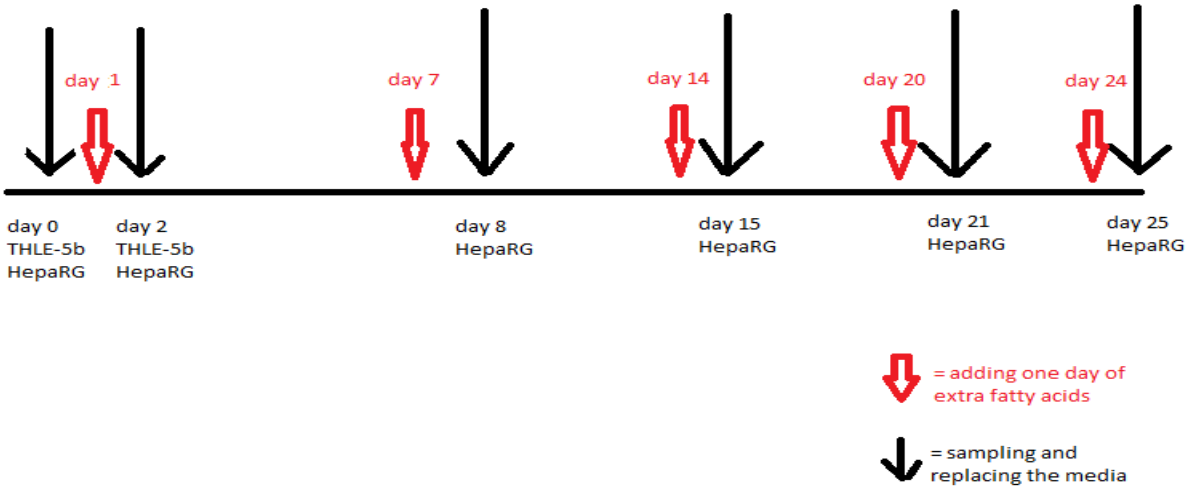


Figure 2: time schedule for the experiments with the HepaRG (and THLE-5b). Normal medium replacement was performed at sampling days.

HepG2

HepG2 cells were cultured in DMEM medium containing 10% FCS, 1% NEAA and 1% penicillin/ streptomycin.

THLE-5b

THLE-5b cells, obtained from the Kanazawa university (Japan), were cultured in DMEM F12 medium containing 10% FCS, 1% penicillin/streptomycin, 5ug/ml insulin (Sigma-Aldrich Company Ltd., The Netherlands) and 0,2uM hydrocortisone hemisuccinate (Sigma-Aldrich). Differentiation was attempted by culturing the cells on a 2.5% poly-2-hydroxyethyl methacrylate (HEMA) coating (Tokiwa et al. 2006). The poly-HEMA was dissolved in ethanol by heating it in a water bath at 50 degrees. For the coating 2ml per 25cm^2 was used. It was dried at 37 degrees.

All cell lines were plated at a density of 2.8*10^4 cells/cm^2. They were cultured at 37 degrees, with 5% CO_2.

HepaRG experiments

	Experiment 1	Experiment 2	Experiment 3	Different FFA concentrations	BSA	HepaRGen HepG2	HepaRG with inhibitors
Day 2	-FFA +FFA (one day)	-FFA +FFA (one day)	-FFA +FFA (one day)	-FFA	-FFA	-FFA +FFA (one day)	-FFA 1dayFFA 1dayFFA+etomoxir 1dayFFA+orlistat 3hours FFA (palmitateD4 and oleate) and etomoxir 6 hours FFA(palmitateD4 and oleate) and etomoxir
Day 8	-FFA +FFA	-FFA +FFA 1dayFFA				-FFA 1dayFFA	-FFA 1dayFFA 1dayFFA+etomoxir 1dayFFA+orlistat 3hours FFA (palmitateD4 and oleate) and etomoxir 6 hours FFA(palmitateD4 and oleate) and etomoxir
Day 15	-FFA +FFA	-FFA +FFA 1dayFFA	-FFA +FFA 1day FFA +FFA + 1dayFFA	-FFA +FFA 1 day FFA 1/10FFA 1/3FFA Oleate BSA 1day FFA + etomoxir	-FFA 1 day FFA BSA 1dayFFA-BSA +oleate dissolved in EtOH +extra FCS 2% FCS 2%FCS+BSA Continues BSA		
Day 21		-FFA +FFA 1dayFFA	-FFA +FFA 1day FFA +FFA + 1dayFFA (all 2x, day 20 and 21)				
Day 25		-FFA +FFA 1dayFFA					

Table 1: a list of the conditions and timepoints used during the different times HepaRGs were cultured. Experiment 1,2 and 3 are the experiments with several types of read-out, qPCR, mass spectrometry, MTT and fluorescent staining. Different FFA concentrations and BSA were only qPCR, HepaRG and HepG2 were mass spectrometry. HepaRG with inhibitors was qPCR, mass spectrometry and fluorescent staining. FFA stands for a dose of extra free fatty acids. BSA: bovine serum albumin.

Organoids

For differentiation of mouse organoïds the following protocol was used, according to the article of (Huch et al. 2013) (see figure 3). Organoïd are cultured in matrigel (BD Biosciences) and receive new media every two days.

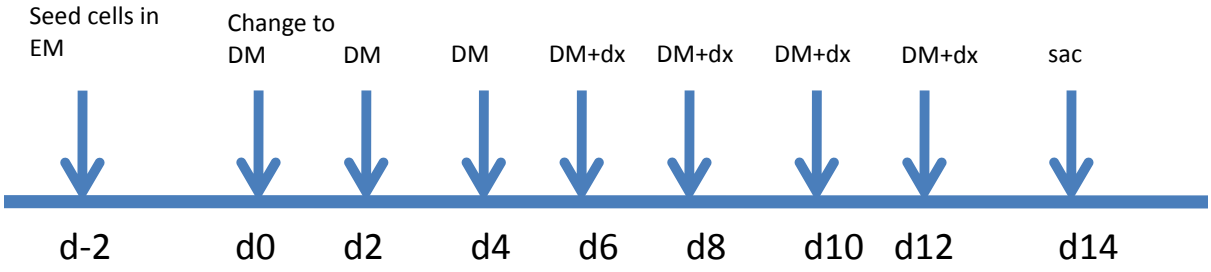


Figure 3: time line for the differentiation of mouse organoids. d stands for day, EM for expansion media, DM for differentiation media and dx for dexamethasone. Figure thanks to S. Nantasanti

Expansion medium consisted of: advanced DMEM (Invitrogen), Rspondin 5% (provided by the Hubrecht institute), NAC (Sigma), mouseEGF (Invitrogen), HGF(peprotech/tebu), FGF10 (peprotech/tebu), GAS (Sigma), glutamax (Invitrogen), penicillin/streptomycin (Gibco), HEPES(Invitrogen), B27 without vitamin A (Invitrogen), N2 (Invitrogen) and NIC (Sigma).
Differentiation medium consisted of advanced DMEM(Invitrogen), penicillin/streptomycin (Gibco), glutamax(Invitrogen), HEPES (Invitrogen), N2 (Invitrogen), B27 without vitamin A (Invitrogen), NAC (Sigma), mouse EGF (Invitrogen), FGF (peprotech/tebu), DAPT (Selleckchem) and A83 (Tocris Bioscience). From day 6 on dexamethasone was added as well.
For the organoïd, washing consists of taking them out of the well by adding ice cold medium to dissolve the matrigel, then spinning down and taking of the supernatant for as many washing steps as required. As long as there is matrigel the samples have to be kept on ice, otherwise the matrigel will become solid again.
Morphological pictures were taken with an Olympus CKX41 microscoop and Cell^{B} imaging software for life science microscopy (Olympus soft image solutions).

Fatty acids

Medium with extra fatty acids had a concentration of 0.2mM oleate and 0.1mM palmitate. Stock solutions of fatty acids were 10mM with 12% BSA.

MTT

20ul MTT solution (5mg/ml, dissolved in PBS, filter sterilized) per 100ul medium was used. This was left incubating at 37 degrees at 5% CO_2 for two hours. After the incubation period the MTT was cast of in a tissue and 50ul DMSO for a 96 wells plate and 200ul DMSO for a 12wells plate was added. The absorbance was measured in a plate reader with Anthos software.

RNA isolation, cDNA and real time quantitive PCR

The cells were washed with Hanks. Organoïd were either taken of with normal medium and washed with Hanks till the matrigel was gone (using a centrifuge for the washing) or the RLT was added

directly unto the matrigel. Samples were taken by dissolving the cells in 350ul RLT with 10ul beta-mercaptoethanol (Qiagen Benelux BV, Venlo, The Netherlands) per 1ml RLT, directly into the plate. RNA was isolated with a RNeasy Micro Kit from Qiagen. cDNA was created using an iScript cDNA Synthesis Kit from Bio-Rad containing a mixture of random hexamers en oligo-dT primers. qPCR reactions were performed using iQ SYBR green Supermix (Bio-Rad). Primer information see attachments.

		Experiment 1	Experiment 2	Experiment 3	Different FFA concentrations	BSA	HepaRG + inhibitors
hepatocyte	Albumin	■	■	■	■	■	■
	CYP3a4	■	■	■	■	■	
	AFP	■	■				
	HNF4a		■	■	■	■	■
	MRP2		■	■			
Progenitor	Oct4	■	■				
	KRT19	■	■	■	■	■	■
	KRT7	■	■	■	■	■	■
Lipid	Perilipin 2			■	■	■	
Cholangiocyte	GGT		■	■			
Mesenchymal	Vimentin		■	■			
	Twist		■	■			
	Zeb1			■			
	Zeb2			■			
	Snail1			■			
Reference (genes used based on genorm)	Rps5	■					
	HPRT	■	■	■	■	■	
	RPL19	■					
	YWHAZ	■	■	■		■	
	GAPDH	■	■	■			
	HMBS		■			■	
	B2M		■		■	■	
	TBP		■	■	■	■	

Table 2: showing for which gene primers were used for qPCR in which experiment with HepaRGs.
KRT7; keratin 7, KRT19; keratin 19, Oct4; octamer-binding transcription factor 4, GGT; gamma-glutamyltransferase, HNF4a; Hepatocyte Nuclear Factor 4 alpha, AFP; Alpha-fetoprotein, CYP3a4; Cytochroom P450 3A4, MRP2; Multidrug resistance-associated protein 2, rps5; ribosomal protein S5, HPRT; hypoxanthine-guanine phosphoribosyltransferase, RPL19; ribosomal protein L19, YWHAZ; tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta, GAPDH; Glyceraldehyde 3-phosphate dehydrogenase, HMBS; hydroxymethylbilane synthase, B2M; beta-2-microglobulin, TBP; TATA Box Binding Protein. FFA; free fatty acids. BSA; bovine serum albumin.

Reference genes	GAPDH, RPS18, beta-actin
genes of interest	Transferrin, TAT, albumin, CYP3a11, MRP2

Table 3: Showing for which gene primers were used for qPCR for the organoïd. GAPDH; Glyceraldehyde 3-phosphate dehydrogenase, rps18; ribosomal protein S18, TAT; tyrosine aminotransferase, CYP3a11; Cytochroom P450 3A11, MRP2; Multidrug resistance-associated protein 2.

Fluorescent staining

Cells were cultured in chamber slides. At the day of fixation they were washed with Hanks and fixed in 4% formaldehyde in PBS for 30 minutes at room temperature. After 30 minutes the 4% formaldehyde was replaced by 1% formaldehyde and wrapped in parafilm at 4 degrees for storage. Cells were washed two times two minutes with PBS. Incubation with 500ul solution of 2% BSA/ 0.1% saponin in PBS for 60 minutes. Incubation with 100ul solution of 1% BSA/ 0.1% saponin in PBS with antibody for 60 minutes. Albumin (1:500), vimentin (1:150) and ZO-1 (1:250). The wells were washed four times five minutes with PBS. Incubation with 100ul solution of 1% BSA/ 0.1% saponin in PBS with secondary antibody, Hoechst (1:500) and LD540 (1:500) for 60 minutes. Wash four times for 5 minutes with PBS. The slides were rinsed in water. 3ul per well FluorSave was added and a coverslip placed on top. Slides were analysed with a Leica Confocal SPE-II microscope.
Antibodies used are albumin (Sigma), vimentin (Abcam) (failed), ZO-1 (Invitrogen)(failed).

Mass spectrometry

Cells were washed three times with PBS. They were scraped off in 1ml PBS. 800ul was used to do a lipid extraction by the Bligh and Dyer method (Bligh E. G. 1959). Separation of TAG fragments and phospholipids was performed using homemade silica columns using acetone for neutral lipid isolation, mass spectrometry was performed as described by Testerink et al (Testerink et al. 2012). Computer analysis was performed using Analyst version 1.6.2.

Extraction of free fatty acids from the media

An internal standard of palmitate D4 (10nmol per sample), was added to 200ul medium. This was dissolved in 800ul water, after which a Bligh and Dyer was performed. After drying the sample under nitrogen 1ml 0.3M NaOH dissolved in methanol/water (2:1) was added. Everything is performed on ice, so only the free fatty acids are captured, not the fatty acids from the TAG as well. Add 1ml of hexane, vortex, discard the upper phase (4x). Add 0.1ml HCL(6N). Repeat adding the 1ml hexane and save the upper phase (3x). Drying under nitrogen, storage at -20 degrees. Mass spectrometry analysis was performed as described by Aardema et al (Aardema et al. 2013).

Statistics

Statistic analysis was performed using R (version 3.1.1). Lineair mixed effect models were made using the nlme package. MTT intensities were compared with the intensities at day 2 untreated. Triacylglycerol amounts were divided by the amounts of cholesterol (as a measurement for the amount of cells in the sample) and compared to the amounts of triacylglycerol at day 2 untreated. The logarithm was taken to ensure a normal distribution. The relative gene expressions were divided by the relative gene expression at day 2 untreated, to obtain the differences in gene expression with differentiation compared to the undifferentiated samples. The model compared the ratios with the ratio of the basic situation at day 2. The logarithms were taken to ensure a normal distribution. Because at day 2 treatment with one day FFA and constant treatment with FFA was the same, the data was used for both conditions. Factors that were taken into account were treatment condition (without, one day with or constantly with extra fatty acids) and the timepoint of sampling. The experiment from which the sample came was considered a random effect. P-values < 0.05 were considered significant.

Results

Differentiation

Gene expression

The HepaRGs cultured for four weeks in the same plate started to express hepatocyte like mRNA levels between day 8 and day 15. The albumin ratio at day 15 was 45 times higher than at day 2 (with p<0.005). There was no effect of the different conditions on this increase (p=0.80 and p=0.30) (see figure 4). The conditions being with constant extra fatty acids and with only one day of extra fatty acids supplemented. HNF4a also increased (with 6 times the ratio between day 15 and day2 (p<0.005) and 5 times the ratio between day 21 and day2 (p<0.005)). Except for day15 with one day extra fatty acids (with 0.37 times (p=0.01)) there was no effect of adding extra fatty acids.

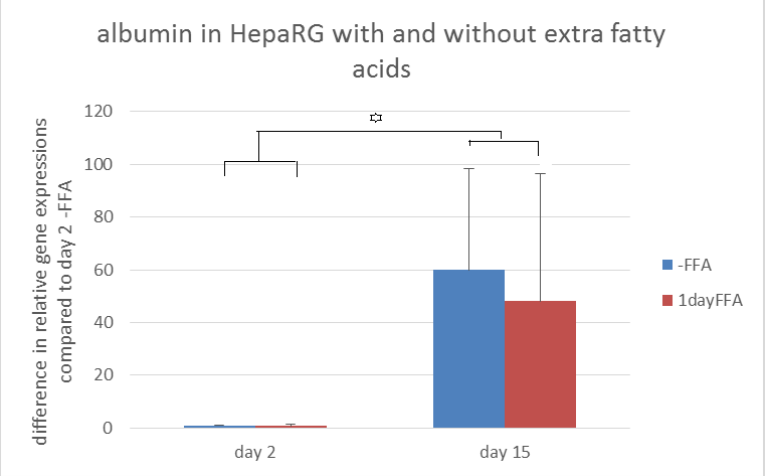


Figure 4: Graf consists of data from five separate experiments, consisting of duplo's or triplo's (see table 1). x-as shows the days after plating before sampling. Y-as the relative gene expression of albumin compared with the relative gene expression at day 2 –FFA. A star means a statistical significant difference.
FFA; extra dose of free fatty acids

GGT (gamma-glutamyltransferase, a cholangiocyte gene) did not show in increase in gene expression over time, at day15 there was 0.74 times the amount of mRNA compared to day 2 (p= 0.02). KRT7 (keratin7, a progenitor cell marker) showed significant increases over time with an interaction with the conditions. At day 15 1.47 times the amount of mRNA compared to day2 (p=0.005), at day 21 2.4 times the ratio (p<0.005). Adding extra fatty acids gave an extra increase in KRT7 expression, at day 21 with constant extra fatty acids 1.8 times (p=0.034). At day 15 with one day extra fatty acids 2.3 times (p<0.005), at day 21 2.5 times (p=0.001). KRT19 decreased at day 21 with 0.71 times the ratio (p=0.03). The treatment was not an influence.
CYP3a4 (a hepatocyte gene involved in drug-metabolism) in the controls came up over time, in the samples with extra fatty acids the expression of CYP3a4 was inhibited (see figure 5). This was both in the samples with constant extra fatty acids (with 0.11 times the ratio day 15/ day2 (p<0.005) and with 0.08 times the ratio day21/day2 (p<0.005)) as in the samples with one day extra fatty acids (with 0.15 times the ratio day 15/ day2 (p<0.005) and with 0.18 times the ratio day21/day2 (p=0.003)). After this inhibition of CYP3a4 expression was shown with experiment 1, 2 and 3, the expression was measured in addition of different concentrations of extra fatty acids (data not shown). Since the extra fatty acids in these concentrations can only be dissolved with addition of BSA, a control with only BSA and no extra fatty acids was performed. As shown in figure 6 the down regulation of CYP3a4 was also observed by the addition of BSA alone.

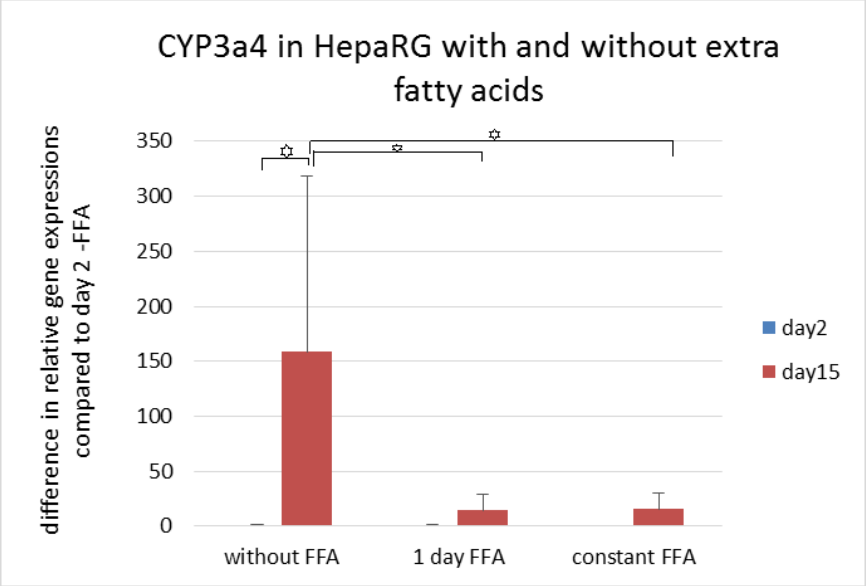


Figure 5: increases in relative gene expression of CYP3a4 compared to the relative gene expression at day 2 –FFA. Day 2 is blue, day 15 is red. There are three conditions, with one day FFA, without FFA and with constant FFA (only at day 15). Data was obtained from five different experiments. (see table 1). A star means a statistical significant difference. FFA; extra dose of free fatty acids

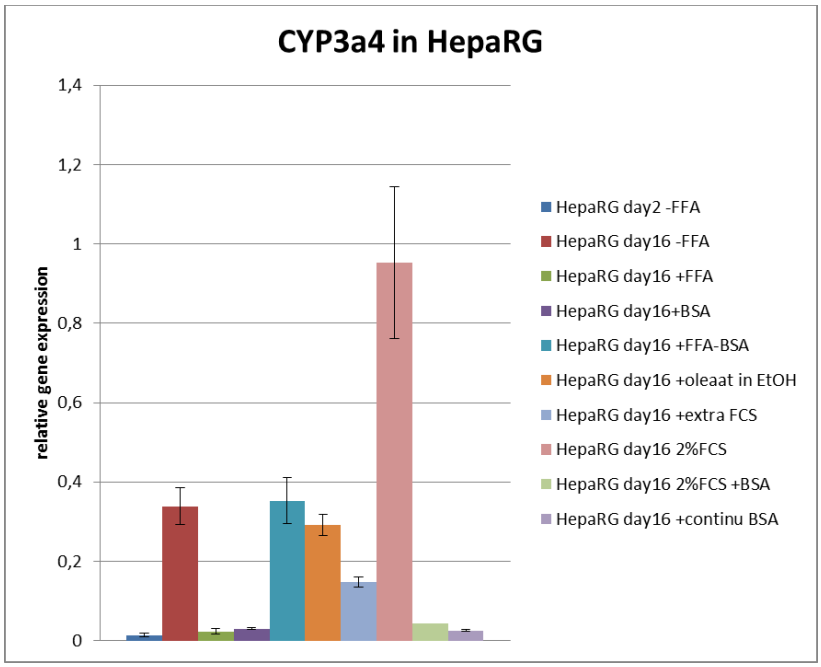


Figure 6: CYP3a4 relative gene expression with several conditions, including only BSA (purple) and fatty acids with BSA(green). Data from BSA experiment, see table 1. BSA; bovine serum albumin, FFA; extra dose of free fatty acids, FCS; fetal calf serum.

To check for LPS contamination of the BSA, our BSA and fatty acids dissolved in BSA were added to TLR4 transfected cells. LPS causes activation of TLR4. Both of the samples, but not the normal HepaRG medium, gave an activation of TLR4. The effect was not inhibited by polymyxin B (see figure 7). There was contamination of a LPS like substance.

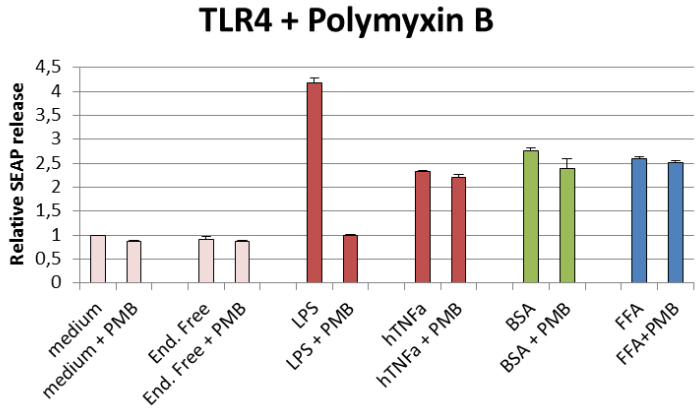


Figure 7: The light pink bars are the negative control. The first ones are from the normal HepaRG medium, which doesn't give an increase in SEAP release. The red bars are the positive controls, they are a TLR4 agonist and a TLR4 independent agonist. They give an increase, and the TRL4 agonist responds to PMB (polymyxin B), where the TLR4 independent agonist does not. Both the BSA and the FFA give an activation, and the PMB has no effect.
SEAP; secreted embryonic alkaline phosphatase, TLR4; Toll-like receptor 4, PMB; polymyxin B, LPS; Lipopolysacchariden, hTNFa; Human Tumor Necrosis Factor-α, BSA; bovine serum albumin, FFA; medium with an extra dose of free fatty acids. Figure thanks to Tom Habraken.

To avoid the BSA effect, several conditions to test the fatty acids alone were tested. Oleate and palmitate added to normal medium did not dissolve. Neither did oleate dissolved in ethanol added to the medium. To check for an effect of BSA contamination, specific to our own, possibly contaminated BSA, we added extra FCS in an amount that was comparable with the amount of BSA when extra fatty acids were added. FCS also contains BSA. This gave an increase in perilipin 2 expression. Except for the perilipin expression most gene expression profiles did not change. Replacing one day before sampling the media with 2% FCS gave an increase in hepatocyte like gene expression. Adding BSA suppressed that effect (see figure 6).

To measure effects of the fatty acids on proliferation a MTT assay was used during experiment 2 and 3 (experiment 1 the MTT was lost due to technical problems). The MTT assay for the HepaRG gave a significant increase between day 2 and day 15 (0.74 with $p<0.005$), and day 2 and day 21 (0.73 with $p< 0.005$), but no significant differences between the culture conditions (0.06 with $p= 0.103$) (see figure 8). Therefore between day 2 and day 8 there is proliferation and there is no effect of constant extra fatty acids on the proliferation.

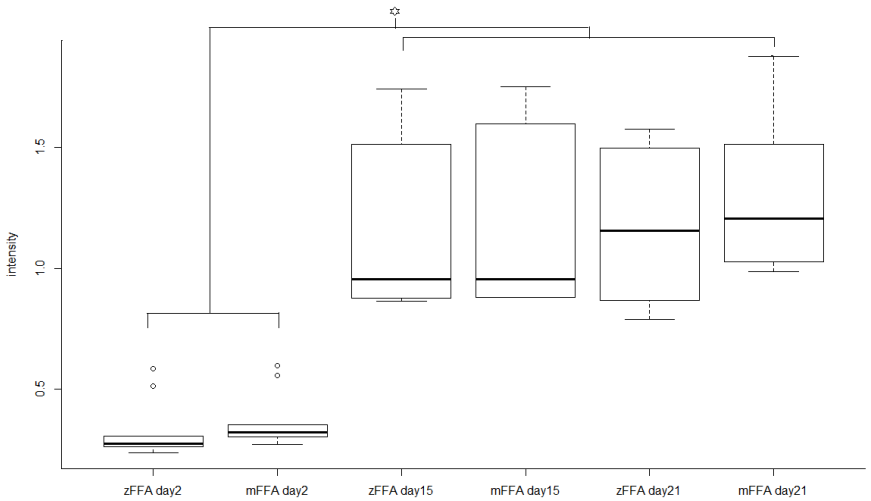


Figure 8: intensity of the MTT assay from two separate experiments (experiment 2 and 3) at day 2, 15 and 21 of culture. The higher the intensity of the MTT, the higher the metabolic activity of the cells. zFFA means culture without extra fatty acids, mFFA with constant extra fatty acids. A star stands for a statistical significant difference.

Morphology

During differentiation the HepaRG cells became bigger, but the image remained very heterogeneous. Between day 15 and 21 the morphology of the HepaRGs cultured constantly in medium with extra fatty acids changed. Groups of cells started to look longer (see figure 9). Our first hypothesis was that it was a mesenchymal change. The mesenchymal genes tested had in the first experiment a slight increase in mRNA levels, but in the second there was no difference in mRNA levels between the +FFA group and the control. Combining those results gives at day15 a significant increase (1.38 times the ratio, p=0.009) for TWIST regardless of the conditions and for vimentin at day21 with constant extra fatty acids (1.64 times higher than without extra fatty acids, p=0.036) (see figure 10). The second time we also tested several other mesenchymal genes, but no clear pattern emerged.

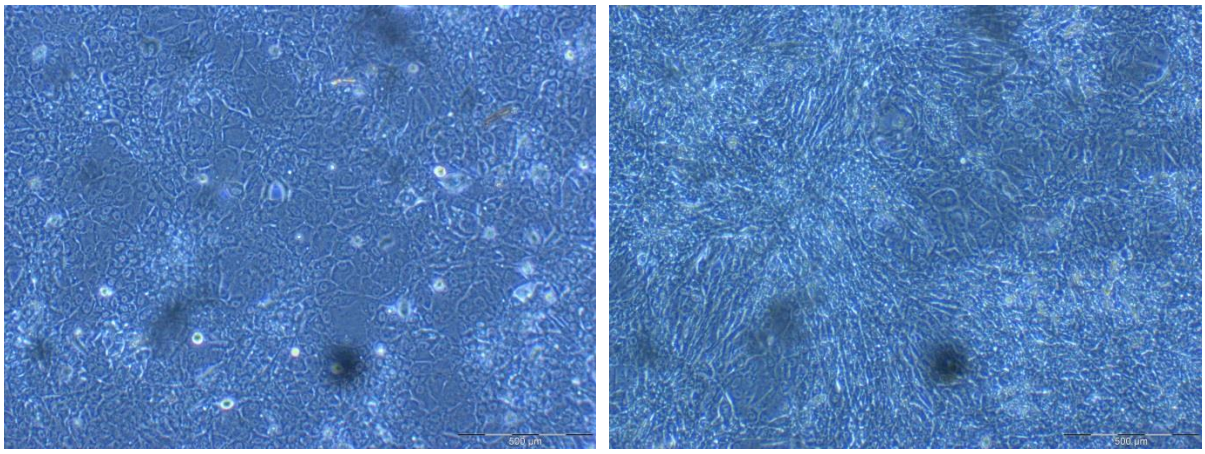


Figure 9: HepaRG cells on day 24 of culture, on the left the control, on the right long time culture in extra fatty acids.

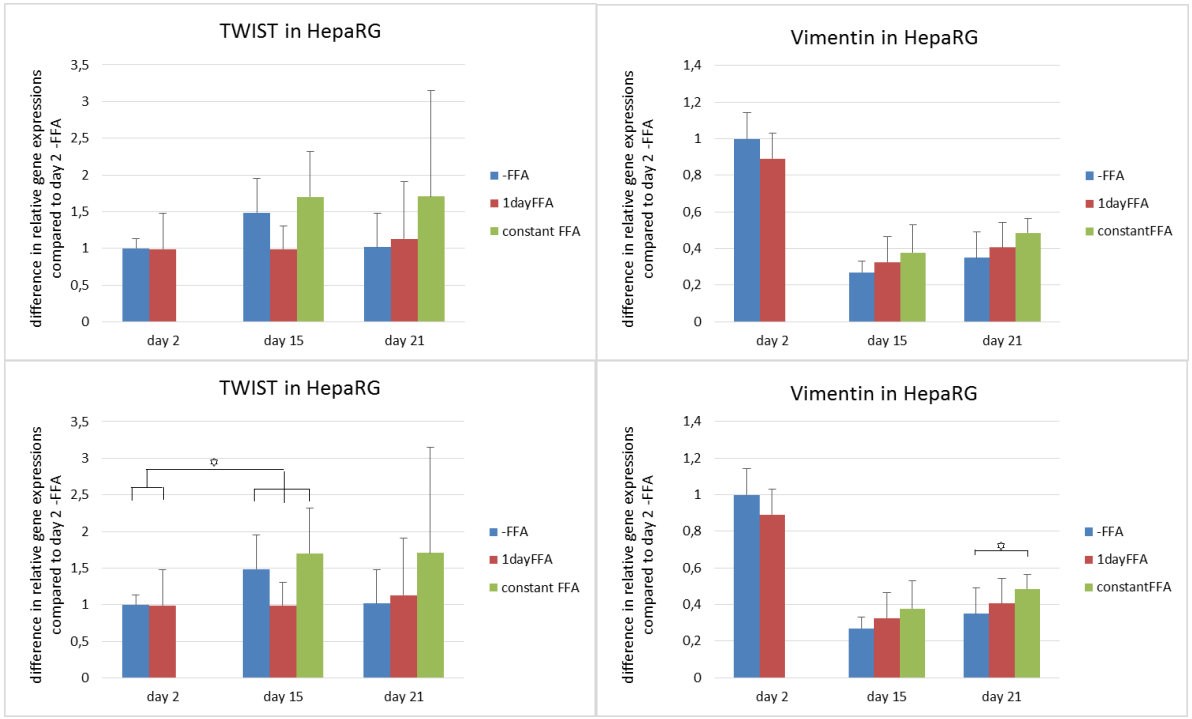


Figure 10: percentage of relative gene expression of TWIST and vimentin at day 2, 15 and 21 of culture compared to the relative gene expression at day 2 –FFA (day2-FFA at 1). For three conditions, the control (blue), the constant culture in medium with extra fatty acids (red) and standard culture with at the day before sampling extra fatty acids added to the media (green). Figure consists of data from two separate experiments. A star stands for a statistical significant difference. FFA; extra dose of free fatty acids

THLE-5b

The differentiation of THLE-5b cells described in the article of (Tokiwa et al. 2006) was not reproducible in our lab. There was a down regulation of KRT7 and KRT19, but no up regulation of hepatocyte like genes (see figure 11 and attachment THLE-5b).

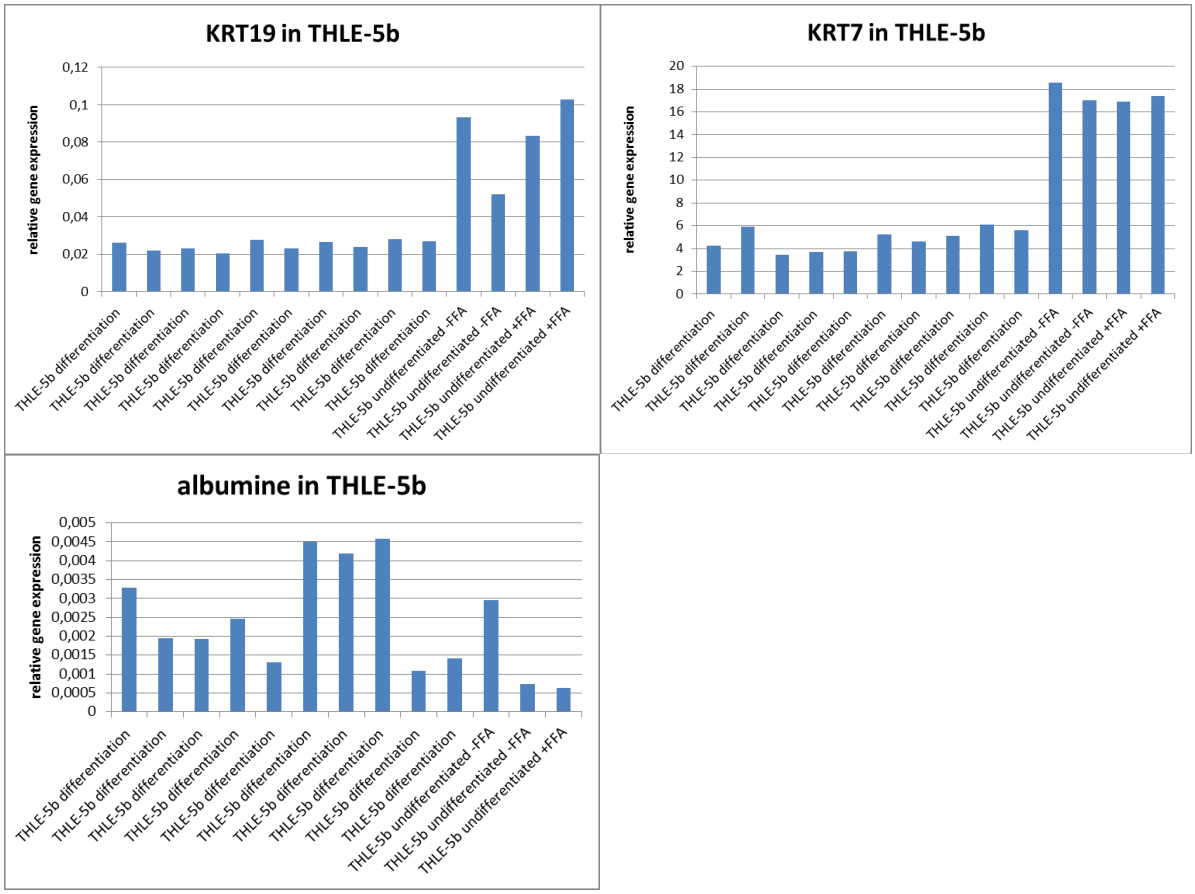


Figure 11: the relative mRNA expression of KRT7, KRT19 and albumin are shown for THLE-5b cells that underwent a differentiation protocol and for cells that were cultured in standard conditions with and without extra fatty acids. KRT19; keratin 19, KRT7; keratin 7, FFA; extra dose of free fatty acids

The organoids

The organoïd differentiation protocol was followed two times. The first time the RNA levels were low. The second time a different way of collection for storage in RLT buffer was used. The RLT was poured directly unto the organoids. This time the RNA levels were higher, just like the quality. Both times the qPCR results showed some deviating peaks (see attachment organoïd) for some samples. Except for those it seemed that the albumin levels increased a little (about 8 times) in the untreated samples, and increased less in the samples treated with extra fatty acids (about 2 times). The albumin levels in the medium showed no increase (see attachment organoïd). The MRP2 relative gene expression had some strange spikes, but on the whole not much seems to happen (see attachment organoïd). The transferrin (a differentiation marker) levels in both experiments decreased between day 3 and day14 (see figure 12).

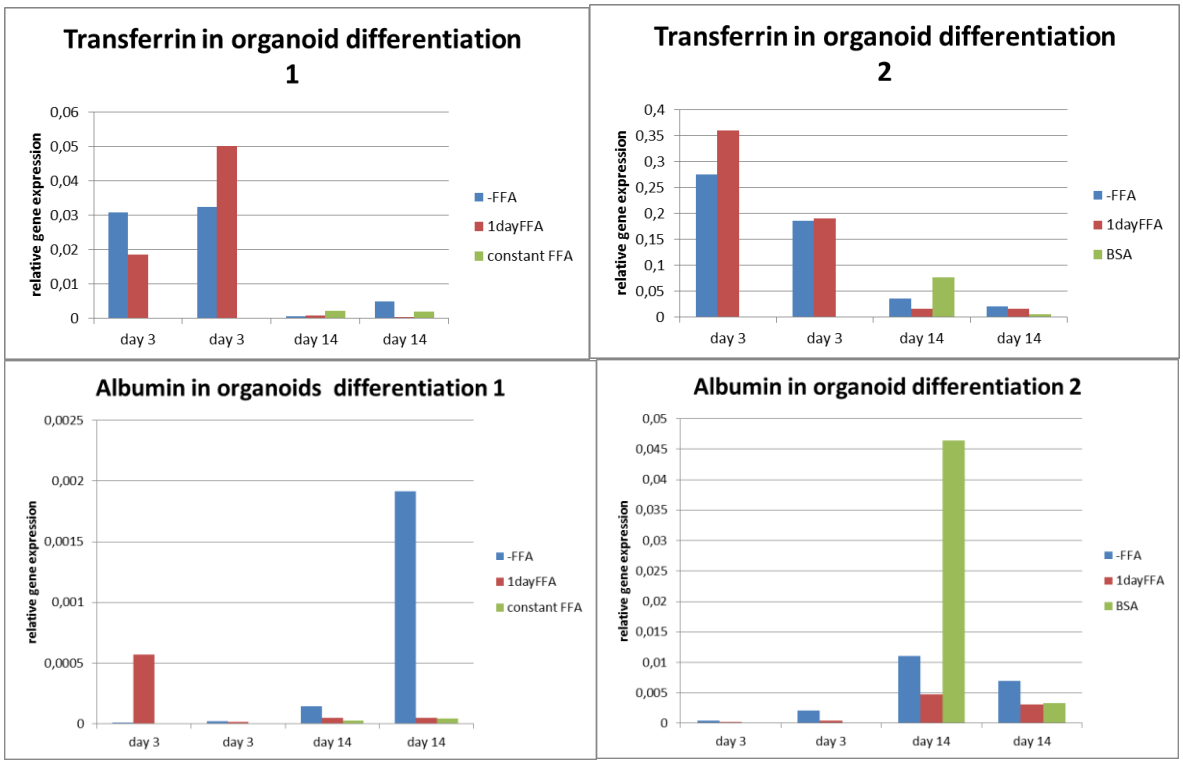


Figure 12: the relative gene expressions on albumin and transferrin for the organoids in the two times the experiment was performed. Time one with the conditions –FFA(without extra fatty acids), with 1day FFA and constant FFA, the second one the constant FFA was replaced by 1dayBSA. FFA; extra dose of free fatty acids, BSA; bovine serum albumin

The organoids during the differentiation protocol started to look darker and the individual cells became visible (see figure 13).

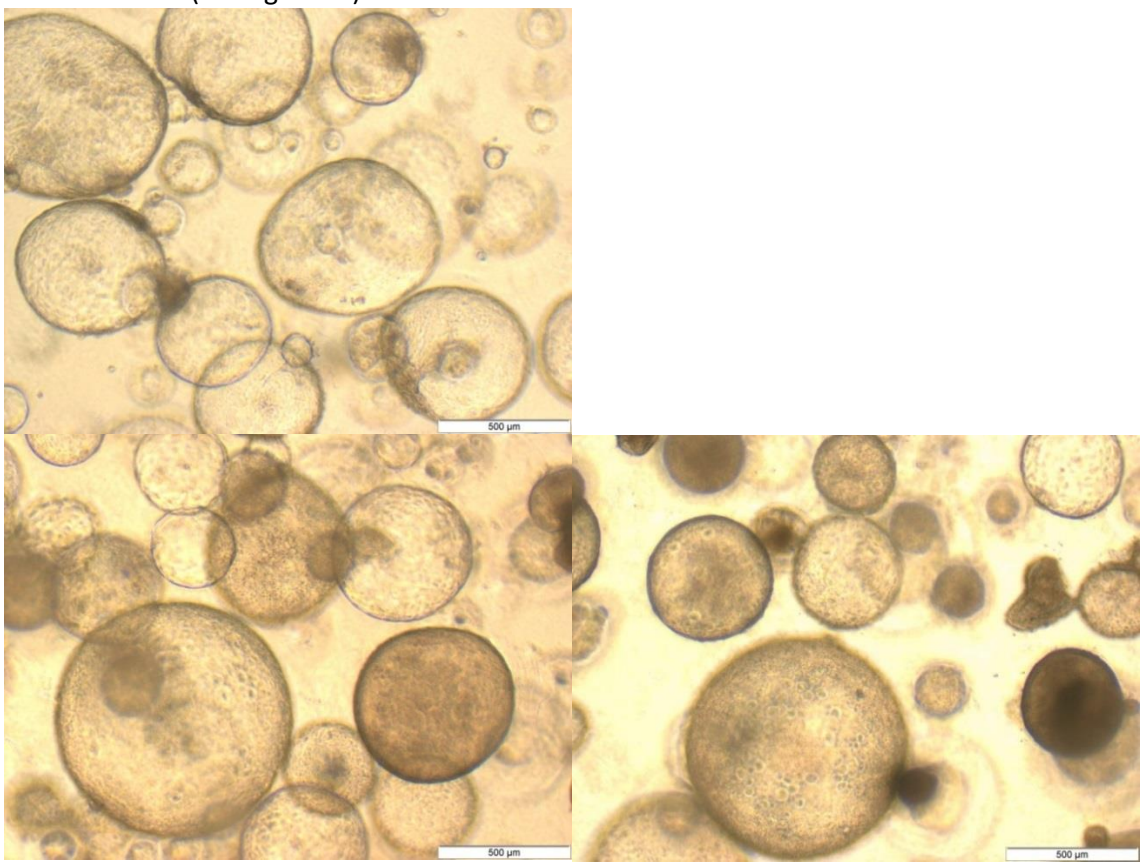


Figure 13: Organoids at day 0 (first picture) and at day 13 (picture two and tree) of the differentiation protocol.

Lipids

HepaRG

The amount of accumulated TAG and the amounts of lipid droplets increase with differentiation in HepaRGs (day 15 compared to day 2 a 7.6 times increase with $p<0.005$, day 21 compared with day 2 a 15 times increase with $p<0.005$). The cells constantly cultured in extra FFA have a bit more TAG and lipid droplets than the controls (at day2 a 7 times increase compared to nontreated with $p<0.005$. At day 15 a 2 times increase with $p=0.01$. At day 21 a 3 times increase with $p=0.0001$). The cells treated with one day extra FFA before sampling had much higher TAG levels and a lot more lipid droplets, except for the undifferentiated HPCs (At day2 a 6.81 times increase compared to nontreated with $p<0.005$. At day15 a 12 times increase with $p<0.005$. At day 21 a 8.6 times increase with $p<0.005$) (see figure 14). The fluorescent pictures confirmed the mass spectrometer data, but also the morphological observation that it's a heterogeneous image. Unfortunately the morphological pictures and the fluorescent pictures are not comparable at the singular cell level. The general image showed an increase in lipid droplets in time, and a bigger increase in cells treated one day with extra fatty acids than in cells cultured constantly in extra fatty acids. Pictures are taken one week after feeding. There was also an increase in albumin staining over time, but there was no consistent overlap between cells stained for albumin and cells stained for lipid droplets (see figure 15).

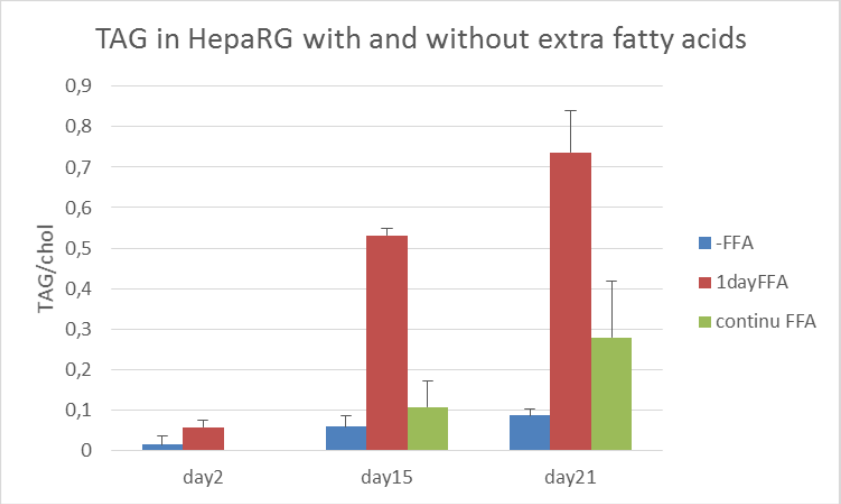
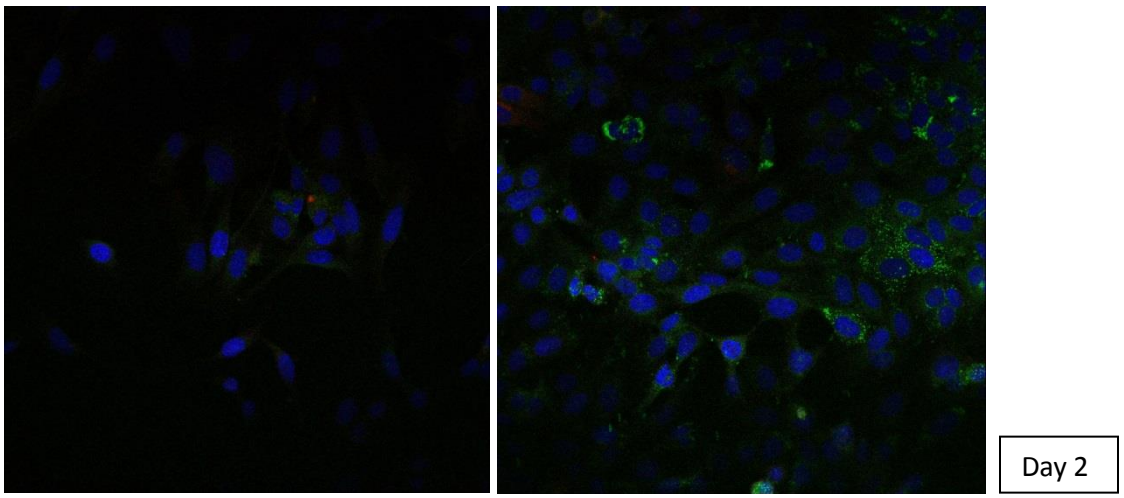


Figure 14: The TAG levels, divided by the amounts of cholesterol. At day 15 and day 21 of culture, for three conditions. The control (blue), standard culture with at the day before sampling extra fatty acids added (green) and continues culture in extra fatty acid containing medium (red). For statistics, see text page 22. Figure is based on data from three separate experiments (experiment 1,2 and 3). FFA; extra dose of free fatty acids, TAG; triacylglycerol, chol; cholesterol.



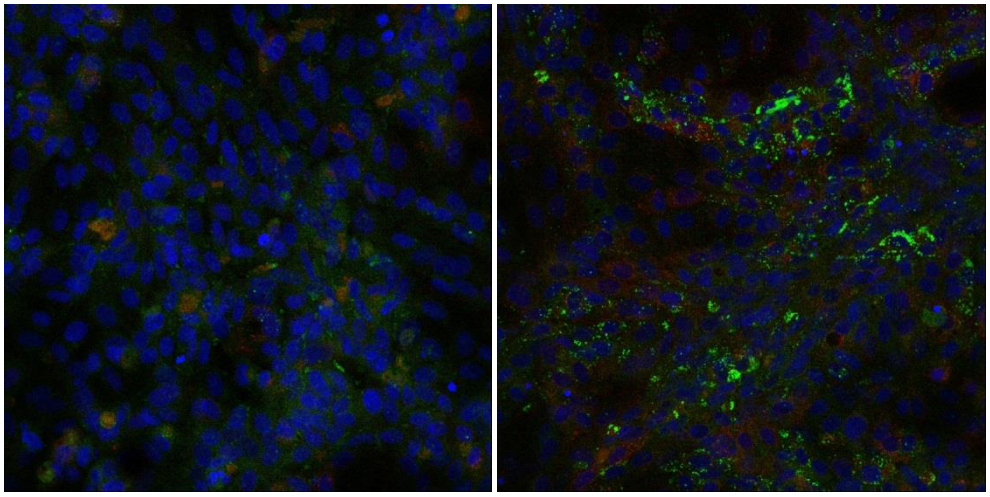


Figure 15: fluorescent pictures of HepaRG at day 2 and day 15, without (left) and with (right) extra fatty acids for one day. Blue is Hoechst staining, red is albumin and green is LD540.

As a large difference in TAG levels between constantly and one day extra fatty acid culture was found, we tested the media to see if there was anything left of the extra fatty acids we added. There was almost nothing left (see figure 16), indicating the fatty acids were somehow consumed by the cells.

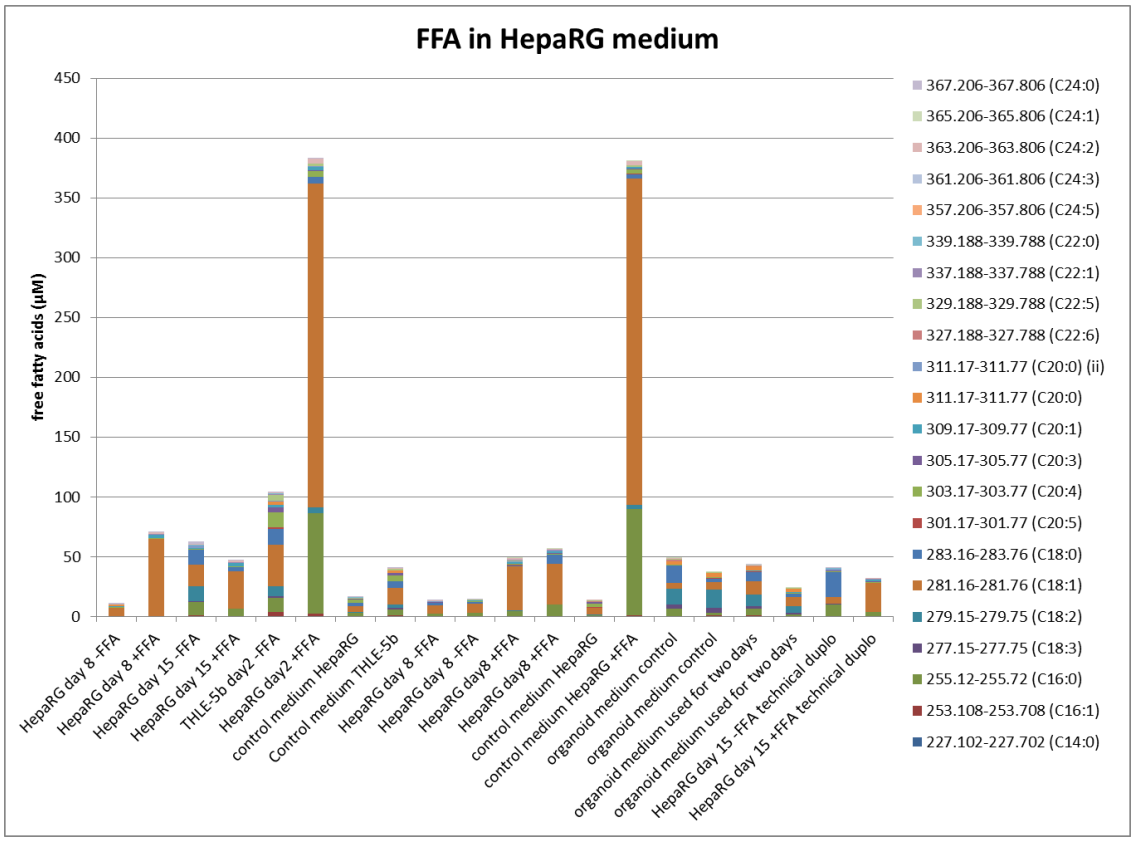


Figure 16: amounts of free fatty acids (FFA) in uM in HepaRG, THLE-5b and organoïd medium. Day stands for the days the cells were in culture, control medium means medium that has never been used on the cells. Colours stand for different fatty acids species. Results are preliminary, have been repeated but still waiting for the results.

We also looked at the expression of perilipin 2. This is a protein present on lipid droplets. The mRNA levels of perilipin 2 increased over time. Between the conditions the levels were higher in the cells that were treated with extra fatty acids only one day before sampling (see figure 17).

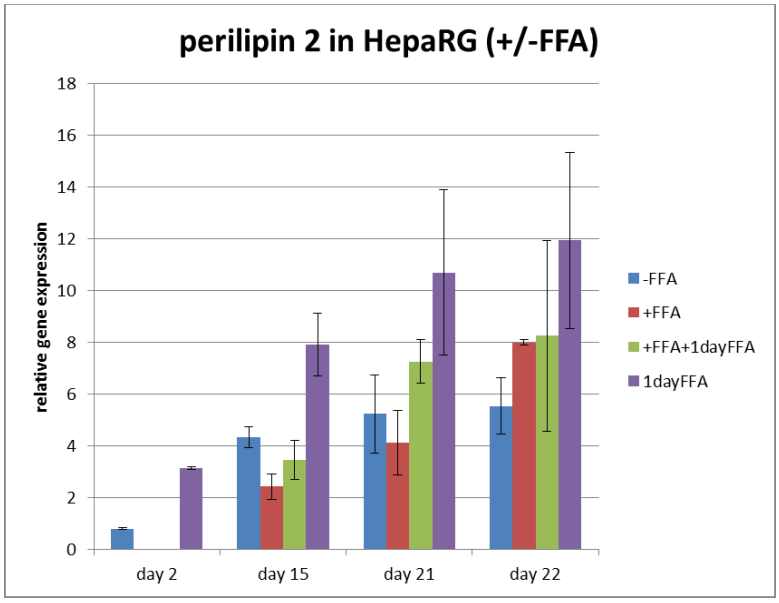


Figure 17: perilipin 2 levels in HepaRG. Conditions are without FFA (blue), with constant FFA(red), with constant FFA and one day FFA. FFA; extra dose of free fatty acids.

HepaRGs as a progenitor cell line and HepG2s as a hepatocyte like cell line were compared. HepaRGs and HepG2s cultured for 8 days showed a difference in the response to extra fatty acids between day 2 and day 8 (see figure 18).

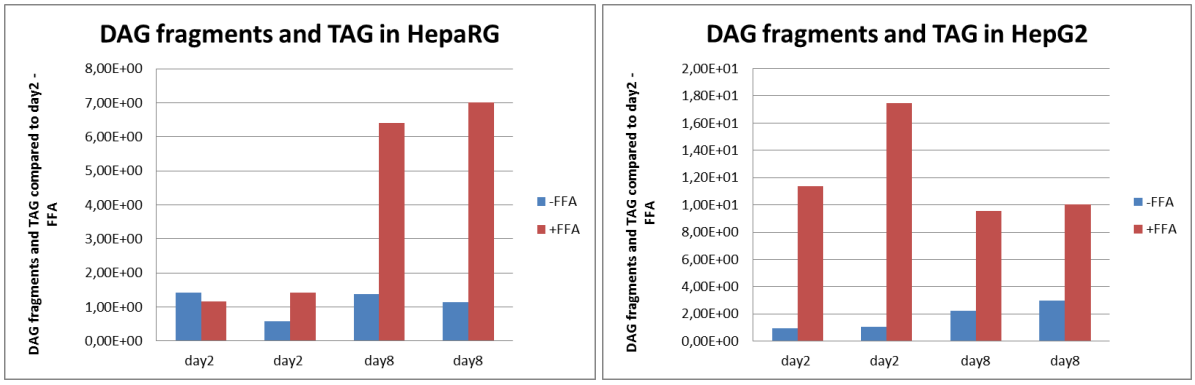
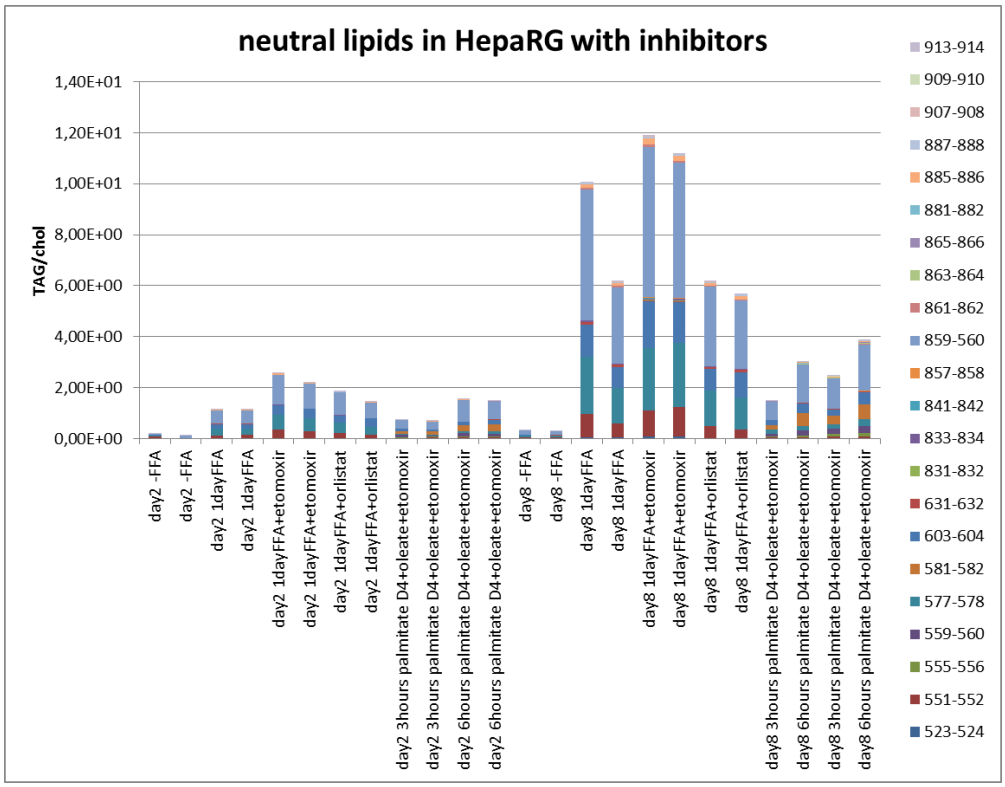


Figure 18: left graph shows the response of HepaRG to extra fatty acids (FFA) to the media, right graph shows the response of HepG2. TAG; triacylglycerol.

To determine what causes the difference between day 2 and the later days, HepaRGs were cultured with one day extra fatty acids and either orlistat (lipase inhibitor) or etomoxir (carnitine shuttle inhibitor). The cells cultured with etomoxir showed a higher increase in triacylglycerol, the LD540 staining seemed to agree with a higher amount of lipid droplets. Orlistat had a smaller, or no effect at all at day8, and a smaller effect at day 2 (see figure 19). Both inhibitors give a decrease in the difference between day 8 and day 2 compared to the control with extra fatty acids, but they do not abolish the effect. Perilipin 2 mRNA levels were highest in the cells treated with etomoxir.
To see if the higher TAG levels after incubation of 24 hrs with extra FFAs at day 8 compared to day 2 are caused by a higher uptake and incorporation of FFAs, the following experiment was performed. HepaRGs were cultured with etomoxir, oleate and palmitateD4 (in the same concentration as the standard palmitate) for 3 and 6 hours. The etomoxir was added to prevent immediate breakdown of the extra fatty acids, the palmitate was labelled to measure the uptake of fatty acids. The short time points were taken to ensure we measure uptake and not breakdown. Because of the palmitateD4 the masses of the triacylglycerol that have the palmitateD4 incorporated become 4 heavier than

normal. Most clearly the 581 (normally 577), the 559 and 555 (normally 551) masses stand out. Together they show a difference in uptake between day 2 and day 8. This ratio is smaller than the ratio for the hepaRGs cultured with extra fatty acids. For these three DAG fragments there is no difference between the ratios at 3 hours or 6 hours (for both the ratio is 2). The total amount of TAG ratio differs a little bit at the different hours of extra fatty acids (day8 is 3 times higher than day2 at 3 hours, at 6 hours that ratio is 2). The ratio between day 8 and day2 for 24 hours with normal palmitate, oleate and etomoxir is 5. Therefore the difference between day 8 and day 2 can not be completely explained by the difference in palmitate D4 uptake.
These are all preliminary data, so they need to be repeated to be able to do statistics on them.



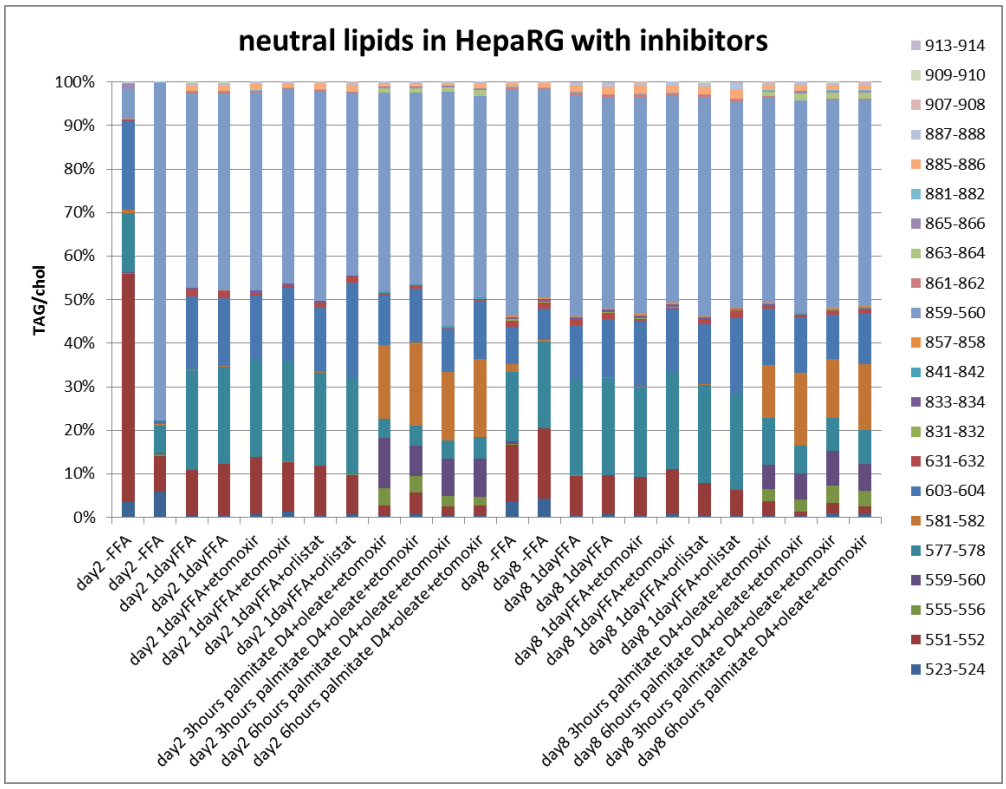


Figure 19: TAG amounts and DAG fragment amounts divided by cholesterol. Colours are the TAG species. TAG; triacylglycerol, DAG; diacylglycerol, FFA; extra dose of free fatty acids, chol; cholesterol

THLE-5b

The THLE-5b cells were only used as an undifferentiated cell type. Compared to the HepaRGs in their undifferentiated state they have more TAG and lipid droplets (see figure 20 and 21). Their response to extra fatty acids seems the same as for HepaRG. There is a small increase in TAG and no apparent differences in TAG species between the conditions.

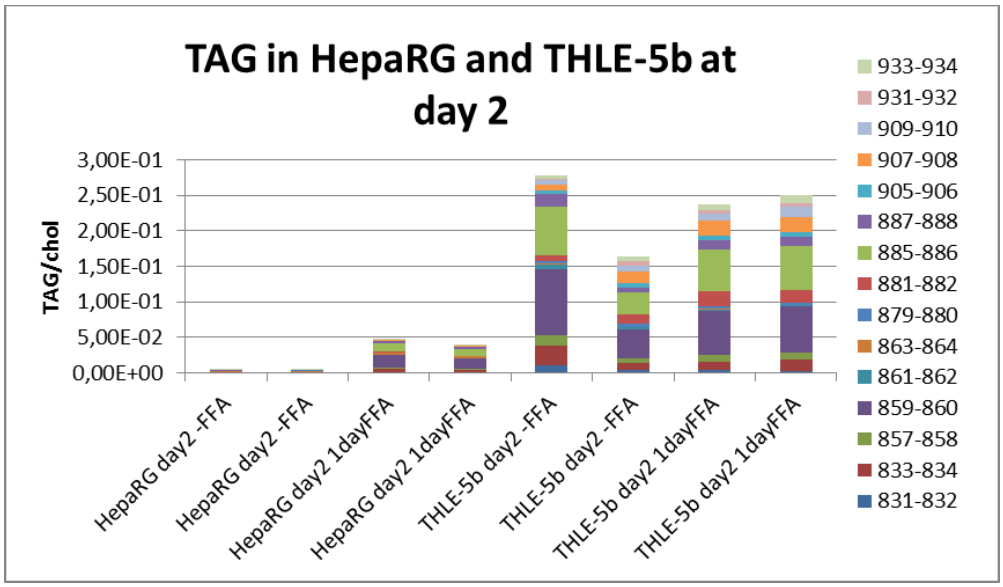
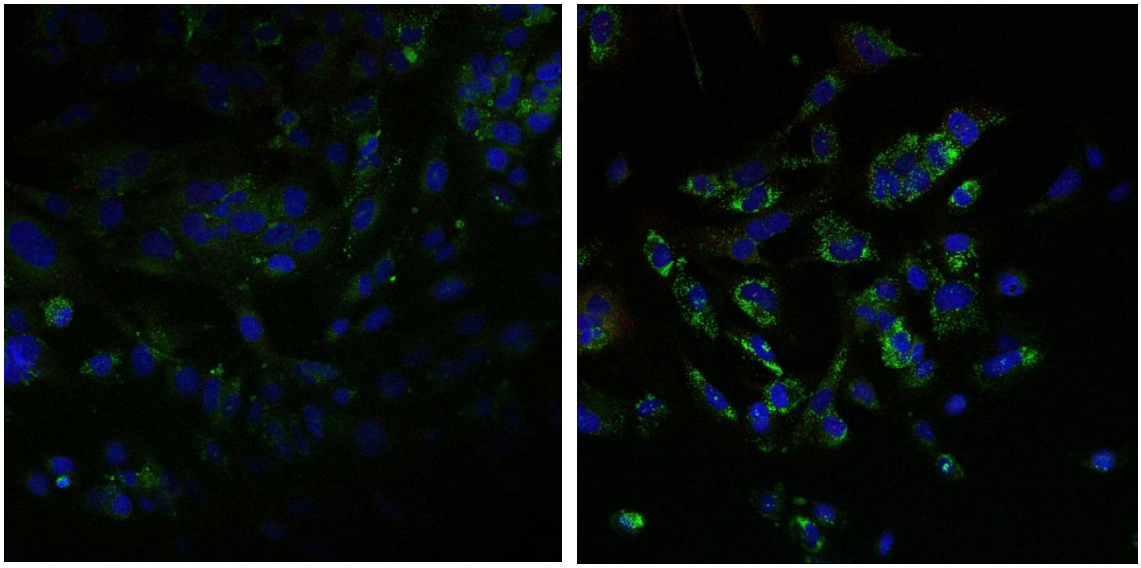


Figure 20: The amounts of Triacylglycerol/cholesterol in HepaRG and THLE-5b cells, undifferentiated. Colours are the different TAG species measured.



Day 2

Figure 21: fluorescent pictures of THLE-5b cells at day 2 of culture. Left the untreated, right the ones treated with extra fatty acids for one day. Blue is Hoechst nuclei staining, green is LD540 lipid staining.

Organoids

In the organoids the levels of TAG increased during the differentiation protocol as well. Only there was no big difference in response of undifferentiated and 'differentiated' organoids to one day of extra fatty acids. Both the conditions responded by a similar relatively large increase in TAG levels. The long-time culture in extra fatty acids showed the highest amount of TAG (see figure 22). Organoids receive new media every two days, in contrast to HepaRGs who receive new media every week. The fluorescent pictures confirmed the mass spectrometer data. They also showed that the organoids develop lipid droplets that sometimes reach a size bigger than the nucleus (see figure 23). In the second differentiation attempt in the samples from day 14, and a bit in the samples from day3 with extra fatty acids there were differences between the organoids (see figure 24). Two types of organoïd were observed. There were some with a lot of space between the nuclei, which had a lot of lipid staining around them. Some others had a lot of nuclei with little space between them, and less lipid staining.
ZO-1 is present on hepatocytes and not on undifferentiated organoïd (Huch et al. 2013). Here the ZO-1 staining failed in the organoids as well as the HepG2 positive control.

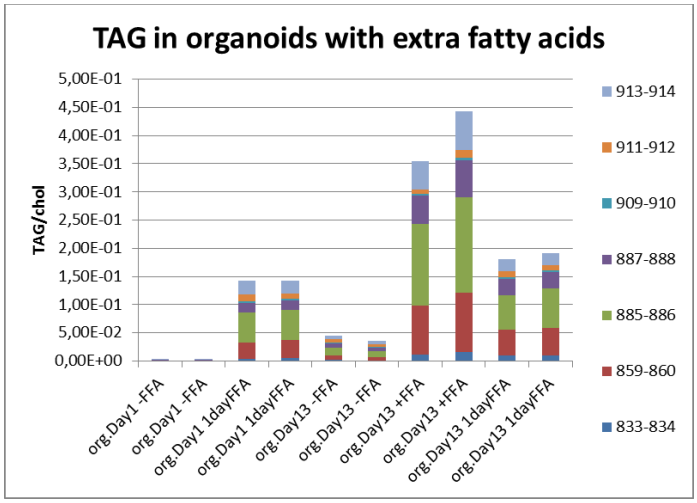


Figure 22: The amount of TAG/chol in undifferentiated organoids (day1) and differentiated organoids (day13) with different conditions. Without extra fatty acids, with one day extra fatty acids and cultured constantly in extra fatty acids. Colours stand for different TAG species. TAG; triacylglycerol, FFA; extra dose of free fatty acids, chol; cholesterol.

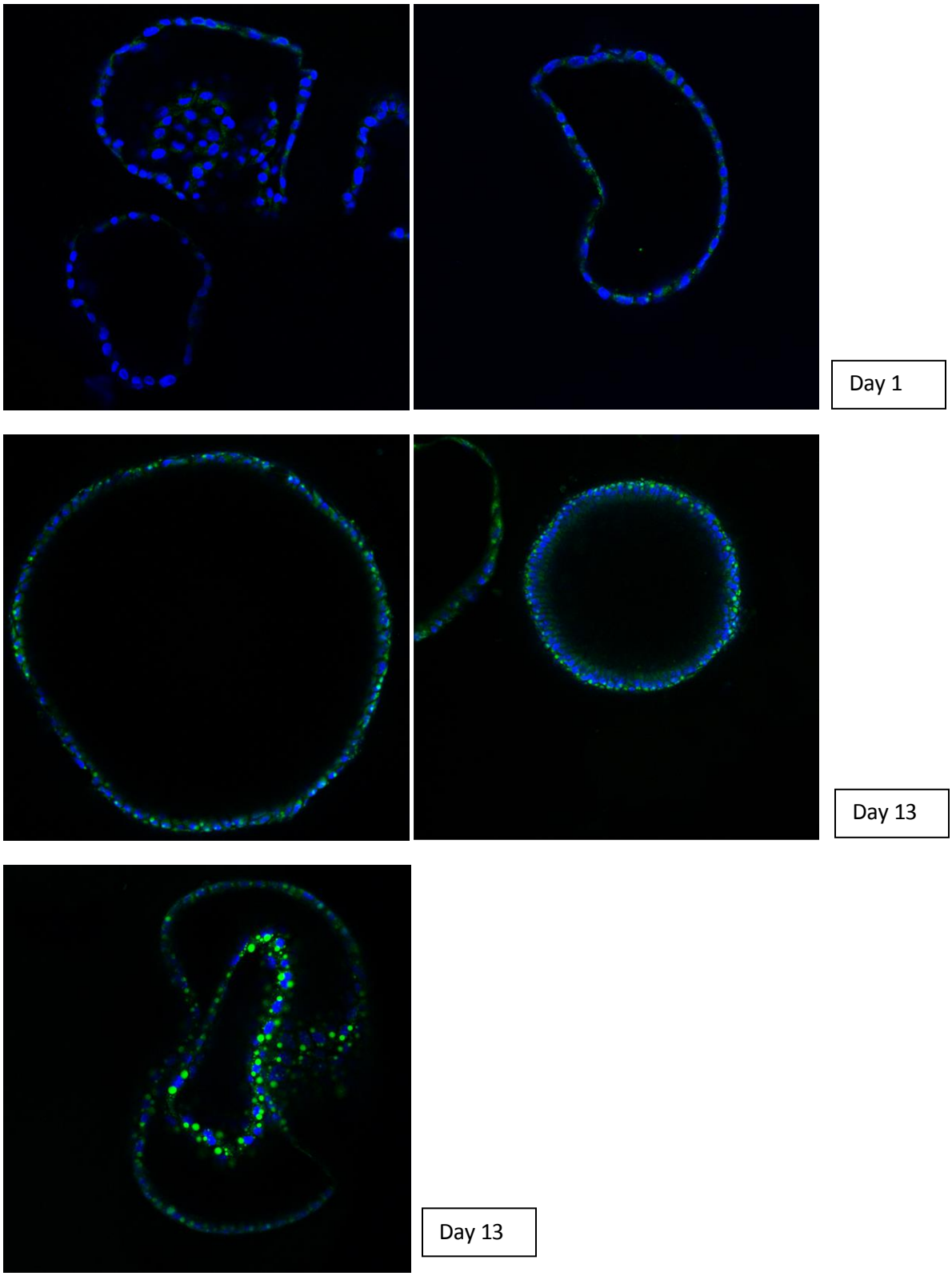


Figure 23: fluorescent pictures of organoids undifferentiated (day1) without (left) and with one day (right) extra fatty acids. Differentiated organoids (day13) without (left), with one day (right) and constant (below) extra fatty acids. Blue is Hoechst staining, green is LD540 staining.

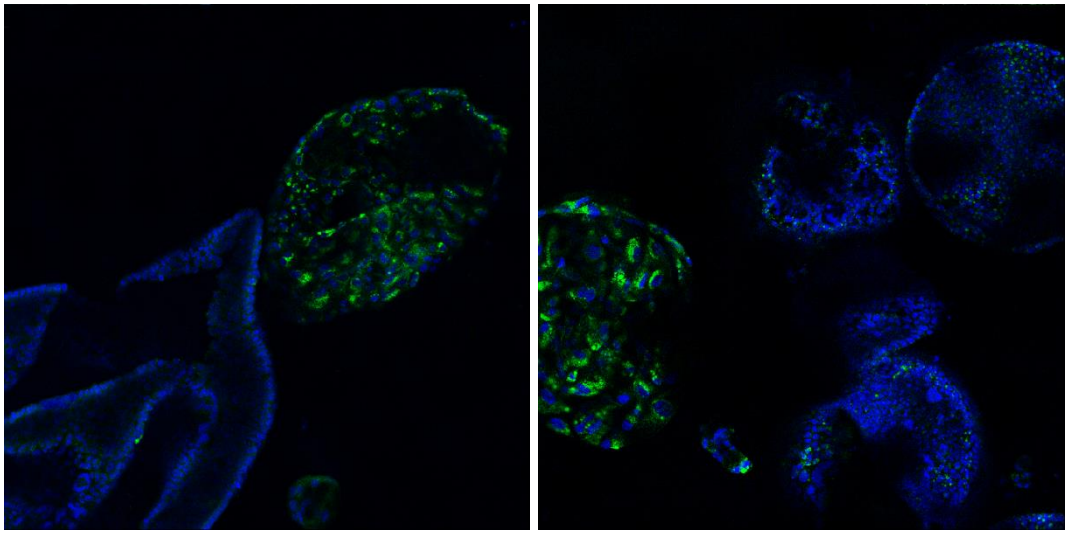


Figure 24: fluorescent pictures from day 14 without (left) and with (right) extra fatty acids. green= LD540 lipid staining, blue= Hoechst nucleus staining.

Discussion

Differentiation

The characteristics of the HepaRGs as a bipotent progenitor cell line and its capability to differentiate towards either cholangiocytes or hepatocytes is well described (Cerec et al. 2007; Andersson et al. 2012; Gripon et al. 2002; Parent et al. 2004). However the differentiation is never complete for all the HepaRG cells in one well. This makes it difficult to connect morphology to qPCR and mass spectrometer data, because those methods look at all the cells together. It is only possible to compare different wells, not differences in areas of cells within one well. Our qPCR results show a significant increase in hepatocyte gene expression. This makes it safe to assume that at least a fraction of the cells has differentiated. However finding a decrease of a fraction is more difficult, because the increase of the hepatocyte markers comes from a starting point of almost zero. Therefore if the markers are present, they are likely to be measured. The markers for the progenitor cells are already present at the starting point, and because of the heterogenic population of cells during differentiation, some cells keep on expressing them. Therefore it is harder to measure a decrease of these markers, because there is heterogeneity at all stages. In our study the expression of KRT19 decreased a little at day 21 and the expression of KRT7 increased over time with a positive interaction with fatty acid treatment. KRT19 and KRT7 are also expressed in cholangiocytes, but the cholangiocyte marker GGT remained stable over time, so it is un likely that a large proportion of the cells differentiated towards the cholangiocyte lineage. KRT7 has been reported to be a less specific progenitor marker than KRT19. KRT7 has even been found on hepatocytes, although in a lesser degree than on biliary cells. KRT19 has only been found on biliary and intermediate cells (Tan et al. 2002).

Activation of HPCs and death of hepatocytes has previously been observed in livers with steatosis (T. Roskams et al. 2003; Nobili et al. 2012). Therefore we expected one of two scenarios: either the extra fatty acids would lead to more HPCs by activation (proliferation, higher MTT) and less hepatocytes (so less differentiation related mRNA) as the HPCs in differentiated state die. . Or the activation would lead to more differentiation in the +FFA group than the –FFA group, because the HPCs would be stimulated to proliferate and then differentiate. Our results showed no significant difference between the cells cultured in extra fatty acids and the controls. From all the hepatocyte gene mRNA levels we tested there was only an effect on CYP3a4. This was most likely not differentiation related, since the other gene expressions were unaffected, and the effect was noticeable in less than 24 hours. Another explanation for the effect on CYP3a4 was needed. The fatty acids we used were dissolved in BSA (bovine serum albumin), to ensure they spread evenly through the medium. In the media of the HepaRG cells albumin was also present, coming from the fetal calf serum (FCS). Albumin is a natural binder of fatty acids (reviewed by (Xenoulis & Steiner 2010)). Possible inhibitors of CYP3a4 expression in hepatocytes include cytokines like lipopolysaccharide (LPS)(Aitken & Morgan 2007). Another study found LPS contamination of fatty acid free BSA (Erridge & Samani 2009). Our results show an activation of TLR4 cells by our fatty acid free BSA, indicating a contamination with an LPS-like substance. Polymyxin B is an antimicrobial peptide (AMP) with a high affinity for LPS(Domingues et al. 2012). Since polymyxin B did not inhibit the activation by BSA, our BSA was very probably contaminated with an LPS like substance, that causes TLR4 activation. Such contamination would explain the CYP3a4 response. Further, since the polymyxin B had no effect, and dissolving the fatty acids in the media without BSA did not work, it is impossible to say that the fatty acids have no effect on CYP3a4. In literature CYP3a4 has been described several times as a lipid sensitive enzyme(reviewed by: (Buechler & Weiss 2011). In nutritionally obese mice CYP3a genes were down regulated (Yoshinari et al. 2006). In human livers a decreasing trend in CYP3a4 activity with progression of NAFLD has been shown, but this has not been statistically significant (Fisher et al. 2009). Other studies did find a significant difference in

CYP3a4 activity in human livers with and without steatosis or NASH (Kolwankar et al. 2007; Donato et al. 2006). The effect of adding extra fatty acids to the media of primary hepatocytes has been measured (Donato et al. 2006; Donato et al. 2007) in two articles, in which stock solutions were prepared with oleate and palmitate (2:1) (50mM) in culture medium containing 1% BSA. If this BSA was already there or that it was added later is unclear. In our case fatty acids were dissolved in 12% BSA. Adding fatty acids to medium containing 0.5% BSA (from the FCS) to create a concentration of 0.3mM fatty acids did not succeeed in dissolving the fatty acids.
Because we could not add the fatty acids without the BSA, we cannot exclude the possibility that extra fatty acids also cause a down-regulation of CYP3a4 in HepaRGs.

The morphological change, which was only seen in HepaRGs cultured for a long period in extra fatty acids, looked, due to the elongated shape of some of the cells, like a mesenchymal change. We could not determine whether the change came from the HPC like cells still there or from the already hepatocyte like cells.
There are several reports on epithelial to mesenchymal transition (Zeisberg et al. 2007) or mesenchymal to epithelial transition (Li et al. 2011). One of the possible types of EMT is organ fibrosis (reviewed by (Kalluri & Weinberg 2009)). Fibrosis is a well-known hallmark of liver damage. It is common in NASH, although it is usually associated with hepatic stellate cells (Brunt 2004). Fibrosis is mostly mediated by fibroblasts. Fibroblasts derived from EMT are positive for fibroblast-specific protein-1 (FSP1). Further hepatocytes exposed to TGF-beta1 show an EMT transition towards fibroblast like cells (Zeisberg et al. 2007). Therefore our initial hypothesis was that the hepatocyte-like cells underwent an EMT like transition in response to long term exposure to extra fatty acids, possibly comparable to fibrosis in NAFLD livers. However the qPCR results were not completely in agreement with the hypothesis. Only the mRNA levels were assessed, not the protein levels. It could be that the protein levels would have shown larger differences then the mRNA levels.
In some cases, depending on several factors, EMT can be partial, with cells only showing a few mesenchymal characteristics (Lamouille S, Xu J 2014). This may be a reason for the difference between vimentin, which has a small but significant difference between –FFA and constant FFA, and TWIST, which has no significant difference between these conditions. However, since it is a morphological visible change, it would be strange if only one gene would show a (small) difference. With the data collected so far there is no convincing evidence for EMT in the hepaRGs constantly treated with extra fatty acids.

We were not successful in differentiating the THLE-5b cells. The morphology of the cells appeared similar to what has previously been described (Tokiwa et al. 2006), our cells also grew in spheroid culture. However the mRNA levels of the hepatocyte and progenitor like genes were different. This in contrast to Tokiwa et all, who showed an upregulation of albumin and HNF4alfa and no upregulation of KRT19. In that paper, two versions are described of the differentiation protocol used: one on a rotator and one in the stove. A difference between our experiment and the one described by Tokiwa was that we couldn't control the CO2 levels in the rotation culture variant. However even their static variant showed an upregulation of hepatocyte like genes. It's unclear in which way our experiments differ from the ones described by Tokiwa, and since there are no other reports on differentiation of THLE-5b cells, some caution on the subject seems prudent. Thus we decided to drop the THLE-5b cell line as a differentiation model.

The organoids were differentiated according to the differentiation protocol from (Huch et al. 2013). The first time the RNA was sampled by first spinning the samples to get rid of the matrigel, then the RLT was added. The second time the RLT was added immediately unto the matrigel. This might influence the samples. The first time the quality and amounts of the RNA for some of the samples were low. The second time they were better. The first time it turned out that one of the components of the differentiation medium, DAPT, was out of date. This might influence the results. The qPCR

results were not very convincing in showing differentiation. The albumin was showing some increase, but the protein levels in the medium did not follow. This might indicate a very small step towards differentiation. Most differentiation models are not capable of getting full differentiation (reviewed by (Sangan & Tosh 2010)). However the transferrin levels were decreasing. Transferrin is a differentiation marker (Zamule et al. 2011; Petropoulos & Zakin 1993). CYP3a11 and TAT gave double meltcurves, so there is some work left to be done. It first needs to be sorted out if there was (some) differentiation. This could be done by testing more genes. If the experiment will be performed once more, it might be usefull to include a paraffin staining. With a paraffin staining it is not possible to stain for lipid droplets, but the ZO-1 staining had been optimised for paraffin and not for fluorescence. Alternatively, ZO-1 staining might be optimised for fluorescence as well.

In HepaRGs adding extra fatty acids in this concentration had no effect on proliferation and differentiation. For the organoids, it is too early to make a conclusion; first, the differentiation needs to be more convincing. This could be done by comparing every small detail of my differentiation protocol with the one that other researchers at the university have used. If this reveals no differences, then we could contact Huch et al to see if there is anything they do differently.
If we assume that the HepaRGs show a similar pattern as the real hepatic progenitor cell, then there is no direct effect of extra fatty acids on the differentiation of the cells. Probably the cells around the HPCs, the HPC niche, with for example hepatic stellate cells (Kordes & Häussinger 2013), respond to the lipid overload by producing factors that stimulate the HPCs to be activated.

Lipid

Our results show a difference in the response to extra fatty acids between HepaRGs in undifferentiated and differentiated form. This is in agreement with the research-group's pilot studies (see attachment: pilot studies). In the pilot studies more lipid droplets were found in hepatocyte like cell lines than in hepatic progenitor like cell lines.
That there was no difference in lipid accumulation between day 2 and day 8 in the hepatocyte-like cell line HEPG2, suggests that the lack of response at day 2 is not an effect of tripsinisation and plating from two days earlier that is general among cell lines. Instead, it may be HPC specific, or HepaRG specific.
The HepaRGs received new medium once a week, and for cells cultured continually in extra FFA this means they received new extra fatty acids weekly. The amount of extra fatty acids was expected to be more than they could metabolise in one week. However the amounts of triacylglycerol in the cells constantly cultured in extra fatty acids were not much higher than the amounts in the controls. This may indicate that the cells become less sensitive to the extra fatty acids, or that they consume them all. Cells cultured in constant extra fatty acids who received another dose of extra fatty acids the day before sampling showed similar TAG levels as the cells who had received their first dose the day before sampling (see attachment HepaRG). The media of the cells contained almost none of the extra fatty acids after one week. Apparently the HepaRGs digest all those fatty acids. Cells cultured with extra fatty acids and orlistat were not much different than the cells cultured with only extra fatty acids, at least at day 8, indicating the extra fatty acids are not metabolised by (extra) lipase activity. The lipases inhibited by orlistat are involved in breaking down TAG into fatty acids (Bénarouche et al. 2014; Carrière et al. 2001)(A.B.Vaandrager, unpublished). The cells cultured with extra fatty acids and etomoxir showed an increase of TAG in the cells, an increase in perilipin 2 levels (a protein found on the surface of lipid droplets in several different tissues including liver (Brasaemle et al. 1997)), and an increase in lipid droplets. This indicates a decrease in the ability of the cells to process the extra fatty acids. Etomoxir is an inhibitor of the carnitine shuttle (Eistetter & Wolf 1986; Lilly et al. 1992) involved in the beta-oxidation. Therefore we suggest that the HepaRG might have a

very rapid beta-oxidation which at least partially allows them to metabolise the extra fatty acids so quickly.
However the etomoxir does not abolish the difference between day 2 and day 8. Since our experiment was only performed once, it will need to be repeated, but the data suggests additional explanations of this difference between day 2 and 8 might also be needed.
The quantities of palmitate D4 uptake are different between day 2 and day 8, but the difference is smaller than the difference between the controls. Therefore a difference in uptake can also add to the difference between day 2 and day 8. A difference in uptake can come from a difference in the quantity or activity of transport proteins on the membrane. A membrane transport protein is for example cluster of differentiation 36/ fatty acid translocase (CD36/FAT), that can work in synergy with fatty acid transport protein 4 (FATP4), an intracellular protein involved in metabolic trapping of fatty acids (Schneider et al. 2014). There can also be a difference in the incorporation of the fatty acids in TAG, because we measured a difference in the incorporation of palmitateD4 in TAG. This could be due to a difference in the activity of diacylglycerol acyltransferase (DGAT) or monoacylglycerol acyltransferase (MGAT) enzymes, enzymes that are involved in the synthesis of triacylglycerol (reviewed by (Shi & Cheng 2009)).

Based on these data, it was expected that the organoids would respond in a similar way. The basal levels indeed increased, even though there is almost no lipid in the media of the organoids, in contrast to the media of the HepaRGs which contains lipids from the FCS. The increase of TAG after the addition of extra fatty acids was similar between undifferentiated and differentiated organoids. The difference in lipid accumulation in the HepaRG cells might be specific to this cell line, and not a HPC characteristic. Because the organoids consist of primary cells and are cultured in 3D they are more likely to represent the HPC. However since there is some doubt about the success of the differentiation of the organoids it might also be that this is the reason for the lack of difference between undifferentiated and 'differentiated'. Once the differentiation part is resolved, it would be interesting to see if the organoids respond similar to etomoxir as the hepaRGs.
The organoïds appeared to have two different types in the fluorescent pictures. Some organoids had the nuclei very close together, with little lipid. Some organoïd had the nuclei further apart, with more lipid staining around them. These nuclei appeared to be bigger, but less intensely stained. These two types were most apparent in the organoïd from day 14, with and without extra fatty acids, and a little in the organoïd from day 3 with extra fatty acids. It is possible that the organoïd with more space between the nuclei were less viable. During the differentiation protocol many organoïd become dark, which might have been a sign of cell death, and although the MTT for the hepaRG showed no negative effect of the extra fatty acids on the viability with these concentrations of fatty acids, this might be different for the organoïd. A viability assay for the organoïd could be performed to determine whether the differentiation protocol and the extra fatty acids added have any effect on viability. The organoïd with the less intense staining do not show nuclei that either fall apart or shrink, as the nuclei of dying cells usually do (reviewed by (Häcker 2000)).
Another explanation might be a mesenchymal differentiation. Mesenchymal cells are elongated compared to epithelial cells, which have nuclei close together in layers (reviewed by (Thiery & Sleeman 2006)). However there are no other indications to support this theory. Co staining with a mesenchymal marker could clarify this issue.
With differentiation the ratio between nucleus and cytoplasm increases as well. HPCs are known as small cells with almost no cytoplasm (reviewed by (Katoonizadeh et al. 2014)). Hepatocytes are bigger, sometimes with multiple nuclei. To check for polynuclear cells a membrane staining could be added.

In conclusion: there is no direct effect of extra fatty acids on the activation and differentiation of hepaRGs. HepaRGs do respond differently to extra fatty acids at day 2 of culture than at day 8. At

day 2, they incorporate less fatty acids from the medium. Furthermore, they consume fatty acids really quickly, most likely by beta-oxidation.
The organoids contain very small amounts of lipid, but with the supplemented extra fatty acids it was clear that they were capable of lipid uptake. It is to early to make any conclusions on the differentiation or the effect of extra fatty acids on this differentiation.
Further research is needed to discover the way in which our differentiation of the mouse organoïd differed from the differentiation protocol used by Huch et all. The experiments with the HepaRGs and the inhibitors need to be repeated to be able to do statistics on them. If the differentiation of the mouse organoïd is satisfying, the experiments on the HepaRG should be performed on the organoïd to determine whether the results found in the HepaRG are specific to this cell line or are specific to HPCs. In the future this might lead to an answer on the question in which way HPCs are protected from lipid overload, offering possible ways of protecting the liver from damage by lipid overload.

References

Aardema, H. et al., 2013. Bovine cumulus cells protect maturing oocytes from increased fatty acid levels by massive intracellular lipid storage. *Biology of reproduction*, 88(6), p.164.

Aitken, A.E. & Morgan, E.T., 2007. Gene-Specific Effects of Inflammatory Cytokines on Cytochrome P450 2C , 2B6 and 3A4 mRNA Levels in Human Hepatocytes. , 35(9), pp.1687–1693.

Andersson, T.B., Kanebratt, K.P. & Kenna, J.G., 2012. The HepaRG cell line: a unique in vitro tool for understanding drug metabolism and toxicology in human. *Expert opinion on drug metabolism & toxicology*, 8(7), pp.909–920.

Araya, J. et al., 2004. Increase in long-chain polyunsaturated fatty acid n - 6/n - 3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. *Clinical science (London, England : 1979)*, 106(6), pp.635–643.

Bénarouche, A. et al., 2014. Using the reversible inhibition of gastric lipase by Orlistat for investigating simultaneously lipase adsorption and substrate hydrolysis at the lipid-water interface. *Biochimie*, 101, pp.221–31.

Bligh E. G., D.W.J., 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, (37), pp.911–917.

Bonagura, J.D. & Twedt, D.C., 2013. *Kirck´s Current Veterinary Therapy XV*, Elsevier Health Sciences.

Brasaemle, D.L. et al., 1997. Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. *Journal of lipid research*, 38(11), pp.2249–2263.

Brunt, E.M., 2004. Nonalcoholic Steatohepatitis. *Seminars in liver disease*, 24(11), pp.3–20.

Buechler, C. & Weiss, T.S., 2011. Does hepatic steatosis affect drug metabolizing enzymes in the liver? *Current drug metabolism*, 12(1), pp.24–34.

Carrière, F. et al., 2001. Inhibition of gastrointestinal lipolysis by Orlistat during digestion of test meals in healthy volunteers. *American journal of physiology. Gastrointestinal and liver physiology*, 281(1), pp.G16–28.

Cerec, V. et al., 2007. Transdifferentiation of hepatocyte-like cells from the human hepatoma HepaRG cell line through bipotent progenitor. *Hepatology (Baltimore, Md.)*, 45(4), pp.957–967.

Department of Health and Human Services, U.S., 2011. 2011 SRTR & OPTN Annual Data Report.

Domingues, M.M. et al., 2012. Biophysical characterization of polymyxin b interaction with LPS aggregates and membrane model systems. *Biopolymers*, 98(4), pp.338–344.

Donato, M.T. et al., 2007. Effects of steatosis on drug-metabolizing capability of primary human hepatocytes. *Toxicology in vitro : an international journal published in association with BIBRA*, 21(2), pp.271–6.

Donato, M.T. et al., 2006. Potential Impact of Steatosis on Cytochrome P450 Enzymes of Human Hepatocytes Isolated from Fatty Liver Grafts. , 34(9), pp.1556–1562.

Duncan, A.W., Dorrell, C. & Grompe, M., 2009. Stem cells and liver regeneration. *Gastroenterology*, 137(2), pp.466–81.

Eistetter, K. & Wolf, H., 1986. Etomoxir. *Drugs of the Future*, (11), pp.1034–1036.

Erker, L. & Grompe, M., 2007. Signaling networks in hepatic oval cell activation. *Stem cell research*, 1(2), pp.90–102.

Erridge, C. & Samani, N.J., 2009. Saturated fatty acids do not directly stimulate Toll-like receptor signaling. *Arteriosclerosis, thrombosis, and vascular biology*, 29(11), pp.1944–9.

Van Eyken, P. et al., 1987. Cytokeratin immunohistochemistry in normal human liver. Cytokeratin pattern of hepatocytes, bile ducts and acinar gradient. *Virchows Archiv. A, Pathological anatomy and histopathology*, 412(1), pp.63–72.

Fausto, N., 2000. Liver regeneration. *Journal of Hepatology*, 32, pp.19–31.

Fisher, C.D. et al., 2009. Hepatic Cytochrome P450 Enzyme Alterations in Humans with Progressive Stages of Nonalcoholic Fatty Liver Disease. , 37(10), pp.2087–2094.

Gómez-Lechón, M.J. et al., 2007. A human hepatocellular in vitro model to investigate steatosis. *Chemico-biological interactions*, 165(2), pp.106–16.

Gripon, P. et al., 2002. Infection of a human hepatoma cell line by hepatitis B virus. *Proceedings of the National Academy of Sciences of the United States of America*, 99(24), pp.15655–60.

Häcker, G., 2000. The morphology of apoptosis. *Cell Tissue Res*, (301), pp.5–17.

Hengstler, J.G. et al., 2005. Generation of human hepatocytes by stem cell technology: definition of the hepatocyte. *Expert opinion on drug metabolism & toxicology*, 1(1), pp.61–74.

Hoekstra, R. et al., 2011. The HepaRG cell line is suitable for bioartificial liver application. *The international journal of biochemistry & cell biology*, 43(10), pp.1483–9.

Huch, M. et al., 2013. In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. *Nature*, 494(7436), pp.247–50.

Kalluri, R. & Weinberg, R.A., 2009. Review series The basics of epithelial-mesenchymal transition. , 119(6).

Katoonizadeh, A., Poustchi, H. & Malekzadeh, R., 2014. Hepatic progenitor cells in liver regeneration: current advances and clinical perspectives. *Liver international : official journal of the International Association for the Study of the Liver*, pp.1–9.

Kolwankar, D. et al., 2007. Association between nonalcoholic hepatic steatosis and hepatic cytochrome P-450 3A activity. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association*, 5(3), pp.388–93.

Kopec, K.L. & Burns, D., 2011. Nonalcoholic fatty liver disease: a review of the spectrum of disease, diagnosis, and therapy. *Nutrition in clinical practice : official publication of the American Society for Parenteral and Enteral Nutrition*, 26(5), pp.565–76.

Kordes, C. & Häussinger, D., 2013. Hepatic stem cell niches. *the journal of clinical investigation*, 123(5).

Kruitwagen, H.S., Spee, B. & Schotanus, B. a, 2014. Hepatic progenitor cells in canine and feline medicine: potential for regenerative strategies. *BMC veterinary research*, 10(1), p.137.

Lamouille S, Xu J, D.R., 2014. Molecular mechanisms of epithelial–mesenchymal transition. *Nat Rev Mol Cell Biol.*, (Mar;15(3)), pp.178–96.

Li, B. et al., 2011. Evidence for mesenchymal-epithelial transition associated with mouse hepatic stem cell differentiation. *PloS one*, 6(2), p.e17092.

Libbrecht, L. & Roskams, T., 2002. Hepatic progenitor cells in human liver diseases. , 13(02), pp.389–396.

Lilly, K. et al., 1992. Effect of etomoxiryl-CoA on different carnitine acyltransferases. *Biochemical Pharmacology.*, 43(2), pp.353–361.

Liu, Q. et al., 2013. Strategies to rescue steatotic livers before transplantation in clinical and experimental studies. *World journal of gastroenterology : WJG*, 19(29), pp.4638–50.

McCormack, L. et al., 2011. Liver transplantation using fatty livers: always feasible? *Journal of hepatology*, 54(5), pp.1055–62.

Michalopoulos, G.K., 1997. Liver Regeneration. *Science*, 276(5309), pp.60–66.

Nobili, V. et al., 2012. Hepatic progenitor cells activation, fibrosis, and adipokines production in pediatric nonalcoholic fatty liver disease. *Hepatology (Baltimore, Md.)*, 56(6), pp.2142–53.

Parent, R. et al., 2004. Origin and characterization of a human bipotent liver progenitor cell line☆. *Gastroenterology*, 126(4), pp.1147–1156.

Petropoulos, I. & Zakin, M.M., 1993. Transferrin gene as a model for liver-specific gene expression. *Biology of the cell*, 77(1), pp.63–65.

Roskams, T. et al., 1998. Hepatic OV-6 expression in human liver disease and rat experiments: evidence for hepatic progenitor cells in man. *Journal of hepatology*, 29(3), pp.455–463.

Roskams, T. et al., 2003. Oxidative stress and oval cell accumulation in mice and humans with alcoholic and nonalcoholic fatty liver disease. *The American journal of pathology*, 163(4), pp.1301–1311.

Roskams, T. a, Libbrecht, L. & Desmet, V.J., 2003. Progenitor cells in diseased human liver. *Seminars in liver disease*, 23(4), pp.385–396.

Sangan, C.B. & Tosh, D., 2010. Hepatic progenitor cells. *Cell and tissue research*, 342(2), pp.131–137.

Schmelzer, E., Wauthier, E. & Reid, L.M., 2006. The phenotypes of pluripotent human hepatic progenitors. *Stem cells (Dayton, Ohio)*, 24(8), pp.1852–1858.

Schneider, H. et al., 2014. Protein mediated fatty acid uptake: synergy between CD36/FAT-facilitated transport and acyl-CoA synthetase-driven metabolism. *Archives of biochemistry and biophysics*, 546, pp.8–18.

Schotanus, B. a, Penning, L.C. & Spee, B., 2013. Potential of regenerative medicine techniques in canine hepatology. *The Veterinary quarterly*, 33(4), pp.207–216.

Shi, Y. & Cheng, D., 2009. Beyond triglyceride synthesis : the dynamic functional roles of MGAT and DGAT enzymes in energy metabolism. *Am J Physiol Endocrinol Metab.*, pp.10–18.

Strick-Marchand, H. & Weiss, M.C., 2002. Inducible differentiation and morphogenesis of bipotential liver cell lines from wild-type mouse embryos. *Hepatology (Baltimore, Md.)*, 36(4 Pt 1), pp.794–804.

Tan, J. et al., 2002. Immunohistochemical evidence for hepatic progenitor cells in liver diseases. *Liver*, 22(5), pp.365–673.

Testerink, N. et al., 2012. Replacement of retinyl esters by polyunsaturated triacylglycerol species in lipid droplets of hepatic stellate cells during activation. *PloS one*, 7(4), p.e34945.

Thiery, J.P. & Sleeman, J.P., 2006. Complex networks orchestrate epithelial-mesenchymal transitions. *Nature reviews. Molecular cell biology*, 7(2), pp.131–142.

Tokiwa, T. et al., 2006. Differentiation potential of an immortalized non-tumorigenic human liver epithelial cell line as liver progenitor cells. *Cell biology international*, 30(12), pp.992–8.

Tokiwa, T. et al., 1998. Restoration of differentiated functions in multicellular aggregates of a human liver epithelial cell line. *Materials Science and Engineering: C*, 6(4), pp.249–252.

Xenoulis, P.G. & Steiner, J.M., 2010. Lipid metabolism and hyperlipidemia in dogs. *Veterinary journal (London, England : 1997)*, 183(1), pp.12–21.

Yoshinari, K. et al., 2006. Hepatic CYP3A expression is attenuated in obese mice fed a high-fat diet. *Pharmaceutical research*, 23(6), pp.1188–200.

Zamule, S.M. et al., 2011. Differentiation of Human Embryonic Stem Cells along a Hepatic Lineage. *Chem.Biol.Interact.*, 190(1), pp.62–72.

Zeisberg, M. et al., 2007. Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. *The Journal of biological chemistry*, 282(32), pp.23337–23347.

Attachment HepaRG

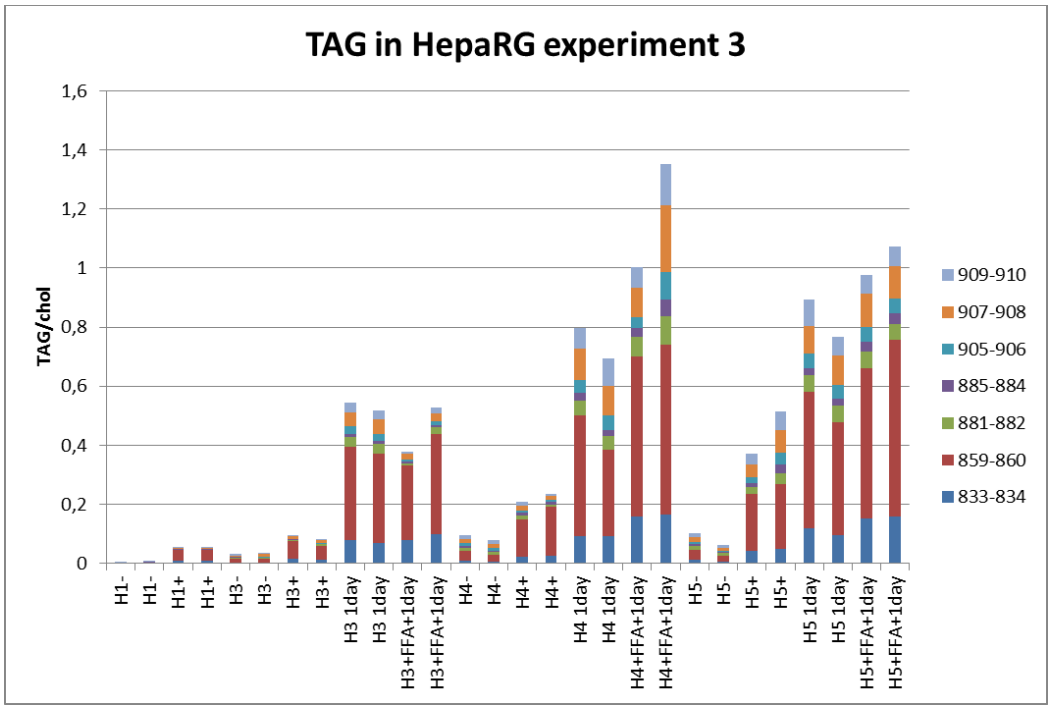
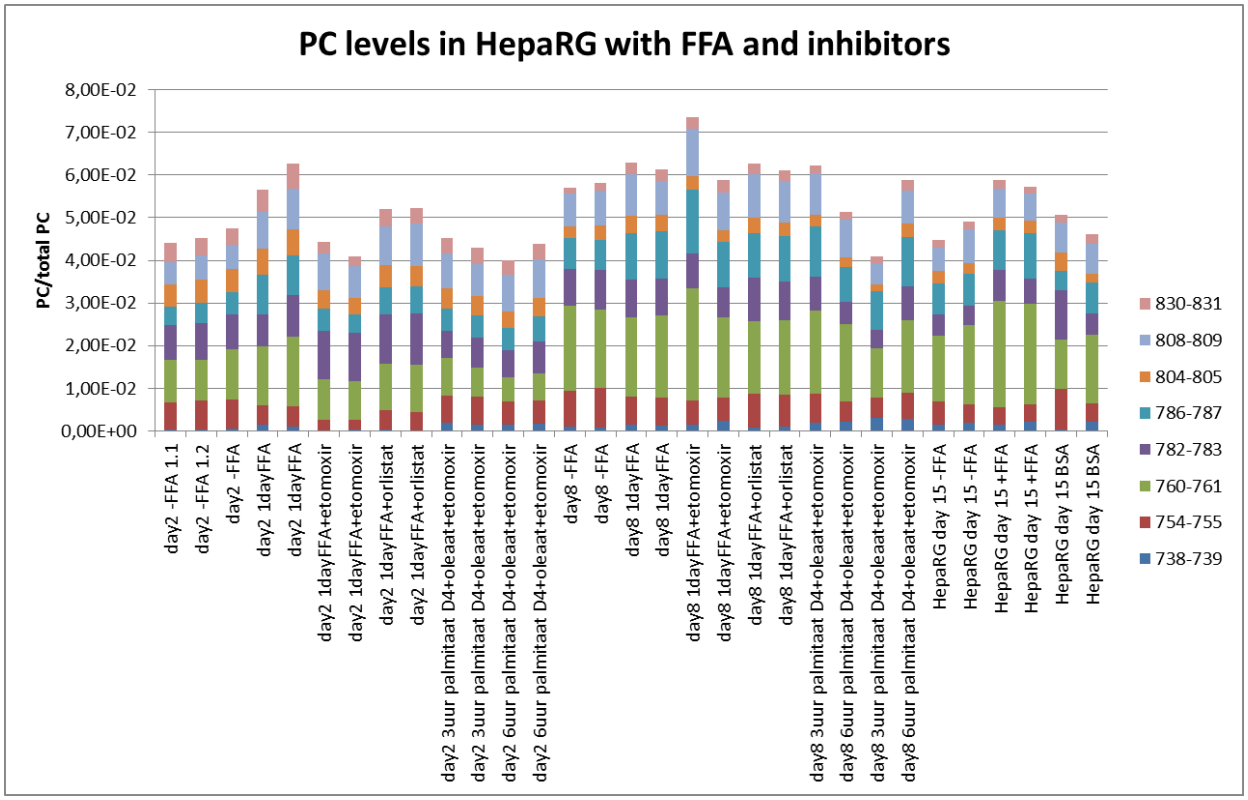


Figure 1 : triacylglycerol levels in HepaRG without extra fatty acids, with constant extra fatty acids, with constant extra fatty acids +1day fatty acids, and one day extra fatty acids. Colours stand for TAG species. H; HepaRG, number; timepoint, -/+; with or without constant extra fatty acids, 1day; 1day extra fatty acids, FFA; extra free fatty acids, TAG; triacylglycerol, chol; cholesterol, experiment 3; see table 1 with experiments



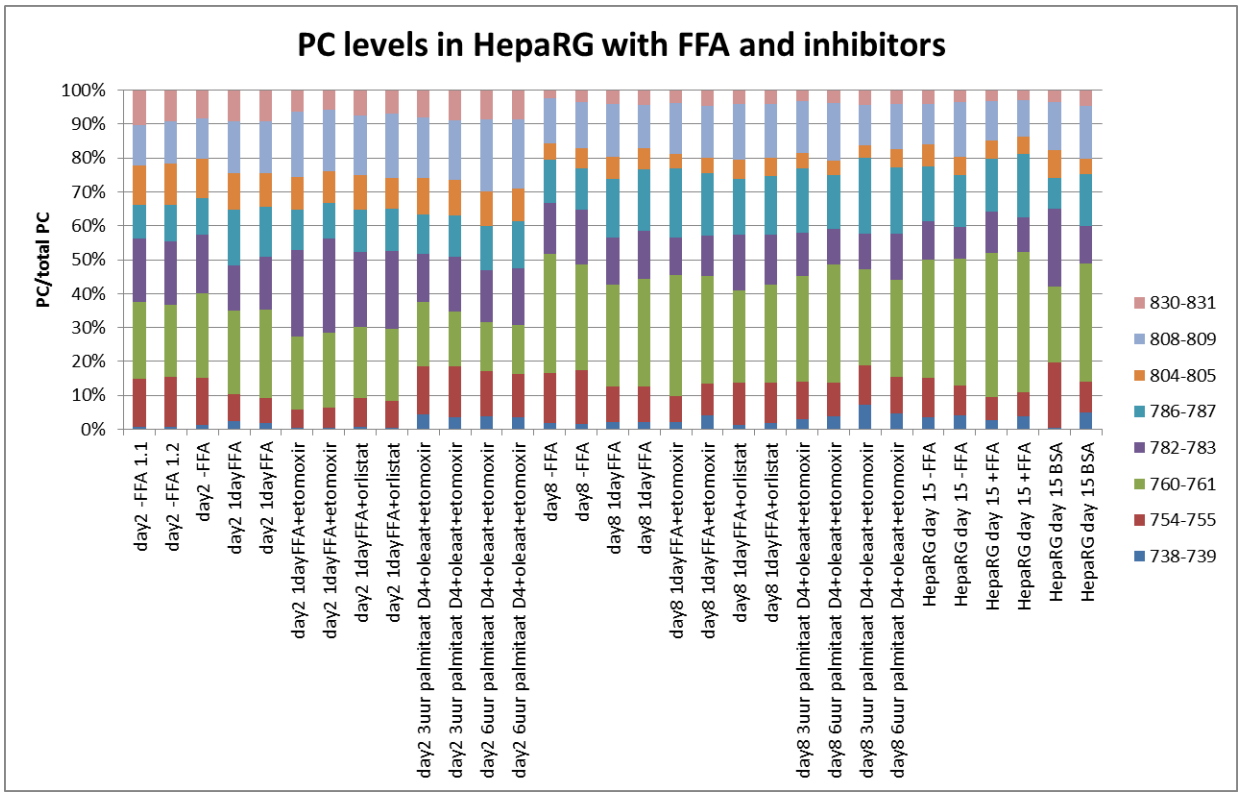


Figure 2: PC levels in HepaRGs treated with extra fatty acids, etomoxir, orlistat or palmitate D4. Colours stand for PC species, PC; phosphatidylcholine, FFA; extra free fatty acids,

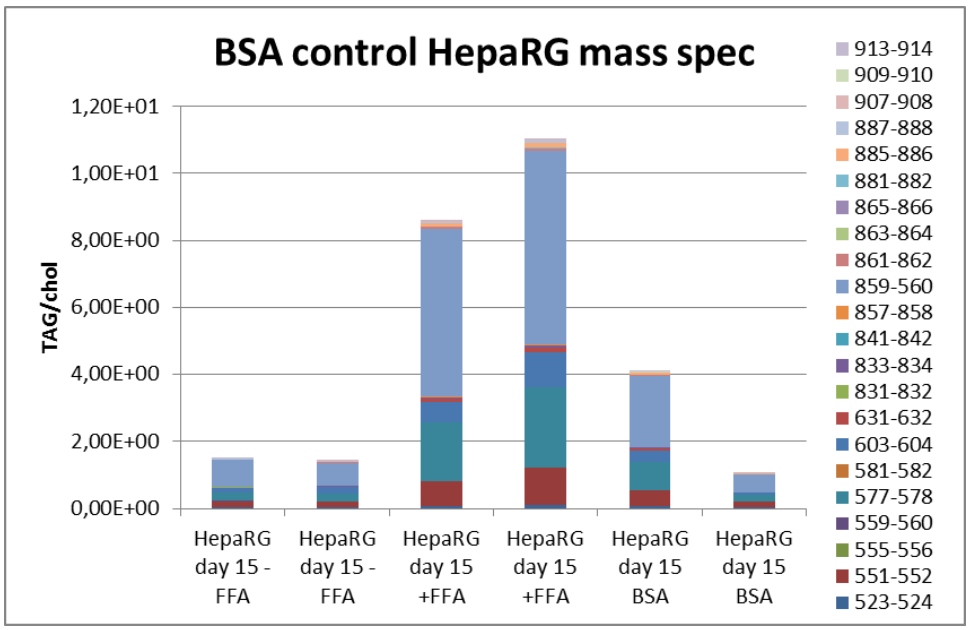


Figure 3: a BSA control for the mass spec data of the HepaRG. Colours stand for the TAG species. Amounts are in TAG/chol. TAG; triacylglycerol, chol; cholesterol, FFA; extra free fatty acids, BSA; bovine serum albumin

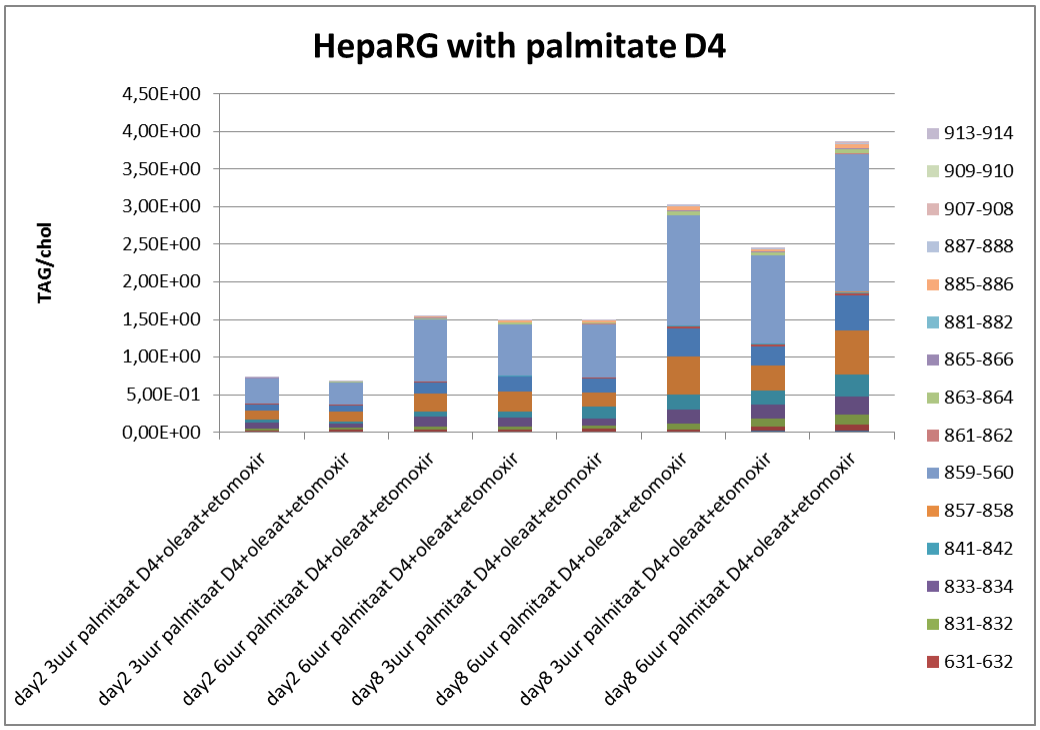
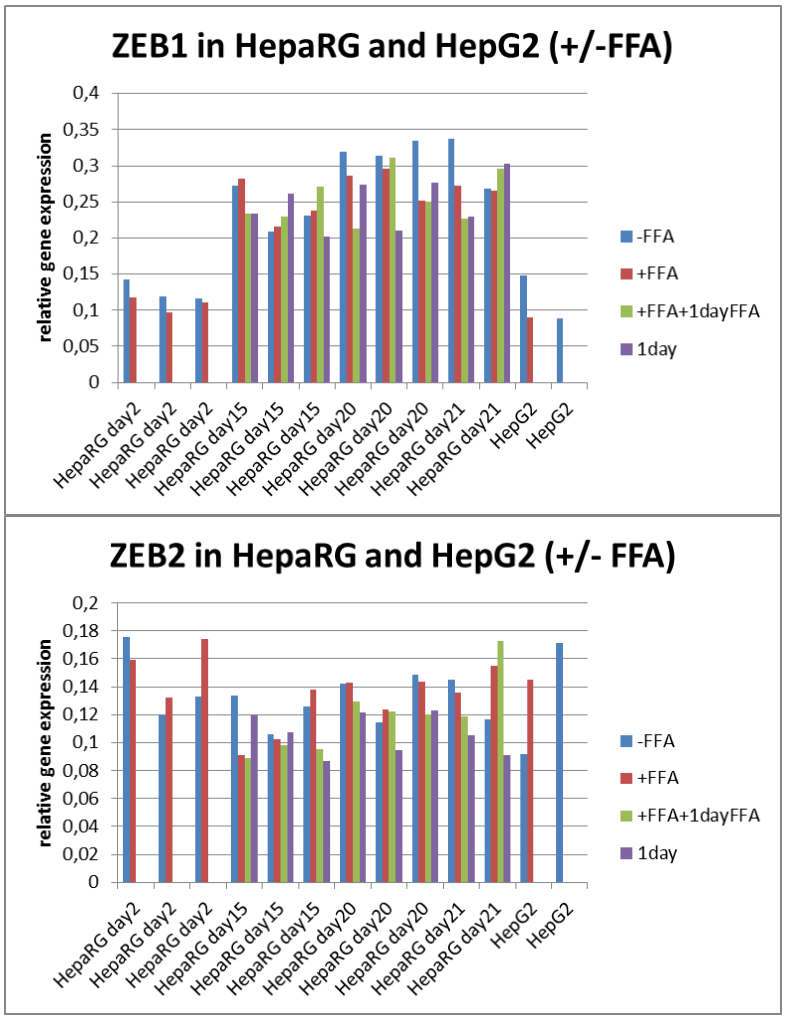


Figure 4: TAG amounts in HepaRGs treated with palmitate D4 to measure uptake. Colours stand for the TAG species. Amounts are in TAG/chol. TAG; triacylglycerol, chol; cholesterol, FFA; extra free fatty acids, BSA; bovine serum albumin



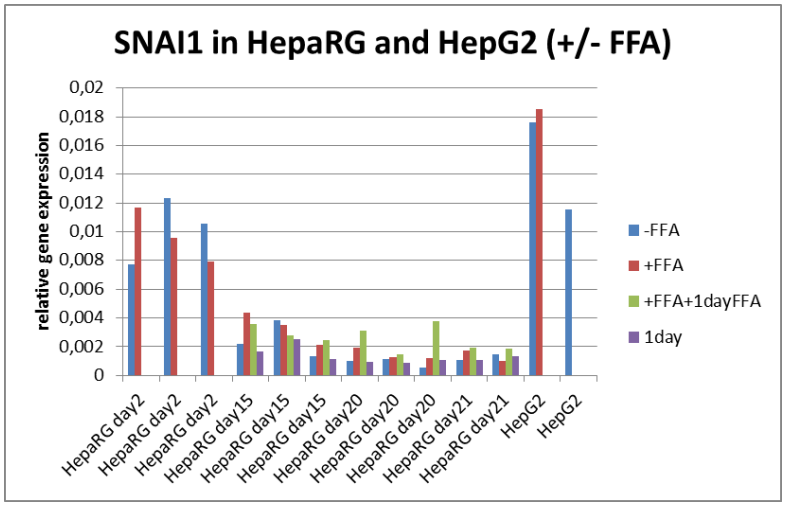


Figure 5: relative gene expression of mesenchymal genes in HepaRG and HepG2. Without extra fatty acids, with constant extra fatty acids, with constant extra fatty acids +1day fatty acids, and one day extra fatty acids.

Attachment THLE-5b

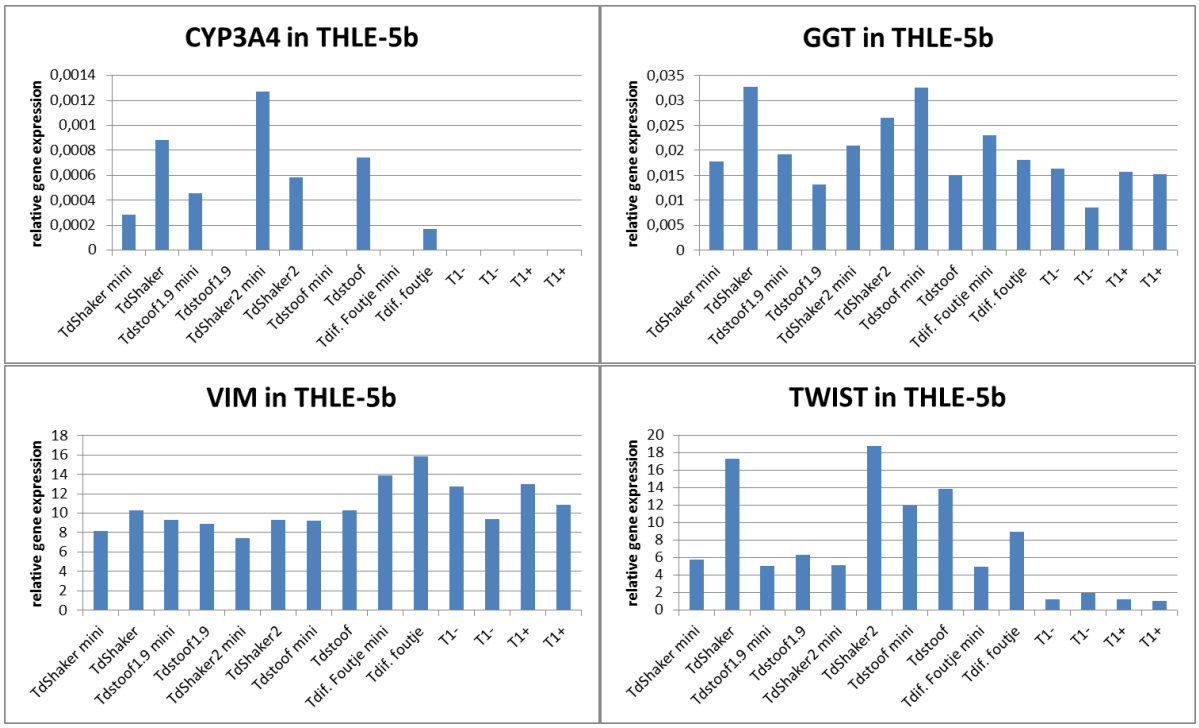


Figure 1: Genes tested on THLE-5b cells that did and did not undergo the differentiation protocol. CYP3a4; cytochrome p450 3a4, GGT; gamma glutamyl transpeptidase, VIM; vimentin. T1; undifferentiated THLE-5b (-/+ with or without extra fatty acids). Td; THLE-5b differentiated in several different ways. Shaker or stoof; stove culture. Mini means with mini RNA isolation kit, others with micro RNA isolation kit.

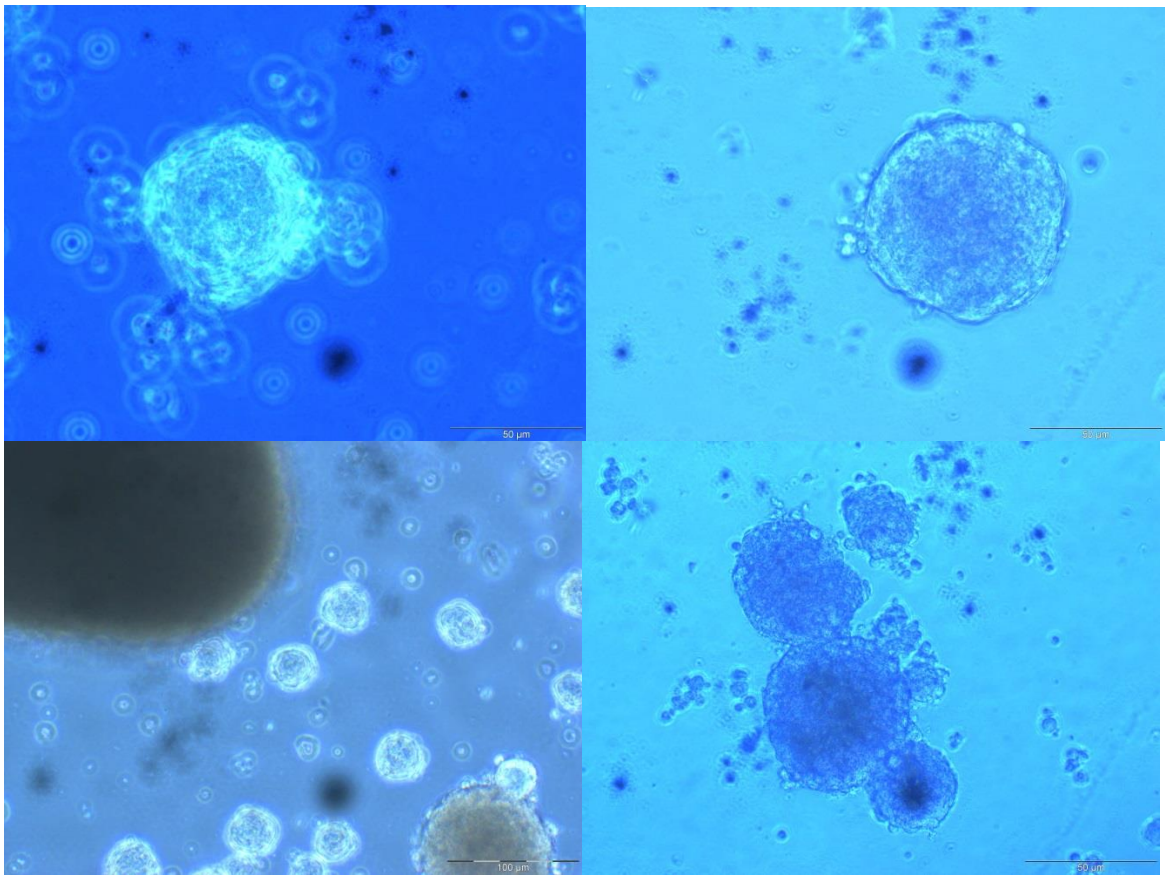


Figure 2: Pictures of the THLE-5b in differentiation protocol

Attachment primers

human

Protein	Extension	Sequence	Tm anealing
TWIST1	U	TGCATGCATTCTCAAGAGGT	60,0
	L	CTATGGTTTTGCAGGCCAGT	
ZEB1	U	GCCAATAAGCAAACGATTCTG	57,2
	L	CTTGTCTTTCATCCTGATTTCC	
ZEB2 (SIP1)	U	AGCCACAAATGAAAGTCCT	60,0
	L	AGCCATCTTCCCAATTCTG	
SNAI1	U	CATCCTTCTCACTGCCATG	60,0
	L	GTCTTCATCAAAGTCCTGTGG	
VIM (vimentin)	U	ACACCCTGCAATCTTTCAGACA	60,5
	L	GATTCCACTTTGCGTTCAAGGT	
HNF4A	U	GTACTCCTGCAGATTTAGCC	56
	L	CTGTCCTCATAGCTTGACCT	
ALB	U	GTTCGTTACACCAAGAAAGTACC	63.7
	L	GACCACGGATAGATAGTCTTCTG	
AFP	U	GATGAAACATATGTCCCTCCTG	64,6
	L	ATGAGAAACTCTTGCTTCATCG	
GGT1	U	CCTCAAAGGGTACAACTTCTC	60
	L	TTGTAGTAGGAGATCGGGTG	
KRT7	U	GGACATCGAGATCGCCACCT	60
	L	ACCGCCACTGCTACTGCCA	
KRT19	U	CTTCCGAACCAAGTTTGAGAC	60
	L	AGCGTACTGATTTCCTCCTC	
hs-TBP_Uq	U	TGCACAGGAGCCAAGAGTGAA	63,5
hs-TBP_Lq	L	CACATCACAGCTCCCCACCA	
hs-HRPT1_Uq	U	TATTGTAATGACCAGTCAACAG	60
hs-HPRT1_lq	L	GGTCCTTTTCACCAGCAAG	
hs-HMBS-Uq	U	GGCAATGCGGCTGCAA	56
hs-HMBS_Lq	L	GGGTACCCACGCGAATCAC	
hs-B2M-uq	U	CTTTGTCACAGCCCAAGATAG	58
hs-B2M_lq	L	CAATCCAAATGCGGCATCTTC	
hs-GAPDH_Uq	U	TGCACCACCAACTGCTTAGC	62
hs-GAPDH_Lq	L	GGCATGGACTGTGGTCATGAG	
hs-YWHAZ_Uq	U	ACTTTTGGTACATTGTGGCTTCAA	64
hs-YWHAZ_Lq	L	CCGCCAGGACAAACCAGTAT	
ADFP	U	TGTCTAGCCCCTTACAGGCA	63,5
	L	GTGAAGACCATCACCTCCGT	
ABCC2 (MRP2)	U	GGGATCTCTTCCACACTGGAT	69
	L	CATACAGGCCCTGAAGAGGA	
CYP3a4	U	CACAGGCTGTTGACCATCAT	62,5
	L	TTTTGTCCTATAAGGGCTTT	
POU5F1 (OCT4)	U	GAGAACCGAGTGAGAGGCAACC	60
	L	CATAGTCGCTGCTTGATCGCTTG	

Table 1: human primers used on the cell lines.

Mouse

Albumin	U	GCAACACAAAGATGACAACC	60
	L	CTTCATGCAAATAGTGTCCCA	

TAT	U	GCTTCCTTAAGTCCAATGCG	65
	L	TCAAATTCTGGGAAGTGCTC	
Transferrin	U	CTCTTGAGAAAGCTGTGTCC	65
	L	AAAGAATGGTTGAGTGGAGG	
Cyp3a11	U	CTCTCATAAAGCCCTTTCTGAC	60
	L	GTGAAGGAAAGTGTGCTACTG	
Mrp2	U	GATAGCCTCATTCAGACGAC	60
(Abcc2)	L	ACCATTATCTTGTCACTGTCCA	
Beta-actin	U	AGCTCCTTCGTTGCCGGTCCA	57
	L	TTTGCACATGCCGGAGCCGTTG	
RPS18	U	GATCCCTGAGAAGTTCCAGCAC	57
	L	ACCACATGAGCATATCTCCGC	
GAPDH	U	GAAGGTCGGTGTGAACGG	61
	L	TGAAGGGGTCGTTGATGG	

Table 2: mouse primers used on mouse organoid.

Attachment organoids

sample	mg/L
medium organoids -ffa day 13; 11/7/14	4
medium organoids -ffa day 13; 11/7/14	4
medium organoids -ffa day 13; 11/7/14	<2
medium organoids -ffa day 2 used	4
medium organoids - diff 13/5/14	4
medium organoids - diff 13/5/14	4
medium organoids -ffa day 2 used	<2
EM 1/7/14 -FFA day3	6
EM 1/7/14 -FFA day3	5
DM	8

Table 1: albumin levels in the medium of organoïd from the two differentiation experiments.

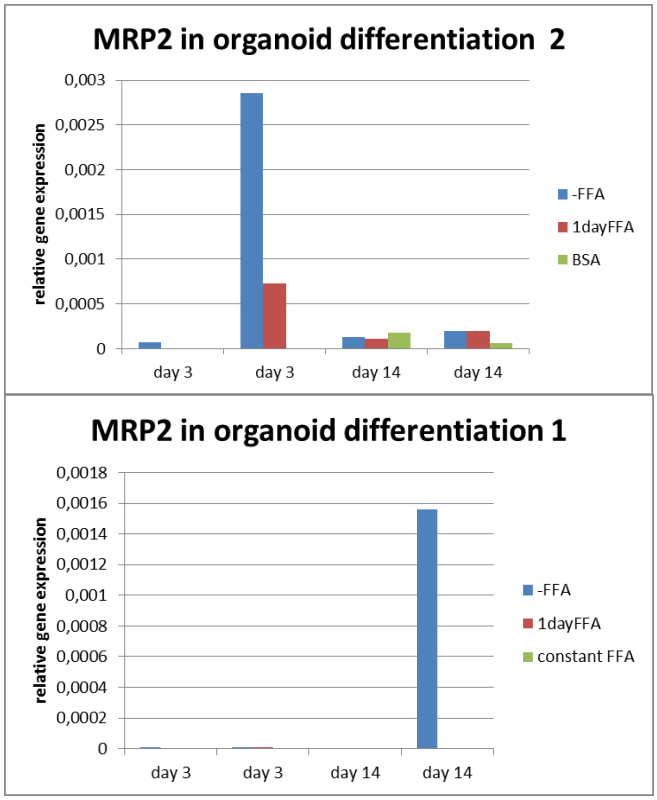
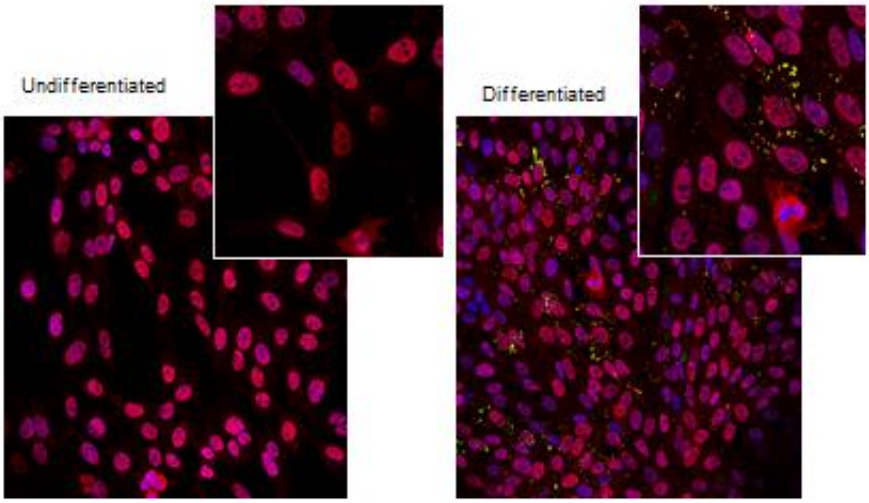


Figure 1: MRP2 expression in organoïd. FFA; extra free fatty acids, x-as; days of culture, BSA; bovine serum albumin. Differentiation 1 or 2: first or second attempt at differentiation.

Attachment pilot studies

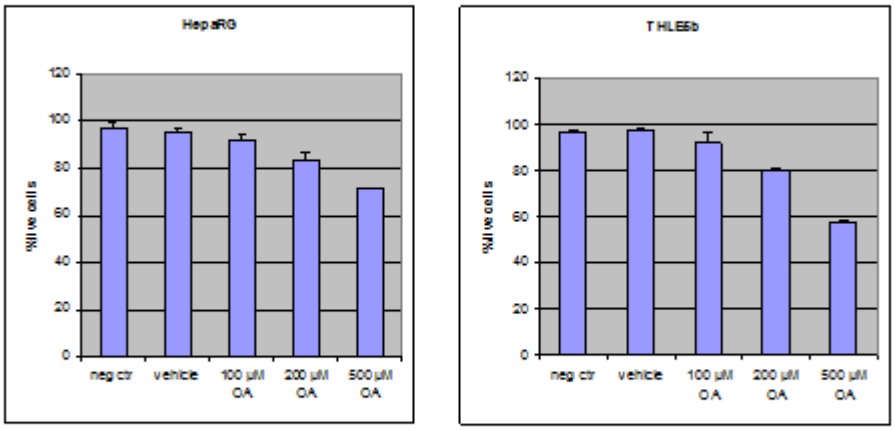
→ No visible lipid droplets in proliferating HepaRGs

→ When HepaRGs start to differentiate, lipid droplets appear.



Hoechst
LD540
ADRP

Viability: results



Conclusion:

- HepaRG and THLE5b viability minimally affected at 100 µM oleate, but more clearly at 200 µM oleate or higher
- also morphological changes visible

This is a summary of the data. Experiments were performed by H.S.Kruitwagen.

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Courses

Modern Methods in Data Analysis
ECTS: 4,5
By: Cas Kruitwagen and Jan van den Broek
Date: January 6-24, 2014
This course consists of morning lectures and afternoon practical computer sessions. Most of it is done in R, some in SPSS. Topics of the lectures are: maximum-likelihood methods, logistic regression, model validation and regression diagnostics, Poisson regression, analysis of `event-history´ data, including an extensive discussion of the Cox proportional hazards regression model and the basics on longitudinal data analysis.

Presenting in English
ECTS: 1
By: Margo de Wolf
Date: April 2014
This course is based on practicing presenting in a small group. With each presentation you receive feedback which you can use during the next presentation. From the other presentations and feedback rounds you can learn to presenting do's and don'ts.

Writing for academic publication in the veterinary and life sciences
ECTS: 3
By: Linda McPhee
Date: April 17 till June 26, 2014
Topics of this course are: how to structure an article, choosing a journal and mostly how to write in such a way that the reader understands what you want to tell him, and not something else.