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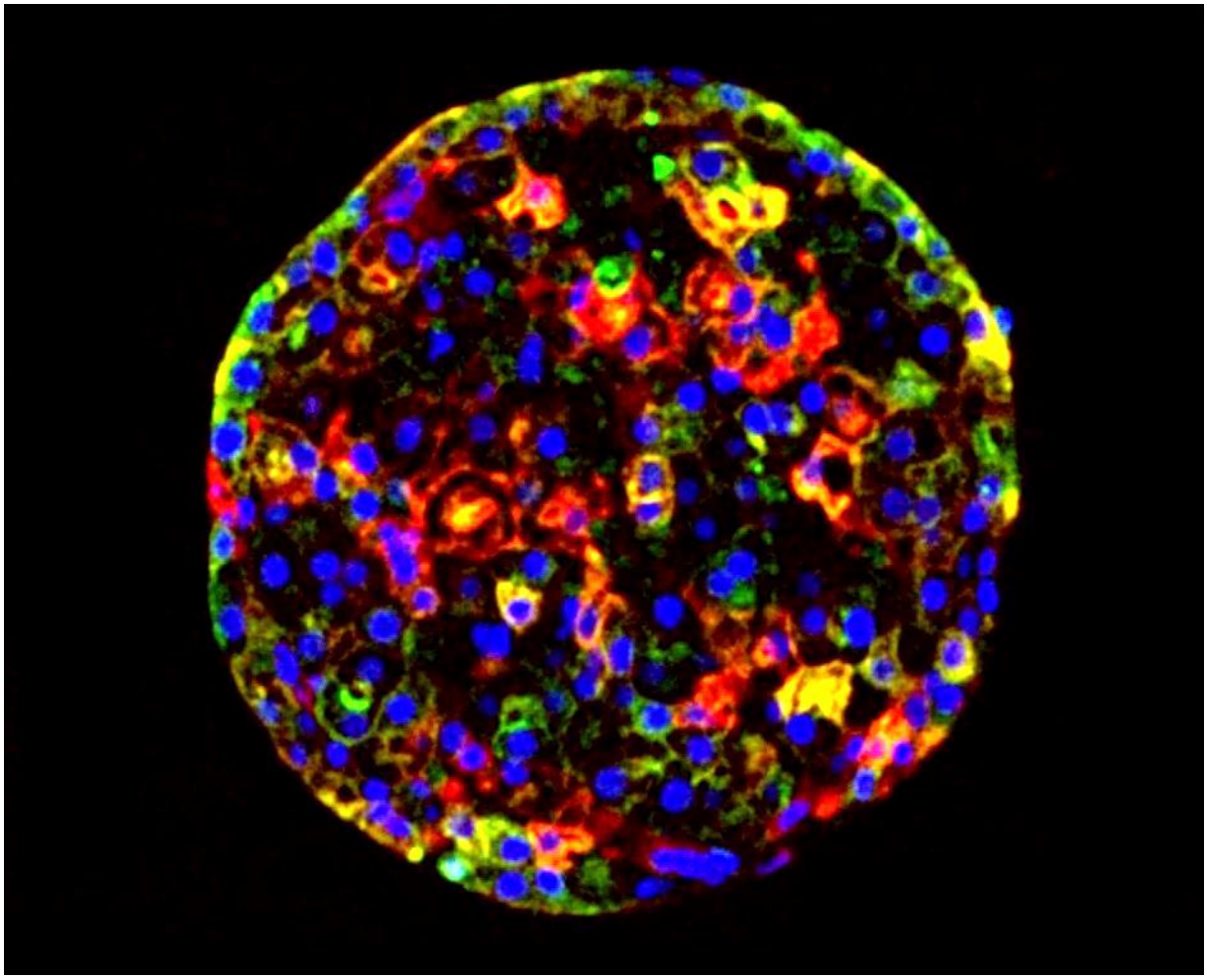

Development and Characterization of a Human Liver Spheroid Culture for Drug Metabolism and Disposition Studies

Master of Science Thesis in Biotechnology

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Department of Physics, Division of Biological Physics
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2016

MASTER'S THESIS 2016

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This thesis was performed at Drug Design and Biotransformation, CVMD iMed DMPK, AstraZeneca, Mölndal under supervision of Senior Research Scientist Kajsa Kanebratt.

Cover: Hepatic spheroid of co-cultured HepaRG and primary human stellate cells stained with antibodies for CK18 (red), CK19 (green) and Hoechst (blue) for the nuclei. Colours are enhanced with Adobe Photoshop.

Acknowledgements

I would like to thank my supervisor Kajsa Kanebratt for giving me the opportunity to perform this thesis project, and also for all the knowledge and good advice I have needed on my way.

Special thanks goes to Linda Andersson for helping me with the analyzes, especially for having the patience to help me with the never ending LC-MS story, and to Matthew O'Hara for his help with figuring out the answers to many of my questions.

I would also like to thank the whole CVMD DMPK group at AstraZeneca in Mölndal for supporting me and making me feel welcome and as a part of the team.

A great thanks also goes to my friends and family for always believing in me.

Abstract

The liver is the main organ of the human body involved in metabolism of xenobiotics. When developing drugs, it is important to have a model that predicts the metabolism and disposition of substances in the liver to be able to ensure the efficacy and safety of the drugs.
In this thesis, a hepatic spheroid model is further developed and evaluated regarding the metabolic functions and genetic expression of the spheroids. The study aims at characterizing the spheroids regarding the gene expression and the activity of specific cytochrome P450 enzymes and over a time period of three weeks. Spheroids were made by culturing different cell types on ultra-low affinity plates on which cells self-assemble and form spheroids. The cell types evaluated for spheroid formation were: HepaRG cells, a co-culture of HepaRG and primary human stellate cells, primary human hepatocytes, and HepatoCells.
HepaRG and co-cultured spheroids of HepaRG and primary human stellate cells were stable in culture for the cultivation period of three weeks. HepaRG spheroids showed results of metabolic activity and gene expression which make them suitable to be used for metabolism and disposition studies. Spheroids of HepatoCells were not stable for three weeks, as they disintegrated after two weeks. HepatoCell spheroids also did not show metabolic activity neither gene expression for any of the enzymes investigated in this study. Primary human hepatocytes did not form spheroids in the scope of this study, hence no evaluation was made. Conclusively, this study has shown the possibility to culture human hepatic spheroids formed from HepaRG cells, applicable for metabolism and disposition studies.

Keywords: Spheroids, 3D model, HepaRG, Co-culture, Metabolism

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1. Introduction

1.1. Background

When developing pharmaceutical drugs, one important aspect is the metabolism of the substances. Metabolism of drugs involves activation, deactivation, detoxification, and kinetics of the substance in the body [1]. It is of high importance to know the specific metabolic activities when developing drugs, to be able to ensure the safety and efficacy of the substances.

The main metabolic organ of the human body is the liver, and it is in the liver where most xenobiotic substances are being metabolized. The metabolic activities of the hepatic tissue are very complex. Finding good models that mimic the metabolic activities that take place in the liver is a difficult task, and most models used today are either not good enough in mimicking the metabolic response or not applicable for long term studies as the activity of the models decline over time [2][3]. It is therefore of high interest to develop hepatic models which are better at predicting the metabolism of compounds in the liver, and which can be used for long term studies.

Previous studies have shown promising results of spheroids cultured from hepatic cells which gives a good estimation of the metabolic activities of human liver tissue [4][5][6][7]. It is indicated that a hepatic spheroid model might have potential to be used for studies of drug metabolism and disposition.

AstraZeneca is currently working on developing a human hepatic spheroid model to be used for metabolism and disposition studies. Thus, this thesis is a part of a larger ongoing project to develop such a model.

1.2. Aim

The aim of this study is to further develop and characterize a human hepatic spheroid model, which is being developed at AstraZeneca. This thesis is a part of a larger project, and the characterization of the spheroids within the thesis will be regarding the metabolic functionality and stability over time.

The metabolic functionality will mainly be characterized regarding the activity of drug metabolizing cytochrome P450 (CYP) enzymes. The activity will be examined using model drug substances, known to be metabolized by specific CYPs in the liver.

The stability over time for the metabolic function of the spheroids will in this thesis be referred to as the stability of the hepatic spheroids.

1.3. Limitations

This thesis is a part of an ongoing project at AstraZeneca in Mölndal, and is aimed at further characterize and develop a human hepatic spheroid model.

A limitation which is made within this project is the choice of cell types. Hepatic tissue is a complex tissue that consists of many different cell types [8][9]. In this thesis the main focus is the hepatic functionality of the spheroids, and therefore the cell types that have been used to form spheroids are chosen to represent the main functions of the liver. Hepatocytes is the cell type responsible for the main metabolic functions of the liver [3][10], and the ultimate goal would be to form spheroids of primary human hepatocytes. Since primary cells are more

expensive, and more difficult to handle than available cell lines, cell lines that have similar functions as hepatocytes are also evaluated in this thesis.
Another limitation made in this project is how the hepatic functionality of the spheroids is evaluated. The functionality will be characterized and evaluated as the activity of specific CYP enzymes. The specific CYP enzymes evaluated in this thesis are chosen to represent CYP enzymes which are important for the drug metabolism in the liver [11]. Gene expression of the specific CYP enzymes together with specific drug transporters will also be evaluated, and histology sections will be stained with antibodies to look for specific proteins.

2. Theory

2.1. Liver

The liver is an important organ for many endogenous processes and it is also the major organ involved in drug metabolism and elimination [8][9][12]. The liver is a very complex organ, and its functionalities are dependent on communication between various cell types and their surroundings in order to maintain the structure and physiology. For a schematic description of liver tissue, see figure 1.

2.1.1. Hepatic cell types

Liver tissue consists of various cell types with different roles, which is part of why the liver is such a complex organ. The hepatic structure and functions rely on cell-matrix interactions as well as cell-cell interactions between different cell types. Hepatocytes are the main cell type in liver tissue, and are also referred to as parenchymal cells. The nonparenchymal cells consists of Kupffer cells, stellate cells, and endothelial cells. Other cell types are also present in the liver, but in a smaller quantity.

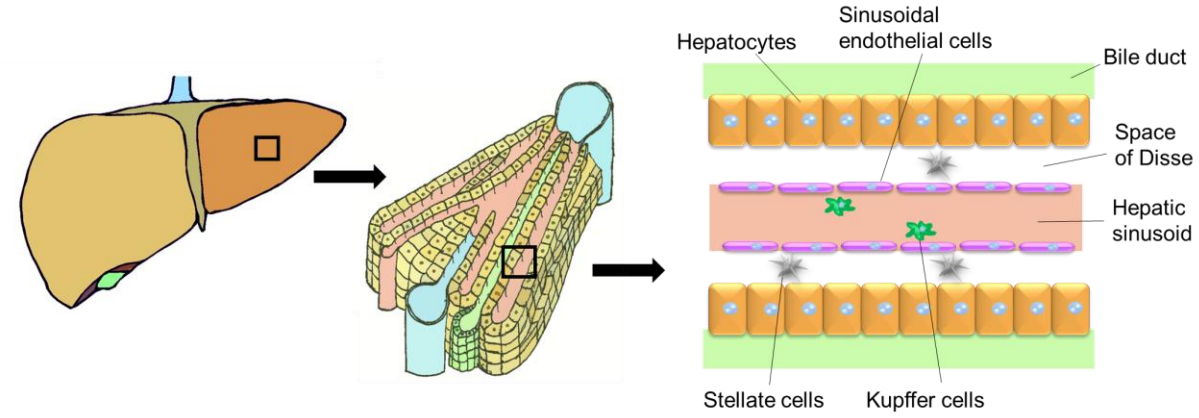

Figure 1. Schematic illustration of hepatic tissue and its cell types.

Hepatocytes are the cells that are responsible for most of the main functions of the liver, and are also the cell type which is represented in highest number. Hepatocytes make up about 80% of the total mass of hepatic tissue [3][9][10].

The functions of hepatocytes are to metabolize proteins, steroids, fats, and toxic substances in the blood. Hepatocytes are responsible for storage of glycogen and fat soluble vitamins and for production of bile, which works as an emulsifier for fats to help the fat digestion in the small intestine [8][10].

Nonparenchymal cells influence the drug response of the hepatic tissue by their signaling and extracellular surroundings [3], and it is important to know how the different cell types in the hepatic tissue work together to understand the full picture.

The hepatic stellate cells are located in the space between the sinusoidal endothelial cells and the hepatocytes, and have a star-shaped morphology. Hepatic stellate cells represent about 5% of the cells in the liver [3]. The stellate cells are responsible for storage of fat and vitamin A, and 80% of the body's retinoids are stored as retinyl palmiate in the lipid droplets of the hepatic stellate cells. The stellate cells regulate the concentration of retinoids in the bloodstream by receptor mediated endocytosis [13]. If a pathological condition occurs, such

as liver cirrhosis, the star-shaped morphology of stellate cells changes to that of fibroblast-like cells. In these pathological conditions the stellate cells also proliferate strongly, loose retinoids and produce a large quantity of extra cellular components [13][14]. Cell-cell interactions between hepatocytes and stellate cells are important in both the developing and the adult liver to maintain stability to the functions of the hepatocytes [15].
Kupffer cells are special liver-macrophages, which act by phagocytosis on materials entering the liver by the blood. The Kupffer cells are located in the sinusoidal walls of the liver, and is the largest population of tissue-specific macrophages in the body [16]. About 12-15% of the hepatic cells are Kupffer cells [3].
The liver sinusoidal cells contribute to about 20% of the total cell number in hepatic tissue [3]. The sinusoidal endothelial cells of the liver are mainly responsible for filtration and transportation of nutrients from the blood [10].
The biliary epithelial cells make up about 3.5% of the hepatic cell mass [10], and are responsible for excretion of bile to the biliary tract of the liver [17].

2.1.2. Cell lines

Primary human hepatocytes are isolated from human liver, and are considered as the "gold standard" for *in vitro* liver model systems [2]. Even though primary human hepatocytes are the closest model available to the native human liver there can be some difficulties when working with these cells. The availability and quality of cells is one problem which also concerns the batch differences that comes from the variation between human donors [18]. Primary cells are also often quite sensitive, which makes culturing over a time period rather complicated, and when culturing primary hepatocytes in 2D they loose their CYP activity very fast [12]. There can also be a difference in the metabolic profiles of primary hepatocytes, which depends on the donor source and the procedures used for preparation of the cells [19].
An alternative to primary hepatocytes are human hepatic cell lines, derived from cancer cells or immortalized hepatocytes. The advantages of such cell lines are that they are more robust to culture and handle, they are cheaper, and they give more reproducible results since there is no inter-individual variability between batches.
A cell line that has shown promising results is HepaRG, which is derived from a hepatocellular carcinoma. HepaRG cells differentiates to two cell types, namely hepatocyte-like cells and biliary epithelial-like cells [3][20][21]. HepaRG cells are shown to be suitable for drug metabolism studies, as they have a genetic expression of CYP enzymes and other liver specific proteins that is close to that of human hepatocytes [4][7][21][22][23][24]. HepaRG are also known to have a low variation between batches as well as a stable phenotype [19].
A new interesting product commercially available on the market is the HepatoCells. HepatoCells are derived from immortalized hepatocytes. The cells are intended as a product for *in vitro* absorption, distribution, metabolism, excretion (ADME), and toxicity studies, and are claimed to have the same properties as hepatocytes [25]. However, the HepatoCells are so new that no data from independent studies has yet been published.

2.2. Hepatic model systems

When studying human drug delivery and metabolism the ideal model system should be able to mimic the physical and biochemical barriers linked to the absorption, distribution, metabolism and excretion of the human body. The model should also be easy to work with and be compatible with high-throughput screening methods. Such an ideal model is off course difficult to obtain, and therefore it is important to find a model that is relevant for the specific purpose. There are different models that have been used and are currently being used, but these models have some limitations.

Animal studies are traditionally used to predict metabolism in the liver. The problem with using animal models is the significant inter-species differences, which makes the results irrelevant for human predictions [2][12][26].

In vitro models with human liver cells is another way to study metabolic effects in liver tissue. However, previously used hepatic 2D culture systems have some challenges when it comes to predict the functionality of hepatic tissue.

For phenotypic gene expression and optimal response to drugs, preservation of normal physiology, and contacts between cells is very important when working with *in vitro* models. Studies with hepatocytes cultured in 2D over time have shown that the differential expression of the major CYP enzymes does not reflect *in vivo* profiles, which indicates that hepatocytes in 2D-culture are limited in their application for studies of drug metabolism [5][7][27]. Culturing cells in 2D also often leads to morphological changes of the cells, which is important since they most of the time are followed by changes in the phenotypic gene expression, which is mainly caused by alterations of the surface receptor-mediated signaling pathways [27]. Hence, loss of the specific phenotype in 2D cultures, is most likely due to loss of polarization and cell-cell contacts [5][7][28]. Primary hepatocytes are known to undergo these phenotypic changes in monolayer cultures [29].

To obtain *in vitro* models that better predict the metabolic activities of the human liver, hepatic cells are cultured in different 3D models. The purpose of a 3D model is to allow the cells to grow in a more "natural environment". In a 3D culture cells are in contact with other cells in all axes, allowing for cell-cell signaling and possibility to polarization. Three dimensional models used today are obtained with different techniques, and are made both with and without scaffold materials such as the sandwich model, entrapment in hydrogel and hepatic spheroids [9]. The sandwich approach is one way to create a 3D culture of hepatic cells, where the cells are grown between two layers of hydrogel [12]. The sandwich technique is used to predict biliary excretion of drugs [12], but has some disadvantages when it comes to the function of phase I and II enzymes as they decline over time [10]. The use of hydrogels for entrapment of hepatic cells can extend the viability and enhance some cell functions, but there are some limitations in transportations of nutrients and it can be difficult to do e.g. activity analyzes, as it is difficult to remove cells from such a hydrogel [10].

A way to further improve the tissue-specific functions of a hepatic 3D model is to co-culture different hepatic cell types. The cell-cell contact of heterotypic cell types improves the hepatocellular phenotype and helps to maintain hepatocytes in their differentiated state [10][12][28].

Three dimensional models, such as spheroids, can also be combined with perfusion and microfluidic systems to further improve the models and overcome problems with limitations

of nutrient transport. Microfluidic systems are small volume systems which can mimic blood flow, and such a system could be suitable for drug metabolism studies [26][30][31].

2.2.1. Spheroids

Hepatic spheroids are multicellular clusters of hepatic cells that assembles into spherical shaped structures. It is known that primary mammalian cells retain the capacity to develop a tissue without the use of a scaffold [28], which makes it possible for cells to form spheroids. Spheroids can be produced with different techniques such as the hanging drop system, a rotating vessel, and self-assembling on non-adhesive plates [3][12], see figure 2 for examples.

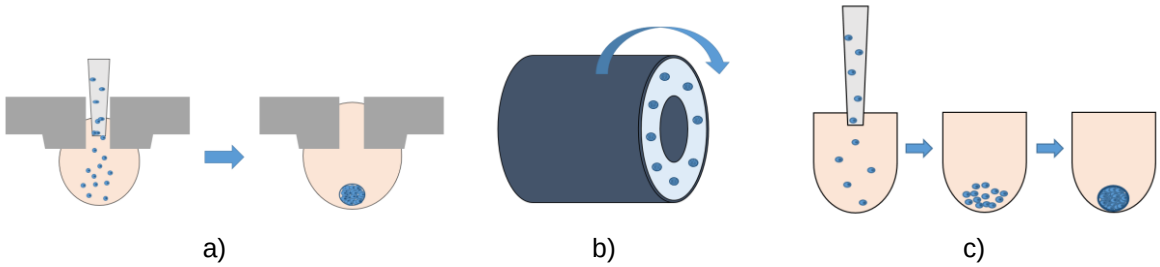

Figure 2. Different techniques used to form hepatic spheroids a) hanging drop technique b) rotating vessel c) self-assembling on non-adhesive plates.

Hepatic cells that have been cultured into spheroids have shown to have improved hepatic functions, and prolonged survival compared to 2D cultures [5][6][10][12]. This is probably due to the cell-cell contact that is established in the spheroid and the presence of extra cellular matrix components that are produced by the cells in and around the aggregates [3]. One of the advantages of self-assembled spheroids, is that there are no need for scaffold materials surrounding the cells, the extra cellular matrix components present are formed by the cells of the spheroid itself [12]. Spheroids can be produced in 96-well plates, which allows for larger scale production of micro-tissues that can be used in drug development.

2.3. Drug metabolism

Drug metabolizing enzymes work to eliminate or detox compounds that can be harmful to the body [32]. It is important to assess the metabolic activity of specific enzymes to be able to predict the drug clearance that will occur *in vivo*. The elimination of drugs consists of four phases. Phase 0 is the transport of the compound into the hepatocyte, phase 1 is the oxidation, reduction, and hydrolysis by e.g. CYP enzymes, phase 2 is a further conjugation by e.g. UDP-glucuronsyltransferases metabolizing enzymes, and phase 3 is the efflux transport out of the cell [12][32]. As metabolizing enzymes are important for the metabolism of substances in the liver, it is important that an *in vitro* model for liver tissue expresses these enzymes in a relevant way.

2.3.1. Cytochrome P450

CYPs is a phase I drug metabolizing superfamily of microsomal enzymes, which are the main enzymes responsible for catalyzing the oxidative biotransformation of most xenobiotics [1][33]. CYPs are divided into families and subfamilies. Amongst the CYPs three gene families are crucial in hepatic metabolism and the elimination of drug compounds, namely

CYP1, CYP2, and CYP3, and the most abundantly found in liver tissue are CYP1A2, CYP3A4, CYP2C9, CYP2C8 and CYP2E1 [32][33]. There is a difference between species in CYP enzyme regulation [27], which suggests that it is important to use human hepatocytes for drug metabolism studies.
Some CYPs are more important for the metabolism of drugs [11], and by studying these one can get an overview of the metabolic activities that take place upon drug delivery.
CYP enzymes chosen for functionality measurements of the metabolic activity in this thesis are CYP1A2, CYP2C9, CYP2D6 and CYP3A4. CYP1A2 is highly expressed in the liver, and therefore plays a clinically important role in metabolism of several clinically important drugs, such as the analgesics and antipyretics acetaminophen, phenacetin and lidocaine [33][34]. CYP2C9 is a major CYP enzyme, which is involved in metabolism of most nonsteroidal anti-inflammatory drugs (NSAIDs), and diclofenac is commonly used as a substrate for phenotyping of CYP2C9 [33]. CYP2D6 has been shown to metabolize about 15-25% of all clinically used drugs, and amongst these are many antidepressants and anti-cancer drugs [33][34]. CYP2D6 is also of importance to study due to the genetic polymorphism of the enzyme [33]. The subfamily of CYP3A enzymes, where CYP3A4 belongs, have a major part in metabolizing about a third of all drugs used clinically [33][34]. There are many drugs commonly used as substrates for measuring the activity of CYP3A4, such as midazolam and erythromycin [33]. Probe substrates used in this thesis, see table 2, are according to recommendations from U.S. Food and Drug Administration [35].

2.3.2. Transporters

Membrane transporters are responsible for transportation of xenobiotic substances over the cell membrane, which makes them an important part of the functionality of a hepatic model [32]. Multidrug resistant protein 1 (MDR1) is an ATP-dependent membrane transporter, which transports endogenous and xenobiotic substances over the cell membrane as an efflux pump [32]. Multidrug resistance-associated protein 2 (MRP2) is another efflux membrane transporter, which is located in the apical membrane of polarized hepatocytes [4], and MRP2 mediated transport leads to excretion of xenobiotic products to the bile [32][36]. In previous studies it has been shown that MDR1 and MRP2 are expressed in the cell membrane in ways that indicates the polarization of the cell, and the presence of bile canaliculi [32][37].

3. Materials and Methods

This thesis was performed to characterize a hepatic spheroid model regarding the metabolic activity of specific CYP enzymes and the gene expression of specific proteins over a time period of three weeks. The experimental part of this thesis project was divided into two experiments. In the first experiment two types of hepatic spheroids were examined, where one consisted of HepaRG cells and the other of a co-culture of HepaRG cells and stellate cells. In the second experiment spheroids were made from primary human hepatocytes and HepatoCells.

3.1 Materials

Differentiated HepaRG cells, additive ADD670 for thawing and seeding, additive ADD620 for culture and maintenance and additive ADD640 were purchased from Biopredict International (Saint Grégoire, France). Human hepatic stellate cells and Stellate Cell Medium were purchased from Tissue Solutions (Glasgow, UK). Cryopreserved HepatoCells, Corning culture medium for HepatoCells, Ultra Low Cluster 96-well plates, Collagen I Cellware 96-well plates and 96-well flat bottom cell culture plates were purchased from Corning (Weisbaden, Germany). William's E with GlutaMAX, Fetal Bovine Serum (FBS), Penicillin-Streptomycin, TRIzol and Superscript III First-Strand Synthesis System were purchased from Invitrogen Life Technologies (Carlsbad, CA). William's E Basal Medium and dexamethasone were purchased from Sigma-Aldrich (St. Louis, MO). Insulin-transferrin-selenium, Pierce BCA Protein Assay Kit and Taqman assay-on-demand were purchased from Thermo Fisher Scientific (Waltham, MA). RNeasy Micro Kit was purchased from Qiagen (Hilden, Germany), and Agilent RNA 6000 Nano Kit was purchased from Agilent technologies (Santa Clara, CA). HistoGel was purchased from Richard-Allan Scientific (Waltham, MA), and antibodies for staining were from Abcam (Cambridge, UK). Primary human hepatocytes, InVitroGRO CP Medium and Torpedo Antibiotic Mix were purchased from BioreclamationIVT (Westbury, NY).

3.2. Experiment I

In the first experiment spheroids of HepaRG cells and spheroids of a co-culture of HepaRG cells and primary human hepatic stellate cells were examined to investigate the effects on the hepatic spheroid model from co-culturing two hepatic cell types. The ratio between HepaRG cells and human hepatic stellate cells in the co-cultured spheroids was 24:1. This ratio was chosen as it previously has been used for spheroid formation with these cell types [31]. Both spheroid types were made in 2000 cell spheroids, and the spheroids were cultured for a time period of three weeks with samples taken on day 3, 7, 10, 14, and 21. A 2D-culture of HepaRG cells were also cultured and sampled at day 7 to be used as reference. Samples were analyzed for CYP activity, gene expression, and presence and localization of specific proteins.

3.2.1. Cell culturing

All media used for the experiment was according to table 1. Differentiated HepaRG cells together with human hepatic stellate cells were thawed with thawing medium according to table 1, and seeded with thawing and seeding medium for HepaRG according to supplier's

recommendations with minor modifications to Ultra Low Cluster 96-well plates. References of 2D-cultured HepaRG cells were seeded to Collagen I Cellware 96-well plates according to supplier's recommendations. On culture day 3, 90% of the medium was replaced with culture and maintenance medium. Thereafter 50% of the culture medium was changed every second to third day throughout the cultivation period of 21 days.
All handling of the spheroids was done under sterile conditions, and the cells were kept in a sterile environment at 37 ˚C, 95% air and 5% CO_2.

Table 1. Different media and its components used in the experiments.

Medium	Components	Experiment
Thawing and seeding, HepaRG	William's E with GlutaMAX, ADD670	I
Thawing, stellate cells	Stellate Cell Medium	I
Culture and maintenance	William's E with GlutaMAX, ADD620	I
Serum-free induction	Williams E, GlutaMAX-I, ADD 640	I
Thawing and seeding, hepatocytes	45 mL InVitroGRO CP Medium, 1 mL Torpedo Antibiotic MIX	II
Thawing and seeding, HepatoCells	Corning culture medium for HepatoCells, 10% FBS, 1% penicillin-streptomycin	II
Culture and maintenance	500 mL William's E, 5 mL penicillin-streptomycin, 5 mL insulin-transferrin-selenium, 1 mL dexamethasone (50 µM)	II

3.2.2. CYP activity

One day prior to sampling, with exception for day 3 samples, 90% medium was changed to serum-free induction medium. On the days of experiments 30 spheroids of each spheroid type was pooled together in separate wells in a 96-well flat-bottom cell culture plate. The spheroids were then incubated in a substrate cocktail consisting of phenacetin, diclofenac, bufuralol, and midazolam, see table 2. Substrates were dissolved in 100% methanol, and the cocktail was made in serum-free induction medium, giving a concentration of 15.6% methanol in the cocktail. The incubation volume was 100 µL. The compounds used are known substrates for specific CYP enzymes, see table 2. Spheroids were incubated with the cocktail for 1 hour. Additionally for sample day 7, 14, and 21 separate pools of 15 HepaRG spheroids were also incubated with the cocktail for 4, 8, and 24 hours. After incubation, 50% of the media was transferred to a new plate, and the activity was terminated by addition of STOP-solution (ratio 1:2), which consisted of acetonitrile with 300 nM 5.5-diethyl-1.3-diphenyl-2-iminobarbituric acid (internal standard) and 0.8% formic acid. The cells were lysed with 0.1 M sodium hydroxide (NaOH) and later used for protein measurements of total protein concentration in the cells, using the Pierce BCA Protein Assay Kit.

Table 2. Probe substrates and their CYP specific metabolites used to measure the CYP activity, together with the specific CYP enzymes of the respective substrate.

Probe substrate	Metabolite	Target CYP	Concentration of substrate in incubation [µM]
Phenacetin	Paracetamol	CYP1A2	26
Diclofenac	4-OH-diclofenac	CYP2C9	3
Bufuralol	1-OH-bufuralol	CYP2D6	20
Midazolam	1-OH-midazolam	CYP3A4	9

The plate with the media samples and STOP-solution was centrifuged at 4000G for 20 minutes, after which 50 µL of the samples were transferred to a new plate and diluted with 50 µL ultrapure water. Samples were centrifuged again at 4000G for 5 minutes, and then analyzed by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) with a triple quadrupole. The UPLC system used was a Waters Acquity UPLC, from Waters Corporation. Separation was performed on a Waters Acquity UPLC® HSS T3 column (50 mm×2.1 mm, 1.8 µm) at 40 °C with a flow rate of 1.0 mL/min and a gradient from 99.8% A (100% water + 0.1% formic acid) to 95% B (100% acetonitrile + 0.1% formic acid). Analysis was performed on a Xevo TQ-S, from Waters Corporation, with electrospray ionization using multiple reaction monitoring (MRM). The analytes were CYP specific metabolites to the substrate drugs used in the incubation, see table 2.

3.2.3. Gene expression

Gene expression analysis was performed by quantitative real-time polymerase chain reaction (qRT-PCR). Each sample day 30 spheroids of the different spheroid types were pooled together to 1.5 mL Eppendorf tubes. For day 7 and 14 2D cultured HepaRG cells were also sampled to be used as reference. RNA was isolated and extracted from the pooled spheroid samples using the RNeasy Micro Kit, and the concentration and purity of the extracted RNA was measured with Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Nano Kit. The RNA was then converted to cDNA using the Superscript III First-Strand Synthesis System. For qRT-PCR Taqman assays were used. Gene assays used were according to table 3. qRT-PCR was performed with a QuantStudio 7 Flex Real-Time PCR System from Life technologies, with the ΔΔCt method. The 2D sample from day 7 was used as a reference for the fold-change calculations.

Table 3. Gene-assays used for RT-qPCR.

Gene	Gene-assay	Function	Experiment
CYP1A2	Hs00167927_m1	CYP enzyme	I/II
CYP2C9	Hs00426397_m1	CYP enzyme	I/II
CYP2D6	Hs00164385_m1	CYP enzyme	II
CYP3A4	Hs00604506_m1	CYP enzyme	I/II
MRP2	Hs00166123_m1	Transporter protein	I/II
CK19	Hs00761767_s1	Biliary cell marker	I/II
Albumin	Hs00910225_m1	Hepatocyte marker	I/II
Vimentin	Hs00958111_m1	Stellate cell marker	I
GAPDH	Hs99999905_m1	Reference gene	I/II

3.2.4. Staining and imaging

Samples for staining and imaging were collected at day 7, 14, and 21. A total of 15 spheroids of each spheroid type were pooled together and collected to tubes respectively. Spheroids were washed twice with PBS and then incubated with 4% paraformaldehyde (PFA) for 24 hours at 4 ˚C. After incubation the spheroids were washed again with PBS twice and then encapsulated with HistoGel according to instructions from manufacturer.
The encapsulated samples were then dehydrated and paraffin embedded and sent to HistoCenter AB in Gothenburg, Sweden, for sectioning and staining with hematoxylin and eosin. When the samples were sectioned and returned from HistoCenter, the samples were first deparaffinized and rehydrated, and then stained with antibodies according to table 4. CYP3A4 together with MRP2, and CK18 together with CK19 were double stained on sections respectively.

Table 4. Antibodies used for staining of histology sections.

Primary Antibody	Secondary Antibody	Function	Experiment
CYP3A4	Alexa Fluor 555	CYP enzyme	I/II
MRP2	Alexa Fluor 488	Transporter protein	I/II
CK18	Alexa Fluor 555	Hepatocyte marker	I/II
CK19	Alexa Fluor 488	Biliary cell marker	I/II
Vimentin	Alexa Fluor 488	Stellate cell marker	I

Rehydrated histology sections were permeabilized by incubation in PBS with 0.25% Triton-X and 0.01% Tween-20 for 15 minutes. After permeabilization the sections were incubated in a serum blocking solution of PBS with 1% bovine serum albumin (BSA), 2% goat serum 22.52 mg/mL glycine, 0.075% Triton-X and 0.01% Tween-20 for 45 minutes. The serum blocking solution was used to prevent unspecific binding of primary antibody. The samples were then incubated with the primary antibodies in PBS with 1% BSA, 2% goat serum, 0.075% Triton-X and 0.01% Tween-20 for 1 hour at room temperature. After incubation with the primary antibodies the sections were washed with PBS with 0.1% Tween-20 3x 5 minutes. The samples were then incubated with secondary antibodies in PBS for 45 minutes at room temperature, and washed again in PBS for 3x 15 minutes. Samples were then counterstained with Hoechst for 1 minute, after which the samples were washed with PBS for 5 minutes and

then sealed with a coverslip. For a schematic presentation of the antibody staining process, see figure 3.

Figure 3. Schematic presentation of the antibody staining process.

Samples were imaged on a Nikon Eclipse TE2000-E microscope and analyzed and processed with E2-CI for Nikon software and ImageJ.

3.3. Experiment 2

In the second experiment spheroids of primary human hepatocytes and of HepatoCells were examined. Both spheroid types were made in 2000 cell spheroids, and the culture period for the spheroids was set to three weeks with sample days on day 7, 14, and 21. Samples were analyzed for CYP activity, gene expression, and presence and localization of specific proteins.

3.3.1. Cell culturing

All media used for the experiment was according to table 1. Cryopreserved HepatoCells and primary human hepatocytes were thawed and seeded according to supplier's recommendations with minor modifications to Ultra Low Cluster 96-well plates. The development of the spheroids was followed by bright field microscopy. For HepatoCell-spheroids on culture day 5, 50% of the medium was replaced with culture and maintenance medium. Thereafter 50% of the culture medium was changed every second to third day throughout the cultivation period. All handling of the spheroids was done under sterile conditions, and the cells were kept in a sterile environment at 37 ˚C, 95% air and 5% CO_2. Unfortunately no spheroids were formed from the hepatocytes, hence the cultures were terminated on culture day 7. Also, the HepatoCell spheroid cultures were terminated on culture day 16, due to decomposition of the spheroids.

3.3.2. CYP activity

On the sample days, triplicates of 5 spheroids of each spheroid type was pooled together in 3 separate wells in a 96-well flat-bottom cell culture plate, respectively. At the same time 3 single spheroids were transferred to separate wells on the same plate. This was done to two equal plates, to measure the activity for 8 and 24 hours. The spheroids were incubated in a cocktail made in culture and maintenance medium according to experiment 1. For the 5 spheroid pools the incubation volume was 100 µL, and for the single spheroid samples, the incubation volume was 50 µL. Sampling and analysis was done according to experiment I, but with 10 nM (instead of 300 nM) 5.5-diethyl-1.3-diphenyl-2-iminobarbituric acid as internal standard in the STOP solution.

3.3.3. Gene expression

Gene expression analysis was performed by real-time polymerase chain reaction (qRT-PCR). Samples for gene expression in primary human hepatocytes were taken on sample day 0 and samples for HepatoCell spheroids were taken on sample day 0, 7, 14, and 16, which was the day the HepatoCell culture was terminated. Each sample day 60 spheroids of the different spheroid types were pooled together in 1.5 mL Eppendorf tubes respectively. RNA was isolated and extracted from the pooled spheroid samples using the TRIzol kit, and the concentration and purity of the extracted RNA was measured with Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Nano Kit. Preparation of cDNA and analysis with qRT-PCR was done according to experiment I with gene assays according to table 3.

3.3.4. Staining and imaging

Samples for staining and imaging were collected at day 7 and 14. Fifteen spheroids were pooled together and collected to an Eppendorf tube. Spheroids were washed twice with PBS and then incubated with 4% paraformaldehyde (PFA) for 24 hours at 4 ˚C. Samples were then sent to HistoCenter AB in Gothenburg, Sweden for preparation, sectioning and staining with hematoxylin and eosin. When the samples were sectioned and returned from HistoCenter, the samples were first deparaffinized and rehydrated, and then stained with antibodies according to table 4. CYP3A4 together with MRP2, and CK18 together with CK19 were double stained on sections respectively. Staining and imaging was done according to experiment I.

4. Results

Spheroids of HepaRG and co-cultured HepaRG and stellate cells were formed at culture day 3, and the spheroids were stabile throughout the culture period of three weeks.
No spheroids were formed from the hepatocytes after 7 days, and the culture was thereby terminated. Hence, no results for hepatocyte spheroids are presented. The intended culture period for the spheroids was 21 days. HepatoCells started to form spheroids at day 3, and they looked nice and stable at day 5. However, the HepatoCell spheroids started to fall apart at culture day 13, and it was therefor decided to end the culture at day 16, since most of the spheroids were decomposed at this time point. Development of HepatoCell spheroids and hepatocytes can be followed in appendix I.

4.1 CYP activity

The activity of specific CYP enzymes was measured by metabolite formation from probe substrates metabolized by the specific enzymes, see table 2.
The levels of paracetamol and 1-OH-bufuralol where below the lower limit of quantification (LLOQ) for the 60 minutes samples for the HepaRG and co-cultured HepaRG and stellate cell spheroids. The CYP2C9 activity, figure 4a, and the CYP3A4 activity, figure 4b, appear to be stabile over the culture period of three weeks for both HepaRG spheroids and co-cultured spheroids of HepaRG and stellate cells. On culture day 7 a peak in activity of CYP3A4 is detected for the co-cultured spheroids and a peak in activity of CYP2C9 is detected for both spheroid types, see figure 4.

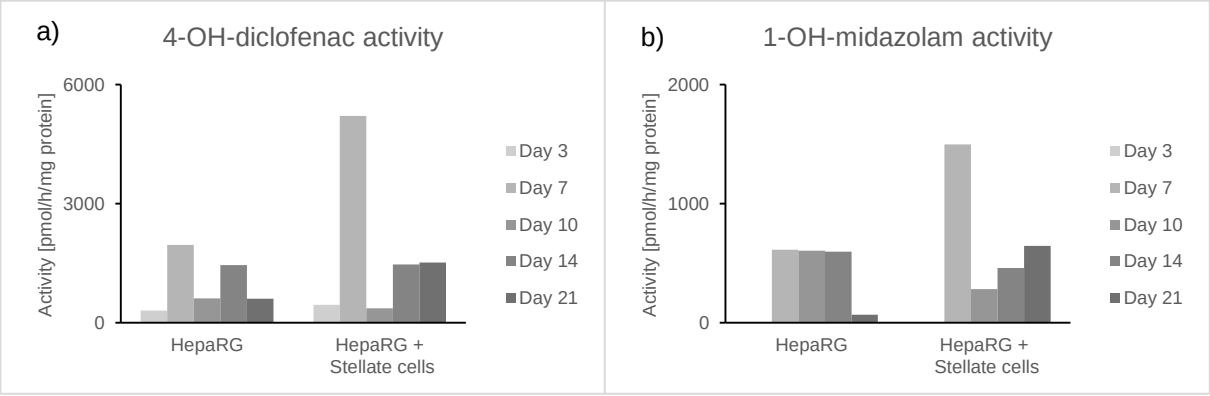

Figure 4. CYP selective activities of HepaRG spheroids and co-cultured HepaRG and hepatic stellate cell spheroids a) formation of 4-OH-diclofenac via CYP2C9 and b) formation of 1-OH-midazolam via CYP3A4. One bar represents one sample of 30 pooled spheroids.

Figure 5 shows the activity of the specific CYP enzymes for HepaRG spheroids after incubations with the cocktail for different time points. For all of the enzymes, the activity goes up after 4 to 8 hours and then goes down again after 24 hours.

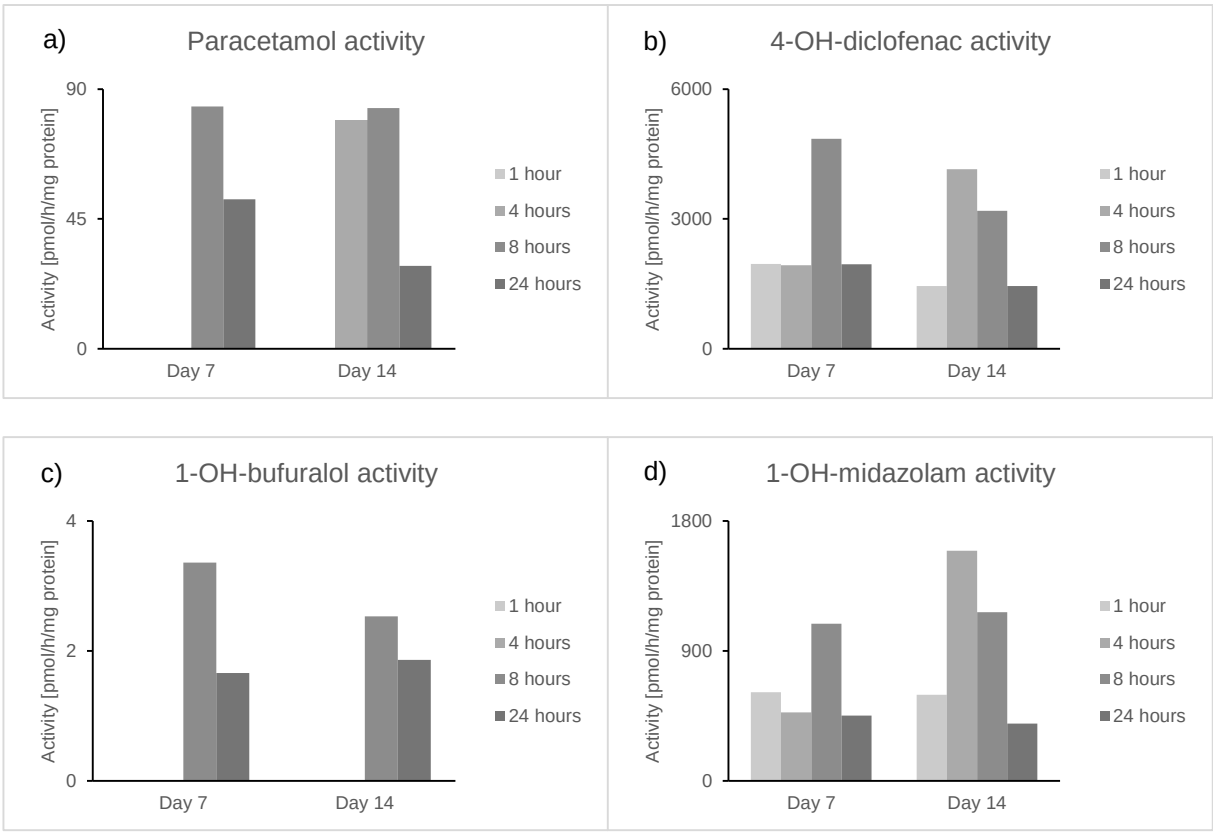

Figure 5. CYP selective activities of HepaRG spheroids using a) formation of paracetamol via CYP1A2, b) formation of 4-OH-diclofenac via CYP2C9, c) formation of 1-OH-bufuralol via CYP2D6, and d) formation of 1-OH-midazolam via CYP3A4 with incubation times of 1, 4, 8 and 24 hours at day 7 and 14. One bar represents one sample of 15 pooled spheroids.

HepatoCell spheroids had low CYP activity compared to HepaRG and co-cultured spheroids, see figure 6. For some samples the concentration of metabolites were below LLOQ, and thereby no analytes were detected. The levels of 1-OH-bufuralol where below LLOQ for all samples. The CYP1A2 activity and the CYP3A4 peaked at day 7, and very little or no activity was detected on day 14, see figure 6a and 6c. The activity for CYP2C9 was higher at day 14 than day 7, see figure 6b. There were no conclusive difference between 5 spheroids incubated together and spheroids that were incubated alone.

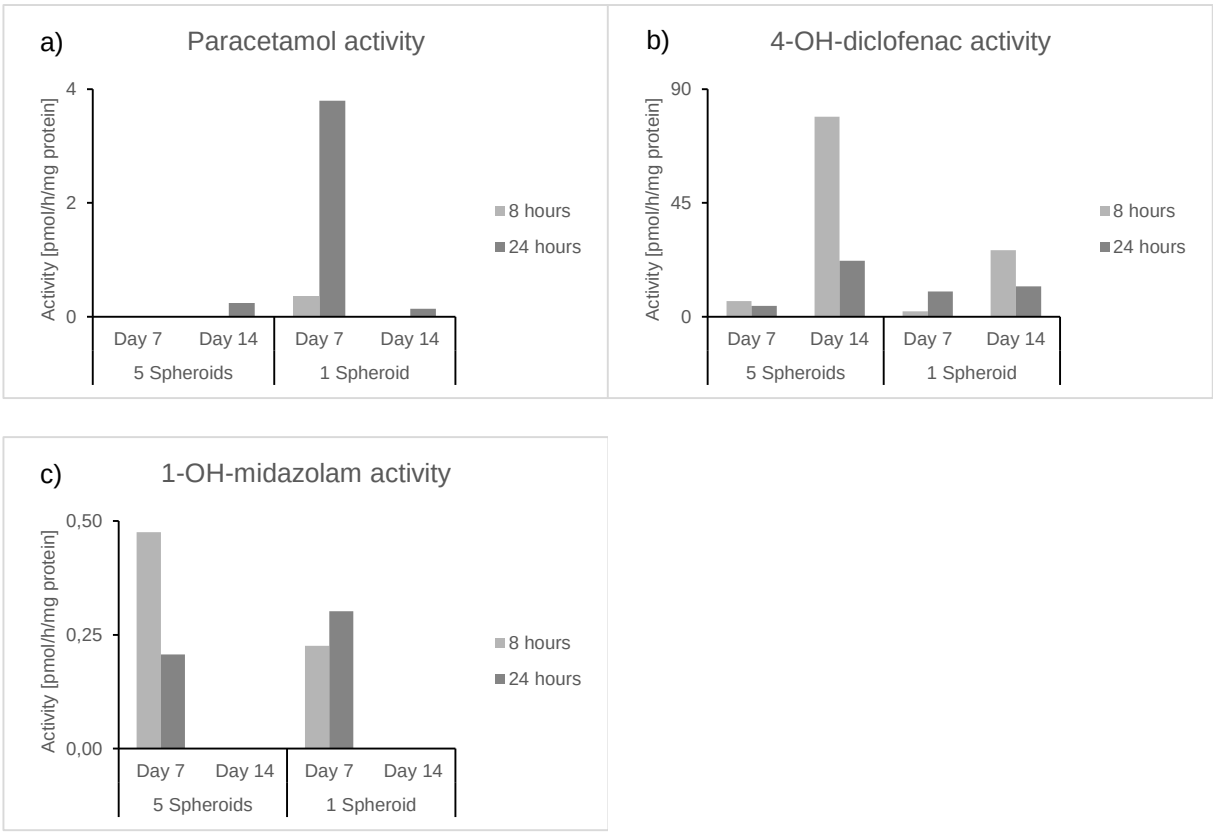

Figure 6. CYP selective activities of HepatoCell spheroids using a) formation of paracetamol via CYP1A2, b) formation of 4-OH-diclofenac via CYP2C9, and c) formation of 1-OH-midazolam via CYP3A4, measured in one or five spheroids incubated for 8 or 24 hours. One bar represents the mean of three measurements.

4.2 Gene expression

Figure 7 shows the gene expression of CYP1A2, CYP2C9, CYP3A4, MRP2, albumin, and CK19 for the different spheroid types compared to a HepaRG 2D reference. Figure 8 shows the gene expression of vimentin for HepaRG and co-cultured spheroids of HepaRG and stellate cells, figure 8a, and the expression of CYP2D6 for hepatocytes and HepatoCell spheroids, figure 8b. See appendix II for the gene expression of all genes investigated at all time points. Hepatocytes have higher expression of all genes except CK19, which is used as a marker for biliary cells, at day 0 compared to the reference. However, hepatocytes were sampled from suspension, and they did not form spheroids. HepatoCell spheroids have low or no expression of all genes investigated compared to the reference, except for CYP2D6 which was higher at day 14, see figure 8b.

Expression of CYP1A2 and CYP2C9 increased for HepaRG spheroids throughout the culture period and decreased for the co-culture spheroids. The expression was higher compared to the reference for both spheroid types, see figure 7a and 7b. For CYP3A4 the expression was lower compared to the reference for both HepaRG and co-culture spheroids, and it was stable during the culture period for the co-culture spheroids and declined for HepaRG spheroids, see figure 7c. The expression of MRP2 was slightly higher for the HepaRG and co-cultured spheroids, with little difference between the spheroid types, and a slight increase in expression for the co-cultured spheroids over time, see figure 7d. The expression of albumin

was higher in the co-cultured spheroids than the HepaRG spheroids with a peak at day 21, which was the only value higher than the reference, see figure 7e. The expression of CK19 was lower in all the spheroid types compared to the reference. It was higher for the HepaRG spheroids than the co-cultured ones, and rather stable throughout the culture period, see figure 7f. No expression of vimentin could be detected in HepaRG spheroids at day 21 and co-cultured spheroids at day 7 and 14, see figure 8a.

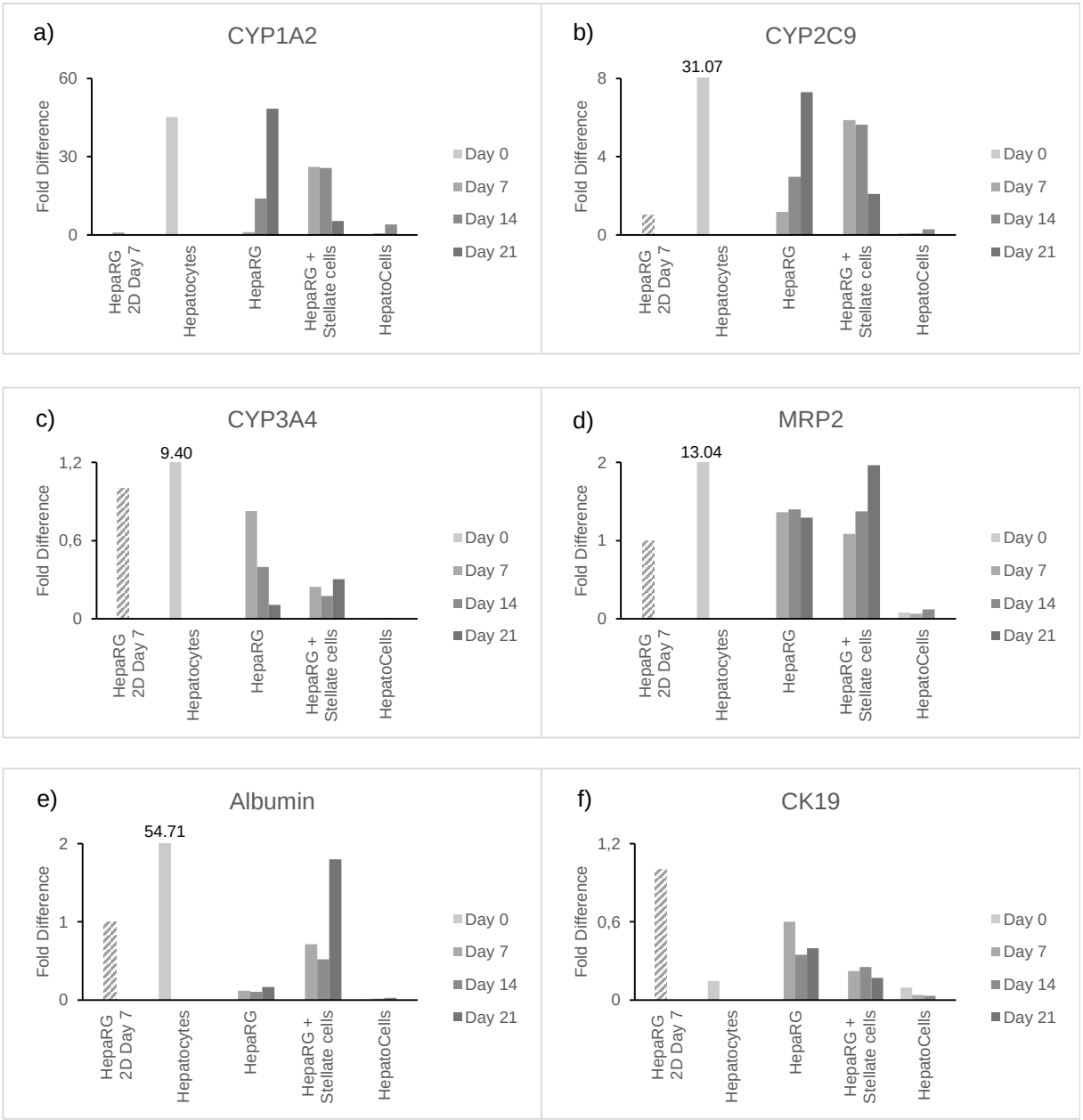

Figure 7. Gene expression of a) CYP1A2, b) CYP2C9, c) CYP3A4, d) MRP2, e) albumin and f) CK19 compared to a reference of 2D cultured HepaRG from day 7 set as 1 for all genes tested. Only hepatocyte and HepatoCell spheroids were sampled at day 0, and no HepatoCell spheroids were sampled at day 21. These results represent the mean of three measurements made from one sample of 60 pooled spheroids.

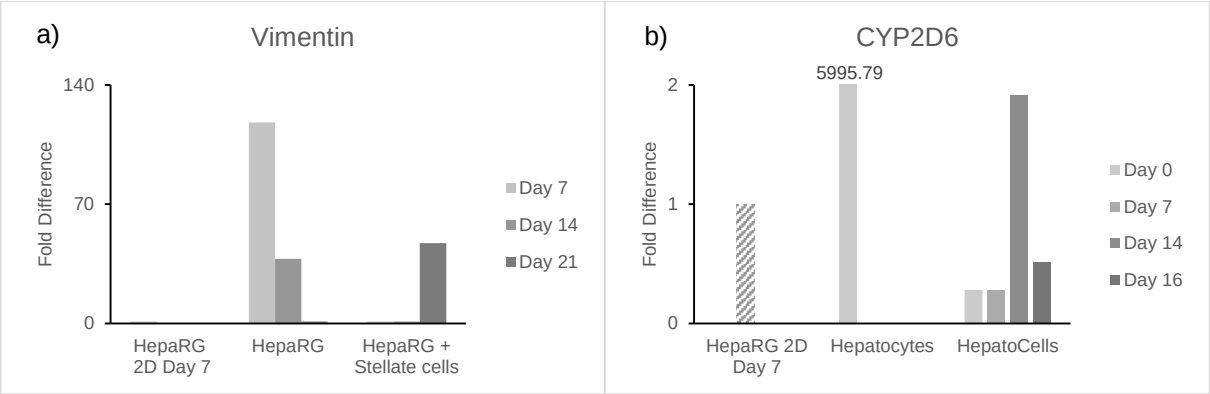

Figure 8. Gene expression of a) vimentin and b) CYP2D6 compared to a reference of 2D cultured HepaRG from day 7 set as 1 for all genes tested. These results represent the mean of three measurements made from one sample of 60 pooled spheroids.

4.3 Staining and imaging

Figure 9 presents histology sections stained with hematoxylin and eosin. Hematoxylin stains nucleic acids in the nucleus deep blue, while eosin stains cytoplasm and extra cellular matrix proteins pink. In figure 9 the nuclei are presented in dark grey, while the cytoplasm is lighter grey surrounding the nuclei. If necrotic cells are present, they are detected by weaker hematoxylin staining of the nucleus, and as can be seen in figure 9, there seems to be no necrotic core present in any of the samples. HepaRG spheroids and co-cultured spheroids of HepaRG and stellate cells seems to be stable in the 21 day samples, see figure 9c and 9d, which reinforces that spheroids of these cell types and of this size can be cultured in this way for up to at least three weeks. In figure 9e it can be seen that HepatoCell spheroids are intact at day 7. However, figure 9f shows that the HepatoCell spheroids were no longer stable on day 14 and that the spheroids had started to disintegrate.

Figure 10 and 11 shows histology sections stained with antibodies. The samples in figure 10 are stained with antibodies for CK18 as a hepatocyte marker (red), CK19 as a marker for biliary cells (green) and Hoechst for the nucleus (blue), except for 10c, which is stained for vimentin as a marker for stellate cells (green). The samples in figure 11 are stained with antibodies for CYP3A4 (red), MRP2 (green) and Hoechst for the nucleus (blue).

In figure 10a and 10b both the HepaRG and co-cultured spheroids of HepaRG and stellate cells contains cells that are positively stained for CK18 (red), and CK19 (green). For the HepatoCell spheroid sections in figure 10d, red fluorescence from cells stained with CK18 can be seen, as well as some green fluorescence from cells stained with CK19. However, in figure 10e only some and no defined fluorescence is detected for any of the proteins, except for one red patch in the middle of the spheroid. The co-cultured spheroid stained with antibodies for vimentin, figure 10c, shows some positive staining, which confirms the presence of stellate cells in the spheroid.

As can be seen in figure 11a and 11b HepaRG and co-cultured spheroids of HepaRG and stellate cells clearly have cells containing CYP3A4, indicating metabolic ability of the spheroids. Green fluorescence can also be detected in the spheroids, indicating presence of MRP2. There seems to be more positive staining for MRP2 in the co-cultured spheroid, however the MRP2 staining is not specific to individual cells in any of the spheroids. In the

HepatoCell spheroid, figure 11c, both red and green fluorescence can be seen indicating presence of CYP3A4 and MRP2, but then the staining is not specific for either of the proteins.

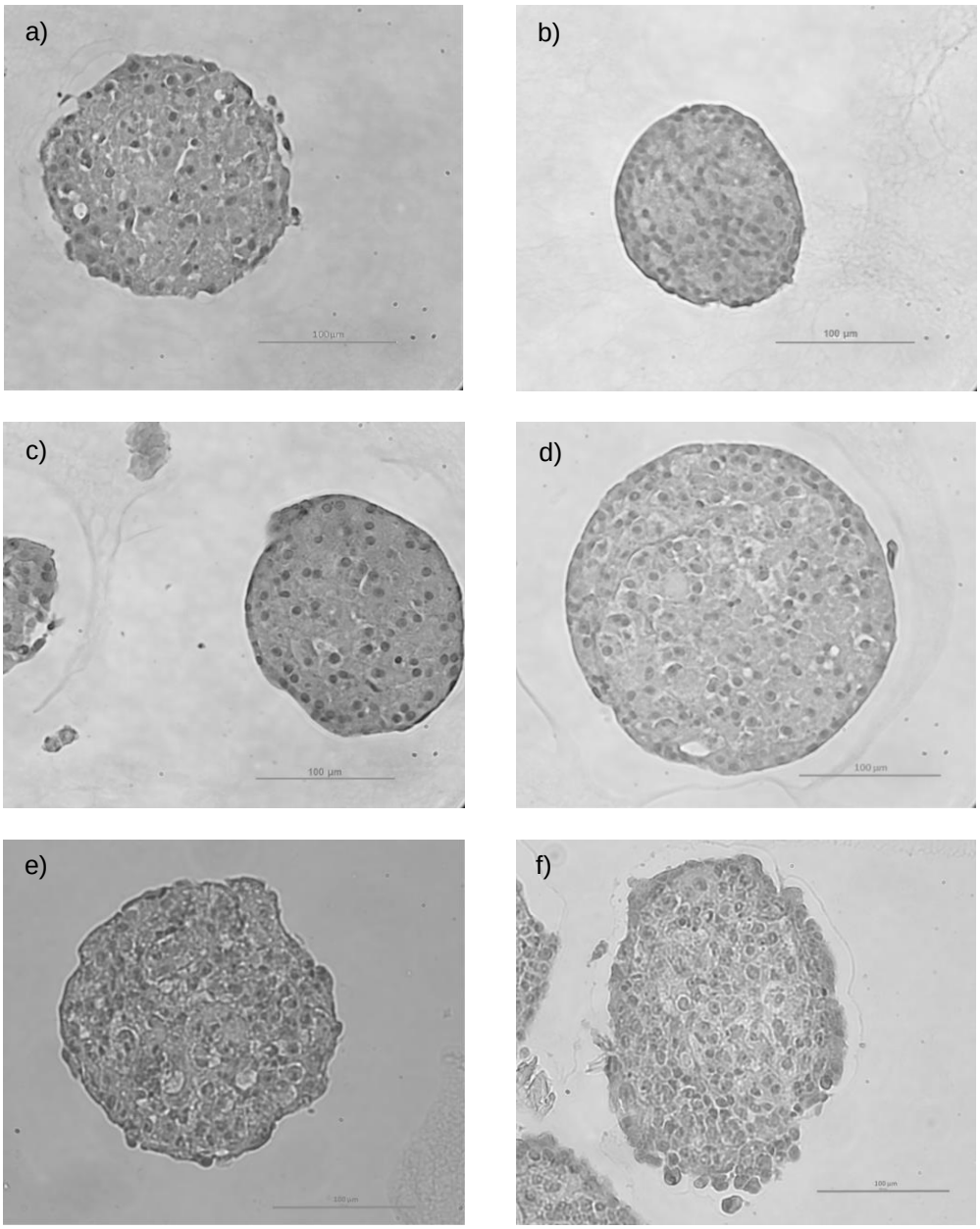

Figure 9. Histology sections of spheroids stained with hematoxylin and eosin a) HepaRG spheroid sampled at day 7. b) Co-cultured spheroid of HepaRG and hepatic stellate cells sampled at day 14. c) HepaRG spheroid sampled at day 21. d) Co-cultured spheroid of HepaRG and hepatic stellate cells sampled at day 21. e) HepatoCell spheroid sampled at day 7. f) HepatoCell spheroid sampled at day 14. The scale bar is 100 µm.

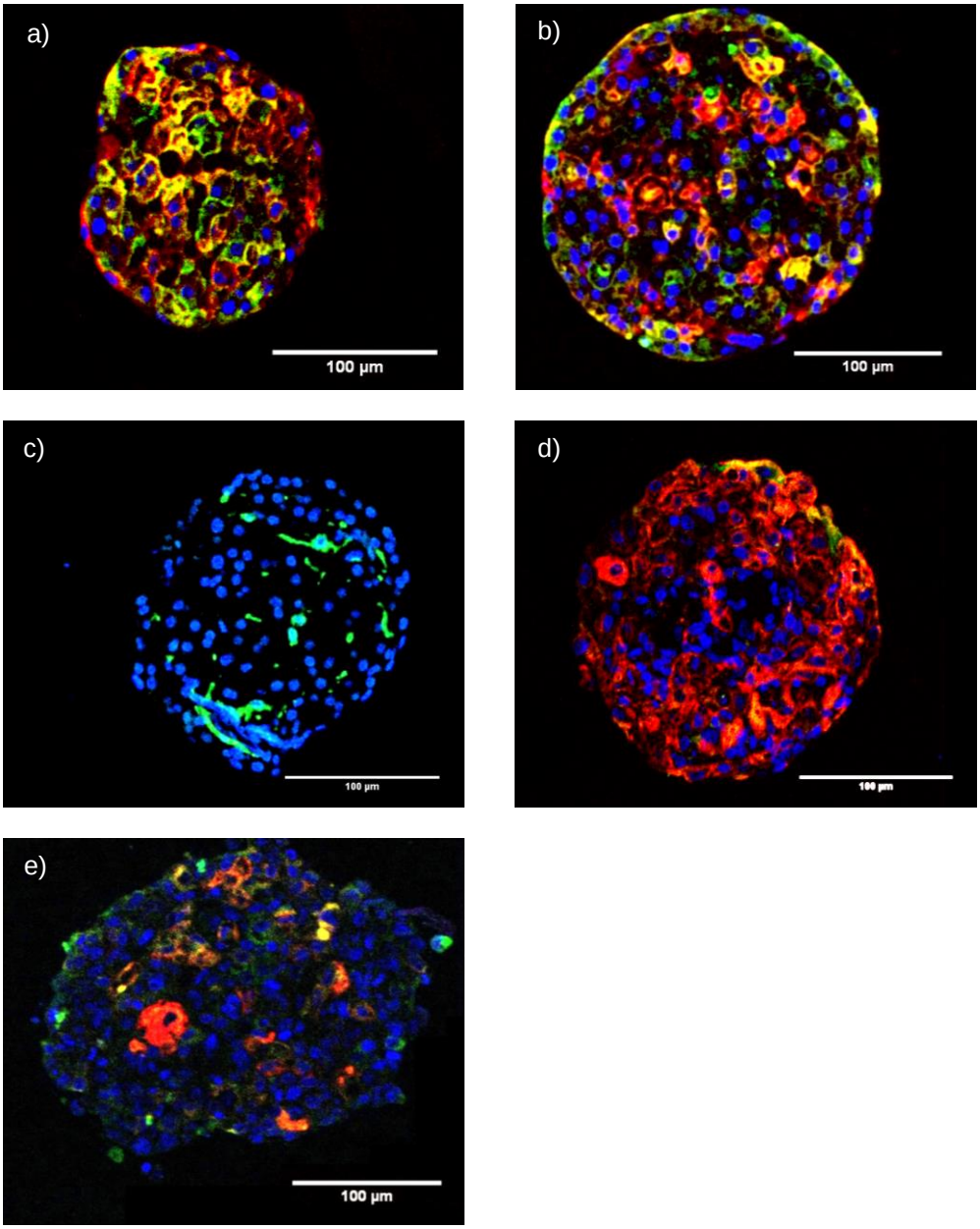

Figure 10. Histology sections of spheroids stained with fluorescent antibodies. All samples are stained for CK18 in red and CK19 in green, except c, which is stained for vimentin in green. Blue colour indicates the nucleus which is stained with Hoechst in all samples. a) HepaRG spheroid sampled at day 21 b) Co-cultured spheroid of HepaRG and hepatic stellate cells sampled at day 21. c) Co-cultured spheroid of HepaRG and hepatic stellate cells sampled at day 21. d) HepatoCell spheroid sampled at day 7. e) HepatoCell spheroids sampled at day 14. The scale bar is 100 µm.

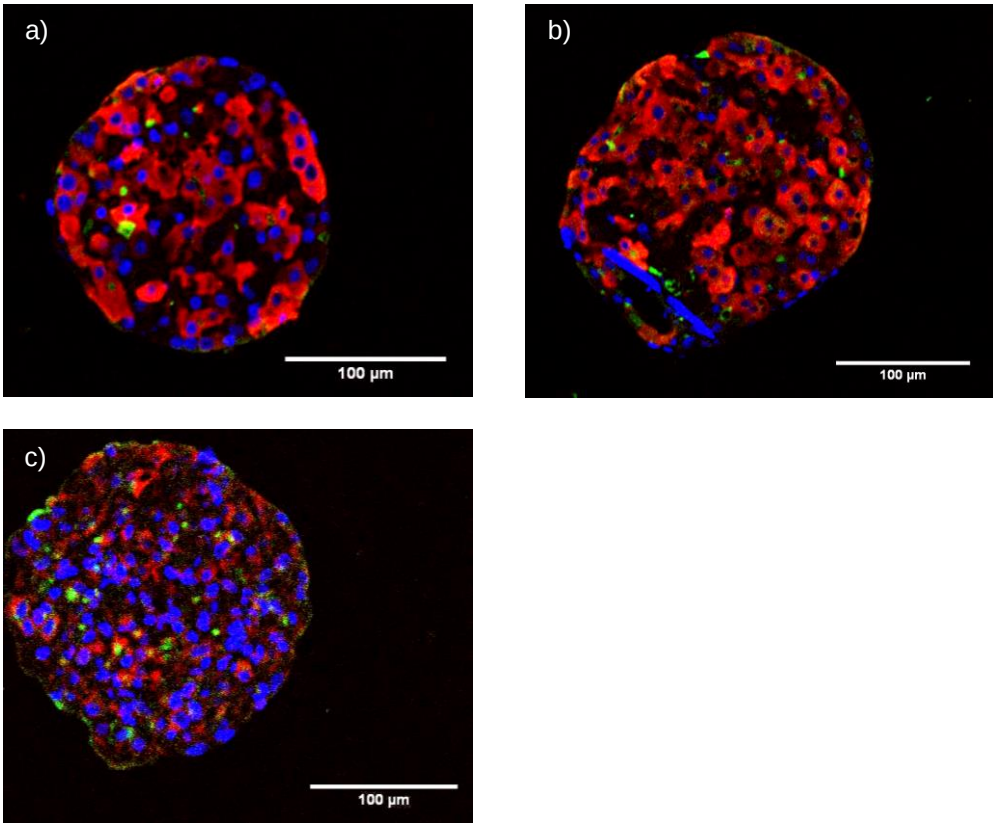

Figure 11. Histology sections of spheroids stained with fluorescent antibodies, red indicates CYP3A4, green indicates MRP2, and blue indicates the nucleus which is stained with Hoechst in all samples. a) HepaRG spheroid sampled at day 7. b) Co-cultured spheroid of HepaRG and hepatic stellate cells sampled at day 21. c) HepatoCell spheroid sampled at day 7.

5. Discussion

It was possible to culture the HepaRG and co-cultured spheroids of HepaRG and stellate cells spheroids for the time period and with the method used in this project. From the histology sections stained with hematoxylin and eosin, figure 9, it can been seen that the HepaRG and co-cultured spheroids look nice and round and that no necrotic core is present, which indicates the viability of the cells in the spheroids throughout the culture period. Looking at figure 10a and 10b, cells stained with antibodies for CK18, marker for hepatocytes, and CK19, marker for biliary cells, are distinguished. Individual cells can be identified, and the nucleus, stained with Hoechst, is visible in blue. There is more positive staining for CK19 in the co-cultured spheroid, though the HepaRG spheroid is from day 7 and the co-cultured from day 21, which might indicate that biliary like cells are more essential the longer the culture is kept. Thus, these results confirms the presence of hepatocyte and biliary like cells in both spheroid types. A spheroid section of co-cultured HepaRG and stellate cells was also stained for vimentin, which is a marker for stellate cells, see figure 10c. Some green fluorescence can be seen in the image, indicating presence of stellate cells.

The primary human hepatocytes unfortunately did not form any spheroids. The primary hepatocytes were cultured in the same way as the HepatoCells, and it was expected to see formation of spheroids after 5-7 days, which was the case for HepatoCells. The reason why no spheroids formed from hepatocytes are not known. It is thought that batch-to-batch differences can affect primary hepatocytes ability to form spheroids, and the batch used in this project might have been a batch not suitable for spheroid formation. The use of hepatocytes for spheroid formation is a quite new application, and hence vendors do not supply any information about the ability of specific batches to form spheroids.
The HepatoCells formed nice round spheroids. However, the spheroids were not stable throughout the culture period, see appendix I, and the culture was terminated at day 16 due to disintegration of the spheroids. Looking at figure 9e and 9f, there is a clear difference between the day 7 and day 14 HepatoCell spheroids, where the day 14 spheroid clearly has started to fall apart. In figure 10d, HepatoCell spheroid sampled at day 7, red fluorescence can be seen. This might indicate a hepatocyte like phenotype of the HepatoCells. However, there is almost no red fluorescence detected in the HepatoCell spheroids sampled at day 14, see figure 10e, indicating that the cells had lost their hepatocyte like phenotype. Some staining for CK19 can be seen for the HepatoCell spheroids, especially in the day 14 spheroid, but the staining does not seem to be specific.

The metabolites measured to determine the activity for CYP1A2 and CYP2D6, were below LLOQ for HepaRG and co-cultured spheroids of HepaRG and stellate cells for the 1 hour samples. Activity was also measured for both enzymes for HepaRG spheroids after longer incubation, see figure 5a and 5c. The gene expression results shows expression of CYP1A2 for both spheroid types, and it has previously been shown that longer incubations gives a higher concentration of metabolites [22]. This indicates that co-cultured spheroids probably had activity of CYP1A2 and CYP2D6, which would have been seen from a longer incubation. As the activity had a peak at 8 hours for all day 7 samples, this time point together with 24 hours were used for the second experiment to make sure that detection of metabolites from

the samples were possible. The activity of CYP1A2 corresponds to previously shown data for HepaRG cells [24], while the activity of CYP2C9 is five times higher, and CYP3A4 is two times higher for the spheroids cultured in this study compared to previous results for HepaRG [24]. Spheroids stained with antibodies for CYP3A4 correlates well with the activity results. In figure 11a and 11b cells stained for CYP3A4 (red) are clearly detected as specific cells.

The activity of CYP2D6 was low for the HepaRG spheroids, figure 5c, and not detected for co-cultured spheroids of HepaRG and stellate cells. It has previously been shown that HepaRG cells have low activity and expression of CYP2D6 [20][21][24][38], and it is assumed that HepaRG cells are derived from a donor who was a poor metabolizer of CYP2D6 [20]. This may be an explanation to the low activity of CYP2D6 for the HepaRG and co-cultured HepaRG and stellate cell spheroids.

The CYP activity was low for all CYP enzymes for the HepatoCell spheroids, see figure 6, which also correlates to the gene expression where almost no expression was found for any of the genes, see figure 7. Also, no specific CYP3A4 could be detected in the histology sections of HepatoCells stained with antibodies, which also strengthens the results that low CYP activity was present in the spheroids. As the HepatoCells are a new product on the market, and no published data about the cells are available, these results cannot be compared to other results for the cell type. However, compared to the HepaRG and co-cultured spheroids the HepatoCell spheroids does not reach the same levels of expression and activity of the CYP enzymes tested in this project. The gene expression for HepatoCells at day 0 can also be compared to the expression of hepatocytes. The day 0 samples were from cells in suspension, and are thereby not representative for spheroids. However, it gives a good prediction of the difference between the cell types. The gene expression was much higher for hepatocytes in suspension at day 0 than for HepatoCells, see figure 7 and 8b. It does not seem like HepatoCell spheroids have functions that match primary human hepatocytes, and may therefore not be suitable for drug metabolism and disposition studies.

The effect on metabolism of co-culturing stellate cells and HepaRG cells for spheroid formation was also investigated in this project. Benefits of co-culturing hepatocytes with stellate cells have previously been shown [15], and in a study by Wagner *et al.* the possibility to form microtissue aggregates of such a co-cultures was proven [31]. Leite *et al.* has also shown that it is possible to form spheroids of HepaRG and primary human stellate cells, and that it was possible to keep the spheroids for 21 days [14]. The results in this project show a slight increase in CYP activity for CYP2C9 and CYP3A4 at day 7, and a higher gene expression for albumin compared to HepaRG spheroids, which indicates a higher hepatic resemblance. Co-cultured spheroid sections stained for vimentin showed some green fluorescence, see figure 10c, which can confirm the presence of stellate cells in the spheroids, although the gene expression of vimentin was not conclusive. Other results show no clear difference between the two spheroid types, thus from this study no conclusions can be drawn about the difference between the spheroids.

The results from this project show that human hepatic spheroids formed from HepaRG cells have CYP activity and gene expression that is relevant for studies of metabolism and disposition. However, as no primary human hepatocyte spheroids were formed, more experiments are needed to confirm the ability to form such spheroids. Since it is thought that batch-to-batch differences has an impact on the ability for hepatocytes to form spheroids, it would also be of interest to investigate what factors that contributes to such difference. Further studies are also needed to give a deeper understanding of the mechanisms involved to give rise to the enhancements of the hepatic functions that occur in the spheroids compared to cells cultured in a monolayer. Regarding the experiments in this thesis, it would be of interest to do more replicates of the spheroid samples for the different time points in order to get better statistics. Looking at co-cultured spheroids with longer incubation times with the substrate cocktail for the CYP activity measurements, and different ratios of the cell types are also things that would be interesting to investigate further.

One way to use the hepatic spheroids in the future is to incorporate them to microfluidic systems, so called organ-chips [26][31]. In such a system microtissues can be cultured while exposed to fluid flow, which allows for nutrients and waste products to be transported to and from the cells in the tissues. Microfluidic systems furthermore allows for accurate control of the microenvironment of the tissue [29]. It is also possible to create a multi tissue system on such organ-chips. This allows for crosstalk between tissues, and the possibility to study the effect on drugs that have been exposed to different tissues in the same system.

6. Conclusion

The aim of this thesis project was to further develop and characterize a human hepatic spheroid model to be used for metabolism and disposition studies.

This study has shown that it is possible to culture hepatic spheroids of HepaRG and a co-culture of HepaRG and primary human stellate cells for at least three weeks, and that these spheroids have a gene expression and CYP activity that are improved compared to HepaRG cells cultured in monolayer.

Primary human hepatocytes were not able to form spheroids in the scope of this project, and spheroids formed from HepatoCell, which is a new product on the market, did not have characteristics to be suitable for use in metabolism and disposition studies.

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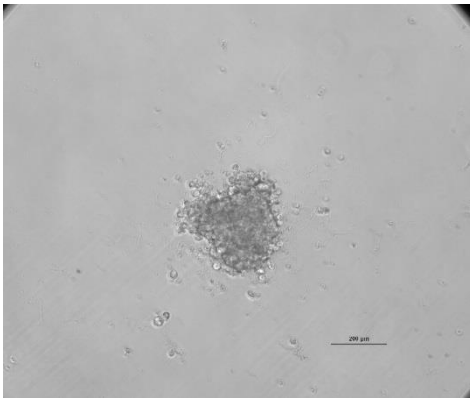
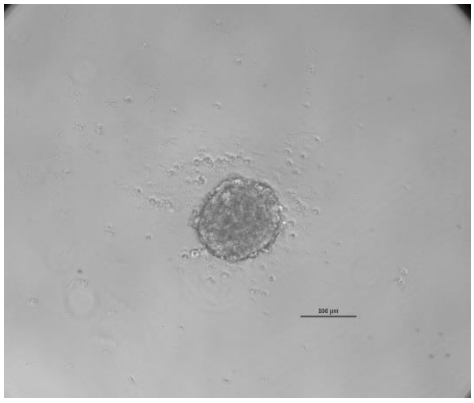
Appendices

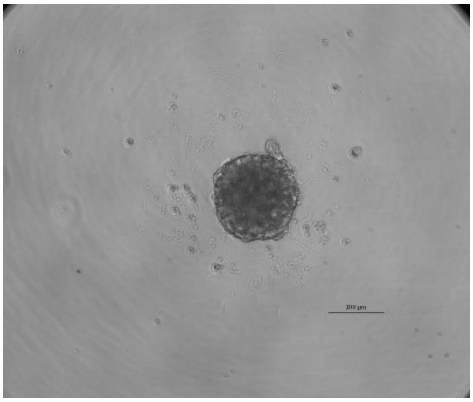
Appendix I



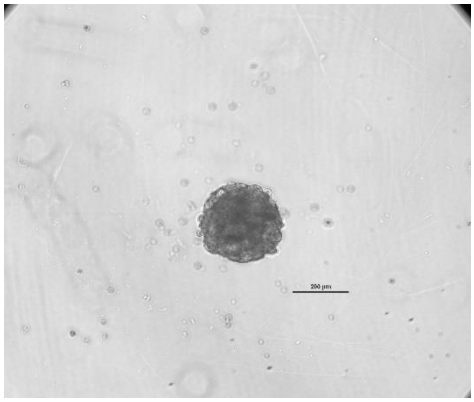
a) Day 2



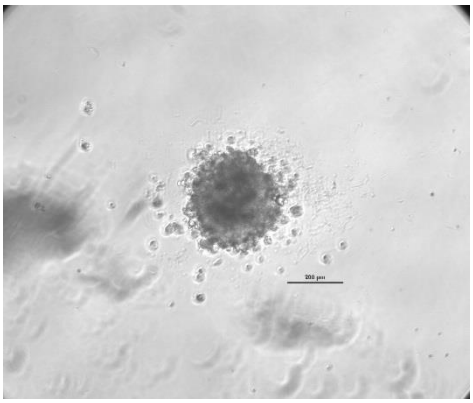
b) Day 3



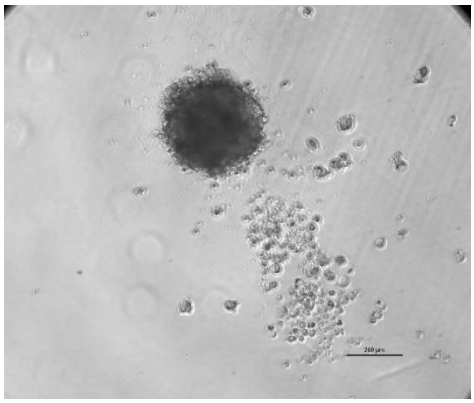
c) Day 5



d) Day 6

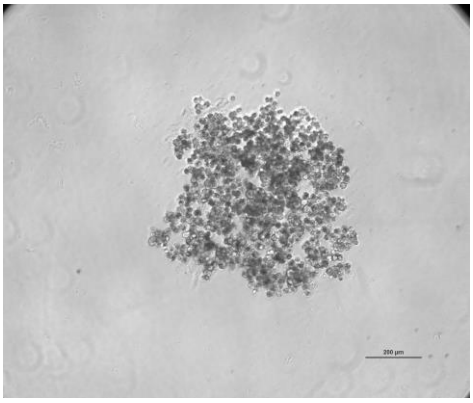


e) Day 13

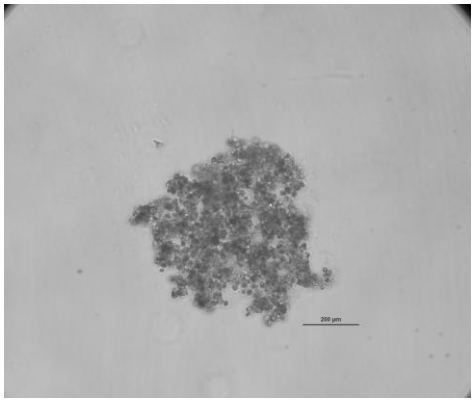


f) Day 15

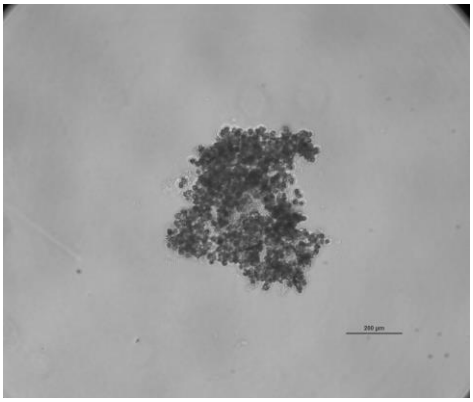
Figure 12. Development of HepatoCell spheroids. Spheroids started to disintegrate at day 13 and therefore the culture was terminated on day 16.



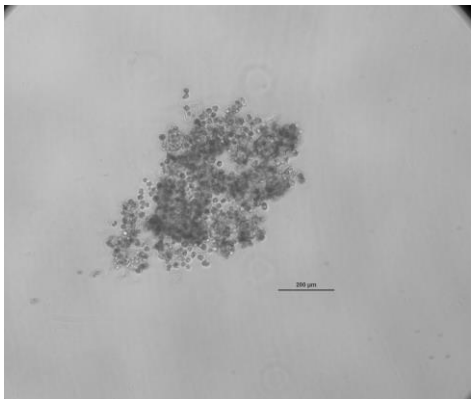
a) Day 2



b) Day 3



c) Day 5



d) Day 6

Figure 13. Development of hepatocyte spheroids. No spheroids were formed, and thereby the culture was terminated at day 7.

Appendix II

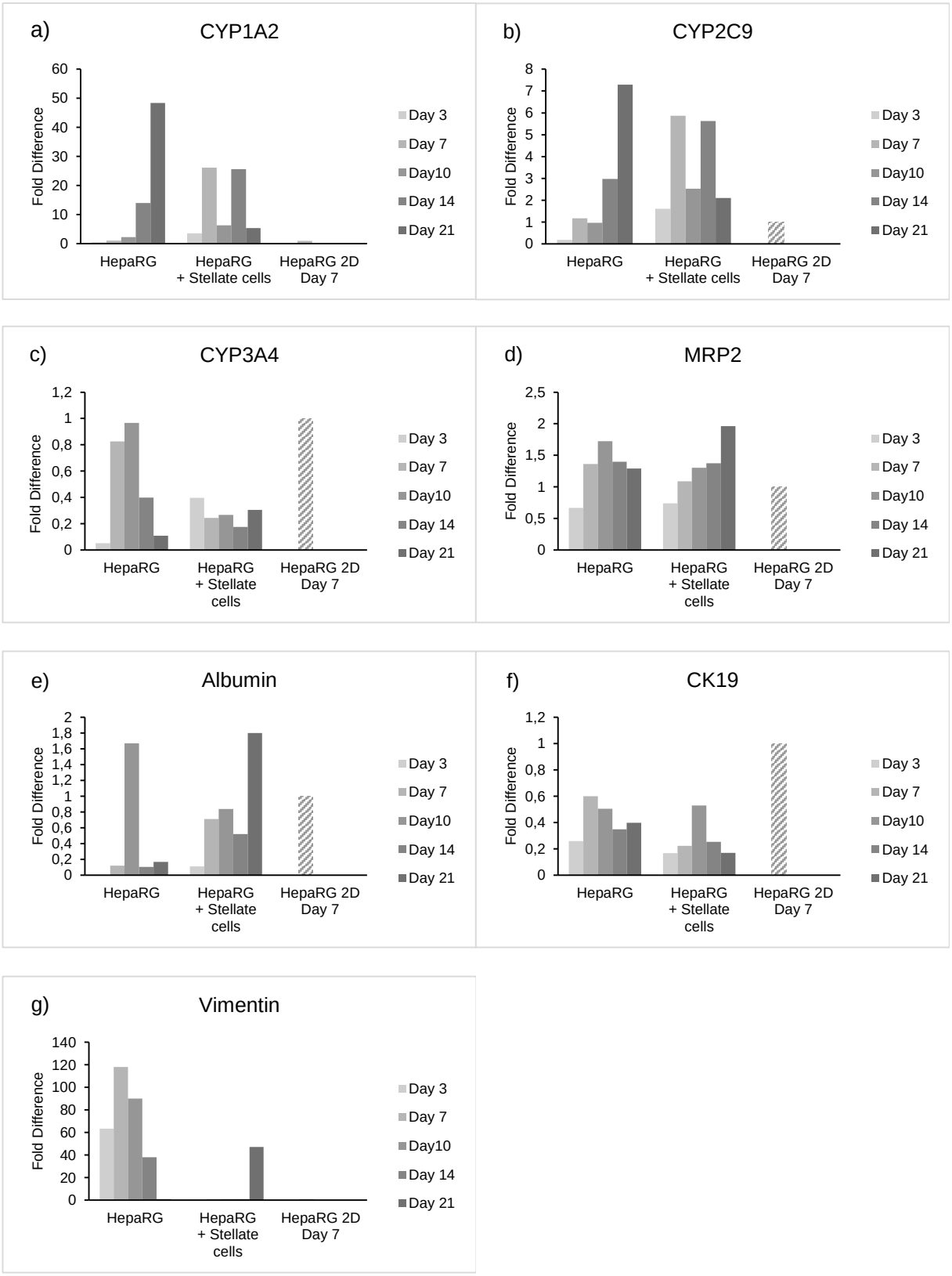


Figure 14. Gene expression of a) CYP1A2, b) CYP2C9, c) CYP3A4, d) MRP2, e) albumin, f) CK19 and g) vimentin compared to a reference of 2D cultured HepaRG from day 7 set to 1 for all genes tested.

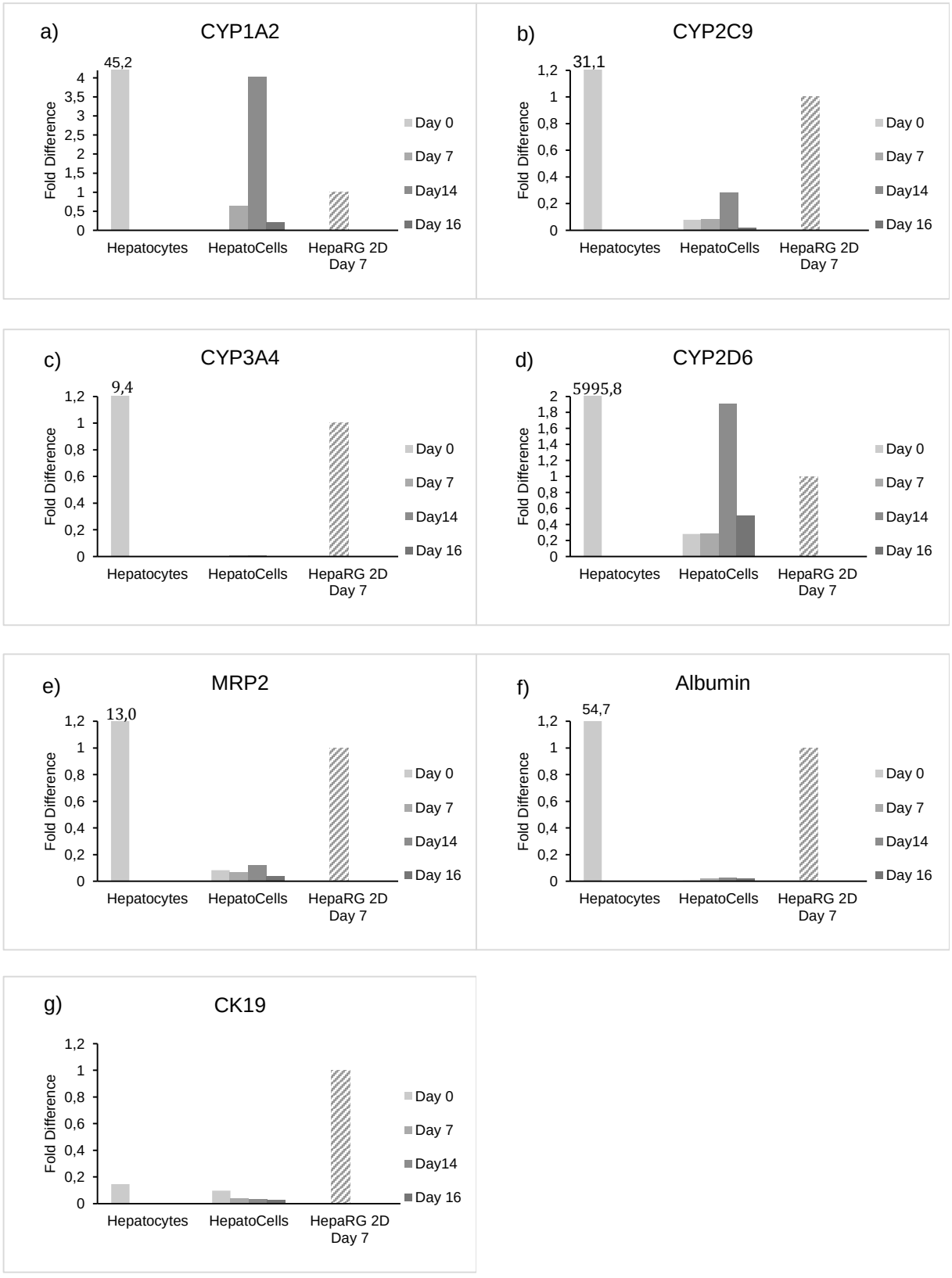


Figure 15. Gene expression of a) CYP1A2, b) CYP2C9, c) CYP3A4, d) CYP2D6, e) MRP2, f) albumin and g) CK19 compared to a reference of 2D cultured HepaRG from day 7 set to 1 for all genes tested.