The role of mitochondria in human liver cell line differentiation for bioartificial livers

Adam, A.A.A.

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The Role of Mitochondria in Human Liver Cell Line Differentiation for Bioartificial Livers

Aziza Abdelrahman Abubakr Adam
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Promotiecommissie:

**Promotor:** Prof.dr. R.P.J. Oude Elferink AMC – UvA

**Copromotores:**
- Dr. R.A.F.M. Chamuleau AMC – UvA
- Dr. R. Hoekstra AMC – UvA

**Overige leden:**
- Prof. dr. A.K. Groen AMC – UvA
- Prof.dr. R.H.L. Houtkooper AMC – UvA
- Prof. dr. P.L.M. Jansen AMC – UvA
- Prof. dr. W.H. Lamers AMC – UvA
- Dr. A.J. Meijer AMC – UvA
- Prof. dr. R.J. Porte Rijksuniversiteit Groningen

Faculteit der Geneeskunde
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Chapter 1

General introduction and thesis outline
General introduction

This thesis focuses on the sources and maturation of human liver cells applicable as biocomponents for bioartificial livers (BALs), which are bioreactors developed for liver support of end-stage liver failure (ESLF) patients, with particular emphasis on the role of energy metabolism.

End-stage liver failure

End-stage Liver Failure (ESLF) is a life-threatening condition of patients with liver disease due to shortage of sufficient functional liver mass. The clinical syndrome of ESLF comprises severely impaired liver function resulting in bleeding risks or thrombosis, disturbed acid-base homeostasis, systemic inflammatory response, hepatic encephalopathy and multi organ failure. Different types of ESLF can be distinguished: Acute Liver Failure (ALF) when ESLF occurs in a person with a previous healthy liver and Acute on Chronic Liver Failure (ACLF) in a patient with an already compromised liver, mostly cirrhosis. A uniform definition of ALF is lacking (1), however, ALF has been defined by the European Association for the Study of Liver (EASL) as the presence of severe hepatic damage (elevated serum transaminases) and impaired liver function (jaundice and coagulopathy), accompanied with clinical manifestations, as hepatic encephalopathy, in the absence of preexisting liver disease (2). Its severity is mostly addressed by King’s College or Clichy-Villejuif criteria (3). ACLF is a recently recognized syndrome in cirrhosis characterized by acute decompensation, organ failure and high short-term mortality (4-6). Its severity is mostly assessed by the CLIF-SOFA score, which includes subscores ranging from 0 to 4 for each of six components (liver, kidneys, brain, coagulation, circulation and lungs) with higher scores indicating more severe organ impairment (4).

The most common causes of ALF are viral hepatitis, drug intoxication (particularly acetaminophen), idiosyncratic drug interactions, acute autoimmune hepatitis and idiopathic liver failure if the triggering event is not known (7). Sepsis, active alcoholism and recurrence of viral hepatitis, are the most frequent predisposition causes to ACLF, however, still in ~50% of cases the cause was not recognized (5). The pathology of ESLF initially relates to the inability of the damaged liver to properly outperform vital functions, such as xenobiotic detoxification, mitochondrial functions and synthesis of plasma proteins, leading to accumulation of toxic substrates, including ammonia, aromatic amino acids and lactate, and coagulopathy (8, 9). In a later stage, liver necrosis causes endoplasmic reticulum stress and the systematic inflammatory response syndrome, which increases the danger of subsequent sepsis and multi-organ failure (10, 11).

ESLF is characterized by multi organ failure and high mortality rate, which can be as high as 80% without liver transplantation depending on the cause and the experience of the Center (12, 13).
Treatment of ESLF

The treatment of ESLF is often carried out in the intensive care unit preferably in a liver transplantation facility. Treatment of these patients is basically symptomatic and supportive therapy to the failing organs. In some cases antiviral medications or medications to reverse intoxication are introduced. Close monitoring particularly of vital complications, such as hepatic encephalopathy and sepsis, is essential. This treatment is defined as standard medical care. The only curative therapy for ESLF is liver transplantation, however, its outcome is dependent on accurate selection of patients who are at eligible phase to liver transplantation (14, 15). Liver transplantation improves patient survival at 1 year up to 77% in ALF (3) and up to 75% for ACLF (16). Yet, the shortage of donor livers often results in a considerably long waiting time; it has been reported in US that 20% of the ESLF patients die while waiting for the transplantation (17). Other risks of liver transplantation are the increased perioperative complications and the long term usage of immunosuppressive therapy to prevent organ rejection. Therefore, there is a high demand for liver support systems to bridge ESLF patients to liver transplant (bridge-to-transplant) or to recovery for those not eligible for transplantation or with sufficient regenerative liver capacity (bridge-to-recovery) (18).

Liver support systems

Many liver support systems have been developed as additional therapy to standard medical care for ESLF. There are non-cell based and cell-based liver support systems. The non-cell based systems rely on albumin dialysis techniques (MARS, Prometheus, ADVOS, SPAD) or replacement of plasma albumin (DIALIVE and High Volume Plasma Exchange). Most of these treatments have shown improvement in biochemical parameters and secondary endpoints. A meta-analysis in 2013, showed that non-cell based liver support systems significantly reduced mortality in ACLF but not in ALF (19, 20). Cell-based liver support systems are bioartificial livers (BALs). BALs are principally bioreactors loaded with viable hepatocytes, to be connected extracorporeally to the circulation of the patient. The AMC-BAL, developed in 1990’s (21), is based on a bioreactor with a spirally wounded non-woven matrix that provides a 3D environment to hepatocytes. For optimal oxygenation of the cells, semi-permeable oxygen capillaries are inserted within the matrix windings which provide a 40%O₂ gas mixture. In contrast to most other BALs, the hepatocytes in the AMC-BAL are in direct contact with the patient’s plasma, without barrier (Fig.1). The culture medium is continuously perfused leading to a dynamic medium flow (DMF).
Figure 1: The AMC-BAL. A) An image of the AMC-BAL, B) Schematic cross section of the AMC-BAL showing the spirally wound matrix in which the liver cells attach in a 3D configuration, positioned between oxygen capillaries for continuous gas (40%O₂, 5%CO₂, and 55%N₂) supply. The bioreactor is continuously perfused with medium, I) Housing, II) Matrix, III) Hollow oxygenation capillaries, IV) Extra-capillary space.

Required criteria for BAL biocomponents

The required functionality criteria for hepatocytes to be applied in the BALs are derived from the ESLF patient’s critical metabolic demands, but are not fully defined, as the requirements may differ per patient. Primary Human Hepatocytes (PHHs), displaying the full spectrum of hepatic functions are the preferred source of cells, but are unfortunately not available in sufficient large amounts and rapidly dedifferentiate in vitro. Alternatively, metabolic profiles of primary porcine hepatocytes are highly similar to that of PHHs. However, due to the risk of xenozoonosis and potential immunological problems, the use of non-human cells in BALs has been forbidden in Europe by a European moratorium in 2001 (22, 23).

Van Wenum et al. listed six functionality benchmarks for BAL biocomponents, including robust hepatic differentiation (suppression of fetal hepatic markers and upregulation of differentiation markers), protein synthesis, ammonia metabolism, xenobiotic detoxification and mitochondrial energy metabolism (24). Furthermore, a sufficient quantity of hepatocytes is required, estimated to be at least 150 g/treatment (25). To achieve this, an unlimited cell proliferative capacity is required.
Proliferative sources of hepatocytes for BAL application

Hepatocytes derived from proliferative sources as human hepatoma cell lines represent an alternative for PHHs as biocomponent for BAL application. Two hepatoma cell lines, HepaRG and C3A, currently serve as BAL-biocomponents for the AMC-BAL and the extracorporeal liver assist device (ELAD), respectively (26, 27).

The C3A cell line, a subclone of HepG2, exhibits an unlimited proliferative capacity and shows a hepatocyte-like morphology (Fig. 2A). Albumin biosynthesis by C3A cells is almost approaching PHHs level, however, several hallmark hepatic functions are extremely suppressed, such as xenobiotic detoxification, and lactate metabolism (28). Moreover, C3A cells produce ammonia instead of eliminating it and lack urea cycle (UC) activity (29, 30).

The HepaRG cell line is a progenitor cell line that develops within 28 days into a mixed heterogeneous monolayer culture with hepatocyte-islands surrounded by cholangiocyte-like cells (Fig. 2B) (31). The proliferative capacity is, however, limited; after 20 passages with a split ratio of 1:5, the HepaRG cells show an increased tendency of transformation and acquisition of a mesenchymal “Warburg” phenotype with aerobic glycolysis, as evidenced by increased lactate production (32, 33) & (Adam et al (in preparation). The general transcriptome of HepaRG cells differs substantially from that of PHHs (34-36). However, the transcriptional profile of HepaRG cells regarding several metabolic processes as xenobiotic detoxification, in several aspects, is comparable to that of PHHs (35). Still, some studies reported limited xenobiotic metabolism by HepaRG cells at the activity level (37, 38), particularly when differentiated in the absence of dimethylsulfoxide (DMSO) (35, 39, 40). DMSO treatment increases biotransformation, however, it negatively affects cell viability and transcript levels of hepatic genes unrelated to biotransformation (26). Several hepatic functions as ammonia elimination, albumin and bile acids production are almost comparable between HepaRG cells and PHHs (26, 41). Yet, other key hepatic functions required for BAL application are limited. The UC activity is significantly reduced compared to PHHs (27, 28), most likely due to decreased expression and activity of one or more -of the five UC enzymes compared to human liver (42). As a result, more than 95% of ammonia elimination occurs through reversible fixation into amino acids, which represents only a temporary elimination, instead of the irreversible clearance through UC. In addition, the expression of critical regulators of xenobiotic and mitochondrial energy metabolism, as the constitutive androstane receptor (CAR), is limited, resulting in a relatively low activity of a set of detoxification enzymes and decreased mitochondrial functions, such as lactate metabolism.

Another important source for hepatocytes are stem cells, which are unique in displaying self-renewal ability and multipotency (43, 44). In addition to the naturally existing embryonic and somatic stem cells, stem cells can be generated from somatic cells, such as fibroblasts, by overexpression of critical pluripotency-regulating transcription factors, generating induced pluripotent stem cells.
(iPSCs) (45). The stem cells can then be directed into hepatocytes like cells (HLCs) after exposure to growth and differentiation factors in the medium, optionally in combination with extracellular matrix (24). Yet, regardless of the cell source, these HLCs lack complex hepatic functions (46). Recently, a new technique, lineage reprogramming, has been established by directly introducing lineage-specific transcription factors into somatic cells to induce distinct cellular characteristics without passing through the pluripotency phase (47). Direct conversion of mouse fibroblasts into so called induced-hepatocytes (iHeps) was achieved by transduction of essential liver-enriched transcription factors (48). Interestingly, the transplanted iHep cells improved survival in fumarylacetoacetate-hydrolase-deficient mice due to sufficient liver repopulation and showed some hepatic characteristics, including cytochrome P450 (CYP) enzyme activity and biliary drug clearance (47, 49, 50). Yet, the iHeps exhibit a narrow range of hepatic functions and hallmark hepatic features, as ammonia elimination and lactate metabolism have not been reported.

A recent comparison of the transcriptome of different sources of human hepatocytes revealed that HepaRG cells are currently, the most suitable substitute for PHHs (35, 41), although there are still some considerable shortcomings in their hepatic functionality. Therefore to meet all requirements for BAL-biocomponents, HepaRG cells need further optimization.

To improve biotransformation properties without DMSO treatment, we recently established a new stable cell line, HepaRG-CAR, by lentiviral overexpression of CAR in HepaRG cells (37). The resulting HepaRG-CAR cells exhibited increased biotransformation activities, including CYP activity and bilirubin conjugation. In addition, albumin production, resistance to DMSO-induced toxicity, and NAD(P)H levels were elevated by CAR overexpression, however, ammonia elimination remained unchanged (37). Nevertheless, HepaRG-CAR cells can currently be considered as the most robust human hepatocyte cell line and very promising for BAL application and in vitro studies.

Figure 2: Morphology of monolayer cultures (without DMSO treatment) of C3A (at day 14) and HepaRG cells (at day 28). A) C3A culture B) HepaRG culture with islands of hepatocytes (I) surrounded by cholangiocyte-like cells (II) (28).
The role of mitochondria in hepatic differentiation

The association between mitochondrial energy metabolism and differentiation of stem cells currently receives much attention in the stem cell field (51). On one hand, undifferentiated stem cells retain pluripotency and unlimited proliferative capacity under conditions mimicking the original relatively anaerobic stem-cell niche. During this phase, the energy is mainly derived through high glycolysis rate coupled with increased lactate production and reduced oxygen consumption, whereas, mitochondria are sub-functional due to immaturity (52, 53). Under conditions of limited oxygenation, glycolysis is more favorable for stem cells as glycolytic intermediates, (mainly glucose-6-phosphate), can be basically directed to feed the pentose phosphate pathway rather than tricarboxylic acid cycle (TCA). Accordingly, more substrates are available for nucleotide synthesis to further facilitate the self-renewal of the stem cells (52).

In general, during stem cell differentiation, energy metabolism switches from high glycolysis towards oxidative phosphorylation (OxPhos), taking place in the mitochondria (52, 54, 55). It is increasingly believed that this shift in energy metabolism is in fact a causal factor to stimulate the differentiation of stem cells into functional cells. Studies have demonstrated that promoting or inhibiting mitochondrial biogenesis or OxPhos severely affect the stemness (53, 56-59). Interestingly, the reprogramming of terminally-differentiated cells into iPSCs and the in vitro dedifferentiation of PHHs are coupled to inverse modifications of the mitochondrial system named “mitochondrial rejuvenation” (54, 57, 60-62). Lauschke et al, in a large-scale transcriptomic and proteomic study, reported that early changes associated with hepatic dedifferentiation related, in part, to inhibition of major metabolic pathways, such as TCA cycle, β-oxidation of fatty acids and OxPhos (63).

Furthermore, as a by-product of energy generation through OxPhos, reactive oxygen species (ROS) accumulate which, at mild levels, play an important role as signaling molecules. High ROS levels are damaging, but at lower level ROS in stem cells cause lineage differentiation and ROS may also have an essential role in posttranscriptional regulation of hepatic functionality (64-66). Low ROS levels are found in cells with relatively low OxPhos, as stem cells, and are essential to maintain the self-renewal capacity and protect the integrity of the genome of these rapidly dividing cells. These findings collectively support the hypothesis that a shift to mitochondrial biogenesis and OxPhos may be a prerequisite in hepatocyte differentiation. Therefore, increased understanding of the molecular background of the involvement of the mitochondrial function and the fate of immature cells is highly recommended to further induce their hepatic differentiation and empower their applicability as BAL-biocomponent or in other fields requiring functional hepatocytes.

The main objective of this thesis, was therefore to study mitochondrial energy metabolism and hepatic differentiation of human liver cell lines, particularly the HepaRG cell line, and improve its potential as biocomponent for BAL application.
Thesis outline

This thesis focuses on the biocomponents applied in bioartificial livers (BALs), the critical factors in their maturation and strategies for improvement with particular emphasis on the role of mitochondrial functions.

**Part I** of this thesis describes the functionality of human liver cell lines HepaRG and C3A, in monolayer and in BAL, and highlights the intimate association between mitochondrial energy metabolism and differentiation.

In **Chapter 2** we compare the hepatic performance of human liver cell lines HepaRG and C3A in conventional monolayer and BAL-cultures. The functionality of both cell lines was highly improved under BAL-culturing with HepaRG cells being most promising for BAL application.

In **Chapter 3** the background is investigated of the improved functionality associated with BAL-culturing of HepaRG cells compared to monolayer culturing using whole genome micro-array analysis. Mitochondrial biogenesis was also studied by measuring mitochondrial abundance, the expression of OxPhos complexes and mitochondrial membrane potential. Similarly, mitochondrial biogenesis was addressed in BAL-cultured C3A cells. The positive effects of BAL-culturing on differentiation may be attributed to 3D configuration, DMF and/or oxygenation. We examined the contribution of these factors to mitochondrial biogenesis.

We continue in **Chapter 4** to further characterize the critical role of pericellular oxygen concentration on the differentiation of HepaRG and, partly, also of C3A cells. We applied 40%O$_2$ (hyperoxia) and hypoxia (5% O$_2$) in addition to the regular 20%O$_2$ concentration during the differentiation phase to test the effect on hepatic differentiation. We also tested long-term hepatic stability upon passaging of HepaRG cells under normoxia and hypoxia.

**Chapter 5** focuses on the importance of DMF, as one of the contributing factors to the enhanced mitochondrial biogenesis under BAL-culturing conditions. To further explore the effect of DMF on hepatic functionality of the HepaRG and C3A cells, we simulate DMF of the BALs in monolayer cultures using a simple practice-changing method based on shaking cultures at 60 rpm during the differentiation phase. Next we assessed hepatic differentiation and mitochondrial energy metabolism.

**Part II** of the thesis focuses on lentiviral correction of critical hepatic functions, particularly xenobiotic detoxification, mitochondrial energy metabolism and ammonia metabolism of HepaRG cells.
In **Chapter 6** we further investigated the effect of lentiviral overexpression of CAR, a master regulator of xenobiotic detoxification and energy metabolism, in HepaRG cells, and its effect on stability over passaging. The functionality and transcriptome of the newly developed HepaRG-CAR cells, as well as the parental HepaRG cells, was assessed in early and late passages to unravel functional and age-related changes in HepaRG and HepaRG-CAR cells.

Furthermore, to improve ammonia detoxification through UC, **Chapter 7** includes investigations, firstly, to identify the limiting factors of UC in HepaRG cells and, secondly, to restore UC to potentiate the usage of HepaRG cells for clinical BAL application. We investigated the role of the mitochondrial carbamoyl-phosphate synthase 1 (CPS1) enzyme, as the first enzyme of the UC, and compared static vs DMF-culturing conditions for the additive effect of mitochondrial-biogenesis stimulating conditions.

Finally, **Chapter 8** provides the summary of the thesis and future perspectives.
References


Part I

Mitochondrial energy metabolism and hepatic differentiation of human liver cell lines
Chapter 2

Selecting cells for bioartificial liver devices and the importance of a 3D culture environment: a functional comparison between the HepaRG and C3A cell lines.

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Martien van Wenum  
Aziza A.A. Adam  
Theodorus B.M. Hakvoort  
Erik J. Hendriks  
Valery Shevchenko  
Thomas M. van Gulik  
Robert A.F.M. Chamuleau  
Ruurdtje Hoekstra
Abstract

Introduction
Recently, the first clinical trials on Bioartificial Livers (BALs) loaded with a proliferative human hepatocyte cell source have started. There are two cell lines that are currently in an advanced state of BAL development; HepaRG and HepG2/C3A. In this study we aimed to compare both cell lines on applicability in BALs and to identify possible strategies for further improvement.

Methods
We tested both cell lines in monolayer- and BAL cultures on growth characteristics, hepatic differentiation, nitrogen-, carbohydrate-, amino acid- and xenobiotic metabolism.

Results
Interestingly, both cell lines adapted the hepatocyte phenotype more closely when cultured in BALs; e.g. monolayer cultures produced lactate, while BAL cultures showed diminished lactate production (C3A) or conversion to elimination (HepaRG), and urea cycle activity increased upon BAL culturing in both cell lines. HepaRG-BALs outperformed C3A-BALs on xenobiotic metabolism, ammonia elimination and lactate elimination, while protein synthesis was comparable. In BAL cultures of both cell lines ammonia elimination correlated positively with glutamine production and glutamate consumption, suggesting ammonia elimination was mainly driven by the balance between glutaminase and glutamine synthetase activity. Both cell lines lacked significant urea cycle activity and both required multiple culture weeks before reaching optimal differentiation in BALs.

Conclusion
Culturing in BALs enhanced hepatic functionality of both cell lines and from these, the HepaRG cells are the most promising proliferative cell source for BAL application.
Introduction

Bioartificial livers (BALs) have been developed as a bridging therapy to liver regeneration or transplantation for patients suffering from acute liver failure (ALF). In the past, BAL systems were mainly based on primary hepatocytes (PHs), most commonly of porcine origin (primary porcine hepatocytes; PPHs) [1]. PPHs pose several problems related to regulatory demands, reproducibility, stability, logistics and, arguably, safety due to the risk of zoonosis [2]. Although there is still at least one group that is in the advanced stage of developing a BAL for clinical use based on PPHs [3], the concept was largely abandoned after the European Union imposed a moratorium on xenotransplantation in 2004, which also includes BALs loaded with xenogeneic cells.

Proliferative hepatocyte-like cell types, such as stem cell derived hepatocyte-like cells and tumor-derived cell lines are alternative BAL biocomponents. These can be expanded under tightly controlled conditions and have a more stable phenotype, yielding a reproducible and well-characterized end-product, in line with the demands posed by the regulatory authorities. However, although differentiation protocols of stem cells become increasingly more sophisticated and effective, the *in vitro* hepatic functionality does not reach an acceptable level [4, 5]. In addition, stem cell technology does not yet allow for affordable large-scale cell expansion.

Currently the biocomponent of choice for BAL application is a highly differentiated human liver tumor-derived cell line. The cell lines that are most suitable for use in BALs are HepaRG and HepG2 sub-clone C3A [6]. C3A was obtained from the hepatocellular carcinoma derived cell line HepG2 by selection on contact inhibition and protein synthesis, leading to a more hepatocyte-like phenotype compared to the parental line [7] (Kelly, JH US Patent 5290684, 1990). C3A cells are used in several BAL systems and the first phase III clinical trial of a C3A BAL has recently been completed (clinicaltrials.gov NCT00973817).

HepaRG cells were also derived from a hepatocellular carcinoma and resemble hepatic progenitor cells in their capacity to differentiate into hepatocytes and cholangiocytes [8].

There are no data available that allows for a comparison between the functionality of C3A and HepaRG cells in BAL systems. Culture conditions have been shown to be of great influence on the performance of both C3A and HepaRG cells [9, 10]. Therefore it is essential to compare the cell lines under identical experimental conditions and to include a BAL system providing medium perfusion, three-dimensional configuration and optimized oxygenation. The cell lines should be evaluated for most important functions, however, the hepatic functions that contribute to improved survival in liver support settings, such as auxiliary liver transplantation in the clinic and BAL-support in animal models, are unknown and may well vary according to etiology and from case to case [5]. Therefore the aim should be a biocomponent that is functionally comparable to
mature PHs as much as possible. In a recent review we identified a set of functional parameters to test the applicability of cell sources for clinical BAL systems [5]. Briefly, these are: protein synthesis, xenobiotic detoxification, ammonia detoxification, carbohydrate metabolism, fetal hepatocyte markers and transcription factors driving hepatic differentiation.

In this study we compared these parameters of HepaRG and C3A cultures in 2D and in laboratory-sized BALs and developed possible strategies for functional improvement.

**Material and Methods**

**Monolayer culture**

HepaRG cells were provided by Biopredic International cultured as described previously [10]. Briefly, cultures were maintained in culture flasks in HepaRG medium (=WE+ medium) and passaged at a split ratio of 1:5 every 2 weeks. To obtain differentiated HepaRG cultures, the cells were seeded in 12-well culture plates (Corning, NY, USA) at 27,000 cells/cm² and cultured for 28 days in WE+ medium. At day 25, three days prior to testing, the WE+ medium was supplemented with 1mM N-carbamoyl-L-glutamate (Sigma Aldrich, St. Louis, USA) to promote carbamoyl phosphate synthetase 1 (CPS1) activity [11].

C3A cells [HepG2/C3A, derivative of Hep G2 (ATCC HB8065)] (ATCC® CRL10741™) were cultured according to the suppliers instructions. Briefly, cultures were maintained in culture flasks in MEM+ medium and passaged 1:10 every week. For experiments, C3A cells were seeded in 12-well plates at 20,000 cells/cm² and unless stated otherwise, cultured in WE+ medium for 7 days, supplemented with N-carbamoyl-L-glutamate three days prior to testing.

**BAL culture**

In this study, we used the previously described scaled-down models of the AMC-BAL [12], with a priming volume of 9 mL, 127 cm² of DuPont™ Spunlaced Nonwoven Fabric- matrix (DuPont, Wilmington, DE, USA), interlaced with 160 gas capillaries for oxygenation (Fig. 1A-D). Nine mL suspensions from 2mL cell pellets were loaded into the BALs, where cells were allowed to attach and subsequently to mature for 3-14 days, as described previously [13]. The BALs were perfused with WE+ medium supplemented with N-carbamoyl-L-glutamate (1mM) at a rate of 5 mL/min and a gas mixture consisting of 5% CO₂, 40% O₂ and 55% N₂ was led through the gas capillaries at 30 mL/min. For this study we cultured four BALs per cell line, however, one C3A BAL was excluded due to a bacterial infection.
SELECTING CELLS FOR BIOARTIFICIAL LIVER DEVICES AND THE IMPORTANCE OF A 3D CULTURE ENVIRONMENT

Figure 1. AMC-BAL Culture AMC-BAL cartridge. (B-C) Schematic cross-section and detail of a HE-staining of a paraffin embedded HepaRG-AMC BAL. Depicted are: I, The outer shell of the cartridge; II, the matrix and attached cells; III, gas capillaries and IV, the intercapillary space through which the culture medium is perfused. (D) Schematic representation of the culture set-up.

Hepatic function tests
The function tests were performed as described [10]. Briefly, monolayer cultures (n=8 per cell line) were exposed to 1 ml, and BAL cultures (C3A n=3; HepaRG n=4) to 120 ml of test medium for 24 h. Test medium was WE+ with 1 mM N-carbamoyl-L-glutamate, 1.5 mM 15NH4Cl, 2.27 mM D-galactose, 2 mM L-lactate and 125 μM testosterone (all compounds from Sigma Aldrich). During the function test, medium samples were taken at 0.75, 6 and 24 h (monolayers) or at 0.5, 1, 2, 8 and 24 h (BALs). In the obtained samples we quantified L-lactate, ammonia, urea, 15N-urea, aspartate aminotransferase
(AST), lactate dehydrogenase (LDH) and glucose, as described [10]. Enzyme leakage was expressed as a fraction of the total cellular content. Human albumin was quantified using the Human Serum Albumin DuoSet ELISA according to instructions of the supplier (R&D systems Inc., Minneapolis, USA) function test samples taken after 0, 6 or 8 (monolayer/BAL) and 24 h. An overview of the data is given in supplemental table S2 and S4.

**High-performance liquid chromatography (HPLC)**

Cytochrome P450 3A4 (CYP3A4) activity was determined in function test samples taken after 45 minutes by quantification of 6β-hydroxylation of testosterone (6β-OH testosterone) through HPLC tandem mass spectrometry. Samples were diluted with the same volume of formic acid 0.1%. The system consisted of an AB Sciex (Framingham, U.S.A) API3200 triple quadrupole mass spectrometer interfaced with an Agilent (Santa Clara, U.S.A.) 1200SL HPLC. Chromatography was performed at 70°C with 20 µL injected into a Zorbax Eclipse XDB C18 column (50 mm x 4.6 mm, 1.5µm particle size), at a flow rate of 1.5 ml/min. The column eluent was split to an electrospray ionization interface, operating at 650°C in positive mode operating in multiple reaction monitoring mode. Q1 mass was 305.3 amu, Q3 mass was 269.2 amu and retention time was 3.0 min. The mobile phase was ammonium acetate 5mM in ultrapure water (A) and 0.3% formic acid in a mixture of methanol and acetonitrile (B). The proportion of the mobile phase B was increased linearly from 30 to 37% in 2.8 min, and then, after 1 min at 99% of B, the column was allowed to re-equilibrate at the initial conditions. The total run time was 5 min. Amino Acids were quantified in function test samples taken after 0.75, 6 and 24 h (monolayer) and after 0, 2, 8 and 24 h (BAL), n=3 per group. One-hundred µl aliquots were added to 4 mg sulfosalicylic acid, snap-frozen in liquid nitrogen and stored at -80°C. Prior to amino acid analysis, proteins were removed by centrifugation at 20.000xG at 4°C. For the separation of the o-phthalaldehyde -derivatized amino acids, two serial coupled BDS Hypersil C18 columns (150 x 4.8 mm, 3 um particles, Thermo Scientific) were used. Flowrate was 0.7 ml/min, solvent A: 12.5 mM sodium phosphate pH7.0 + 0.005% tetrahydrofuran, solvent B: 6 mM sodium phosphate pH7.0 + 0.07% tetrahydrofuran + 40% acetonitrile. For normalization purpose we used norvaline and methylargenine (respectively IS-1 and IS-2 in the chromatogram, Fig. S1). A typical chromatogram is depicted in supplemental figure S1 and an overview of the data is given in supplemental table S6.

**Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)**

For gene expression analyses we obtained matrix samples (T-bags) from the BALs at different time points as described [14]. RNA from BALs (C3A: n=3, HepaRG: n=4) and monolayers (C3A: n=6, HepaRG: n=4) were isolated using the RNEasy Mini Kit (Qiagen, Hilden, Germany). qRT-PCR was performed using a touchdown annealing temperature protocol, as previously described [10]. Transcript levels were normalized for 18S ribosomal RNA and are expressed as % of the average level of two human liver samples isolated from the healthy parenchyma in liver resection material.
from female patients, aged 40 and 41, with liver adenomas and no signs of liver damage. The transcript levels of the tested genes differed no more than 1.45 fold between these samples, except for CEBPA, AFP and CYP3A7 which differed 1.88, 2.19 and 4.24 fold respectively. The procedure was in accordance with the ethical standards of the institutional committee on human experimentation (protocol number 03/024) and the Helsinki Declaration of 1975. Primer sequences and amplicon sizes are listed in Table 1. An overview of the transcript data is given in supplemental tables S2, S3 and S5.

**Statistical analyses**
We performed one-way ANOVA and Holm Sidak’s multiple comparisons test (alpha = 0.05) to assess differences between more than 2 groups, and multiple t-tests with Sidak-Bonferroni’s multiple testing correction for the comparison between two groups on multiple parameters. Statistical analyses were performed in Prism version 6 (GraphPad Prism Inc. San Diego, CA, USA). Data are presented as mean ± SD. The clustering and graphical representation of amino-acid metabolism were performed with City-block distance analysis in Gene-E (Broad-institute, Cambridge, MA, USA).

**Results**

**Standardization of the culture medium for C3A and HepaRG cells**
In order to standardize the culture conditions between the cell lines, we compared the performance of C3A monolayer cultures in their standard medium (MEM+) and in HepaRG medium (WE+). There was no difference in growth rate as determined by total protein content at day 7. In both culture media ammonia was produced rather than eliminated at equal rates (Fig. 2A). In MEM+, urea production was higher, however, this coincided with a relatively high transcript level of Arginase 2 (ARG2) (Fig. 2B), and the lack of incorporation of mass enriched 15N-ammonia into 15N-urea. This indicates that urea is formed through degradation of arginine by ARG2, which is not hepatocyte-specific and does not contribute to ammonia detoxification [15]. The MEM+ and WE+ cultures showed similar transcript levels of a panel of hepatic genes (Fig. 2B). Therefore, we concluded that WE+ is an appropriate culture medium for both C3A and HepaRG cells and all further experiments in this study were performed in WE+ medium to maximally standardize the testing conditions.
BAL culturing of C3A cells promotes differentiation in time in contrast to monolayer culturing

We then established the optimal maturation time of C3A cells in monolayer and BAL cultures. In the past the optimal maturation time of HepaRG cultures was found to be 28 days in monolayer and 14 days in the AMC-BAL [10]. In C3A monolayers, the transcript levels of genes associated with immature liver, encoding serum protein Alpha Fetoprotein (AFP) and Cytochrome P450 3A7 (CYP3A7), decreased after day 7 (Fig. 3A, supplemental table S3). However, the transcript levels of eight out of sixteen tested mature hepatic genes were also downregulated >2-fold, and thus hepatic differentiation could not be confirmed. These genes encoded for hepatic transcription factors (HNF4A, CEBPA, PXR, CAR), as well as for proteins involved in the urea cycle (OTC, CPS1), plasma protein synthesis (TF) and transport (NTCP). The other tested mature hepatic genes that remained stable in their expression (<2-fold up/down) were involved in plasma protein synthesis (ALB, FVII), nitrogen metabolism (GLUL, ARG1) biotransformation (CYP3A4, CYP2B6) and transport (SLCO1B1). Moreover, the panel of tested hepatic functions (ammonia elimination, urea production, albumin synthesis) did not increase after day 7 (Table1).

In C3A BALs, we assessed hepatic functionality (Table 1) and transcript levels (Fig. 3A, supplemental table S3) of hepatic genes at 3, 7 and 14 days. There was a trend of AFP and CYP3A7 downregulation (>2-fold), however, in contrast to C3A monolayer cultures, none of the investigated mature hepatic genes were downregulated >2-fold, and four were upregulated >2-fold (OTC, CYP3A4, CYP2B6 and SLCO1B1, p<0.05). Albumin production increased 1.3-fold between day 7 and 14, the urea production increased ~10-fold between day 3 and day 14, while lactate production converted into elimination. Glucose consumption, ammonia production and CYP3A4 activity did not change significantly between day 3 and 14, while enzyme leakage increased 8-fold.
In the following part of the study we compared HepaRG cultures with C3A cultures at their optimal maturation times, being 28 days and 7 days for the HepaRG and C3A monolayers respectively, and 14 days for both HepaRG and C3A BALs.

**HepaRG cells exhibit contact inhibition in monolayers, in contrast to C3A cells**

As previously reported, HepaRG monolayers followed a distinct proliferation- and differentiation pattern (1). After seeding, the cells proliferated for ~14 days, after which they differentiated into hepatocyte-like clusters surrounded by cholangiocyte-like cells during the following 2 weeks (Fig. 3B). HepaRG cells strictly grew in monolayers and did not proliferate during the last 2 weeks, as determined by total protein/culture well, indicating strong contact inhibition (Fig. 3C). C3A cells are reported to exhibit increased contact inhibition compared to their parental HepG2 cell line (Kelly, JH US Patent 5290684, 1990). In our hands, C3A monolayers cultured for seven days contained areas of cells in monolayer, cells overgrowing each other and non-confluent patches, while after 14 days the cultures were completely overgrown (Fig. 3B). The total protein per culture well continuously increased in time (Fig. 3C). These results indicate that, in monolayers, HepaRG cells exhibit contact inhibition and C3A cells do not.
## Table 1. Hepatic functionality and integrity of C3A monolayers and BALs in time

<table>
<thead>
<tr>
<th>Function</th>
<th>Unit</th>
<th>C3A monolayer</th>
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<tr>
<td></td>
<td></td>
<td>7</td>
<td>14</td>
</tr>
<tr>
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<td>days</td>
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NM=not measured
Figure 3. Time course of differentiation, morphology and proliferation. (A) Transcript levels of hepatic in time in C3A cells cultured in monolayer and BALs (B) Morphology of monolayer cultures at days 7 and 14 for C3A and days 7 and 28 for HepaRG. C3A cells form confluent monolayer patches (I), flanked by open spaces (II) and areas with overgrowing cells (III) at day 7. HepaRG cells form strict monolayers with islands of hepatocytes (I) surrounded by cholangiocyte-like cells (II) at day 28. (C) Total protein per culture well of C3A and HepaRG monolayer cultures in time.
HepaRG cells have lower AFP transcript levels and are more robust than C3A cells

To compare the differentiation status of C3A and HepaRG cells in monolayer and BAL cultures, we quantified the transcript levels of genes of immature liver, *i.e.* AFP and CYP3A7. AFP was highly expressed in C3A monolayers and BALs, ~400- to 800-fold higher than in HepaRG cells and ~5000-fold higher than in human liver (Fig. 4C). CYP3A7 expression did not differ significantly between cell lines or culture platforms, with expression levels between 54% and 154% of human liver (Fig. 4D). Gene transcript levels of the hepatic transcription factors HNF4A and CEBPA were within the range of 2-fold down to 4-fold up compared to human liver (Fig. 4E-F). The only significant difference for the transcript levels of these two genes between cell lines and culture platforms was a ~3-fold higher level for CEBPA in C3A compared to HepaRG monolayers (Fig. 4F).

Leakage of AST and LDH was measured as a marker for cell death in BAL cultures upon 24 h exposure to ammonia and lactate. C3A BALs leaked a significantly higher proportion of intracellular enzymes amounting to 13%-14% of total cellular content against ~10% per 24 hours for HepaRG BALs (Fig. 4A-B). In conclusion, AFP, but not CYP3A7 expression was significantly higher in C3A compared to HepaRG cultures, while the tested hepatic transcription factor expression was within the physiological range for all cultures. Therefore, HepaRG cells show a tendency to higher maturation compared to C3A cells.
Figure 4. Differentiation and integrity. Comparison of HepaRG and C3A cultures in monolayers and BALs for (A-B) enzyme leakage and (C-D) transcript levels of fetal hepatic genes and of (E-F) hepatic transcription factors. b=p≤0.05 compared to C3A cells in the same culture platform.
CHAPTER 2

BAL culturing improves nitrogen metabolism of both C3A and HepaRG cells

In monolayer cultures, C3A cells produced ammonia, in contrast to HepaRG cells that consistently eliminated ammonia (Fig. 5A). When cultured in BALs, ammonia elimination increased ~4-fold in HepaRG cells. BAL culturing reduced ammonia production ~17-fold in C3A cells, yet conversion into elimination was not established (Fig. 5A).

Next, we investigated the contribution of the urea cycle (UC)-activity to ammonia elimination. Urea production alone is not an adequate measure of UC-activity, since urea can also be formed by the degradation of arginine into ornithine and urea through (non-hepatic) Arginase 2 activity [15] (Fig. 5H). The production of $^{15}$N-urea after a challenge with $^{15}$N-ammonia is a measure of the contribution of UC-activity to urea production (Fig. 5H). Urea production in BALs compared to monolayers was ~3-fold higher for HepaRG cultures and not different in C3A cultures. In HepaRG cells compared to C3A cells, urea production was ~2 and 13-fold higher in monolayers and BALs respectively (Fig. 5B).

In line with a previous report, we found that $^{15}$N-urea enrichment in C3A monolayers was around the detection limit (Fig. 5C) [15]. However, BAL culturing induced a trend towards higher $^{15}$N-urea enrichment (~2-fold) and higher expression of urea cycle genes $CPS1$, $ARG1$ and $OTC$ (2- to 5-fold) (Fig. 5B-F). For HepaRG cells, BAL culturing increased the transcript levels of two of the three tested urea cycle genes ($OTC$ and $ARG1$, up to 4-fold) and the $^{15}$N-urea enrichment (6-fold) (Fig. 5C-F).

$^{15}$N-urea enrichment was 10- to 20-fold higher compared to C3A cultures and the transcript levels of the tested urea cycle genes were 5- to 77-fold higher (Fig. 5C-F). $ARG2$ expression was highest in C3A cultures and lowest in HepaRG BALs, explaining the high production of non- $^{15}$N enriched urea (Fig. 5G-H). Because, even in HepaRG BALs, the estimated urea cycle activity amounts to <10% of total ammonia elimination, we conclude that the urea cycle does not play a significant role in ammonia detoxification in either of the cell lines.
SELECTING CELLS FOR BIOARTIFICIAL LIVER DEVICES AND THE IMPORTANCE OF A 3D CULTURE ENVIRONMENT

Two pathways that lead to the production of urea:

1: **Arginase 1** in the cytosol, as part of the urea cycle detoxifies ammonia as determined by incorporation of 15N ammonia into urea.

2: **Arginase 2** in the mitochondria degrades arginine into ornithine and urea, this does not detoxify ammonia.

**Figure 5. Nitrogen metabolism.** Comparison of HepaRG and C3A cultures in monolayers and BALs for (A-C) functional parameters and (D-G) gene transcript levels. Diagram H illustrates the two pathways producing urea. 

a=p≤0.05 compared to same cell line in monolayer, b=p≤0.05 compared to C3A cells in the same culture platform.
Ammonia elimination is a function of glutamine metabolism in both C3A and HepaRG cells

The other main route through which ammonia can be eliminated next to the irreversible fixation into urea by UC-activity, is the reversible fixation into glutamine through glutamine synthetase (enzyme: GS, gene: GLUL) activity (Fig. 6G). Conversely, ammonia can be produced during the degradation of glutamine, mainly through the activity of Glutaminase I (GLS1; non-liver specific isoform) and II (GLS2; liver-specific isoform). The ammonia concentration in culture media is a product of both ammonia production and elimination. To gain an insight into these processes, we quantified ammonia, glutamine and glutamate, as well as GLS1, GLS2 and GLUL transcript levels at 24 hours after ammonia exposure in culture media (Fig. 6A-F). Ammonia elimination in HepaRG BALs was limited by substrate availability (data not shown) and reaction products are subject to further processing, therefore the results obtained should be considered as a qualitative rather than a quantitative representation. Net ammonia elimination was associated with net glutamine production and glutamate elimination in HepaRG cultures (Fig. 6A-C). In contrast, C3A cultures produced ammonia, consumed glutamine and produced glutamate (Fig. 6A-C). Glutamate production was associated with higher GLS1 transcript levels which were >3-fold higher in C3A cells compared to HepaRG cells, both in monolayers and in BALs (Fig. 6C-E). GLUL transcript levels varied less between both cell lines (Fig. 6F). BAL culturing of C3A cells shifted the glutamate metabolism towards elimination and glutamine metabolism towards production, in contrast to HepaRG cells (Fig. 6B-C). On transcript level, the differences between BAL and monolayer cultures were non-significant, except for a 2-fold upregulation of GLS2 expression in C3A BALs compared to monolayers. (Fig. 6D-E).
SELECTING CELLS FOR BIOARTIFICIAL LIVER DEVICES AND THE IMPORTANCE OF A 3D CULTURE ENVIRONMENT

Figure 6. Nitrogen balance. Comparison of HepaRG and C3A cultures in monolayers and BALs after 24 hours exposure to culture medium containing 1.5 mM of ammonia and 2 mM lactate for (A-C) ammonia, glutamine and glutamate balance, and for (D-F) transcript levels of GLS, GLS1 and GLUL. Diagram G illustrates the reversible conversion of glutamine into glutamate. a=p≤0.05 compared to same cell line in monolayer; b=p≤0.05 compared to C3A cells in the same culture platform.

BAL culturing shifts the metabolism of amino acids in C3A cells towards the HepaRG phenotype
To further investigate the amino acid metabolism, we quantified the resultant of production and consumption for the main amino acids, depicted these in a heat map and performed a cluster analysis (Fig. 7A). C3A monolayer cultures clustered separately from the three other groups. This indicates that BAL culturing induces a shift in amino acid metabolism of C3A cells towards the HepaRG phenotype. Several samples could not be analyzed reliably for all amino acids due to overlapping peaks; these data points were excluded.

Aromatic amino acids (AAA: phenylalanine, tryptophan, histidine, and tyrosine) and branched chain amino acids (BCAA: leucine, isoleucine and valine) are of interest, as an increased AAA/BCAA ratio has been proposed as a causal factor in the development of hepatic encephalopathy [16]. Both AAAs and BCAAs were consumed more in C3A monolayers compared to all other conditions. In HepaRG BALs, there was no net change of the measured AAAs and a consumption of BCAAs. In C3A BALs, consumption levels of the measured AAAs and BCAAs were lower and comparable to HepaRG BALs respectively (Fig. 7A, supplemental table S6).

BAL culturing converts lactate production into elimination in HepaRG cultures
Glucose consumption was 1.5-fold higher in C3A compared to HepaRG monolayers (Fig. 7B). In BAL cultures this difference between cell lines increased to 5-fold. HepaRG and C3A monolayer cultures both produced lactate at similar rates (Fig. 7C). When cultured in BALs, lactate production seized in
C3A cells and converted to elimination in HepaRG cells. This suggests that both C3A and HepaRG cells depended on anaerobic glycolysis in monolayers, which was ameliorated by BAL culturing, albeit more so in HepaRG than in C3A BAL cultures.

Changes in the metabolism of amino-acids, ammonia and lactate upon BAL culturing of C3A cells imply a shift in phenotype towards that of PHs, although not to the extent of BAL-cultured HepaRG cells.

Figure 7. Amino acid and carbohydrate metabolism. HepaRG and C3A cells, cultured in monolayers and BALs, were analyzed for metabolism of (A) amino acids, several amino acids could not be analyzed in a number of samples due to overlapping peaks, these data points were excluded from analysis and depicted as a black box in the heat map. (B) glucose consumption and (C) lactate. a=p≤0.05 compared to same cell line in monolayer, b=p≤0.05 compared to C3A cells in the same culture platform.
Protein synthesis is similar between culture platforms and cell lines

As a measure of hepatic protein synthesis we quantified albumin production in culture media and transcript levels of three genes encoding plasma proteins (\textit{ALB}, \textit{TF} and \textit{FVII}) (Fig. 8A-D). Albumin synthesis rate was not statistically different between HepaRG and C3A cultures, and there was no difference between culture platforms (Fig. 8A).

There was no consistent pattern in transcript levels of \textit{ALB}, \textit{TF} and \textit{FVII} between the four groups (Fig. 8B-D). The \textit{ALB} transcript level was higher in HepaRG BALs compared to C3A BALs, \textit{FVII} transcript levels were upregulated in C3A BALs compared to all other groups and transcript levels of \textit{TF} showed a trend towards upregulation in both monolayer groups compared to BAL groups. Combined, these results do not indicate a difference in protein synthesis between cell lines and/or culture platforms.

![Figure 8](image)

**Figure 8.** Protein synthesis in culture medium. Comparison of HepaRG and C3A cultures in monolayers and BALs for (A) albumin synthesis and (B-D) transcript levels of genes encoding proteins related to hepatic protein synthesis. a=p≤0.05 compared to same cell line in monolayer, b=p≤0.05 compared to C3A cells in the same culture platform.

Drug detoxification is enhanced in HepaRG vs C3A and in BALs vs monolayers

Transcript levels of \textit{CYP3A4} and \textit{CYP2B6} relative to human liver were highest in HepaRG BAL cultures (20%-75%) followed by HepaRG monolayers (≈8%), C3A BALs (≈0.02%) and C3A monolayers (under the detection limit) (Fig. 9 B-C). For genes encoding basolateral membrane transporters
NTCP and SLCO1B1 a similar trend was observed, with transcript levels ranging from undetectable in C3A monolayers to 13%-28% of human liver in HepaRG BALs (Fig. 9D-E). The transcript levels of two genes encoding regulators of xenobiotic detoxification, CAR and PXR, ranged from 1%-45% and 22%-87% of human liver, respectively (Fig. 9F-G). The only statistically significant difference between cell lines in the same culture platform or between culture platforms of the same cell line was a ~9-fold higher transcript level of CAR in HepaRG BALs vs C3A BALs (Fig. 9F). On a functional level, CYP3A4 activity, as determined by testosterone 6β-hydroxylation, was tested in BAL cultures only and was 6-fold higher in HepaRG BALs compared to C3A BALs (Fig. 9A).

Figure 9. Xenobiotic metabolism, Comparison of HepaRG and C3A cultures in monolayers and BALs for (A) CYP3A4 activity and transcript levels of genes encoding (B-C) enzymes related to xenobiotic metabolism (D-E) basolateral transporter proteins, and (F-G) nuclear hormone receptors. ND= not detectable. a=p≤0.05 compared to same cell line in monolayer, b=p≤0.05 compared to C3A cells in the same culture platform.
Discussion

We compared hepatic differentiation and functionality of the two main candidate cell lines to be applied in BALs: C3A and HepaRG. HepaRG BALs had the most favorable outcome on xenobiotic detoxification, nitrogen metabolism and lactate elimination, while protein synthesis was not different between cell lines. Therefore we conclude that HepaRG is currently the most promising cell line to be applied in BALs. Both cell lines differentiated markedly in AMC-BALs compared to monolayers, hallmarked by significant differences in hepatic gene expression levels and xenobiotic-, nitrogen-, carbohydrate-, and amino acid metabolism. This confirms that cells can only be properly assessed for their applicability as a BAL biocomponent when cultured in a BAL device.

When cultured in AMC-BALs, HepaRG cells eliminated ammonia and lactate at pathophysiological concentrations, whereas C3A BALs did not eliminate lactate and produced ammonia. Cyp3A4 activity and UC-activity were 6- and 110-fold higher in HepaRG BALs, while glucose consumption was 5-fold lower and albumin synthesis did not differ from C3A BALs. Out of the 16 tested mature hepatic genes, 6 were expressed equally, 2 were highest in C3A BALs and 8 were highest in HepaRG BALs.

HepaRG BALs consumed BCAAs, which might lead to an increase in AAA/BCAA ratio in patients plasma when applied clinically. This ratio has been proposed to be associated to HE in ALF, although this remains debated. [16, 17] A recent meta-analysis on the effects of BCAA supplementation on hepatic encephalopathy in chronic liver diseases showed a small beneficial effect on encephalopathy grade, but not survival or morbidity [18]. We propose that this is not a contraindication for the use of HepaRG cells in BALs, but that BCAA supplementation might be considered.

High expression of \( AFP \) against relatively low expression of \( ALB \) in C3A cells suggests that C3A cells are less differentiated on the foetal-mature hepatocyte axis compared to HepaRG cells. Interestingly, although \( CYP3A7 \) is considered a foetal hepatocyte marker, expression was not increased in C3A cells compared to HepaRG cells. We propose that since \( CYP3A7 \) is regulated similarly to mature CYP enzymes, the low expression level has to be considered in extension of the generally minimal expression of detoxification genes in C3A cells [19].

C3A cells have been studied in the context of BAL application by others in the past, although one should exercise caution in comparing outcomes due to differences in test-conditions, culture time, culture platforms and normalization methods [20-24]. One study reported conditions similar to this study, except for a difference in culture time. In this study, urea and albumin synthesis were 2 fold lower and 3-fold higher respectively, indicating external validity of the data in our current research [24].
C3A monolayers did not exhibit contact inhibition, in contrast to HepaRG monolayers. This gives rise to the concern that C3A cells are tumorigenic and may compromise safety. From studies in immunocompromised mice, HepaRG cells are known to have some remnant tumorigenicity when undifferentiated, but not when differentiated [25]. Data on tumorigenicity of C3A are not available to the best of our knowledge. The parental cell line of C3A, HepG2, is known to be highly tumorigenic [26]. Since HepaRG cells become strictly non-tumorigenic only when differentiated, it is conceivable that tumorigenicity in C3A cells decreases and contact inhibition increases with increased differentiation upon BAL-culture. Due to technical limitations, contact inhibition could only be assessed in monolayers and therefore we feel we cannot draw firm conclusions on the tumorigenicity of C3A cells in BAL cultures. To eliminate the risk of tumorigenicity in patients, plasma should be passed over multiple barriers before re-entering the body in order to prevent cells from entering the circulation [27].

Ammonia is believed to be an important agent in ALF disease progression and therefore ammonia detoxification is regarded as a key function of BALs [3, 5, 28, 29]. Our data indicate that HepaRG cells have a higher ammonia elimination capacity compared to C3A cells, and that ammonia balance in both cell lines is associated with the conversion of glutamate into glutamine and vice versa. These processes are driven by the balance between the enzymes GS and GLS I (non-liver specific isoform) or II (liver-specific isoform). The activity of these enzymes is linked to the metabolic state through regulation by nucleotides. For example, adenosine diphosphate (ADP) is a prototypical activator of glutaminases and capable of inhibiting GS [30, 31]. As lactate production correlated negatively with ammonia elimination in this study, we propose that the metabolic state drives the net ammonia eliminating capacity in hepatocyte cell lines that rely on GS activity for ammonia detoxification. If this is indeed the case, proliferation is likely to have a negative impact on ammonia elimination capacity due to its large demand for energy. As HepaRG cells showed contact inhibition, but C3A cells continued to proliferate, the observed difference in ammonia elimination capacity could be explained through this mechanism. A possible strategy to solve this issue would be to physically limit proliferation by space restriction, e.g. in hydrogel beads, which has indeed been shown to increase hepatic functions in C3A as well as in HepaRG cells [32, 33]. This may also limit differentiation time, which should be minimal to effectively control logistics and finance of clinical BAL therapy.

Another way to improve ammonia detoxification would be to increase UC-activity up to the level of PHs. In vivo, PHs exhibit a specific functional phenotype depending on their distance from the portal- and central vein [34]. The GS activity is restricted to the pericentral region, whereas UC-activity takes place in the intermediate and portal zones. Recently it was described how this metabolic zonation is driven by the Wnt signalling pathway: proliferative pericentral hepatocytes are high in b-catenin expression, maintained by Wnt factor secreting endothelial cells adjacent to the central vein. The hepatocytes lose GS expression and gain CPS expression as they migrate away from the central vein [35]. Wnt signalling poses an interesting target to induce UC-activity in hepatocyte cell lines for BAL application.
Conclusion

We conclude that culturing proliferative hepatocyte sources in BALs enhances hepatic functionality and that the cell line HepaRG is currently the most promising proliferative cell source for BAL application. In addition, we identified two strategies for functional improvement. First, UC-activity may be improved though manipulation of the Wnt signalling pathway. Second, the capacity for fixation of ammonia into amino acids may be improved by decreasing basal energy expenditure, e.g. by physically limiting proliferation.
REFERENCES


### Supplemental Material

**Table S1. Primer sequences**

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<td>GGCGCTAGGATGGGCTACAC</td>
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<tr>
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<td>CAGGTTGATCATCTGGCCC</td>
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<tr>
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<td>GGGAAAGGAAGAAAAGTGAG</td>
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<td>CATGTTGGGCGAGGAGAAG</td>
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<tr>
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</table>

Primer sequences used in the RT-PCR analyses with resulting amplicon sizes. Primers are indicated from 5’ to 3’. bp, base pairs; rRNA, ribosomal RNA; AFP, Alpha fetoprotein; ALB, Albumin; ARG1, arginase 1; ARG2, arginase 2; CEBPA, enhancer-binding protein alpha; CAR, constitutive androstane receptor; CPS1, carbamoyl phosphate synthetase; CYP2B6, cytochrome p450 2B6; CYP3A4, cytochrome p450 3A4; CYP3A7, cytochrome p450 3A7; FVII, factor seven; GLS, glutaminase; GLS2, glutaminase 2; GS, glutamine synthetase; HNF4A, hepatic nuclear factor alpha; NTCP, sodium taurine co-transporting peptide; SLCO1B1, Solute carrier organic anion transporter family member 1B1; OTC, ornithine transcarbamylase; PXR, pregnane x receptor; TF, transferrin.
Table S2. C3A hepatocyte functions and gene transcript levels in MEM+ and WE+ culture medium

<table>
<thead>
<tr>
<th></th>
<th>MEM+ mean</th>
<th>SD</th>
<th>WE+ mean</th>
<th>SD</th>
</tr>
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<tr>
<td><strong>Functions, normalized to MEM+</strong></td>
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<td></td>
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<tr>
<td>Total protein/well</td>
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<td>1.09</td>
<td>0.05</td>
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<tr>
<td>Ammonia production</td>
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<td>0.23</td>
<td>0.94</td>
<td>0.23</td>
</tr>
<tr>
<td>Urea production</td>
<td>1</td>
<td>0.17</td>
<td>0.05</td>
<td>0.09</td>
</tr>
<tr>
<td>Lactate production</td>
<td>1</td>
<td>0.10</td>
<td>0.88</td>
<td>0.29</td>
</tr>
<tr>
<td>15N urea enrichment</td>
<td>N.D</td>
<td>N.D</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Transcript levels, normalized to MEM+</strong></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>CPS1</td>
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<td>ALB</td>
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<td>0.96</td>
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<tr>
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N.D.= Not determined
### Table S3a. Gene transcript levels in C3A cells in BALs and in monolayers

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<th>C3A monolayer T=14d</th>
<th>C3A monolayer T=21d</th>
<th>C3A BALs T=3d</th>
<th>C3A BALs T=7d</th>
<th>C3A BALs T=14d</th>
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</thead>
<tbody>
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<td><strong>SD</strong></td>
<td><strong>mean</strong></td>
<td><strong>SD</strong></td>
<td><strong>mean</strong></td>
</tr>
<tr>
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<td>1.10</td>
<td>0.44</td>
<td>0.83</td>
<td>0.21</td>
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<td>0.42</td>
<td>0.18</td>
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<td>0.77</td>
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<td>2.15</td>
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<tr>
<td>NTCP</td>
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<td>0.32</td>
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<td>0.09</td>
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<td>0.12</td>
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</tr>
<tr>
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<td>C3A T=7 d</td>
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<td>HepaRG T=1 d</td>
<td>HepaRG T=4 d</td>
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<td>0.03</td>
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* = P < 0.05  ** = P < 0.01  *** = P < 0.001  **** = P < 0.0001

Table S3b. Total protein in monolayers
Table S4. HepaRG and C3A hepatocyte functions in monolayer and in BALs

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<tr>
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<th>C3A monolayer</th>
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<th>HepaRG BAL</th>
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<td>SD</td>
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<tr>
<td>% of total per 24 hours</td>
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<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
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<tr>
<td>AST leakage</td>
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<td></td>
</tr>
<tr>
<td>% of total per 24 hours</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
</tr>
<tr>
<td>Ammonia elimination</td>
<td>µmol × h-1 × g protein-1</td>
<td>-33.54</td>
<td>9.59</td>
<td>-3.26</td>
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<tr>
<td>Urea production</td>
<td>µmol × h-1 × g protein-1</td>
<td>1.42</td>
<td>0.34</td>
<td>0.79</td>
</tr>
<tr>
<td>15N urea enrichment</td>
<td>% of urea produced</td>
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<td>0.15</td>
<td>0.90</td>
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<td>Glutamine balance</td>
<td>mmol × g protein-1 × 24 hours -1</td>
<td>-75.54</td>
<td>5.47</td>
<td>-10.81</td>
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<td>Glutamate balance</td>
<td>mmol × g protein-1 × 24 hours -1</td>
<td>75.93</td>
<td>9.18</td>
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<td>Ammonia balance</td>
<td>mmol × h-1 × g protein-1</td>
<td>156.55</td>
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<td>Lactate production</td>
<td>µmol × h-1 × g protein-1</td>
<td>88.94</td>
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<td>Albumin synthesis</td>
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N.D. = Not determined
Table S5. HepaRG and C3A gene transcript levels in monolayer and in BALs

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<td>SD</td>
<td>MEAN</td>
<td>SD</td>
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<tr>
<td><strong>AFP</strong></td>
<td>% of human liver</td>
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<td>250756.80</td>
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<tr>
<td><strong>CYP3A7</strong></td>
<td>% of human liver</td>
<td>154.49</td>
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<td>99.54</td>
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<tr>
<td><strong>HNF4A</strong></td>
<td>% of human liver</td>
<td>310.94</td>
<td>86.84</td>
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<td><strong>CEBPA</strong></td>
<td>% of human liver</td>
<td>202.44</td>
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</tr>
<tr>
<td><strong>CPS1</strong></td>
<td>% of human liver</td>
<td>7.70</td>
<td>1.95</td>
<td>10.33</td>
</tr>
<tr>
<td><strong>ARG1</strong></td>
<td>% of human liver</td>
<td>0.16</td>
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<tr>
<td><strong>OTC</strong></td>
<td>% of human liver</td>
<td>0.18</td>
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</tr>
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<td><strong>ARG2</strong></td>
<td>% of human liver</td>
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<td>33.12</td>
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<td>% of human liver</td>
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N.D.= Not determined
Table S6. HepaRG and C3A amino acid metabolism rates in millimole h\(^{-1}\) mg protein\(^{-1}\)

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AMC-Bio-Artificial Liver Culturing Enhances Mitochondrial Biogenesis in Human Liver Cell Lines: the Role of Oxygen, Medium Perfusion and 3D Configuration

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Aziza A.A. Adam
Martien van Wenum
Vincent A. van der Mark
Aldo Jongejan
Perry D. Moerland
Riekelt H. Houtkooper
Ronald J.A Wanders
Ronald P. Oude Elferink
Robert A.F.M. Chamuleau
Ruurdtje Hoekstra
Abstract

Background
Human liver cell lines, like HepaRG and C3A, acquire higher functionality when cultured in the AMC-Bio-Artificial Liver (AMC-BAL). The three main differences between BAL and monolayer culture are the oxygenation (40% vs 20%O₂), dynamic vs absent medium perfusion and 3D vs 2D configuration. Here, we investigated the background of the differences between BAL-cultures and monolayers.

Methods
We performed whole-genome microarray analysis on HepaRG monolayer and BAL-cultures. Next, mitochondrial biogenesis was studied in monolayer and BAL-cultures of HepaRG and C3A. The driving forces for mitochondrial biogenesis by BAL-culturing were investigated in representative culture models differing in oxygenation level, medium flow or 2D vs 3D configuration.

Results
Gene-sets related to mitochondrial energy metabolism were most prominently up-regulated in HepaRG-BAL vs monolayer cultures. This was confirmed by a 2.4-fold higher mitochondrial abundance with increased expression of mitochondrial OxPhos complexes. Moreover, the transcript levels of mitochondria-encoded genes were up to 3.6-fold induced and mitochondrial membrane potential activity was 8.3-fold increased in BAL vs monolayers. Culturing with 40%O₂, dynamic medium flow and/or in 3D increased the mitochondrial abundance and expression of mitochondrial complexes vs standard monolayer culturing. The stimulatory effect of the BAL culture on mitochondrial biogenesis was confirmed in C3A cells in which mitochondrial abundance increased 2.2-fold with induction of mitochondria-encoded genes.

Conclusions and General Significance
The increased functionality of liver cell lines upon AMC-BAL culturing is associated with increased mitochondrial biogenesis. High oxygenation, medium perfusion and 3D configuration contribute to the up-regulation of the mitochondrial biogenesis.
Introduction

There is an unmet need for highly differentiated human hepatocytes from proliferative sources to serve as predictive in vitro hepatocyte models and as biocomponents for Bio-Artificial Livers (BALs) (1). BALs are bioreactors housing functional hepatocytes, developed to support patients with end-stage liver failure (2). Basic hepatic functions, including the elimination of ammonia and lactate and the detoxification of toxins, should be present in these cells at levels ideally comparable to those of mature human hepatocytes (MHHs). Currently, the human liver cell line HepaRG is the proliferative biocomponent of choice for many in vitro liver studies and also for the AMC-Bio-Artificial liver (AMC-BAL) (3, 4) (Fig 1A-B). HepaRG cells, which originate from a female hepatocellular carcinoma patient, closely mimic MHHs in various hepatic functions. HepaRG monolayer cultures differentiate during 28 days from a progenitor cell culture into a mixed culture with hepatocyte islands surrounded by bile duct-like cells (Fig 1C). The differentiation of the cells increases particularly during the differentiation phase (the last 2 weeks) preceded by 14 days of proliferation (3, 5). Interestingly, HepaRG cells cultured in the AMC-BAL reach maximal differentiation within 2 to 3 weeks when loaded with freshly isolated or cryopreserved cells, respectively (6). Moreover, the cell integrity and hepatic functionality are higher compared to HepaRG monolayers. For instance, cell leakage is 4-fold lower and ammonia elimination, urea cycle activity and cytochrome p450 (CYP) 3A4 activity are 3.2-, 1.4- and 7.9-fold higher, respectively (4, 7, 8). Moreover, HepaRG-BAL cultures eliminate lactate, whereas HepaRG monolayers produce lactate and consume more glucose (8). Notably, lactate elimination is a hallmark of highly differentiated hepatocytes, and is absent in currently available proliferative sources of hepatocytes (8). The stimulatory effect of BAL culturing on hepatic functionality was also confirmed in another hepatoma cell line, C3A, which is a sub-clone of the HepG2 hepatoma cell line (9). This cell line is used as biocomponent in the extracorporeal liver assist device (ELAD). By BAL culturing of C3A cells, the production of ammonia was reduced compared to monolayer cultures, and lactate production ceased, however urea cycle activity remained unchanged (8).

There are three major differences between culture conditions in AMC-BAL and regular monolayer which may inflict these changes in functionality. Firstly, the oxygen supply of 40%O2 in the AMC-BAL vs 20%O2 in monolayer. Secondly, the dynamic medium flow in the AMC-BAL vs static medium in the monolayer culture. Thirdly, the 3-dimensional (3D) cell configuration in the AMC-BAL vs 2-dimensional (2D) configuration for cells grown in monolayer.

In this study, we investigated the factors that might lead to the improved metabolic functioning of HepaRG cells, cultured in the AMC-BAL, with the goal to improve available in vitro models for human hepatocytes or potentially further improve the HepaRG-BAL culture. We compared the whole-genome expression profiles of the HepaRG monolayers (HepaRG-MONO) with HepaRG-BAL cultures. Expression profiles of the HepaRG-MONO and HepaRG-BAL groups were compared to two
reference sources: MHHs and primary human fetal liver cells (HFLCs). Gene-set enrichment analysis showed that the majority of gene-sets up-regulated in the HepaRG-BAL group were involved in energy metabolism and mitochondria. To assess which aspect of BAL culturing increased the mitochondrial biogenesis in HepaRG cells, we tested three culture conditions that deviated in one or two aspects from the standard monolayer culture conditions: 1) monolayer cultures under 40%O\textsubscript{2} supply, 2) monolayer cultures with dynamic medium flow and 3) 3D cultures with dynamic medium flow, in a system called BAL-In-A-Dish (BALIAD).

To evaluate whether the effect of BAL culturing on mitochondrial biogenesis was exclusive for HepaRG cells, we also tested mitochondrial biogenesis in the human liver cell line C3A in monolayer and BAL cultures.

**Figure 1:** The AMC-Bio-Artificial Liver and HepaRG cells. A) Laboratory model of the AMC-BAL. B) Schematic cross section of the AMC-BAL showing the spirally wound non-woven polyester matrix in which the HepaRG cells attach in a 3D configuration, positioned between oxygen capillaries for continuous gas (40%O\textsubscript{2}, 5%CO\textsubscript{2}, and 55%N\textsubscript{2}) supply. The cells inside the BAL receive continuous medium perfusion at a rate of 5 mL/min. C) HepaRG monolayer morphology, arrow A indicates the hepatocytes islands, arrow B indicates the bile duct-like cells.
Materials and methods

HepaRG and C3A monolayer culture
HepaRG cells were kindly provided by Biopredic International (Rennes, France). HepaRG cells were maintained in William’s E–based culture medium (HepaRG medium), as described (3, 5). One group of HepaRG monolayers was kept for 4 weeks in 20%O₂ under regular conditions, i.e. static in an humidized atmosphere of 95% air and 5% CO₂ (HepaRG-MONO). A second group was kept for the first 2 weeks under regular conditions and during the last 2 weeks under an atmosphere of 40%O₂, 5%CO₂ and 55%N₂ (HepaRG-40%O₂). A third group (monolayer with dynamic medium flow or HepaRG-DMF) was also cultured for the first 2 weeks under regular conditions, followed by 2 weeks shaking at 60 rpm under the same atmosphere. C3A cells (ATCC® CRL10741™) were cultured as described (8). For testing, C3A cells were seeded in 12-well plates and maintained in HepaRG medium for 2 weeks under regular culture conditions (C3A-MONO). All cultures were maintained at 37°C.

HepaRG and C3A AMC-BAL culture
A laboratory-scale version of the third generation AMC-BAL with an internal volume of 9 mL was used (Fig. 1). These AMC-BALS were loaded with 2 mL pellet of cryopreserved HepaRG or freshly isolated C3A cells suspended in 9 mL HepaRG medium, as described (10). The BAL cultures were incubated at 37°C and continuously oxygenated with a mixture of 40%O₂, 5%CO₂, 55%N₂. After a 3h-attachment phase, the AMC-BALs were continuously perfused at a rate of 5 mL/min with recirculating HepaRG culture medium. The medium was refreshed every 3 to 4 days (4). HepaRG-BAL and C3A-BAL cultures were analyzed after 21 and 14 days of culturing, respectively.

HepaRG-BALIAD culture
HepaRG cells were cultured on 6 mm (0.28 cm²) rounded discs DuPont™ Spunlaced Nonwoven Fabric- matrix, which is also used in the AMC-BAL, to provide a 3D configuration to the cells. To that goal, around 200.000 of HepaRG cells were seeded in matrices positioned in 96-well plates in 100 uL of HepaRG medium. After a 3h-attachment phase the matrices were moved to 1 mL HepaRG medium/well in 12-well plates and cultured under regular conditions for the first 2 weeks, followed by shaking at 60 rpm for the next 2 weeks in new plates. The medium was changed twice weekly.

HFLCs and MHHs isolation and culture
Human fetal livers were obtained from elective abortions. Gestational age was determined by ultrasonic measurement of the skull diameter and ranged from 14 to 18 weeks. The use of this tissue was approved by the Medical Ethical Committee of the Academic Medical Center, Amsterdam/ Netherlands, subjected to informed consent in compliance with the Helsinki Declaration. We isolated HFLCs on three independent occasions; in each case four fetal livers were pooled. Cells were isolated as described previously (11, 12). HFLCs were seeded in DMEM culture medium
(Dulbecco’s modified Eagle’s medium, BioWhittaker) containing 10% heat-inactivated fetal bovine serum (FBS, BioWhittaker), 2 mM L-glutamine (BioWhittaker), 1 μM dexamethasone (Sigma), 10 μg/mL insulin, 5.5 μg/mL transferrin, 6.7 ng/mL selenium-X (ITS mix, Life Technology), 100 U/mL penicillin, 100 μg/mL streptomycin (penicillin/streptomycin mix, BioWhittaker) at a density of approximately 3*10^5 cells/cm^2 in Primaria 6-well plates (BD Falcon). Cells were kept for 2 days at 37°C in a humidified atmosphere (95% air, 5% CO_2) before harvesting the total RNA. The purity of the isolation was confirmed by the measurement of transcript level of immature hepatic-markers including GSTт, AFP and CYP3A7, for more details refer to (11).

MHHs were isolated from tumor-free liver tissue of three patients undergoing partial hepatectomy, because of metastatic carcinoma. The procedure was approved by the Medical Ethical Committee of the Academic Medical Center subjected to informed patient consent. The hepatocyte isolation method was adapted from the protocol described by Seglen (13), as previously described (11). The MHHs were cultured in William’s E culture medium containing 4% heat-inactivated FBS, 2mM L-glutamine, 1uM dexamethasone, 20 mU/mL insulin (Novo Nordisk), 2mM ornithine (Sigma-Aldrich), 100U/mL penicillin, 100ug/mL streptomycin. Cells were kept for 2 days at 37°C in a humidified atmosphere (95% air, 5% CO_2) before harvesting the total RNA.

**Microarray analysis**

Total RNA was isolated from HepaRG monolayers cultured for 4 weeks (HepaRG-MONO), from freshly isolated HepaRG cells cultured for 2 weeks in the AMC-BAL (HepaRG-BAL), and from MHHs and HFLCs monolayers cultured for 2 days, according to the protocol of the RNeasy Mini Kit (Qiagen® RNeasy Mini Kit) from 3 independent cultures/group. Quality control, RNA labeling, hybridization, and data extraction were performed at ServiceXS (Leiden, the Netherlands). Biotinylated cRNA was prepared using the Illumina TotalPrep RNA Amplification Kit (Ambion, Inc.) according to the manufacturer’s specifications with an input of 200 ng total RNA and hybridized to Illumina HumanHT-12 v4 microarrays. Scanning was performed on the Illumina iScan. Image analysis and extraction of raw expression data were performed with Illumina GenomeStudio v2011.1 Gene Expression software with default settings (no background subtraction and no normalization). Analyses were performed with Bioconductor packages (version 2.12) using the statistical software package R (version 3.0.0). Raw data normalization was performed on the Illumina sample and control probe profiles by a normexp-by-control background correction, quantile normalization, and log2 transformation using the limma package (version 3.16.5). Based on quality control using the arrayQualityMetrics package (version 3.16.0) all arrays were deemed to be of sufficient quality. Probes with a detection P value > 0.05 (non-expressed) on all arrays (16,939 of 47,323 probes) were filtered out. Differential expression between the cell types was assessed with an empirical Bayes moderated t-test using the linear model framework from the limma package. Resulting P values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate (FDR).
Corrected $P$ values <0.05 were considered as statistically significant. Probes were reannotated using the IlluminaHumanv4.db package (version 1.18.0). Low-quality probes that according to the updated probe annotation match repeat sequences, intergenic or intronic regions, or are unlikely to provide specific signal for any transcript (6,626 of 30,384) were filtered out. Gene sets were retrieved from the Molecular Signatures Database (MSigDB) v5.2. We selected the Hallmark and C5 (Gene Ontology) collections and the liver-specific gene set HSIAO_LIVER_SPECIFIC_GENES (C2), for more details see Hsiao et al 2001 (14). Gene set enrichment analysis was performed using CAMERA (limma package) with preset value of 0.01 for the inter-gene correlation using the same linear model as above. In case multiple probes mapped to the same Entrez Gene ID according to the updated probe annotation, the probe with highest standard deviation of its expression values was chosen. $P$ values were calculated for each gene set for two alternative hypotheses (up or down). Hierarchical clustering of the whole-genome expression profiles and the profiles of the set of liver-specific genes was performed using Pearson correlation as distance measure and complete linkage as agglomeration method.

Quantitative reverse transcription PCR (RT-qPCR)

Total Quantitative RT-PCR was performed as previously described (7, 15). Transcript levels were normalized for 18S ribosomal RNA and expressed as a % of the average of two human liver samples. Primer sequences and amplicon sizes are given in Table 1.

Mitochondrial vs nuclear DNA ratio

The mitochondrial DNA (mtDNA) vs nuclear DNA (nucDNA) ratio was assessed as a measure of the mitochondrial abundance. Total DNA was isolated from AMC-BAL, BALIAD and monolayer cultures using the QIAamp DNA kits (QIAGEN® DNA Mini Kit) protocol.

Quantitative PCR was performed on 20 ng DNA/sample, using primers of 2 nuclear genes, i.e. CCAAT/enhancer binding protein alpha (CEBPa) and N-acetyl transferase (NAT), and 2 mitochondria-encoded genes, i.e. mitochondrial-NADH dehydrogenase subunit 1 (MT-ND1) and mitochondrial cytochrome c oxidase subunit 3 (MT-CO3). The input concentration of the different genes was calculated using the LinRegPCR program (16). Next, the ratio of the geometric mean of the concentration of mitochondrial genes to nuclear genes was calculated, then ratio data were transformed into log10 data to calculate the geometric mean of each group.

Mitochondrial inner membrane (MIM) complexes immune-blotting (WB)

Mitochondrial abundance was further determined by Western blot analysis of different mitochondrial proteins. Cell lysates of the different HepaRG cultures were obtained after incubation at 4°C in RIPA buffer (25 mM Tris (Bio-Rad), 150 mM NaCl (Merck), 0.1% SDS (Merck), 0.5% sodium deoxycholate (Sigma), 1% Triton X-100 (Bio-Rad), pH 7.4) containing 1% of protease inhibitor...
cocktail (Roche) for 30 min. The protein concentration of the samples was determined using the BCA protein assay kit (Pierce Biotechnology). Proteins were separated (40 µg of protein/sample) by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred by semi-dry electro-blotting on polyvinylidene difluoride membranes (Invitrogen). Membranes were blocked overnight at 4°C in TBST (150 mM NaCl, 50 mM Tris, 0.1% Tween 20 (Applichem), pH 7.5) with 5% milk powder (Nutrilon). Next day, membranes were incubated with the primary antibodies (1:2000 in 5% milk/TBST) for 4 h at 4°C. Primary antibodies used were mouse IgG antibodies directed against mitochondrial inner membrane (MIM) complexes, Complex II -Flavoprotein subunit of succinate dehydrogenase (ABCAM), MIM complex IV Cytochrome C oxidase subunit (ABCAM) and MIM complex V ATP synthase subunit 5A (ABCAM). Rabbit-anti-actin antibody (Sigma) was used to detect actin as loading control. Then the membranes were washed 3x with TBST and subsequently incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG antibody (Bio-rad) in 5% milk/TBST. The blots were incubated with a homemade enhanced chemiluminescence mix (100 mM Tris-HCl pH 8.5, 1.25 mM luminol (Fluka), 0.2 mM p-coumarin (Sigma) with freshly added 3 mM H₂O₂ (Merck) and proteins were visualized by using ImageQuant LAS 4000 (GE Healthcare Life Sciences). The expression level of the proteins quantified using ImageJ software (http://imagej.nih.gov/ij/).

**Mitochondrial membrane potential activity _JC-1 staining**

JC-1 staining was used to analyze the mitochondrial membrane potential. JC-1 a cationic dye, that exhibits potential-dependent accumulation in the mitochondrial matrix, indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm). Monolayer cultures, matrices of BAL–cultures and BALIADs were incubated with 0.5 mL of 4 µM JC-1 (Invitrogen) for 30 min at 37°C. Simultaneously, the cells were incubated with 1 µM verapamil (Sigma) to inhibit JC-1 efflux through activity of ATP binding cassette subfamily B member 1 (17). The JC-1 mitochondrial aggregates to monomer cytosolic form ratio was quantified by calculating red to green fluorescence (Fλ585/Fλ510), as measured by using a NOVOstar plate reader (BMG Labtech). The ratio data were transformed into log10 data to calculate the geometric mean of each group.

**Statistical analysis**

We performed Student’s t-tests for the comparison between two groups and one-way ANOVA test for comparison between >2 groups with Dunnett’s post hoc test comparing the mean of the control group (HepaRG-MONO) with the mean of each of the other groups, and Tukey’s post hoc test to compare the mean of each group with the mean of every other group, using Prism version 7 (GraphPad Prism Inc.). Significance was indicated by # = P value <0.05, ## = P value <0.01 and ### = P value <0.001. Data was represented as mean ± SD.
Results

The hepatic expression profile of the HepaRG cells is more similar to that of MHHs than HFLCs

The whole-transcriptome profiles of the HepaRG-MONO, HepaRG-BAL, MHHs and HFLCs groups were determined using Illumina HumanHT-12 v4 microarrays (n=3/group). Hierarchical clustering analysis of the transcriptome profiles revealed that the HepaRG-MONO and HepaRG-BAL groups were most similar to each other and less similar to the MHHs and HFLCs groups (Fig. 2A). Considering that hepatic functionality is of major interest for clinical BAL applications, we further investigated the expression levels of a previously described set of 346 probes targeting human liver-specific genes (HSIAO_LIVER_SPECIFIC-GENES) in the four groups. The expression profile of the liver-specific genes in the HepaRG cells was more similar to the MHHs than to the HFLCs. Whereas the majority of the liver-specific genes showed a significantly higher expression in HepaRG-MONO and HepaRG-BAL compared to HFLCs, yet, when relating to MHHs, the expression of liver-specific genes in the HepaRG-MONO and HepaRG-BAL groups was relatively low. Again, of the four groups, the HepaRG-MONO and HepaRG-BAL groups were most similar in their expression profile of the liver-specific genes (Fig. 2B).

Figure 2: Transcriptome analysis of HepaRG cells, cultured in monolayer and BAL, and of primary hepatocytes, cultured in monolayer. A) Hierarchical clustering based-dendrogram of the whole-genome expression profiles (n=3/group). B) Heatmap of hierarchical clustering on the expression profiles of a previously described set of 346 probes targeting human liver-specific genes (HSIAO_LIVER_SPECIFIC-GENES), (n=3/group). The key color bar indicates standardized gene expression levels.
AMC-BAL culture positively regulates the expression of genes involved in energy metabolism and mitochondria in HepaRG cells

To determine which factors could explain the differences in metabolic functioning between HepaRG-BAL and HepaRG-MONO cells, we first identified probes that were differentially expressed between the two groups. Only 175 out of 23,758 probes (~0.7%) were identified as differentially expressed (adjusted $P$ value <0.05) between HepaRG-BAL and HepaRG-MONO. Among the top 20 up-regulated genes (Table S2), in the HepaRG-BAL compared to HepaRG-MONO were liver-specific genes, including those encoding apolipoprotein A2 (ApoA2), which is the second most abundant protein of the high density lipoprotein particles, CYP3A4; a cytochrome p450 enzyme involved in the metabolism of approximately 50% of the drugs in use (18), and oxidative stress-induced growth inhibitor 1 (OSGIN1), which is stimulated by oxidized phospholipids to regulate cell response to oxidative stress (19). Recently, OSGIN1 was found to regulate mitochondrial structure and function through a direct interaction with P53 (20).

In addition, the gene encoding Dishevelled binding antagonist of beta catenin 3 (DACT3), an epigenetic and negative regulator of Wnt/beta-catenin signaling pathway, was induced under BAL culture. The Wnt/beta-catenin signaling pathway regulates stem cell pluripotency and cell fate decisions, and is associated with carcinogenicity (21-23). This signaling pathway also plays a central role in governing the hepatic zonation along liver sinusoids (24). The top 20 down-regulated genes contained several cancer-related genes, including H19, IGFBP5, MCF2, SPANXA1, SPANXE and SPANXD (Table S3).

Gene-set enrichment analysis was performed using CAMERA (25) to identify gene-sets that are highly ranked in terms of differential expression in HepaRG-BAL vs HepaRG-MONO relative to genes not in the set. In total 85 gene-sets with FDR< 0.05 were identified, among which 48 were up-regulated and 37 were down-regulated in HepaRG-BAL compared to HepaRG-MONO. The majority of the up-regulated gene-sets were directly related to energy metabolism and mitochondria, whereas, most of the down-regulated gene-sets were involved in cell-cycle regulation, (Fig. 3A-B).

A representative example of an up-regulated gene-set related to mitochondria is shown in (Fig. 3C) with a selection of up- and down-regulated genes highlighted. Up-regulated genes included NDUFB9 and UQRC1 which encode proteins related to OxPhos complex I and III, respectively, and CS, ACO2 and CPT1A which are involved in TCA cycle and beta-oxidation. In contrast, the down-regulated gene SLC25A14 (known also as UCP5) is a mitochondrial uncoupling protein that functions to separate OxPhos from ATP generation with the energy dissipated in form of heat. Also down-regulated is ABCA12 which is involved in the regulation of cellular cholesterol metabolism at posttranscriptional level (26). This clear shift towards up-regulation of genes involved in mitochondrial energy metabolism by BAL culturing prompted us to further investigate mitochondrial biogenesis in the context of the BAL culturing.
AMC-BAL culturing enhances mitochondrial biogenesis in the HepaRG cells, an effect driven by the 3D configuration, dynamic medium perfusion and higher oxygenation (40%O₂).

Mitochondria provide the cell with ATP through oxidative phosphorylation (OxPhos) activity (27), and therefore the abundance of mitochondria is finely tuned to meet cell-and state-specific energy needs (28). When compared to other subcellular organelles, the mitochondrion is unique in possessing a separate autonomously replicating genome and its own translation machinery (29).
We investigated the effect of BAL culturing on mitochondrial biogenesis. As outlined in the introduction, the three major differences between the AMC-BAL culture and the regular monolayer comprise the oxygenation (40% O₂ in the AMC-BAL vs 20% O₂ in monolayer), presence (AMC-BAL) or absence (monolayer) of medium perfusion and culture configuration (3D in the AMC-BAL vs 2D in monolayer).

To study the effect of the different factors, we applied different culture conditions to the differentiation phase. We tested the effect of oxygenation in HepaRG monolayers cultured under 40% O₂ (HepaRG-40%O₂) and the effect of dynamic medium flow on monolayers cultured under 60 rpm supplied with 20% O₂ (HepaRG-dynamic medium flow (HepaRG-DMF). To study the joint effect of 3D configuration and DMF on mitochondrial biogenesis, we developed the BAL-in-a-dish culture platform (HepaRG-BALIAD). The BALIAD system consists of 6 mm diameter discs of BAL matrix that are placed in cell culture plates under continuous shaking with 20% O₂ supply, a graphic representation of different culture conditions is shown in (Fig. 4A).

Parameters of mitochondrial biogenesis of these 3 different culture conditions and HepaRG-BAL cultures were compared to those of control static HepaRG monolayer cultures supplied with 20% O₂ (HepaRG-MONO).

The relative mitochondrial abundance was determined by measuring the ratio between mitochondrial DNA to nuclear DNA copies (mtDNA/NucDNA ratio). The relative mitochondrial abundance was significantly increased up to 2.4-fold in the HepaRG-BAL compared to the HepaRG-MONO group (Fig.4B). Culturing of the HepaRG cells with 40% O₂, DMF or BALIAD, positively affected the abundance of the mitochondria as indicated by a 2.1-, 3.3- and 5.1-fold increase, respectively. Noteworthy, mitochondrial abundance was not significantly different between HepaRG-BAL, HepaRG-40%O₂, HepaRG-DMF and HepaRG-BALIAD.

In line with this, the expression of the mitochondrial inner membrane (MIM) complex II was 2.4-, 1.9-2.3- and 2.1-fold increased in HepaRG-BAL, HepaRG-40%O₂, HepaRG-DMF and HepaRG-BALIAD, resp., while, MIM complex IV was 2.2- and 2.5-fold increased in HepaRG-BAL and HepaRG-DMF, compared to HepaRG-MONO cultures. The expression of MIM complex V was 2.3-, 1.9 and 2.3-fold induced in HepaRG-BAL, HepaRG-40%O₂ and HepaRG-DMF cultures. Noteworthy, no significant differences was observed in the level of MIM complexes when HepaRG-BAL was compared to HepaRG-40%O₂, HepaRG-DMF or HepaRG-BALIAD, (Fig. 4C-D).

Furthermore, the transcript levels of the mitochondria-encoded genes MT-ND5, MT-CYB and MT-ATP6 were 2.9-, 3.6- and 3.6-fold induced in HepaRG-BAL compared to HepaRG-MONO, reaching 93.1%, 142.6% and 142.1% of human liver transcript levels, respectively (Fig. 4E). On the other
Figure 4: Mitochondrial biogenesis in HepaRG cells cultured in monolayer and BAL; the role of higher oxygenation (40%O2), DMF and 3D configuration A) Graphic representation of different culturing conditions. B) Mitochondrial abundance measured by mtDNA/NucDNA ratio (n=4 for HepaRG-BAL and n=8-11/other groups). C) Mitochondrial abundance measured by the expression level of mitochondrial inner membrane complexes (MIM complexes) complex II, IV and V, as determined by immune-blotting (n=4-9/group).
Figure 4, continued. D) Quantification of the immunoblots using ImageJ software. E) Transcript level of mitochondria-encoded genes (n=6-8/group).
**AMC-BIO-ARTIFICIAL LIVER CULTURING ENHANCES MITOCHONDRIAL BIOGENESIS IN HUMAN LIVER CELL LINES**

**Figure 4, continued. F)** Quantification of the JC-1 staining, by calculating red (mitochondrial JC-1 fraction) to green (cytosolic JC-1 fraction) fluorescence ratio (n=3-12/group).

**G)** Imaging of mitochondrial JC-1 staining in HepaRG-MONO, HepaRG-40%O2, HepaRG-DMF and HepaRG-BALIAD from top to bottom respectively. The scale bar is 50 µm for the upper 3 panels and 100 µm for the lowest panel (BALIAD). To elucidate the 3D configuration, the arrow indicates a BALIAD matrix fiber to which the HepaRG cells attach.
hand, the transcript levels of *MT-ND5, MT-CYB* and *MT-ATP6* were only significantly increased in the HepaRG-DMF vs HepaRG-MONO cultures by 1.8-, 1.9- and 2-fold respectively and remained unchanged in the HepaRG-40%O₂ and HepaRG-BALIAD cultures.

Next, we assessed whether the increased mitochondrial biogenesis by BAL culturing was also associated with an increase in mitochondrial membrane-potential activity (MMPA), through JC-1 staining. MMPA was 8.3- and 3.2-fold increased in the HepaRG-BAL and HepaRG-BALIAD compared to the HepaRG-MONO group, while it remained comparable to HepaRG-MONO in HepaRG-DMF and HepaRG-40%O₂ groups (Fig. 4F). The JC-1 staining showed cluster patterns in the cultures (HepaRG-BAL cultures not included), which were not specifically confined to hepatocyte islands (Fig. 4G).

Taken together, these data provide evidence that BAL culturing increases mitochondrial abundance and MMPA in HepaRG cells. The 3D configuration, DMF and high oxygenation exert a positive effect on mitochondrial biogenesis as confirmed by both mtDNA/NucDNA ratio and western blot results, however only BALIAD cultures, which are closely mimicking the BAL platform by combining DMF with 3D configuration, exhibited increased MMPA as measured by JC-1 staining.

**Promotion of mitochondrial biogenesis by BAL culturing is a general phenomenon**

To assess whether the effect of the AMC-BAL culturing on mitochondrial biogenesis is a general effect of the BAL culture or HepaRG cells-dependent, mitochondrial biogenesis was also studied in the hepatoma cell line C3A. BAL-cultured C3A cells showed 2.2-fold increased mitochondrial abundance compared to C3A monolayers (Fig. 5A), which was a similar fold up-regulation compared to HepaRG cells. Yet, the mtDNA/nucDNA ratio was 1.7- and 1.8- fold higher in HepaRG-MONO and HepaRG-BAL compared to C3A-MONO and C3A-BAL, respectively.

**Figure 5: Mitochondrial biogenesis in human liver cell line C3A cultured in monolayer and BAL. A) Mitochondrial abundance measured by mtDNA/NucDNA ratio. B) Transcript level of mitochondria-encoded genes (n=3/group).**
Moreover, the transcript levels of mitochondria-encoded genes, specifically, *MT-ND1*, *MT-ND5*, *MT-CO3*, *MT-ATP6*, *MT-ATP8* and *MT-CYB*, were 3.8-, 2.6-, 4.0-, 3.6-, 4.1- and 3.2-fold induced in the C3A-BAL compared to the C3A-MONO group, reaching 92.5%, 57%, 94.9%, 72.4%, 90.8% and 59.1% of human liver transcript levels, respectively (Fig. 5B). Thus, the stimulatory effect of AMC-BAL culturing on mitochondrial biogenesis is a general phenomenon and not restricted to HepaRG cells.

**Discussion & Conclusions**

*In vitro* models of highly functional, and well-differentiated hepatocytes from proliferative sources are needed. Usability of *in vitro* cultured primary hepatocytes are limited by scarcity of the material and rapid dedifferentiation, while hepatocytes from proliferative sources still fail to show the full functionality spectrum (1). Therefore it is essential to improve the hepatic differentiation methods of proliferative hepatocyte sources. Previously, we found that AMC-BAL culturing improves the hepatic functionality of HepaRG cells compared to monolayers (7, 8). In the current study we found that BAL culturing additionally enhances the mitochondrial biogenesis in HepaRG cells. The contributing factors to this increased mitochondrial biogenesis are 3D configuration, DMF and high oxygenation (40%O2). All of the three investigated factors exerted a stimulatory effect on mitochondrial biogenesis and hence contributed, probably in a synergistic way, to the positive effect of the BAL culturing on mitochondrial biogenesis. In fact, these 3 factors jointly, closely model the liver *in vivo* where the hepatocytes receive continuous perfusion of highly oxygenated blood from the hepatic arterio-venous system with a mean oxygen saturation of 72.9% (30) and the cells are in a 3D intimate contact with other non-parenchymal liver cells. In addition, the stimulatory effect of the AMC-BAL culture on mitochondrial biogenesis was confirmed in another hepatoma cell line, C3A, indicating that it is a general effect of the BAL culture and not a HepaRG-dependent effect.

Our observation that BAL culturing of HepaRG cells increased mitochondrial biogenesis and activity is in line with our previous finding that HepaRG monolayer cultures produced lactate, while HepaRG-BAL cultures eliminated lactate and consumed glucose at a lower rate (7, 8), indicating a shift in energy metabolism from glycolysis towards OxPhos. In addition, lactate production by C3A cells in monolayer was completely abolished by BAL culturing (8). The hepatic functionality of the C3A cells is relatively low compared to HepaRG cells. Similarly, we found that HepaRG monolayers and BALs displayed a doubled mitochondrial abundance compared to C3A monolayers and BALs respectively. Therefore, we find a strong association between mitochondrial energy metabolism and hepatic differentiation of these two liver cell lines which may apply to hepatogenic differentiation in general.
Currently, the strong association between mitochondrial energy metabolism and cell differentiation receives much attention in stem cell studies. Undifferentiated stem cells retain pluripotency and unlimited proliferative capacity under conditions ideally mimicking the original relatively anaerobic stem-cell niche. In this state, energy metabolism relies on high glycolysis and mitochondria are immature (31, 32). Upon differentiation, mitochondrial remodeling takes place leading to a shift of energy metabolism towards OxPhos (31, 33, 34). Evidence is accumulating that this shift in energy metabolism is in fact a causal factor for the differentiation of stem cells. Promoting or inhibiting mitochondrial biogenesis or function severely impact the stemness (32, 35-38). The reprogramming of somatic cells into induced pluripotent cells and the in vitro dedifferentiation of primary hepatocytes, on the other hand, are coupled to inverse modifications of the mitochondrial system, in a process called “mitochondrial rejuvenation” (33, 36, 39-41). In a large-scale transcriptomic and proteomic study, Lauschke et al, observed that early changes associated with hepatic dedifferentiation related, in part, to inhibition of major metabolic pathways such as TCA cycle, β-oxidation and OxPhos (42). These findings collectively render the hypothesis that a shift to mitochondrial biogenesis and OxPhos is a causative factor in hepatocyte differentiation, very plausible.

In the past, high oxygenation, DMF, and 3D culturing have been applied to increase the differentiation of hepatocyte or liver cell line cultures. Our group showed that increasing oxygenation level by increasing the number of gas capillaries inside the BAL system as well as by increasing oxygen pressure from 135-150 mm Hg (20%O₂) to 235-250 mm Hg (40%O₂) both enhanced hepatic functions and transcript level of liver-specific genes in primary pig hepatocytes (43). In addition, rat hepatocyte spheroids maintained under different oxygen tensions exhibited increased urea and albumin production under 21%O₂ vs 6% O₂. However, the use of a high oxygen tension (95%O₂) without supplementation of antioxidants negatively affected the functionality of the spheroids, due to accumulation of reactive oxygen species (ROS) (44). Yet, another study reported that high oxygen (95%O₂) supply to primary rat hepatocytes co-cultured with either fibroblast (3T3-J2) or endothelial cells, positively affected their functions, including ammonia elimination, urea and albumin production and the detoxification capacity (45, 46).

DMF has also previously been recognized as an important factor, in addition to oxygenation, that stimulates hepatic differentiation. Rat hepatocytes co-cultured with 3T3-J2 fibroblasts under DMF supplemented with an internal oxygenation system showed elevated rates of albumin synthesis and urea production (47, 48). We previously found that the functionality of the HepaRG cells in the AMC-BAL system strongly depends on optimal medium perfusion at 5 mL/min perfusion rate. Lower perfusion rates (0.3 and 1.5 mL/min) decreased functionality, while a higher rate (10 mL/min) increased cell damage (49).
In addition, culturing in 3D configuration, most often utilizing spheroids, has been found to stimulate hepatic functionality in primary hepatocytes (50, 51) and in liver cell lines (52). A combination of 3D culturing and DMF positively affected the differentiation of the liver cell lines HepG2 and C3A, as assessed by morphology and albumin synthesis (53, 54). However, none of these studies related the enhanced hepatic differentiation to increased mitochondrial biogenesis. We provide for the first time evidence that these differentiation promoting culture conditions are tightly associated with mitochondrial biogenesis. In particular, the combination of DMF and 3D culturing, as established in the BALIAD culture set-up, proved to substantially increase mitochondrial biogenesis.

When comparing mitochondrial biogenesis parameters between different culturing platforms, we found a discrepancy between mitochondrial abundance (mtDNA and the expression of the OxPhos complexes) from one side and mitochondrial activity (MMPA) from the other side. While mitochondrial abundance increased in the HepaRG-BAL, HepaRG-40%O₂, HepaRG-DMF and HepaRG-BALIAD compared to HepaRG-MONO, only HepaRG-BAL and HepaRG-BALIAD exhibited an increased MMPA. MMPA is critical for maintaining the physiological function of the respiratory chain to generate ATP. A significant loss of MMPA renders cells depleted of energy and subsequently induces apoptosis (55). Explanations for this unexpected finding may be related to either increased ROS generation or mitochondrial immaturity under 40%O₂ and DMF cultures. Furthermore, the regulation of mitochondrial polarization might be different in 3D cultures (BAL and BALIAD) compared to 2D monolayers (HepaRG-MONO, HepaRG-40%O₂ and HepaRG-DMF). However, this requires further investigations.

Despite the substantial improvement in the hepatic functions of the HepaRG cells upon AMC-BAL culturing, the transcriptional profile of a set of liver-specific genes remained to some extent comparable to that of monolayer cultures, which suggests that post-translational modifications play an important role in the gain of functionality. We postulate that two major consequences of the shift in energy metabolism may affect the hepatic functionality, including a shift in the concentration of nutrient metabolites and ROS. Firstly, the change in energy metabolism yields a different spectrum of nutrient metabolites that could directly change flux rates of metabolic pathways. For instance, amino acids regulate many critical metabolic pathways, such as gluconeogenesis as well as the urea and TCA cycles (56). Previously we already showed that BAL-cultured HepaRG and C3A cells display an amino acids metabolome profile different from that of monolayer cultures (7, 8). Furthermore, accumulation of metabolites mainly detoxified through mitochondria, such as lactate and ammonia, may severely impact hepatocyte viability and functions (57). BAL-cultured HepaRG cells efficiently eliminate lactate and ammonia, whereas monolayer cultures produce lactate and eliminate ammonia to a lesser extent. Secondly, as a by-product of energy generation through OxPhos, ROS may play a role in post-transcriptional regulation of hepatic functionality by oxidation of lipids, proteins and RNA (58). Low ROS levels
are found in cells with reduced mitochondrial metabolism, as stem cells, to conserve the self-renewal capacity and to protect the genome of these rapidly dividing cells. On the other hand, increased levels of ROS favor cell differentiation (59, 60). The transcript level of OSGIN1 was induced in BAL-cultured HepaRG cells, reflecting the increased OxPhos activity, and subsequently increased ROS production (19). Recently, OSGIN1 was also identified as a tumor suppressor that is down-regulated or mutated in human hepatocellular carcinoma tissues (61). It will be, however, difficult to distinguish between the different players that may post-transcriptionally increase the hepatic functionality during BAL culturing, due to interconnections between the processes regulated by the concentration of energy metabolites and ROS.

Interestingly, the hepatic transcriptome of the HepaRG was more similar to MHHs than to HFLCs. The relatively high similarity of HepaRG cells with MHHs was previously also found in a global transcriptome study comparing HepaRG, MHHs and HepG2 cells (62). A recent study by Gao et al, compared the global gene expression profiles of human induced pluripotent stem cells subjected to hepatogenic-differentiation protocol (iPSC-Heps) to that of MHHs and several human hepatoma cell lines (HepaRG, HuH-7, HepG2, and HepG2/C3A). Among all other studied hepatoma cell lines and iPSC-Heps, the HepaRG cells were most similar to MHHs (63). Yet, when compared to the whole-transcriptome profile of MHHs, the profile of the HepaRG cells clearly deviates, indicating that there is still room for further optimization of HepaRG cells. New culture platforms that are capable to enhance mitochondrial metabolism and subsequently the functionality through 3D culture and medium perfusion, such as the BALIAD system, could provide in this need. Besides optimizing the culture platform, interfering with the mitochondrial system by over-expression of master regulators of mitochondrial biogenesis, such as Peroxisome proliferator-activated receptor gamma coactivator 1-alpha or addition of chemical compounds that stimulate mitochondrial biogenesis, e.g. S-nitrosoacetylpenicillamine or metformin may boost the differentiation process (64-66).

Taken together, the data presented in this paper demonstrate that AMC-BAL culturing enhances mitochondrial biogenesis in two different human liver cell lines. These findings establish an association between hepatic differentiation and mitochondrial biogenesis in the context of BAL culturing. Importantly, we identified three factors related to culture conditions that positively regulate mitochondrial biogenesis: oxygenation level, medium flow and 3D configuration of the culture. In particular the BALIAD culture set-up yielded improvement of mitochondrial biogenesis as well as MMPA. This culture set-up is easily applicable and scalable, and may therefore be a substantial improvement to the current monolayer set-up for obtaining higher levels of hepatic differentiation of human liver cell lines or stem cells.

Acknowledgements
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References


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48. Tilles AW, Baskaran H, Roy P, Yarmush ML, Toner M. Effects of oxygenation and flow on the viability


63. Gao X, Liu Y. A transcriptomic study suggesting human iPSC-derived hepatocytes potentially offer a


## Supplementary Material

### Table S1: Primers used in the qPCR and RT-qPCR and amplicon size

<table>
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<th>Gene</th>
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<td>18S rRNA</td>
<td>TTCGGAACTGAGCCCATGAT</td>
<td>CGAACCTCCGACTTTCTGTTCT</td>
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<td>CEBPa</td>
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<td>MT-ATP6</td>
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### Table S2: Top 20 up-regulated genes in HepaRG-BAL vs HepaRG-MONO

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<th>Adjusted P value</th>
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<tr>
<td>ILMN_1688543</td>
<td>APOA2</td>
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<td>ILMN_1801205</td>
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<td>ILMN_1668924</td>
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<td>ILMN_1658333</td>
<td>ECM1</td>
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<td>ILMN_1791647</td>
<td>ASIP</td>
<td>Agouti signaling protein</td>
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<td>ILMN_1816342</td>
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<td>ILMN_1662587</td>
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<td>ILMN_1767129</td>
<td>ABCC8</td>
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<td>ILMN_1737298</td>
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### Table S3: Top 20 down-regulated genes in HepaRG-BAL vs HepaRG-MONO

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<td>ILMN_2148527</td>
<td>H19</td>
<td>Imprinted maternally expressed transcript (non-protein coding)</td>
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<td>ILMN_2313672</td>
<td>IL1RL1</td>
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<td>ILMN_2132982</td>
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<td>ILMN_1682176</td>
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Chapter 4

Oxygen drives hepatocyte differentiation and phenotype stability in liver cell lines.

*These authors contributed equally
Abstract

Background & aims
The in vitro generation of terminally differentiated hepatocytes is an unmet need. We investigated the contribution of oxygen concentration to differentiation in human liver cell lines HepaRG and C3A.

Methods
HepaRG cells were cultured under hypoxia (5% O₂), normoxia (21% O₂) or hyperoxia (40% O₂). Cultures were analyzed for hepatic functions, gene transcript levels, and protein expression of albumin, hepatic transcription factor CEBPα, hepatic progenitor marker SOX9, and hypoxia inducible factor (HIF)1α. C3A cells were analyzed after exposure to normoxia or hyperoxia.

Results
In hyperoxic HepaRG cultures, urea cycle activity, bile acid synthesis, CytochromeP450 3A4 (CYP3A4) activity and ammonia elimination were 165-266% increased. These effects were reproduced in C3A cells. Whole transcriptome analysis of HepaRG cells revealed that 240 (of 23,223) probes were differentially expressed under hyperoxia, with an overrepresentation of genes involved in hepatic differentiation, metabolism and extracellular signaling. Under hypoxia, CYP3A4 activity and ammonia elimination were inhibited almost completely and 5/5 tested hepatic genes and 2/3 tested hepatic transcription factor genes were downregulated. Protein expression of SOX9 and HIF1α was strongly positive in hypoxic cultures, variable in normoxic cultures and predominantly negative in hyperoxic cultures. Conversely, albumin and CEBPα expression were highest in hyperoxic cultures. HepaRG cells that were serially passaged under hypoxia maintained their capacity to differentiate under normoxia, in contrast to cells passaged under normoxia.

Conclusions
Hyperoxia increases hepatocyte differentiation in HepaRG and C3A cells. In contrast, hypoxia maintains stem cell characteristics and inhibits hepatic differentiation of HepaRG cells, possibly through the activity of HIF1α.
Introduction

There is a need for terminally differentiated hepatocytes that can be maintained in vitro. Continuous efforts in unravelling the processes underlying hepatocyte differentiation, have led to an increased understanding of critical transcription factors [1, 2], signaling pathways [3, 4], mechanical forces and paracrine stimuli [4], and of ways to influence these in vitro through co-culturing [5, 6], culture platforms [7], small molecules [8] [4] and extracellular matrix constructs [9, 10]. However, terminal differentiation in vitro remains out of reach, leading to continuation of the search for contributing factors and strategies to improve differentiation grade.

Oxygen concentration is a known morphogen that can direct cell differentiation through factors such as which the hypoxia-inducible factors (HIFs) (reviewed in [11] and [12]). Little is known about the role of oxygen concentration in hepatocyte differentiation; there are limited data that suggest that atmospheric hypoxia may stimulate hepatic progenitor cell differentiation from embryonic stem cells (13). Data on the effects of atmospheric hyperoxia on cultured primary hepatocytes are contradicting, some reporting improvement [5, 14, 15] and other deterioration [16], of hepatic functions. This may be explained by differences in experimental set-up leading to a difference in oxygen flux at equal starting concentrations, as well as the use of primary hepatocytes, which display biological variability and enter a condition of stress and dedifferentiation after harvesting, leading to significant batch-to-batch variation [17].

HepaRG is a human hepatic progenitor cell line that expresses most progenitor markers and has the capability to reproducibly differentiate into highly functional hepatocyte-like cells [7, 18]. These cells acquire a proliferative progenitor phenotype when plated subconfluently, and, after reaching confluence, differentiate into islets of hepatocyte-like cells, surrounded by cholangiocyte-like cells (19). The phenotype of HepaRG cells remains stable for ~20 passages, after which they lose their ability to differentiate [20]. HepaRG cells represent primary hepatocytes [21] to high extent and were therefore selected to study the effects of oxygen on hepatocyte differentiation.

In this study we show that ambient hyperoxia drives HepaRG hepatocyte differentiation, and suggest this might be a general finding for human hepatocyte cell lines by showing the same phenomenon with the human liver cell line C3A. We also show that hypoxia maintains HepaRG cells in a progenitor state and increases their stability.
CHAPTER 4

Material and methods

Cells and culture procedure

Primary human hepatocytes (PHHs) were isolated from the healthy parenchyma in liver resection material from three patients, aged 40, 68 and 70, with liver adenomas or colorectal cancer metastases and no macroscopic signs of liver damage, by a modified 2-step collagenase perfusion technique as described [22]. Cells were snap-frozen directly after isolation and kept in liquid nitrogen until RNA isolation. The procedure was in accordance with the ethical standards of the institutional committee on human experimentation (protocol number 03/024) and the Helsinki Declaration of 1975. Ethical approval was obtained from the ethics committee of the Academic Medical Center Amsterdam, and informed consent was obtained from all three patients. HepaRG cells (Biopredic) were maintained under normoxia at 37°C as described previously [20]. For experiments, cells were plated 1:5 in 6-well plates (for immunofluorescence) or 12-well plates (other experiments) (Corning) and cultured without dimethylsulfoxide. Three gas compositions were applied to the cells: 5% O$_2$ (=hypoxic; 5% O$_2$, 5%CO$_2$ and 90%N$_2$), 21% O$_2$ (=normoxic; 5%CO$_2$, 21% O$_2$, 75%N$_2$) and 40% O$_2$ (=hyperoxic; 40% O$_2$, 5%CO$_2$, 55%N$_2$) (Linde Gas) in gastight incubator chambers at 37°C. For hypoxic and normoxic culturing, the HepaRG cells were immediately after seeding exposed to these gas compositions and cultured for 4 weeks; for hyperoxic culturing the cells were cultured under normoxic conditions during the first 2 weeks and then transferred to the hyperoxic conditions for the following 2 weeks. After four weeks of culturing under the different gas regimes, RNA was harvested and function tests were performed (2 independent experiments, n=3 per experiment). To test the stability of the cells at serial passaging, the cultures were split at passage 17 from isolation, and transferred to the normoxic and hypoxic incubator. Cultures were passaged at a regular 1:5 ratio once per two weeks, and for every 2 passage (passage 19, 21 and 23) cells were seeded in 12-well culture plates, cultured under normoxia for 4 weeks and tested for functionality and transcript levels (3 independent experiments, n=3 per experiment).

C3A cells (ATCC, CRL10741) were maintained as described [23]. For experiments, cells were plated 1:10 in 12-well plates (Corning) and kept under normoxia for 7 days until testing (normoxic cultures) or transferred to the hyperoxic incubator after 24 hours (hyperoxic cultures) until testing at day 7 (2 independent experiments, n=3 per experiment).

Function tests

The elimination and/or production of ammonia, lactate, glucose and total bile acids, as well as urea cycle activity were tested as described [24]. Briefly, cultures were exposed to HepaRG culture medium supplemented with 1 mM N-carbamoyl-l-glutamate, 1.5 mM $^{15}$NH$_4$Cl, 2.27 mM D-galactose, 2 mM L-lactate and 125 μM testosterone (all compounds from Sigma Aldrich), and samples were taken after 0.75, 8 and 24 hours and analysed for concentrations of ammonia, lactate, glucose, $^{15}$N urea and total bile acids. Next, the accumulation or disappearance rates could be calculated. The
accumulation of $^{15}$N-urea was used as a measure for urea cycle activity. Cytochrome P450 (CYP)3A4 activity was quantified with CYP3A4 P450-Glo™ Assays (Promega) according to the manufacturer’s instructions. For optional CYP3A4 induction, the cultures were pre-exposed to 4 µM rifampicin (Sigma Aldrich) for 3 days, and subsequently washed with fresh culture medium before testing.

After testing, the cells were lysed in 1 mL 0.2 M NaOH and total protein content per well was determined using the Bio-Rad Protein Assay (Bio-Rad) for normalization of the functionality data.

### Oxygen measurement
HepaRG cells were seeded in 24-well culture plates (OxoDish®) with oxygen sensor spots at the bottom of the well and subjected to the different gas regimes, as described above. Oxygen concentration at the bottom of the wells was measured real-time and non-invasively through the transparent bottom of the OxoDish® plates after 2 hours equilibration, with and without 4 week old cultures, inside the incubators with different oxygen compositions, using the SDR SensorDish® Reader, which was kindly made available by Applikon Biotechnology (n=24/experiment).

### RNA extraction, qRT-PCR and microarray analysis
Cells were lysed in 600 ml RLT buffer (RNaseq; QIAGEN). RNA was extracted according to manufacturer’s instruction. RT-PCRs were performed using gene-specific RT-primers and a touch-down qPCR protocol as described previously [25]. Primers and template dilutions are listed in table S1. For microarray analysis, cRNA, obtained from freshly isolated PHHs and from HepaRG cells cultured under normoxia or hyperoxia (n=3/group) was labelled (cRNA labelling kit for Illumina, Ambion), and hybridized after sample randomization to Illumina HumanHT-12 v4 arrays according to manufacturer’s instructions. Image analysis and extraction of raw expression data was performed with Illumina GenomeStudio v2011.1 Gene Expression software with default settings (no background subtraction and no normalization).

Microarray data were analyzed with Bioconductor packages (v2.12) using the statistical software environment R (v3.0.0). Raw data normalization was performed on the Illumina sample and control probe profiles by normexp-by-control background correction, quantile normalization, and log2 transformation using the limma package (version 3.16.8). The arrayQualityMetrics package (version 3.16.0) was used to assess the quality of the microarray data. Probes with a detection $P$-value > 0.05 (non-expressed) on all arrays (16,863 of 47,231 probes) were filtered out. Differential expression was assessed using a moderated t-test using the linear model framework from the limma package. Resulting $P$-values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate. Probes were reannotated using the IlluminaHumanv4.db package (version 1.18.0). Upstream regulator analysis was performed using the web-based Ingenuity Pathway Analysis package (QIAGEN). Statistical significance of the overlap between
the list of genes from our dataset (non-adj. \( P < 0.01 \) between normoxia and hyperoxia) and target genes in transcription regulator datasets was calculated using the Fisher’s Exact test. The minimum number of overlapping genes was set to 5.

**Western Blotting**

Cells were lysed in ice-cold nuclear extraction buffer (420 mM NaCl, 20% (w/v) glycerol, 5 mM MgCl\(_2\), 5 mM EGTA, 0.5% Nonidet-P40, 20 mM Tris-HCl, pH 8.0) freshly supplemented with complete EDTA-free protease inhibitor cocktail (Roche), and 1mM dithiothreitol. Next, the samples were centrifuged at 14,000g for 10 minutes at 4°C. The supernatant was harvested for SDS-PAGE. Sixty micrograms of protein per sample were electrophoresed on an 8% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane (Invitrogen) by semi-dry blotting and blocked overnight in 5% non-fat milk / PBST (phosphate-buffered saline with 0.05% (w/v) Tween 20). For immunodetection, the PVDF membrane was incubated with a rabbit polyclonal antibody against HIF1α (Abcam, ab2185) diluted 1:1000 for 1 hour at room temperature, washed 3x with TBST (Tris-buffered saline with 0.05% (w/v) Tween 20), incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Bio-rad) for 1 hour and washed 3x with TBST. All antibodies were diluted in 5% non-fat milk / PBST. The PVDF membrane was developed with home-made enhanced chemiluminescence reagents (100 mM Tris-HCl pH 8.5, 1.25 mM luminol, 0.2 mM p-coumarin and freshly added 3 mM H\(_2\)O\(_2\)) and detected by ImageQuant LAS 4000 (GE Healthcare Life Sciences). For loading control, the PVDF membrane was stripped and reprobed with horseradish peroxidase-conjugated monoclonal rabbit anti-vinculin antibody (Cell Signaling #18799).

**Immunofluorescent staining**

HepaRG monolayers were fixed with 2% formalin (VWR) for 2-5 minutes at room temperature prior to permeabilization with 0.3% Triton X-100 (Bio-Rad) in ice-cold PBS for 20 minutes and blocked for 1 hour with 10% fetal calf serum in PBS on ice. The cells were incubated with 1:200 diluted primary antibodies in PBS overnight at 4°C followed by 3x wash with ice-cold PBS, and then incubated with 1:1000 diluted secondary (fluorescent) antibody in PBS for two hours at 4°C. Finally, the monolayers were washed 3x with ice-cold PBS and mounted with DAPI-containing Vectashield (Vector Laboratories). Imaging was performed on a Leika DCF450 microscope. Primary antibodies were: Goat anti-human albumin (Bethyl Laboratories), Rabbit anti-human SRY-box 9 (SOX9) (Millipore), Rabbit anti-human HIF1α (Abcam), Goat anti-human CCAAT/enhancer-binding protein alpha (CEBPα) (Santa-Cruz). Fluorescent secondary antibodies: Donkey anti-Rabbit, Alexa Fluor-546 (Invitrogen), Donkey anti-Goat, Alexa Fluor-448 (Molecular Probes), Donkey anti-Goat, Alexa Fluor-546 (Invitrogen). Negative controls were performed under the same experimental conditions, without primary antibodies, and were imaged at the same setting (Fig. S1).
Statistical analyses
Values are given as mean ± standard deviation. Data were analysed and displayed graphically using Prism 7.02 (GraphPad). Student’s t-tests, corrected for multiple testing using the Holm-Sidak method were used when comparing two groups. Two-way ANOVA analysis with Dunnett’s multiple comparison procedure was used when comparing more than two groups. Adjusted P-values < 0.05 were considered statistically significant.

Results

HepaRG cells do not acquire hepatocyte morphology under hypoxic conditions and develop more discrete hepatocyte clusters under hyperoxic conditions
HepaRG cells were cultured under Normoxia (21% ambient O₂), Hypoxia (5% ambient O₂) or Hyperoxia (40% ambient O₂). The actual oxygen concentration at the bottom of the culture wells was determined in the absence and presence of 4-week-old cultures. The concentrations were 82±1.8, 181±2.4 and 339±3.9 µM O₂ without cells and 52±19.1, 93±10.4 and 275±26.7 µM O₂ with cells (Fig. 1A). Total protein analysis revealed no significant difference in proliferation or viability between the cultures (Fig. 1B).

Figure 1. Ambient oxygen concentration influences HepaRG cell morphology but not growth. (A) Pericellular oxygen concentration in culture medium with- and without 4-week old HepaRG cultures and (B) total protein content of 4-week old cultures. There was a marked difference in morphology of HepaRG cells cultured under: (C) normoxia, (D) hypoxia or (E) hyperoxia. Scale bar= 100 µM
After four weeks in culture, normoxic cultures differentiated into patches of polygonal hepatocytes, surrounded by flat cholangiocyte-like cells, as described (Gripon et al. 2002), (Fig. 1C), while hypoxic cultures consisted of stretched cells without hepatocyte islands (Fig. 1D). When cultures were exposed to ambient hyperoxia from day 1 viability was lost, and therefore cultures in the hyperoxic group were subjected to hyperoxia only after reaching confluence at day 14. After 28 days, the polygonal hepatocyte clusters were more clearly delineated compared to the normoxic cultures (Fig 1E).

Protein expression of stem cell marker SOX9 and hepatic transcription factor CEBPα was assessed by immunocytochemistry. A staining was performed for albumin to visualize hepatocyte-like cells (Fig 2A). Hypoxic cultures were SOX9-positive in most nuclei and partly albumin-positive, while normoxic cultures developed clusters of albumin-positive cells that were partially nuclear SOX9-positive. Hyperoxic cultures formed larger clusters of albumin-positive cells that were in majority SOX9-negative. In contrast, nuclei of hyperoxic cultures were CEBPα-positive, while hypoxic and normoxic cultures were less positive (Fig 2B).

On the basis of these observations we hypothesized that oxygen is an important factor in determining the differentiation state of HepaRG cells; hypoxia promotes stem cell characteristics, whereas hyperoxia induces hepatic differentiation.
Figure 2. Nuclear SOX9 expression is downregulated, while CEBPa is upregulated at increased oxygen levels. (A) HepaRG cells cultured under normoxia (top), hypoxia (middle) and hyperoxia (bottom), stained for: DAPI (blue), Albumin (green) and SOX9 (red). Arrows A&B indicate nuclear translocation of SOX9 in HepaRG-Hypoxia, and to a lesser degree in HepaRG-Normoxia. Arrow C indicates mainly cytosolic SOX9 expression in HepaRG-Hyperoxia, with SOX9-negative nuclei. (B) HepaRG cells cultured under normoxia (top), hypoxia (middle) and hyperoxia (bottom), stained for: DAPI (blue) and CEBPa (red). Arrow A indicates positive nuclear staining. Scale bar = 50 µM

**Hyperoxia augments hepatocyte functionality in HepaRG and C3A cells**

To confirm that hyperoxia increases hepatic differentiation, we analyzed hepatic functions and hepatocyte-specific gene transcript levels of HepaRG monolayers cultured under normoxia or hyperoxia. Hyperoxic HepaRG cultures exhibited significantly higher levels of urea cycle activity (266±118%), bile acid synthesis (230±93%), CYP3A4 activity (174±20%) and ammonia elimination (156±34%) compared to normoxic cultures (Fig 3A), while lactate and glucose metabolism did not differ significantly (Fig. 3B).

Under hyperoxia, transcript levels of the hepatic genes CYP3A4 and Arginase1 (ARG1) were significantly higher (433±457% and 305±203% respectively), while transcript levels of Carbamoyl-phosphate synthase (CPS1), Transferrin (TF), CYP2B6 and Glutamine synthase
(GS) were unchanged (Fig. 3C). Transcript levels of hepatic transcription factors Hepatic nuclear factor 4α (HNF4A), Pregnane X receptor (PXR) and CEBPA were significantly induced at 119±25%, 155±50% and 242±90% respectively, whereas Constitutive androstane receptor (CAR) transcript levels were unchanged (Fig. 3D).

To exclude that the effects of ambient hyperoxia are cell-line specific, we repeated the experiments for the hepatoblastoma cell line C3A [26]. There was no evident effect on morphology (not shown), however, urea cycle and CYP3A4 activity were induced up to 298±115% and 1008±464%, respectively, compared to normoxic cultures (Fig 3E). In contrast to HepaRG, C3A cells produce rather than eliminate ammonia [23]. Under hyperoxia, ammonia production was reduced to 19±13% and lactate production to 48±14%. CYP3A4 transcript levels were significantly reduced (to 44±19%). The transcript levels of ARG1, TF and GS did not differ significantly and HNF4A transcript levels were increased (236±32%) (Fig. 3F).

These results confirm that hyperoxia augments hepatic differentiation in hepatic cell lines in general.

**Hyperoxia induces upregulation of transcription factors involved in hepatocyte differentiation**

To analyse the transcriptional activity underlying the increased hepatic functionality under hyperoxia, a whole-genome transcriptome analysis was performed on freshly isolated PHHs and HepaRG cells cultured under normoxia or hyperoxia. Of the 23,223 probes, 240 were differentially expressed in hyperoxic vs normoxic HepaRG cultures (adj. P<0.05), 66 were upregulated and 174 were downregulated (Fig. 4). Of these 66 upregulated genes, 54 were also upregulated in PHHs vs normoxic HepaRG. The top-10 upregulated genes in hyperoxic vs normoxic HepaRG cells were involved in haemostasis (4/10), amino acid metabolism (2/10), signal transduction (2/10), detoxification (1/10) and carbohydrate metabolism (1/10) (Table 1). The top-10 downregulated genes were involved in extracellular matrix (ECM) and anchorage (4/10), innate immune-response (2/10) and transmembrane transport (2/10), as well as carbohydrate metabolism (1/10) and detoxification (1/10), the latter being CYP4B1, which is expressed in lung- rather than in liver-tissue, and is presumed inactive in humans [27] (Table 2).
Figure 3. Ambient hyperoxia augments hepatic differentiation of HepaRG and C3A cells. HepaRG monolayers cultured under ambient normoxia or hyperoxia were tested for hepatic functions (A), glucose consumption and lactate production (B), as well as transcript levels of hepatic genes (C) and transcription factor genes (D). C3A monolayers cultured under normoxia and hyperoxia were tested for hepatic functions (E) and hepatic gene transcript levels (F). *\(P<0.05\) compared to HepaRG normoxia.

Genes that were differentially expressed between normoxic and hyperoxic cultures were cross-referenced against the Gene Ontology transcription factor gene set [28], and seven transcription factors were identified (Table 3). Hepatic transcription factors One cut homeobox 2 (ONECUT2), Forkhead box A3 (FOXA3), and CEBPG were upregulated in hyperoxic vs normoxic cultures. The
downregulated transcription factors were not known to be involved in hepatic differentiation and included Orphan nuclear receptor estrogen related receptor gamma (ESRRG), TSC22 domain family member 4 (TSC22D4), Heat-shock factor 4 (HSF4) and Ankyrin Repeat Domain 30A (ANKRD30A). To further explore possible regulators of transcriptional changes, a gene signature composed of genes that were differentially expressed between normoxic HepaRG cultures and both freshly isolated PHHs and hyperoxic HepaRG cultures (non-adjusted $P<0.01$) was analysed using Ingenuity Pathway Analysis upstream regulator analysis. Two transcription regulator target gene sets were found to overlap significantly with this gene signature: Hepatic nuclear factor 1α ($HNF1A$) was predicted to be activated ($P=1.14\cdot10^{-12}$), whereas V-myc avian myelocytomatosis viral oncogene homolog ($MYC$) was predicted to be inhibited ($P=4.45\cdot10^{-3}$) in PHH and in HepaRG under hyperoxia compared to HepaRG under normoxia. $HNF1\alpha$ is an established hepatic transcription factor, involved in -amongst others- bile, cholesterol, and glucose metabolism [29], whereas MYC is an important proto-oncogene involved in immortalization and proliferation and associated with dedifferentiation [30].

These data suggest that hyperoxia induces upregulation of genes that are predominantly involved in hepatic differentiation, metabolism and extracellular signaling.

**Figure 4.** Whole-transcriptome microarray analysis on HepaRG cells cultured under normoxia or hyperoxia and primary human hepatocytes. Venn diagram of differentially expressed genes (adj. $P<0.05$) between freshly isolated PHHs and HepaRG cells cultured under ambient normoxia or hyperoxia.
Table 1. Top-10 upregulated genes in hyperoxic versus normoxic HepaRG cultures

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<th>Gene</th>
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<th>Adj. P-value</th>
<th>Protein</th>
<th>Function</th>
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<td>ASNS</td>
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<td>F9</td>
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<td>TRIB3</td>
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<td>3.37·10⁻⁴</td>
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<td>Signal transduction</td>
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<td>PPP1R1A</td>
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Table 2. Top-10 downregulated genes in hyperoxic versus normoxic HepaRG cultures.

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<td>SLC10A1</td>
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<td>SPINT3</td>
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<td>REG3G</td>
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<td>2.04·10⁻³</td>
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Table 3. Differentially expressed genes encoding transcription factors in hyperoxic versus normoxic cultures.

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<td>ONECUT2</td>
<td>2.16</td>
<td>1.03·10⁻³</td>
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Hypoxia blocks hepatocyte differentiation of HepaRG cells

Hypoxia markedly reduced hepatic functions of HepaRG cells: ammonia elimination converted into minor production, basal CYP3A4 activity was under the detection limit, and after rifampicin...
induction CYP3A4 activity was 0.6% of non-induced normoxic cultures (Fig 5A). Lactate production increased up to 251%, while glucose consumption did not change significantly (Fig 5B). Hypoxia reduced the transcript levels of all tested structural hepatic genes to 3% - 35% of the normoxic group (Fig. 5C). Transcript levels of hepatic transcription factors HNF-4A and PXR were downregulated to 22% - 24% of normoxic cultures, while CEBPA transcript levels did not change significantly (Fig. 5D). These data confirm the opposite effects of hyperoxia and hypoxia on hepatic differentiation of HepaRG cells.

Figure 5. HepaRG cells cultured under hypoxia lose hepatic gene transcription and functionality. HepaRG monolayers cultured under ambient normoxia or hypoxia were tested for hepatic functions (A), glucose consumption and lactate production (B), as well as transcript levels of hepatic genes (C) and transcription factor genes (D). *=P<0.05 compared to HepaRG normoxia.

**Hypoxia-induced effects correlate with HIF1α protein expression**

HIF proteins are known to be important in the cellular adaptive response to oxygen. Under hypoxia, ubiquination of cytosolic HIF1α is inhibited and the protein can translocate into the nucleus and activate the transcription of hypoxia-responsive proteins [31]. When oxygen is abundant, HIF1α is prevented to translocate to the nucleus by proteasomal degradation. Because SOX9 is a known target of HIF1α, [32], we hypothesized that SOX9 expression would correlate with HIF1α expression. Immunostaining revealed a clear nuclear translocation of HIF1α protein in hypoxic HepaRG cultures, while HIF1α was
mainly cytosolic (and thus not able to activate transcription of hypoxia-responsive proteins) in hypoxic cultures (Fig. 6A). In normoxic cultures, HIF1α was present in both nuclei and the cytosol. Western blotting of HepaRG samples revealed that HIF1α was most abundant in hypoxic cultures (Fig 6B). There was no clear difference between the protein expression levels of normoxic and hyperoxic cultures. These data show that the hypoxia induces nuclear HIF1α expression in HepaRG cells in our experimental set-up, which correlates with SOX9 expression.

Figure 6. Nuclear HIF1α expression decreases at increased oxygen levels (A) HepaRG cells cultured under normoxia (top), hypoxia (middle) and hyperoxia (bottom), stained for DAPI (blue) and HIF1α (red). Arrow A indicates nuclear expression of HIF1α, arrow B indicates cells that appear free of nuclear HIF1α. Scale bar = 50 µM (B) Western blot showing expression of HIF1α (top lanes), and vinculin as loading control (bottom lanes). Targets and positive controls are parts of the same image.
**Hypoxia stabilizes HepaRG cultures**

The HepaRG cell line cannot be expanded indefinitely; after undergoing 20 passages from isolation with a split ratio of 1:5 the cells lose their capacity to fully differentiate [20]. We hypothesized that since HepaRG cells maintain their progenitor characteristics under hypoxia, this may reduce stress on the cells and improve the long-term stability. To test this, we split HepaRG lineages into two sub-lines at passage 17: one propagated under normoxia and the other one under hypoxia. Every other passage, cells from both sub-lines were cultured and differentiated under normoxia and compared head-to-head (Fig. 7A).

We confirmed that above passage 20, the normoxic-maintained lines showed a marked decrease in the hepatocyte hallmark function ammonia elimination, as well as the transcript levels of CYP3A4, ARG1, CPS1, TF and CYP2B6 compared to passage 17, and that there was a trend towards an increase in total protein ($P=0.08$ for passage 23) (Fig. 7 B-E). In hypoxia-maintained lines, which were differentiated and tested under normoxia, neither ammonia elimination, total protein content nor hepatic gene transcription reduced significantly. In addition, the transcript levels of CYP3A4 and CYP2B6 were higher at passage 22 compared to passage 17.

We found no significant changes in lactate production over the passages and no significant difference between normoxic-maintained or hypoxic-maintained lines when differentiated and tested under normoxia (Fig 7F). Glucose consumption was significantly lower in hypoxic-maintained sub-lines at passage 23, at which point glucose consumption turned into production (Fig 7G).

In conclusion, these data show that maintaining HepaRG cultures under hypoxia stabilizes the capacity to hepatic differentiation under normoxia, thus increasing the total amount of HepaRG cells that can be produced from the original isolate.
Figure 7. Expansion under hypoxia stabilizes the HepaRG phenotype during serial passaging. HepaRG cells at passage 17 (P17) from isolation were split into sub lines that were maintained under ambient hypoxia or normoxia. Cultures were passaged every other week, every other passage samples from both cultures were cultured under normoxia for 4 weeks and tested (A). Cultures were tested on ammonia elimination (B), total protein content (C), transcript levels of hepatic genes (D) and transcription factor genes (E), lactate production (F) and glucose consumption (G). *=P<0.05 compared to normoxia maintained line of same passage number. $= P<0.05$ compared to P17.
Discussion and conclusions

In this study we show that oxygen has a significant effect on the differentiation state of HepaRG cells; hypoxia promotes stem cell characteristics with increased cell line stability, whereas hyperoxia induces hepatic differentiation. In addition, hyperoxia increases the hepatic differentiation of C3A cells, which makes it highly conceivable that hyperoxia could be routinely supplied to induce hepatic differentiation in proliferative cell sources.

Hyperoxia increased all tested hepatic functions in HepaRG and C3A cells compared to normoxia-maintained cultures, and the transcript levels of most tested hepatocyte-specific genes. Whole-transcriptome analysis revealed that transcriptional changes between hyperoxic and normoxic cultures were modest, indicating a significant role of underlying post-transcriptional regulation. However, we identified several potentially contributing up- and downregulated transcription factors. Upregulated transcription factors, included ONECUT2, which is involved in liver cell faith and hepatoblast migration [33, 34], FOXA3, a crucial transcription factor driving hepatocyte differentiation [2, 35], and CEBPG, implied to stimulate oxidative phosphorylation in the liver [36]. In addition, CEBPγ can inhibit other members of the C/EBP family through dimerization [37]. Interestingly, both CEBPA gene transcript levels and CEBPα nuclear protein expression were upregulated in hyperoxic cultures, indicating higher hepatocyte differentiation grade. Upregulation of CEBPG gene expression may therefore indicate a negative feedback loop. The most interesting downregulated transcription factors under hyperoxia were MYC (predicted downregulated), a proto-oncogene [38], ESRRG, a key regulator of hepatic gluconeogenesis [39], and TSC22D4, which plays a role in lipid metabolism. TSC22D4 overexpression reduces VLDL release, while inhibition leads to hypertriglyceridemia through the induction of hepatic VLDL secretion [40]. Urea cycle activity was induced under hyperoxia, while transcription of the rate-limiting enzyme CPS1 was not affected, indicating that the effects of hyperoxia on CPS1 are post-transcriptional. Hypoxia kept HepaRG cells undifferentiated, as indicated by negligible hepatic functionality, low transcript levels of hepatic genes and nuclear expression of progenitor marker SOX9 [4], which is in line with previous observations that hypoxia is beneficial for the development of hepatic progenitor cells from embryonic stem cell-derived endoderm [13]. SOX9 has been described to induce hepatocyte dedifferentiation, while CEBPα can counteract this effect, resulting in both transcription factors functioning as reciprocal repressors [41].

Hypoxia induced HIF1α expression and nuclear translocation in HepaRG cultures, which likely accounted for the observed HIF-associated effects, including the nuclear translocation of SOX9, the increased lactate production, and maintenance of stem cell characteristics. A vast range of effects have been attributed to HIFs, as an adaptation of mitochondrial respiration to hypoxia, amongst which the downregulation of free fatty acid synthesis (mainly HIF2α), stimulation of anaerobic glycolysis and inhibition of pyruvate dehydrogenase (mainly HIF1α) [31]. These may, at
least in part be modulated by MYC, as MYC-target gene transcription negatively correlated with oxygenation grade. In addition to HIFs, MYC is reported to be upregulated under hypoxia and is also a downstream target of HIFs. However, the interplay with HIFs is complex, as HIF may also repress MYC activity during small and severe hypoxic events [42, 43]. In addition to HIFs, other less-studied mechanisms may play a role, such as activation of the Raf-ERK pathway by NDR3-stabilization through increased lactate levels [44].

The pericellular oxygen concentration in both normoxic (181±2.4 µM) and hyperoxic cultures (339±3.9 µM) in this study is beyond the physiological concentration that ranges between 65 µM periportally and 35 µM pericentrally [45, 46]. It has been described that primary mouse hepatocytes retain their functionality longer after isolation under 40% ambient oxygen, and that oxygen consumption was three-fold higher in vitro vs in vivo [15]. Also, primary porcine hepatocytes cultured in bioartificial livers under medium perfusion showed higher functionality & stability at 250 µM (40%O₂) vs 130 µM (20% O₂). Clearly, the optimal oxygen concentration in vitro depends on the developmental state of the cells; as HepaRG cells lost viability under hyperoxia when applied in the proliferation phase, and not when applied in the differentiation phase [18]. In addition, propagation of the HepaRG cells was optimal under hypoxia, while differentiation was clearly inhibited by low oxygen concentration. This indicates that oxygen concentration has to be optimized for each developmental stage in a culture-protocol.

The experiments in this paper were all performed in tumour-derived cell lines. Primary hepatocytes are not a suitable model, since their phenotypes are not stable in vitro [17] and increase in oxygen tension does not revert this phenomenon [14]. Although stem cell derived hepatocyte-like cells hold great promise, differentiated HepaRG are the closest representation of human hepatocytes available at the moment [7, 21]. Both the total supply of HepaRG cells and –relevant for therapeutic application– batch sizes are limited by the maximum number of passages. Increase in the expansion potential provides a significant benefit to the development of new cell-based medicinal therapies. In conclusion, we show that oxygen is a driving factor in hepatocyte differentiation in hepatocyte cell lines, and that higher levels of oxygen correspond to lower nuclear expression of SOX9 and HIF1α, highlighting the importance of adjusted pericellular oxygen tension to the development stage of in vitro liver cell cultures. In addition, we show that hypoxia improves the propagation capacity of HepaRG cells.
References


Supplementary materials

A

B

C

Figure S1. Negative controls for immunofluorescent stainings. Negative controls (secondary antibody only), performed during the same experiments and taken at the same settings as the stainings in Fig 2A (A. Albumin and SOX9) Fig 2B (B. CEBPα) and Fig 6A (C. HIF1α).
Chapter 5

A practice-changing culture method relying on shaking substantially increases mitochondrial energy metabolism and functionality of human liver cell lines

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Aziza A.A. Adam
Vincent A. van der Mark
Joanne Donkers
Manon E. Wildenberg
Ronald P. Oude Elferink
Robert A.F.M. Chamuleau
Ruurdtje Hoekstra
Abstract

Practice-changing culturing techniques of hepatocytes are highly required to increase their differentiation. Previously, we found that human liver cell lines HepaRG and C3A acquire higher functionality and increased mitochondrial biogenesis when cultured in the AMC-Bioartificial liver (BAL). Dynamic medium flow (DMF) is one of the major contributors to this stimulatory effect. Recently, we found that DMF-culturing by shaking of HepaRG monolayers resulted in higher mitochondrial biogenesis. Here we further investigated the effect of DMF-culturing on energy metabolism and hepatic functionality of HepaRG and C3A monolayers. HepaRG and C3A DMF-monolayers were incubated with orbital shaking at 60 rpm during the differentiation phase, while control monolayers were maintained statically. Subsequently, energy metabolism and hepatic functionality were compared between static and DMF-cultures.

DMF-culturing of HepaRG cells substantially increased hepatic differentiation; transcript levels of hepatic structural genes and hepatic transcription regulators were increased up to 15-fold (Cytochrome P450 3A4) and nuclear translocation of hepatic transcription factor CEBPα was stimulated. Accordingly, hepatic functions were positively affected, including ammonia elimination, urea production, bile acid production, and CYP3A4 activity. DMF-culturing shifted energy metabolism from aerobic glycolysis towards oxidative phosphorylation, as indicated by a decline in lactate production and glucose consumption, and an increase in oxygen consumption. Similarly, DMF-culturing increased mitochondrial energy metabolism and hepatic functionality of C3A cells.

Conclusions: Simple shaking of monolayer cultures substantially improves mitochondrial energy metabolism and hepatic differentiation of human liver cell lines. This practice-changing culture method may prove to prolong the in-vitro maintenance of primary hepatocytes and increase hepatic differentiation of stem cells.
Introduction

Highly differentiated human hepatocytes from proliferative sources are needed to serve as predictive hepatocyte model in vitro and as biocomponent for Bio-Artificial Livers (BALs). However, to date, hepatocytes deriving from different proliferative sources, as stem cells, induced pluripotent stem cells and liver cell lines, are deficient in complex hepatic functions (1).

HepaRG is the human liver progenitor cell line increasingly used as human liver model for the prediction of hepatotoxicity and human liver infections, and is also applied as biocomponent in the AMC-Bio-Artificial liver (AMC-BAL), (2-4) as the functionality is relatively high, and its transcriptome closely resembles that of primary human hepatocytes (5). HepaRG cultures develop during 28 days into a mixed heterogeneous culture with hepatocyte-islands and bile duct-like cells. Treating HepaRG cells with 2% dimethylsulfoxide (DMSO) during the last two weeks of culturing, enhances their hepatic differentiation and the detoxification properties, however, it also increases cell damage (3, 6).

Interestingly, HepaRG cells cultured in the AMC-BAL platform have increased hepatic functionality and integrity compared to HepaRG monolayer cultures (7). Of particular interest, BAL-cultured HepaRG cells efficiently eliminate lactate, while monolayer-cultured cells produce lactate (8-10). Lactate elimination is a hallmark function of highly differentiated hepatocytes, and is lacking in the available hepatocyte culture models. Consistent with this, we recently found that BAL-culturing enhances mitochondrial biogenesis and mitochondrial activity, resulting in a shift of energy metabolism towards oxidative phosphorylation (OxPhos) (11). The stimulatory effect of the AMC-BAL culture on mitochondrial biogenesis also applied to another human liver cell line, C3A, a sub-clone of the HepG2 hepatoma cell line (12). Among the driving factors underlying this metabolic shift is the presence of dynamic medium flow (DMF) in the BAL system (13). We mimicked the DMF of the BAL by placing monolayer cultures into a shaking incubator (at 60 rpm) during the differentiation phase. Culturing of HepaRG monolayers with DMF increased their mitochondrial abundance 3.3-fold and the expression levels of mitochondrial inner membrane OxPhos complexes (11). Recent studies reported a strong, likely causal, relationship between mitochondrial energy metabolism and differentiation of stem cells. On one hand, inhibition of OxPhos augments the expression of pluripotency markers in stem cells (14, 15). On the other hand, increased mitochondrial biogenesis with a shift in cellular energy metabolism towards Oxphos is associated with the differentiation of stem cells, e.g. into hepatocyte-like cells (16).

In the current work, we investigated whether the increased mitochondrial biogenesis in HepaRG DMF-monolayers is also positively associated with hepatic functionality, and extended the study for cell line C3A.
Materials and methods

Cell culture
HepaRG cells were kindly provided by Biopredic International (Rennes, France). HepaRG cells and C3A cells (ATCC® CRL10741™) were maintained in HepaRG medium, as described (3, 4, 10). For testing, the HepaRG and C3A cells were seeded in 12-well culture plates (Corning, Corning, USA), unless indicated otherwise, and cultured under either Static or DMF-regimen. The Static groups were cultured under conventional conditions, i.e. static in a humidified atmosphere of 95% air and 5% CO₂. One group was supplemented with 2% DMSO for the last two weeks of culturing (HepaRG-DMSO-Static), and the other was maintained without DMSO (HepaRG-Static). The DMF-group was kept for an initial phase (proliferation phase) under conventional conditions, being 14 days for HepaRG and 3 days for C3A cells, followed by a 14 days (HepaRG cells) or 11 days (C3A cells) shaking-phase under the same atmosphere in a shaking incubator (Eppendorf, Westbury, USA) with 60 rpm orbital-shaking frequency. Three days prior testing, the cultures were supplemented with 1 mM carbamoyl-glutamate (CAG) (Sigma-Aldrich, St. Louis, USA) to stimulate carbamoyl phosphate synthetase 1 (CPS1) activity (6). All cultures were maintained at 37°C and were negative for mycoplasma.

Hepatic function test
The cultures were tested for their functionality, as described (9). Briefly, monolayer cultures were exposed to 1.5 ml test medium, based on HepaRG medium supplemented with 1 mM CAG, 1.5 mM NH₄Cl, 2.27 mM D-galactose, 2 mM L-lactate and 125 μM testosterone (all compounds from Sigma-Aldrich). During the function test, medium samples were taken at 45 min (0.5 mL) and at 24 h (1 ml), in which L-lactate, ammonia, urea, glucose, bile acid and human albumin concentrations were measured. L-lactate determination was performed using L-lactic acid assay kit (Megazyme, Wicklow, Ireland). Ammonia was assessed using Ammonia assay kit (Megazyme). Urea production was measured according to the blood urea nitrogen protocol (Sigma). Glucose concentration was determined with a glucosemeter (Contour® next) (Bayer, Leverkusen, Germany). In addition, total bile acid concentration was determined using the DIAZYME total bile acid assay kit (DIAZYME Laboratories, Dresden, Germany). Human albumin was measured using the Human Serum Albumin DuoSet ELISA according to supplier’s instructions (R&D systems Inc., Minneapolis, USA).

Metabolic rates were determined by calculating the changes in concentration in medium in time and then normalized to the protein content per well. The cultures were also tested for baseline and induced CYP3A4 activity using the P450-Glo™ CYP3A4 (Luciferin-IPA) Assay kit (Promega, Madison, USA). CYP3A4 activity was optionally induced by a 3-day treatment with 10 μM rifampicin (Sigma-Aldrich) prior to testing. The measured CYP3A4 activity, expressed as Relative Luminescence Units (RLU), was normalized to protein content per well. Total protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Irvine, USA), according to manufacturer’s protocol.
**Oxygen consumption determination**

HepaRG-Static and DMF-cultures were fully differentiated in 24-well culture plates (OxoDish®, PreSens, Regensburg, Germany) with oxygen sensor spots at the bottom of the well. Pericellular oxygen concentrations were measured through the bottom of the culture plates using the SDR SensorDish® Reader, which was kindly made available by Applikon Biotechnology, Delft, the Netherlands. The measured oxygen consumption was normalized to protein content per well.

**Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)**

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and qRT-PCR was performed as described (9). Transcript levels were normalized for 18S ribosomal RNA. Primer sequences and amplicon sizes are given in supplementary S1 Table. Transcript levels of Static and DMF-cultures of HepaRGand C3A were expressed as a % of the average of two human healthy liver samples of two female patients aged 40 and 41 years undergoing liver resection, in S2 and S3 Table resp.

**Taurocholate uptake assay**

Taurocholate (TC) uptake was measured as described in (17), using chemicals from Merck (Darmstadt, Germany), unless indicated otherwise. The cultures were washed twice with uptake buffer (5 mM KCl, 1.1 mM K2HPO4, 1 mM MgCl2 (Sigma-Aldrich) 1.8 mM CaCl2, 10 mM D-Glucose, 10 mM HEPES, 136 mM NaCl, pH 7.4), and then exposed to 500 µL uptake buffer containing 10 µM 3H-labelled TC (PerkinElmer, Waltham, USA) for 2 min at 37°C. Subsequently, the cells were washed 4x with ice-cold PBS and lysed in 0.05% SDS for 30 min at room temperature. Radioactivity was measured by liquid scintillation counting using the TRI-CARB 2900 TR (PerkinElmer) and data was normalized to protein content per well.

**Immunofluorescence staining**

Cultures in 6-well plates were fixed with 2% formalin (VWR, Radnor, USA) for 2-5 min at room temperature prior to permeabilization with 0.3% Triton-X100 (Bio-Rad) in cold PBS, for 20 min on ice. The monolayers were blocked for 1h with 10% fetal calf serum (BioWhittaker, Walkersville, USA) in PBS on ice before overnight incubation with the primary antibody diluted 1:200 in PBS at 4°C. Cultures were washed 3x with cold PBS, incubated 2h at 4°C with secondary fluorescent antibody diluted 1:1000 in PBS, then washed 3x with cold PBS before incubation with DAPI-containing Vectashield (Vector Laboratories, Burlingame, USA). Imaging was performed with a DM6000B fluorescent microscope (Leica Microsystems, Wetzlar, Germany) and images were processed using ImageJ software (http://imagej.nih.gov/ij/).

Primary antibodies: Goat anti-human albumin (Bethyl Laboratories, Montgomery, USA), Rabbit anti-human SOX9 (Millipore, Billerica, USA), Goat anti-human CEBPa (Santa-Cruz, Dallas, USA),
Rabbit anti-rat OATP1a1 (Alpha Diagnostics, San Antonio, USA), Mouse anti-human MRP2 (Enzo Life Sciences, Oyster Bay, USA).

Fluorescent secondary antibodies were obtained from Invitrogen, unless indicated: Donkey anti-rabbit/Alexa Flour-546, Donkey anti-goat/Alexa Flour-448 (Molecular Probes, Eugene, USA), Donkey anti-goat/Alexa Flour-546, Goat anti-rabbit/Alexa Flour-448, Goat anti-mouse/Alexa Flour-546.

**Cellular polarization assay**

To assess cellular polarization, the cultures were incubated with 20 μM 5-carboxyfluorescein diacetate (CFDA; Molecular Probes, Eugene, USA) in PBS at 37°C for 15 min to allow its internalization and subsequent translocation into the canalicular lumen by the multidrug resistance protein 2 (MRP2) ATP-binding cassette transporter. Then the cultures were washed 2x with PBS and mounted with DAPI containing Vectashield. Imaging was performed with fluorescent microscope (Leica Microsystems).

**Statistical analyses**

Statistical analyses were performed in Prism version 7 (GraphPad Prism Inc. San Diego, USA) using Student’s t-tests for the comparison between two groups.

**Results**

**DMF-culturing enhances HepaRG cell differentiation**

We assessed whether DMF-culturing increased the hepatic differentiation of HepaRG cells compared to conventional static culturing by co-staining of two markers; albumin (ALB), as a marker of differentiated hepatocytes, and Sry (sex determining region Y)-box 9 (SOX9), as a progenitor cell marker. SOX9 is a transcription factor, highly expressed in pluripotent, fetal, and adult stem and progenitor cells that maintains their undifferentiated-status (18, 19). HepaRG-DMF cultures exhibited decreased SOX9 expression compared to HepaRG-Static cultures (Fig 1A and S4 Fig for higher resolution). In addition, HepaRG-Static cultures showed predominant SOX9 nuclear translocation, which was less prominent in HepaRG-DMF cultures. Furthermore, the expression of SOX9 was also studied in HepaRG-Static controls supplemented with 2% DMSO (HepaRG-DMSO-Static). Similarly, the expression of SOX9 was clearly reduced in HepaRG-DMSO-Static cultures compared to HepaRG-Static (S4 Fig). The expression of albumin was confined to hepatocyte islands and was not different between Static and DMF-cultures (Fig 1A and S4 Fig). In line with this, human albumin synthesis measured by ELISA was comparable in HepaRG-DMF and HepaRG-Static cultures and significantly higher than the level of primary human hepatocytes (PHHs) (Fig 1B, S4 Table).
Furthermore, the transcript levels of critical hepatogenic differentiation regulators (20) hepatic nuclear factor 4 (HNF4), constitutive active receptor (CAR), pregnane X receptor (PXR), small heterodimer partner (SHP) and CCAAT/enhancer-binding protein (CEBPα) were increased by 1.5-, 2.3-, 1.8-, 1.8- and 2.9-fold, resp. in HepaRG-DMF vs HepaRG-Static (Fig 1C, S2 Table). Noteworthy, the transcript levels of HNF4, CAR and PXR were not changed in static HepaRG cultures supplemented with 2% DMSO compared to HepaRG-Static controls (6). The expression of CEBPα, a nuclear factor that regulates hepatic maturation and the urea cycle, was increased with more nuclear translocation in HepaRG-DMF compared to HepaRG-Static cultures (Fig 1D and, S5 Fig for higher resolution).
DMF-culturing improves nitrogen metabolism in HepaRG cells

We further assessed the effect of DMF-culturing on ammonia elimination, as one of the hallmark functions of hepatocytes. There are 2 principal routes of ammonia elimination in hepatocytes, either by irreversible conversion into urea through urea cycle activity or by reversible fixation into amino acids, primarily through the activity of glutamine synthase (GS) (21). The urea cycle consists of 5 enzymatic reactions catalyzed by 5 enzymes with Carbamoyl Phosphate Synthetase I (CPS1) and Arginase 1 (ARG1) being two of the most critically expressed proteins in HepaRG cells (6). ARG1
catalyzes the last step yielding urea and ornithine through hydrolysis of arginine, however, a similar reaction can be catalyzed by an extra-hepatic mitochondrial isoform, ARG2 (22, 23). DMF-culturing induced the transcript levels of CPS1, ARG1 and GS 1.9-, 6.0- and 1.6-fold, resp. (Fig 2A, S2 Table). Consistent with this, ammonia elimination and urea production were 2.0- and 2.4-fold increased in the DMF-cultures resp., ammonia elimination was restored to the physiological level (Fig 2B-C, S4 Table). As ARG2 transcript levels remained unchanged under DMF-culturing (Fig 2A, S2 Table), it is likely that the increased urea production under DMF can be attributed to increased urea cycle activity. In DMSO-treated static monolayers, the transcript levels of CPS1 and GS were 28.2- and 2.4-fold decreased compared to HepaRG-Static monolayers. However, the ureagenesis and ammonia elimination were 1.6- and 1.5-fold upregulated compared to HepaRG-Static cultures (6).

In brief, DMF-culturing improved ammonia metabolism of the HepaRG cells to higher extent compared to static cultures with or without DMSO supplementation.

Figure 2. DMF-culturing improves nitrogen metabolism in HepaRG cells. (A) Transcript levels of genes involved in nitrogen metabolism, n=6/group. (B-C) Ammonia elimination and urea production, n= 9-18/group. Significance Static vs DMF: # = P value <0.05, ## = P value <0.01, ### = P value <0.001 and #### = P value <0.0001.
DMF-culturing substantially increases the detoxification properties and bile acid production of HepaRG cells

DMF-culturing increased markers of detoxification at all three phases; phase 1, the alteration of the chemical structure to more water-soluble moiety, executed primarily by CYPs, phase 2 involving the conjugation of the hydrophilic moiety (24, 25), and phase 3, the transport into the canalicular space or circulation (26), as well as bile acid synthesis, which is essential for the secretion phase. The transcript level of \(\text{CYP3A4} \), a CYP enzyme, involved in the detoxification process of 50% of prescribed drugs (27), was substantially upregulated up to 15-fold by DMF-culturing, whereas the transcript levels of \(\text{CYP2B6} \) and \(\text{UDP-glucuronosyltransferase (UGT1a1)} \) were 2.5- and 1.8-fold increased (Fig 3A, S2 Table). In the past, we found that DMSO-treatment increased the transcript levels of \(\text{CYP2B6} \) and \(\text{CYP3A4} \) 20.3- and 6.9-fold resp. (6). Furthermore, the transcript level of cytochrome P450 oxidoreductase (\(\text{POR}\)), that donates electrons to CYPs, and is therefore essential for the biosynthesis of bile acid, as well as the metabolism of more than 80% of drugs in use (28, 29), was 1.6-fold upregulated in DMF-cultures (Fig 3A, S2 Table). The substantial induction of \(\text{CYP3A4} \) transcript levels was in line with a 7.6- and 3.8-fold increase in the baseline and induced CYP3A4 activity (Fig 3B-C). Bile acid synthesis was 2.6-fold augmented in the DMF-cultures (Fig 3D, S4 Table). The transcript levels of hepatic uptake transporters \(\text{Na}^+\)-taurocholate co-transporter poly peptide (\(\text{NTCP}\)) and \(\text{organic anion transporter poly peptide (OATP1b1)} \) were 2.7- and 2.3-fold increased under DMF-culturing, reaching 36.7% and 6.4% of human liver level, resp. (Fig 3E, S2 Table). In contrast, the already very low transcript level of \(\text{OATP1b3} \) compared to human liver being 1.2% for HepaRG-Static, was 2-fold further decreased by DMF-culturing. On the other hand, the transcript level of the efflux transporter multidrug resistance-associated protein \(\text{MRP2} \), was 1.4-fold increased with DMF-culturing (Fig 3E, S2 Table).

Unexpectedly, despite the significant increase of the transcript level of \(\text{NTCP} \), no difference between DMF- and Static-cultures was observed in the taurocholate-uptake function of NTCP (Fig 3F). One of the important factors determining transporter function, is the polarization of the hepatocytes, resulting into distinct luminal and basolateral domains that execute the import and export, for e.g. proteins and bile acid (30). The polarization of Static- and DMF-cultures was studied by immune-staining of OAPT1a1 and MRP2. The expression of OATP1a1 and MRP2 was increased in the DMF-cultures (Fig 3G-H). However, the localization of these hepatic transporters was predominantly cytosolic with minimal membrane localization, indicating that the polarization of the HepaRG cells was absent in both Static- and DMF-cultures. The disturbed polarization of the hepatic transporters was further confirmed by CFDA polarization assay. CFDA, upon entering hepatocytes, is converted into green fluorescent carboxyfluorescein (CF) by intracellular esterases, which then is effluxed to the canalicular side mainly through MRP2. Although CF-signal was increased in the DMF-cultures compared to Static-cultures, no clear canalicular localization was observed under both conditions, confirming that the polarization of the HepaRG cells was not yet established (Fig 3I).
Figure 3. DMF increases detoxification properties of HepaRG cells. (A) Transcript levels of detoxification genes, n=6-9/group. (B-C) CYP3A4 baseline- and induced-activity, n= 6-15/group. (D) Bile acid production, n=6-15/group. (E) Transcript levels of hepatic transporter genes, n=6-9/group. (F) TC uptake, n=6/group. Significance Static vs DMF: # = P value <0.05, ## = P value <0.01, ### = P value <0.001 and #### = P value <0.0001.
Figure 3. continued. (G-H) Staining of hepatic transporters OATP1a1 (green) and MRP2 (red) with DAPI counter-staining for the nuclei (blue). (I) Visualization of CFDA (green) with DAPI counter-staining of the nuclei (blue), scale bar=50μm.
DMF-culturing induces a shift in cellular energy metabolism towards more mitochondrial-dependent in HepaRG cells

Previously, we found that culturing of HepaRG cells under DMF increased mitochondrial abundance and OxPhos complexes (11). Here, we further assessed cellular-energy metabolism and mitochondrial functions. In agreement with the previous findings, the transcript level of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), the master regulator of mitochondrial biogenesis, was 1.4-fold increased under DMF (Fig 4A, S2 Table). Moreover, the transcript levels of mitochondrially-encoded cytochrome B gene (MT-CYB) was 1.7-fold enhanced (Fig 4A, S2 Table). Furthermore, lactate production and glucose consumption were 1.9- and 2.5-fold reduced, whereas oxygen consumption was 1.6-fold increased in DMF-HepaRG vs HepaRG-Static cultures (Fig 4B-D). Collectively, these data confirm that DMF-culturing enhances mitochondrial functions and exerts a shift in cellular-energy metabolism towards OxPhos in HepaRG cells.

Figure 4. DMF increases mitochondrial energy metabolism of HepaRG cells. (A) Transcript levels of PGC1α and MT-CYB, n=6/group. (B-D) Lactate production, glucose and oxygen consumption, n=9-15/group. Significance Static vs DMF: # = P value <0.05, ## = P value <0.01, ### = P value <0.001 and #### = P value <0.0001.
DMF-culturing substantially stimulates hepatic functions and mitochondrial metabolism of C3A cells

To assess whether the effect of DMF-culturing is cell line (HepaRG) specific, we also compared Static and DMF-cultures of the C3A cell line. Compared to C3A-Static, C3A-DMF relied more on mitochondrial energy metabolism, as indicated by a 3.2- and 2.3-fold reduced lactate production and glucose consumption, resp. (Fig 5A-B). In addition, DMF-culturing increased hepatic differentiation, as indicated by a 1.9-, 3.1- and 5.6-fold induction of the transcript levels of HNF4, CAR and CEBPα, while PXR and SHP exhibited a positive trend (Fig 5C, S3 Table). Unexpectedly, Albumin synthesis was reduced in C3A-DMF cultures compared to C3A-Static (Fig 5D, S4 Table). On the other hand, the baseline and induced-CYP3A4 activity was 2.5- and 4.7- fold increased by DMF-culturing (Fig 5E-F). In contrast to HepaRG cells, C3A cells produce ammonia and are not capable of eliminating ammonia. C3A cells maintained under DMF-cultures displayed an improved ammonia metabolism as shown by induction of the transcript levels of the genes encoding urea cycle enzymes CPS1 and OTC, 3.0- and 4.0-fold, resp., whereas the levels of ARG1 and GS remained comparable to that of Static-cultures (Fig 5G, S3 Table). Accordingly, ammonia metabolism was positively modulated, resulting in a 3.3-fold less ammonia production, while urea production remained unchanged (Fig 5H-I).
Figure 5: DMF-culturing enhances mitochondrial energy metabolism and hepatic functions of C3A cells. (A-B) Lactate production and glucose consumption, n=6/group. (C) Transcript levels of genes encoding hepatic differentiation regulators, n=6/group. (D) Albumin synthesis. (E-F) CYP3A4 activity, n=3/group.
Figure 5 continued: (G) Transcript levels of genes involved in nitrogen metabolism, n=6/group. (H-I) Ammonia elimination and urea production, n=3-6/group. Significance Static vs DMF: # = $P$ value <0.05, ## = $P$ value <0.01, ### = $P$ value <0.001 and #### = $P$ value <0.0001.
Discussion

There is an unmet need for a hepatocyte culture platform that either maintains the hepatic functionality of primary hepatocytes and/or induces hepatic functionality of proliferative sources of hepatocytes, such as stem cells and cell lines. This study presents a practice-changing culture method comprised of simply shaking of monolayer cultures after reaching confluence, which substantially increases the hepatic functionality and shifts energy metabolism towards OxPhos of two hepatocyte cell lines, HepaRG and C3A.

Recently, DMF was found to be one of the factors contributing to the enhanced mitochondrial biogenesis under AMC-BAL-culturing (11). In this study, we further established that DMF-culturing exerted a clear shift in energy metabolism from aerobic glycolysis towards OxPhos, as indicated by the significant decline in lactate production, and glucose consumption in both HepaRG and C3A cell lines and, only tested in HepaRG cells, an increase in oxygen consumption. Additionally, DMF upregulated hepatic transcription factors and hepatic functions of HepaRG and C3A cells, including ammonia metabolism and CYP3A4 activity. More extended studies, in HepaRG cells, showed that DMF induced the transcript levels of various hepatic genes, bile acid synthesis, whereas the expression of the stem cell marker SOX9 was reduced, and its localization was shifted from nuclear towards cytoplasmic, indicating a higher degree of hepatic differentiation under DMF. Therefore, we established a simple modification to conventional culturing methods that upregulates mitochondrial energy metabolism and hepatic differentiation of liver cell lines.

Solid association between mitochondrial energy metabolism and differentiation has recently been established, particularly in stem cell research. Undifferentiated stem cells retain pluripotency and high proliferative potentials under glycolysis-stimulating conditions (31, 32). Furthermore, reverse mitochondrial remodeling occurs during reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) and during dedifferentiation of primary hepatocytes (16). Conversely, differentiation of stem cells is associated with mitochondrial remodeling and a shift of energy metabolism towards OxPhos (16, 33).

Although, the hepatic functions of the C3A cells were significantly increased under DMF-culturing, yet ammonia production was not converted into elimination rendering the usage of this cell line as a biocomponent in BALs to support patients with liver failure, questionable. Importantly, the effect of DMF-culturing on the hepatic differentiation of different liver cell lines may vary in its magnitude dependant on the basal hepatic differentiation of the specific cell line under static conditions.

The importance of DMF for HepaRG cells was already shown in the AMC-BAL culture platform (34). A medium flow rate of 5 ml/min optimally stimulated hepatic functions, whereas lower and higher flow rates resulted in decreased functionality and increased cell damage, resp. Here, we
mimicked DMF of the BAL system in monolayers using a simple technique by continuously shaking the cultures at 60 rpm rate during the differentiation phase. Lower shaking rates, being 5 and 25 rpm, were not effective (Supplementary Fig S1-3). In addition, DMF of less dense (sub-confluent) cultures was detrimental to the cells (results not shown).

DMF has also been applied for hepatocyte culturing by others, relying on complex systems, such as microfluidic chambers (35-38). Importantly, unlike DMF by simple shaking applied in our study, in all above mentioned DMF platforms, an internal oxygenation system was crucial for the viability and functionality of the hepatocytes in agreement with the assumption that improved oxygenation under shaking, stimulates mitochondrial biogenesis and thereby hepatic differentiation. Consistent with this, we recently established that oxygenation with 40%O$_2$ instead of 20%O$_2$ increased mitochondrial biogenesis (39), and hepatic differentiation of HepaRG cells Static-monolayers, however not to the level of DMF-culturing (11, 40) A combination of DMF and 40%O$_2$ culturing, is however, detrimental to the HepaRG cells (results not shown), suggesting a shared underlying mechanism, relying, at least in part, on improved oxygenation, however this requires further investigations.

Interestingly, culturing of the HepaRG cells under DMF increased their hepatic differentiation superior to DMSO-treatment, without inducing any cytotoxic effect, as found for DMSO (3, 6). Ammonia metabolism and the transcript levels of several hepatic genes (e.g HNF4, PXR, CAR and CYP3A4) were relatively high in the HepaRG-DMF cultures, while only the transcript level of CYP2B6 among all tested hepatic genes, was more upregulated by DMSO-treatment vs. DMF culturing. Yet, the combination of DMSO treatment and DMF culturing, however, exhibited an toxic effect on HepaRG cells (data not shown).

The DMF-induced gain of hepatic functionality is probably mediated through upregulation of hepatogenic transcription factors. DMF increased the transcript levels of HNF4, CEBPa and CAR in HepaRG and C3A cells, whereas PXR and SHP, were induced in HepaRG cells. SHP mediates FXR signaling as a small heterodimer partner. Among other functions, FXR acts as a transcriptional regulator of bile acid biosynthesis and transport in liver (41). Interestingly, Godoy et al. found that three of these regulators, i.e. CAR, FXR and PXR, were, together with HNF1, strongly repressed in cultivated hepatocytes, suggesting that the current in vitro culturing models lack stimuli required to maintain gene expression of these regulators in hepatocytes (42), which can be counteracted by DMF-culturing. The upregulation of HNF4 and CEBPa will also contribute to hepatic functionality, as these two transcription factors play a major role in governing hepatic development and regulating a number of critical metabolic pathways, including the urea cycle (43, 44). CEBPa overexpression in combination with HNF4 and FOX2a in adult-liver derived progenitor cells resulted in increased hepatic differentiation (45).
Of note, in HepaRG cells, the production of bile acids, being versatile signaling molecules, was 2.6-fold augmented by DMF-culturing, which may further upregulate FXR, CAR, PXR and other relevant pathways, as cAMP synthesis, and protein kinase C activation (46). Interestingly, DMF-culturing did not increase albumin synthesis in HepaRG cells and resulted in a reduced albumin production by C3A cells. This unexpected observation might be related to the fact that albumin production is already higher or comparable to the level of human primary hepatocytes in HepaRG and C3A cells cultures resp and could not be further increased by DMF-culturing.

The expression of several hepatic transporters, that were relatively low expressed in HepaRG-Static monolayers, such as NTCP, was increased in HepaRG-DMF cultures, however probably due to limited polarization, NTCP function remained unchanged. Hepatocyte polarization is a complex process, leading to an organized localization of extracellular, cytoskeletal and tight junction molecules (47). The polarization is more established in HepaRG monolayers when treated with 1.7% DMSO for the last two weeks of culturing (48). Of note, the combination of DMSO treatment and shaking exerted a negative effect on the functionality of HepaRG cells (data not shown). In the past increased polarization of HepaRG cells was achieved by AMC-BAL culturing of HepaRG cells (26). Therefore, new culture techniques combining DMF by simple shaking and 3D configuration may thus represent a potential window for further improving in vitro hepatocyte culturing.

Conclusions

The current study demonstrates that DMF-culturing substantially enhances mitochondrial energy metabolism and hepatic functions in two different human liver cell lines. These findings strongly support a role of mitochondria in regulating the differentiation of human liver cells. Importantly, we developed a new easily applicable and scalable culture platform that substantially enhances the functionality of human liver cell lines. Considering the high similarity of responsiveness to DMF-culturing between two hepatic cell lines, it is likely that DMF will also exert positive effects on hepatic differentiation of stem cells and maintenance of differentiation of primary hepatocytes.

Acknowledgement

We thank Erik Hendriks for cell culturing and Applikon Biotechnology for providing the SDR SensorDish® Reader.
References


Supporting information

Tables

S1 Table: Primers used in the RT-qPCR and amplicon size.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense sequence</th>
<th>Anti-sense sequence</th>
<th>bp</th>
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</thead>
<tbody>
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<td>18S</td>
<td>TTCGGAACTGAGCCATGAT</td>
<td>CGAACCTCCGACTTTCGTTCT</td>
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</tr>
<tr>
<td>ARG1</td>
<td>TTGGCAAGGTATGGAAGAAACA</td>
<td>CCTCCCGAGCAAGTCCGAAAAACAA</td>
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<tr>
<td>ARG2</td>
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<td>GCCATCAACCCAGACAACACAACAA</td>
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<td>CAR</td>
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<td>CEBPa</td>
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<td>GCCGCCGGCGGCTGGTA</td>
<td>254</td>
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<td>ACCACCCGGGCGCAAGAAACAC</td>
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<td>CYP3A4</td>
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<td>GCCACCTCCCACCTTCAACAG</td>
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<td>CYP2B6</td>
<td>CCCCCTCTCTGCCCTTTTGA</td>
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</tr>
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<td>GS</td>
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<td>GCCGCCCTTGAGTGGAGTTG</td>
<td>162</td>
</tr>
<tr>
<td>HNF4</td>
<td>TCCGGGCTGGCATGAGAAAGG</td>
<td>CCAAGGGGAGCTGCGAAAGAGG</td>
<td>321</td>
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<tr>
<td>MT-CYB</td>
<td>AACCTGGCGATCCATCTCCCTGG</td>
<td>CCGATCGTAGGAGAAAGGC</td>
<td>204</td>
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<tr>
<td>MRP2</td>
<td>AGACCGAATATCCACGCTTCT</td>
<td>ATCCGGCCTGGGGTGTGGT</td>
<td>205</td>
</tr>
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<td>NTCP</td>
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<td>GGGGAAGGAAGAAAGATGTG</td>
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<td>OATP1b1</td>
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<td>OATP1b3</td>
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<td>OTC</td>
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<tr>
<td>PGC1a</td>
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<td>SHP</td>
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<td>TGCTCATACAGGCTTGGCC</td>
<td>175</td>
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<tr>
<td>UGT1a1</td>
<td>CCCATTCTCTACCTGCGCCAGG</td>
<td>GGTCCAGTGTAAGGCACAGAGG</td>
<td>457</td>
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### S2 Table: Transcript levels of genes in HepaRG cultures as a % of human livers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>HepaRG-Static</th>
<th>HepaRG-DMF</th>
<th>P value, Static vs DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARG1</td>
<td>2.7 ± 0.9</td>
<td>16.2 ± 6.1</td>
<td>0.000</td>
</tr>
<tr>
<td>ARG2</td>
<td>1406.8 ± 952.0</td>
<td>1413.5 ± 308.7</td>
<td>0.987</td>
</tr>
<tr>
<td>CAR</td>
<td>2.2 ± 1.5</td>
<td>4.4 ± 2.2</td>
<td>0.000</td>
</tr>
<tr>
<td>CEBPa</td>
<td>17.1 ± 8.4</td>
<td>41.1 ± 13.2</td>
<td>0.000</td>
</tr>
<tr>
<td>CPS1</td>
<td>30.3 ± 19.1</td>
<td>56.0 ± 20.7</td>
<td>0.049</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>2.3 ± 1.0</td>
<td>33.7 ± 19.6</td>
<td>0.003</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>4.5 ± 3.7</td>
<td>11.1 ± 6.1</td>
<td>0.047</td>
</tr>
<tr>
<td>GS</td>
<td>486.5 ± 154.7</td>
<td>800.4 ± 231.2</td>
<td>0.030</td>
</tr>
<tr>
<td>HNF4</td>
<td>242.5 ± 62.5</td>
<td>351.6 ± 74.2</td>
<td>0.020</td>
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<tr>
<td>MT-CYB</td>
<td>58.2 ± 26.3</td>
<td>108.0 ± 65.5</td>
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<td>MRP2</td>
<td>64.9 ± 20.4</td>
<td>83.4 ± 20.3</td>
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<tr>
<td>NTCP</td>
<td>16.4 ± 13.4</td>
<td>36.7 ± 24.1</td>
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<tr>
<td>OATP1b1</td>
<td>3.2 ± 2.5</td>
<td>7.1 ± 5.4</td>
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<tr>
<td>OATP1b3</td>
<td>1.2 ± 0.7</td>
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<td>PGC1a</td>
<td>462.9 ± 257</td>
<td>573.6 ± 361.9</td>
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<td>POR</td>
<td>40.5 ± 21.7</td>
<td>58.4 ± 24.7</td>
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<td>PXR</td>
<td>9.7 ± 5.7</td>
<td>16.5 ± 6.3</td>
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<td>SHP</td>
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<tr>
<td>UGT1a1</td>
<td>46.6 ± 17.1</td>
<td>74.2 ± 21.6</td>
<td>0.006</td>
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### S3 Table

Transcript levels of genes in C3A cultures as a % of human livers.

<table>
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<tr>
<th>ID</th>
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<th>C3A-DMF</th>
<th>P value, Static vs DMF</th>
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<tr>
<td>ARG1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
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<td>CAR</td>
<td>0.8 ± 0.5</td>
<td>2.4 ± 1.2</td>
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<tr>
<td>CEBPa</td>
<td>15.2±6.4</td>
<td>85.6±55.6</td>
<td>0.011</td>
</tr>
<tr>
<td>CPS1</td>
<td>2.9 ± 1.0</td>
<td>8.7 ± 4.8</td>
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<td>GS</td>
<td>406.3 ± 100.0</td>
<td>289.4 ± 131.4</td>
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</tr>
<tr>
<td>HNF4</td>
<td>1.3 ± 0.4</td>
<td>2.5 ± 1.2</td>
<td>0.039</td>
</tr>
<tr>
<td>OTC</td>
<td>0.4 ± 0.2</td>
<td>1.6 ± 0.9</td>
<td>0.029</td>
</tr>
<tr>
<td>PXR</td>
<td>20.1 ± 5.5</td>
<td>27.6 ± 13.4</td>
<td>0.321</td>
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<tr>
<td>SHP</td>
<td>215.6 ± 65.3</td>
<td>368.1 ± 213.6</td>
<td>0.194</td>
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### S4 Table

Hepatic functions of primary human hepatocytes (PHHs), HepaRG and C3A static and DMF-cultures. Values are given as mean±SD, for more details (6, 49).

<table>
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<tr>
<th>Hepatic function</th>
<th>HepaRG-Static</th>
<th>HepaRG-DMF</th>
<th>C3A-Static</th>
<th>C3A-DMF</th>
<th>PHHs</th>
</tr>
</thead>
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<tr>
<td>Ammonia elimination (nmol/h/mg protein)</td>
<td>30±10.5****</td>
<td>59.6±22.4 NS</td>
<td>-12.5±5.4****</td>
<td>-3.8±2.2****</td>
<td>95±5.0</td>
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<tr>
<td>Urea production (nmol/h/mg protein)</td>
<td>1.7±0.9 ****</td>
<td>4.1±1.3 ****</td>
<td>0.9±0.3 **</td>
<td>1.0±0.7 **</td>
<td>91±34</td>
</tr>
<tr>
<td>Bile acid production (nmol/h/mg protein)</td>
<td>0.1±0.0 ****</td>
<td>0.18±0.0****</td>
<td>ND</td>
<td>ND</td>
<td>1.5±1.1</td>
</tr>
<tr>
<td>Albumin production (ng/h/mg protein)</td>
<td>51.5±10.3**</td>
<td>48.4±5.3*</td>
<td>34.2±12.3 NS</td>
<td>18.6±11.6**</td>
<td>37.7±7.8</td>
</tr>
</tbody>
</table>

**ND**: not determined
**NS**: not significant
* indicates significance vs PHHs
Supplementary figures

S1 Fig.

Ammonia elimination (nmol/nl/mg)

S2 Fig.

Urea production (nmol/nl/mg)

S3 Fig.

Lactate production (nmol/nl/mg)

S1-S3 Figures: Optimization of the shaking rate for DMF-cultures. Briefly, HepaRG monolayers were kept statically for two weeks (the proliferation phase), then cultures were moved to a shaking incubator with 5, 25 or 60 rpm during the differentiation phase (the last two weeks of culturing). Hepatic functionality of different DMF-cultures was compared to HepaRG-Static cultures. S1 Fig) Ammonia elimination. S2 Fig) Urea production. S3 Fig) Lactate production.
**S4 Figure:** Higher resolution staining of albumin (green), and SOX9 (red), with DAPI counter-staining of the nuclei (blue) in HepaRG-Static, HepaRG-DMSO-Static and HepaRG-DMF cultures. The arrow indicates the nuclear translocation of SOX9 in HepaRG-Static, scale bar=50µm.
**Figure S5:** Higher resolution staining for CEBPα (red), with DAPI counter-staining of the nuclei (blue) in HepaRG-Static, HepaRG-DMSO-Static and HepaRG-DMF cultures. The arrow indicates nuclear translocation of CEBPα, observed in HepaRG-DMF, scale bar=50µm.
Part II

Lentiviral correction of critical hepatic functions of HepaRG cells
Chapter 6

Genome-wide expression profiling reveals increased stability and mitochondrial energy metabolism of the human liver cell line HepaRG-CAR

Submitted

Aziza A.A. Adam
Aldo Jongejan
Perry D. Moerland
Vincent A. van der Mark
Ronald P. Oude Elferink
Robert A.F.M. Chamuleau
Ruurdtje Hoekstra
Abstract

There is an increasing demand for a proliferative source of human hepatocytes that differentiate into highly functional hepatocytes. Human liver cell line HepaRG is a well-known source of hepatocytes which, however, displays limited biotransformation and a tendency to transform after 20 passages. The new HepaRG-CAR cell line overexpressing constitutive androstane receptor (CAR, NR1I3), a regulator of detoxification and energy metabolism outperforms the parental HepaRG cell line in various liver functions. To further characterize this cell line and assess its stability we compared HepaRG-CAR with HepaRG cells at different passages in terms of their expression profile, ammonia and lactate metabolism, bile acid and reactive oxygen species (ROS) production.

Transcriptomic profiling of HepaRG-CAR vs HepaRG early-passage revealed downregulation of hypoxia, glycolysis and proliferation and upregulation of oxidative phosphorylation genesets. In addition CAR overexpression downregulated the mTORC1 signaling pathway, which, as mediator of proliferation and metabolic reprogramming, may play an important role in the establishment of the HepaRG-CAR phenotype. The ammonia and lactate metabolism and bile acid production of HepaRG-CAR cells was stable for 10 additional passages compared to HepaRG cells. Interestingly, bile acid production was 4.5-fold higher in HepaRG-CAR vs HepaRG cells, whereas lactate and ROS production were 2.7- and 2.0-fold lower, respectively. Principal component analysis showed clustering of HepaRG-CAR (early- and late-passage) and HepaRG early-passage and not with HepaRG late-passage indicating that passaging exerted larger effect on the transcriptional profile of HepaRG than HepaRG-CAR cells. In conclusion, overexpression of CAR in HepaRG cells improves their bile acid production, mitochondrial energy metabolism, and stability, with the latter possibly due to reduced ROS production.

At this moment, the HepaRG-CAR cell line seems therefore the best available alternative to human hepatocytes.
Introduction

Highly differentiated human hepatocytes from proliferative sources are needed for hepatocyte-based *in vitro* models of human liver and for several clinical applications, such as Bio-artificial Livers (BALs). Yet, at present, hepatocytes deriving from different proliferative sources, as stem cells, induced pluripotent stem cells and liver cell lines, are lacking complex hepatic functions (1). The human liver cell line HepaRG displays a relatively high hepatic functionality and its transcriptome profile resembles that of primary human hepatocytes (PHHs) most closely compared to other proliferative sources of human hepatocytes (2, 3). During culturing, HepaRG cells progress in a period of four weeks from progenitor cells into a mixed population of hepatocyte-islands surrounded by cholangiocyte-like cells (4). In our lab, we successfully applied the HepaRG cell line as a biocomponent of the AMC-BAL (5). However, one of the major obstacles that limit the usage of HepaRG cells, is their tendency to transform after undergoing 20 passages from isolation with a split ratio of 1:5. The HepaRG cells then gradually lose their hepatic differentiation and acquire a mesenchymal phenotype with glycolysis-dependent energy metabolism, as evidenced by increased lactate production (6, 7). This instability represents a hurdle towards the long-term and large-scale usage of HepaRG cells, as required for BAL application. In addition, the biotransformation properties, also deemed essential for BAL application, although higher compared to other proliferative sources of human hepatocytes, are, in part, still limited in absence of dimethylsulfoxide (DMSO) (3, 8, 9). DMSO treatment increases biotransformation, but negatively affects cell viability and transcript levels of hepatic genes unrelated to biotransformation (10). To improve biotransformation properties without DMSO treatment, we recently established a new stable cell line, HepaRG-CAR, by lentiviral overexpression of the constitutive androstane receptor (CAR, NR1I3) in HepaRG cells (11). The resulting HepaRG-CAR cells exhibited increased biotransformation, including cytochrome P450 (CYP) activities and bilirubin conjugation. In addition, also albumin production, resistance to DMSO-induced toxicity, and NAD(P)H levels were elevated by CAR overexpression, however ammonia elimination remained unchanged (11).

To further investigate its potential as proliferative hepatocyte source we compared the transcriptional profile of the newly established HepaRG-CAR cell line and its parental cell line HepaRG using next generation RNA sequencing (RNA-seq) and assessed their stability upon serial passaging.

Materials and Methods

Cell culture

HepaRG cells were kindly provided by Biopredic International (Rennes, France). The HepaRG-CAR line was developed by stable lentiviral overexpression of the nuclear receptor CAR, as described
HepaRG and HepaRG-CAR cells were maintained in William’s E–based culture medium (HepaRG medium) in a humidized atmosphere of 95% air and 5% CO₂, as described (4, 10), and the medium was refreshed twice/week. To test the stability of the cells upon serial passaging, the cultures were propagated starting at passage 15 (P15) for HepaRG and P19 for HepaRG-CAR from isolation. The cultures were passaged at a regular 1:5 ratio once per two weeks, and for every two passages (passage 15, 17, 19, 21, 23, 25, 27, 29 and 31) cells were seeded in parallel in 12-well culture plates for testing functionality and obtaining RNA after four weeks culturing.

RNA isolation
Primary human hepatocytes (PHHs) were isolated from the healthy parenchyma in liver resection material from three patients, aged 40, 68 and 70, with liver adenomas or colorectal cancer metastases and no macroscopic signs of liver damage, by a modified 2-step collagenase perfusion technique, as described (12). Cells were snap-frozen directly after isolation and kept in liquid nitrogen until RNA isolation. The procedure was in accordance with the ethical standards of the institutional committee on human experimentation (protocol number 03/024) and the Helsinki Declaration of 1975. Total RNA was isolated using the RNeasy kit (QIAGEN) from the three PHH isolates and 12 samples of fully differentiated cultures, including three samples from HepaRG early-passage cultures (P15 and P16), three HepaRG late-passage cultures (P21), three HepaRG-CAR early-passage cultures (P17) and three HepaRG-CAR late-passage cultures (P22), for more information refer to Table S1. The quality of the RNA was assessed by Agilent RNA 6000 Nano-Bioanalyzer. Samples with RNA integrity number above 7 were used for RNA-seq (all samples).

cDNA preparation and RNA-seq
A cDNA library was prepared from ribosomal-depleted RNA (50 ng input/sample) according to the Ovation® RNA-Seq System V2 kit (Nugen) protocol. Next, the cDNA was fragmented, blunt ended, ligated to indexed (barcoded) adaptors and amplified with PCR using the Ovation® Ultralow System V2 kit (Nugen) according to manufacturer’s protocol. Prior to RNA-seq analysis, the final library size distribution was determined using Agilent Bioanalyzer 2100. Fifteen cDNA libraries were prepared with one library per RNA sample. Next, all cDNA libraries were pooled and single-end sequenced (50 nucleotides) on two lanes of the Illumina HiSeq4000 platform.

RNA sequencing data analysis
Raw sequencing data were subjected to quality control using FastQC and trimmed using Trimmomatic (v0.32). Reads were aligned to the human reference genome (hg38) using HISAT2 (v2.0.4). Gene level counts were obtained using HTSeq (v0.6.1) and the human GTF (gene transfer format) file from Ensembl (release 85). Samples from a different well, but from the same cell line, seeded from the same culture were considered to be technical replicates and their counts were summed, therefore (n=1 or 2)/group, refer to Table S1. One of the PHHs sample was excluded,
as it clearly exhibited a cancerous rather than hepatic transcriptional profile. Based on principal component analysis (PCA), one of the HepaRG-CAR late samples was identified as outlier and therefore excluded from downstream analysis. Statistical analyses were performed using the edgeR and limma R (v.3.4.1) and Bioconductor (v3.5) packages. Genes with more than one count in one or more samples were retained. The two most abundant genes (MT-RNR1 and MT-RNR2) were removed in order to stabilize the scaling factors. Count data were transformed to log2-counts per million (logCPM), normalized by calculating scaling factors using the trimmed mean of M-values method and precision weighted using voom. Differential expression was assessed using an empirical Bayes moderated t-test within limma’s linear model framework including the precision weights estimated by voom. Resulting P-values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate (FDR). Additional gene annotation was retrieved from Ensembl (release 91) using the biomaRt R/Bioconductor package. PCA was performed on the logCPM values of the 5000 most variable genes (function prcomp). The variance explained by the first two principal components was calculated as percentage of the total variance. Geneset enrichment analysis was performed using CAMERA (limma package) with preset value of 0.01 for the inter-gene correlation using the Hallmark, C1, C2, C3, C5, C6 and C7 geneset collections retrieved from the Molecular Signatures Database (v6.0; Entrez Gene ID version). P-values were calculated for each geneset for two alternative hypotheses ('up' or 'down') and adjusted using the Benjamini-Hochberg FDR. Geneset variation analysis (GSVA) was performed using the GSVA package. Sample-specific geneset enrichment scores calculated by GSVA were clustered using Euclidean distance and complete linkage as agglomeration method (function hclust).

**Quantitative reverse transcription PCR (RT-qPCR)**

Quantitative RT-PCR was performed as previously described (13, 14). Transcript levels were normalized for 18S ribosomal RNA and expressed as a % of the average of two human liver samples. Primer sequences and amplicon sizes are given in Table S2.

**Hepatic function test**

HepaRG and HepaRG-CAR fully-differentiated monolayer cultures of different passages were tested in 12-well plates (Corning) for their functionality, as described (14). Briefly, cultures were exposed to 1.5 mL test medium based on HepaRG medium supplemented with 1 mM carbamoyl glutamate, the allosteric activator of the urea cycle enzyme carbamoyl phosphate synthase 1, 1.5 mM NH₄Cl, 2.27 mM D-galactose, 2 mM L-lactate and 125 μM testosterone (all compounds from Sigma Aldrich). During the function test, medium samples were taken at 0.75 h (0.5 mL) and at 24 h in which L-lactate, ammonia and bile acids were measured, as described (15). At termination, the cells were washed with phosphate buffered saline and total protein/well was determined, as described (15). Metabolic activities were calculated on basis of the changes in concentration in medium in time, normalized to the protein content per well.
Mitochondrial superoxide detection (MitoSOX)
MitoSOX based flow cytometric assay was used to detect mitochondrial superoxide in 4-week cultures of low-passage HepaRG and HepaRG-CAR cells in 24-well plates. MitoSOX targets to mitochondria, where oxidation by superoxide results into red fluorescence (16). The cells were incubated with 250 µL of Hanks’ balanced salt solution (Gibco) containing 10 mM HEPES (pH7.4, Sigma) and 5 µM freshly prepared MitoSOX (Thermo Fisher) for 0.5 h. Subsequently, the cells were trypsinized and fluorescence was analyzed by flow cytometry.

Statistical analysis
We performed one-way ANOVA test with Dunnett’s post hoc test to compare the mean of the baseline passage of either HepaRG (P15) or HepaRG-CAR (P19) with the mean of every other passage analyzed within the same cell line. Furthermore, Student’s t-test was applied to compare the corresponding passages (P19-P25) in HepaRG vs HepaRG-CAR. Analyses were performed with Prism version 7 (GraphPad Prism Inc). Data are expressed as mean ± SD; P-value < 0.05 was considered as significant.

Results
HepaRG-CAR cell line exhibits more stable hepatic functionality upon passaging
To compare the stability of the HepaRG and HepaRG-CAR cell lines, the cultures were passaged every two weeks, and their hepatic functionality was assessed once every two passages. HepaRG cultures showed gradual morphological changes over passaging. At early passage (P15) the cultures displayed well-delineated hepatocyte-islands surrounded by flat cholangiocyte-like cells (Fig. 1A). The island structure was partially maintained after five passages (P20) (Fig.1B), and was totally disrupted at P25 (Fig. 1C), and the cell size was reduced. In contrast, the island structure of HepaRG-CAR cultures showed no evident changes up to P30 (Fig. 1D-F), indicating increased morphological stability upon serial passaging at least up to 10 passages above the critical passage P20 of HepaRG cells.

Furthermore, we compared functionality and total protein content of HepaRG and HepaRG-CAR monolayers upon passaging. Total protein content/well exhibited an increasing trend in both cell lines upon passaging (Fig. 1G). At P25, the total protein content was 1.6-fold higher in HepaRG vs HepaRG-CAR cells. At late passages (P27-P31), the total protein content/well was significantly increased in the HepaRG-CAR line compared to baseline level. Ammonia elimination, which is a hepatocyte hallmark function, severely deteriorated with the passaging of HepaRG cells and converted into marginal ammonia production at P25 (Fig.1H). In contrast, ammonia elimination by HepaRG-CAR cells displayed more stable profile through all tested passages, except for P21
Figure 1: HepaRG-CAR morphology and functions are more stable upon passaging. A-F) Morphology. Scale bar= 100 µM, G) Protein content/well, H) Ammonia elimination, I) Lactate production, J) Bile acid production, (n=3-9/1-3 independent experiments). Significance is indicated by # vs HepaRG P15, * vs HepaRG-CAR P19 and & vs HepaRG at the same passage.

which was 2.1-fold improved vs baseline (P19) level. Notably, ammonia elimination by HepaRG-CAR cells was exceeding the level of HepaRG cells starting from P21. Similarly, bile acid synthesis, another essential hepatocyte function, declined 3.9-fold during the passaging of HepaRG cells
from P15 until P25 (Fig. 1I) and was stable in HepaRG-CAR cells. In addition, bile acid synthesis was 4.5-fold higher in HepaRG-CAR cells vs HepaRG cells at P19. Lactate production, as a measure of mitochondrial dysfunction, raised gradually in HepaRG cells up to 2.8-fold difference at P25 vs P15 (Fig. 1J). Again, HepaRG-CAR cells exhibited a stable lactate production during passaging. At P19 the lactate production in HepaRG-CAR cells was 2.7-fold lower when compared to P19 HepaRG cultures, suggesting that HepaRG-CAR cells possess an enhanced and sustainable mitochondrial function.

Collectively, these results show that HepaRG cultures lose their hepatic functionality upon passaging whereas HepaRG-CAR cells display a stable phenotype for 10 passages above the critical P20 in the parental cells and also show improved basal bile acid production and lactate metabolism, whereas ammonia clearance was comparable to HepaRG cells.

**The effect of passaging on the transcriptional profile of HepaRG-CAR cells is limited**

To investigate the molecular background of the increased hepatic differentiation and stability of HepaRG-CAR cells, RNA-seq was performed of the HepaRG and HepaRG-CAR fully differentiated cultures at different passages (early, *i.e.* P15-P17 vs late, *i.e.* P21-P22) and their gene expression profiles were compared with those of two PHH isolates that served as gold standard. PCA revealed that the expression profiles of HepaRG and HepaRG-CAR cells, regardless of the passage number, were different from those of PHHs (Fig. 2A). Interestingly, expression profiles of HepaRG-CAR (early- and late-passages) cells were similar and clustered to some extent with those of the HepaRG early-passage cells, and deviated considerably from those of HepaRG late-passage cells (Fig. 2A).

Next, we identified differentially expressed genes (DEGs) between HepaRG and HepaRG-CAR cells. There were 171 upregulated genes and 209 downregulated genes between HepaRG vs HepaRG-CAR at early-passage (Fig. 2B). Analysis of DEGs as a measure of the passaging effect on gene expression, revealed far less changes in HepaRG-CAR late- vs early-passage with only 93 upregulated and 344 downregulated genes, compared to HepaRG late- vs early-passage with 870 and 929 genes up- and downregulated respectively (Fig. 2B).
Figure 2: The effect of passaging on the transcriptome of the HepaRG-CAR cell line is limited

A) Principal component analysis of gene expression data. Each symbol represents an individual sample. PC1 and PC2 indicate principal components 1 and 2. B) Venn diagram of DEG, cut-off P-value 0.01 (not adjusted). Numbers shown in the center of the Venn diagram represent the significantly upregulated (red) and downregulated (blue) DEG; the total number of up- and downregulated genes included in the analysis is depicted in the lower right corner.
Upon passaging, the transcriptional profile of HepaRG cells shifts towards dedifferentiation and cell proliferation with enhanced hypoxia

Geneset enrichment analysis was employed to identify genesets enriched in DEGs. Tables S2-S6 show the results of the CAMERA analysis covering the full spectrum of genesets for all comparisons. To reduce overlap and cover a wide range of biological processes, here we discuss the results for the Hallmark genesets include 50 genesets derived from the Molecular Signature Database (MSigDB) (17), and the HSIAO liver-specific geneset, which contains 238 liver-specific genes (18), in more detail. Furthermore, we visualized the relatedness of the different groups for ten selected discriminative genesets involved in energy metabolism, hepatic differentiation and cell proliferation in a heatmap (Fig. 3). These genesets were selected from differentially regulated Hallmark genesets of early- and late-passages from both HepaRG and HepaRG-CAR cells and PHHs. Among the top 19 differentially regulated (FDR<0.1) genesets, there were 18 upregulated genesets and only one geneset that was downregulated in HepaRG early-passage vs HepaRG-CAR early-passage, (Table 1). Among the upregulated genesets, six genesets were related to cell proliferation and four genesets were involved in inflammatory response. Interestingly, hypoxia and glycolysis Hallmark genesets were upregulated, whereas oxidative phosphorylation (OxPhos) was downregulated in HepaRG early-passage vs HepaRG-CAR early-passage (Fig. 3). This, together with the relatively high lactate production suggests that HepaRG cells highly depend on glycolysis to obtain their energy, whereas HepaRG-CAR cells are more dependent on mitochondrial energy metabolism. Despite the improvement of some hepatic functions (albumin synthesis, bile acid synthesis, lactate metabolism, and, in part, biotransformation) (11), neither the HSIAO liver-specific geneset (FDR=0.41), nor Hallmark genesets related to hepatic functions were overrepresented in early passages of HepaRG-CAR vs HepaRG cells.
Table 1: Top differentially regulated genesets (19 genesets) in HepaRG early-passage vs HepaRG-CAR early-passage.

<table>
<thead>
<tr>
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Furthermore, to assess the degree of hepatic and metabolic competence of HepaRG cells, we compared the transcriptional profile of HepaRG early-passage to that of PHHs. In the top 20 differentially regulated genesets, 15 and five genesets were up- and downregulated, resp. (Table 2). Nine of the 15 upregulated genesets were involved in cell proliferation. Other upregulated genesets appearing in the top 20 list were related to unfolded protein response, protein secretion and UV response. The five downregulated genesets were all associated to liver functions, including the HSIAO liver-specific geneset, the xenobiotic detoxification, fatty acid oxidation, bile acid metabolism and coagulation genesets. This outcome indicates the lower hepatic differentiation grade of HepaRG cells compared to PHHs.
The top 20 differentially regulated genesets in HepaRG-CAR early-passage vs PHHs was highly similar to that of the HepaRG early-passage vs PHH comparison with 15 upregulated genesets including nine related to cell proliferation and the same five downregulated genesets (Table 3). In line with the observation that HepaRG-CAR cells show relatively high lactate metabolism, the OxPhos was more induced in HepaRG-CAR early-passage than in PHHs (Table 3 & Fig. 3), however, it should be noted that PHHs replicates displayed a variable OxPhos profile, which complicates the interpretation of the heatmap (Fig. 3). In brief, these data show that the hepatic differentiation of HepaRG-CAR cells is also still underdeveloped when compared to PHHs.

<table>
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<th>Gene set</th>
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<th>Direction</th>
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Table 2: Top 20 differentially regulated genesets in HepaRG early-passage vs PHHs.
To study the effect of passaging on the transcriptional profile of HepaRG and HepaRG-CAR cells, we compared the transcriptional profile of the Hallmark genesets and HSIAO liver specific-geneset of early-passage to that of late-passage within the same cell line.

In HepaRG late-passage vs HepaRG early-passage, only 16 differentially regulated genesets, with FDR < 0.1, were identified, including five upregulated and 11 downregulated genesets (Table 4). Three of the upregulated Hallmark genesets were linked to cell cycle and proliferation (3/5), in agreement with the trend of increased protein synthesis at late passages. The same liver function-associated genesets differentially regulated between early passages of HepaRG and HepaRG-CAR vs PHHs were downregulated in the top differentially regulated genesets of HepaRG late-passage vs HepaRG early-passage, indicating loss of hepatic differentiation upon passaging of HepaRG cells, which was further confirmed by upregulation of the epithelial-mesenchymal transition geneset. In line with increased lactate production upon passaging, the bioenergetics profile of HepaRG cells was shifted to suppression of OxPhos and induction of hypoxia related genesets. The shift in
energy metabolism by decreasing the OxPhos activity was further reflected by the downregulation of the geneset related to reactive oxygen species (ROS) (Fig. 3). Furthermore, two immunity-related, interferon-response genesets were downregulated upon passaging of HepaRG.

<table>
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<th>Gene set</th>
<th>N Genes</th>
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<td>3.43E-02</td>
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There were only eight differentially regulated genesets in HepaRG-CAR late-passage vs HepaRG-CAR early-passage of which six were upregulated and two downregulated (Table 5). All of the six upregulated genesets were related to cell proliferation, compatible with the increased protein synthesis at later passage. Interestingly, upon passaging of HepaRG-CAR cells the Hallmark geneset epithelial mesenchymal transition was downregulated, and there was no effect on hepatic differentiation as the transcriptional profile of the HSIAO liver-specific-geneset was not significantly altered (FDR= 0.43), and none of the genesets associated to hepatic functions appeared in the top differentially regulated genesets. In addition, the angiogenesis process, which is critically important for cancer cells to meet their increasing demand for nutrient supply (19-21), was downregulated upon the passaging of HepaRG-CAR cells. These results are pointing to a more stable hepatic and energy metabolic profile of HepaRG-CAR cells upon passaging compared to HepaRG cells, in line with the functional data.
Table 5: Top differentially regulated genesets (8 genesets) in HepaRG-CAR late-passage vs HepaRG-CAR early-passage.

<table>
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<td>1.02E-08</td>
</tr>
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<td>Up</td>
<td>1.20E-08</td>
</tr>
<tr>
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<td>198</td>
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<td>1.52E-05</td>
</tr>
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<td>HALLMARK_DNA_REPAIR</td>
<td>147</td>
<td>Up</td>
<td>1.54E-05</td>
</tr>
<tr>
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<td>58</td>
<td>Up</td>
<td>3.45E-03</td>
</tr>
<tr>
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<td>6.31E-02</td>
</tr>
<tr>
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<td>35</td>
<td>Down</td>
<td>9.23E-02</td>
</tr>
<tr>
<td>HALLMARK_MTORC1_SIGNALING</td>
<td>199</td>
<td>Up</td>
<td>9.55E-02</td>
</tr>
</tbody>
</table>

The heatmap (Fig. 3), confirms the general picture of a sustained stability of HepaRG-CAR cells upon passaging, in contrast to the parental HepaRG cells, where passaging exerted alterations in favor of loss of differentiation and epithelial-mesenchymal transition. In agreement with the PCA analysis (Fig. 2), the heatmap showed a clear clustering of the HepaRG-CAR cells (regardless of passaging) and the HepaRG early-passage, whereas the HepaRG late-passage cells clustered separately. Furthermore, CAR overexpression shifted the transcriptional profile of HepaRG cells from glycolysis towards mitochondrial energy metabolism, however, the effect on hepatic differentiation at transcript level seems low, resulting in a considerable gap in hepatic differentiation between HepaRG±CAR cells and PHHs.

In contrast to HepaRG, HepaRG-CAR cells produce less ROS

Oxidative stress is a driving force in aging processes, as well as diverse liver pathologies (22,23). Oxidative stress reflects the balance between the generation of ROS, as byproduct of aerobic energy metabolism and the antioxidant system which detoxifies these reactive molecules (24-25). Important enzymes involved in ROS detoxification include superoxide dismutase (SOD) 1 and 2 and catalase (CAT) (26-28).

We tested whether the difference in stability between HepaRG and HepaRG-CAR may be associated with oxidative stress changes. Interestingly, the transcript levels of CAT and SOD1 were significantly higher in HepaRG-CAR vs HepaRG cells at early-passage, whereas SOD2 showed a positive trend (Fig. 4A). Furthermore, mitochondrial superoxide level was 2.0-fold lower in HepaRG-CAR vs HepaRG cells (Fig. 4B), indicating that HepaRG-CAR cells are less exposed to ROS than HepaRG cells.
Figure 3: Upon passaging, the transcriptional profile of HepaRG cells shifts towards dedifferentiation and cell proliferation with enhanced glycolysis. Heatmap of the sample-specific geneset enrichment scores determined by CAMERA analysis on selected genesets from top altered (FDR < 0.1) Hallmark genesets and the HSIAO liver-specific geneset of different comparisons.

Figure 4: HepaRG-CAR cells produce less ROS. A) The transcript levels of antioxidant genes expressed as a % of human livers. Also shown is the level of 18S ribosomal RNA, which was not changed in HepaRG-CAR line vs HepaRG cells and was comparable to human liver level, and was used as reference gene for normalization of the RT-qPCR data (29). B) ROS production (n=4/2 exp). Data was represented as mean ± SD; P-value < 0.05 was considered as significant.
Discussion

There is an increasing demand for terminally-differentiated hepatocyte cultures to function as a hepatocyte-based in vitro model for human liver as well as biocomponent of BALs. The most functional proliferative source of human hepatocytes to date is the human liver cell line HepaRG, which shows a relatively broad spectrum of liver functions (4, 10). In this study we demonstrate that, compared to the parental HepaRG cell line, the newly developed HepaRG-CAR cell line displays higher mitochondrial function, and, in part, higher hepatic differentiation, as well as increased stability upon passaging, and therefore outperforms the parental HepaRG cell line as proliferative source of human hepatocytes.

Previously it was established that HepaRG-CAR cells showed increased biotransformation, particularly at activity level, and albumin production compared to HepaRG cells (11). The current study additionally shows increased mitochondrial function, as reflected by lower lactate production, and also a higher bile acid production. Accordingly, the RNA-seq expression analysis confirmed the induction of OxPhos in HepaRG-CAR vs HepaRG cells whereas glycolysis, hypoxia and proliferation processes were downregulated.

CAR is implicated not only in biotransformation through regulating the transcript level of a large array of genes involved in biotransformation, however, it also is involved, among others, in bile acid synthesis and in energy homeostasis by governing lipogenesis and gluconeogenesis (30-33). The upregulation of bile acid synthesis is in line with our previous findings that several CYP enzymes are induced at transcript and/or activity level in HepaRG-CAR cells (11), as CYP enzymes are involved in the synthesis of bile acids from cholesterol (32-34).

Several other studies suggested CAR as a critical transcription factor for regulating hepatocyte differentiation. CAR forms a transcriptional regulatory network with other nuclear receptors that are involved in hepatic differentiation, as hepatic nuclear factors 1 and 4 (35). Chen et al, demonstrated that lentiviral CAR-overexpression promotes the differentiation and maturation of human embryonic stem cells into hepatocyte-like cells (36). CAR further stimulates hepatic differentiation indirectly by promoting the synthesis of bile acids which, on their turn, activate the farnesoid X receptor and small heterodimer partner (FXR/SHP) signaling axis (37-39). Godoy et al, indicated FXR, CAR, pregnane X receptor and hepatic nuclear factor 1 as key transcription factors of a cluster of hepatic genes with low, deregulated, expression levels in stem cell-derived hepatocyte like cells and dedifferentiated PHHs relative to fully differentiated PHHs (40, 41). The genes overrepresented in this cluster were involved in biotransformation processes, most often CYPs (40). These findings were confirmed by proteomic analyses showing that PHH dedifferentiation is particularly associated with changes in CYP levels (42). Interestingly, the other group of proteins associated with PHH dedifferentiation comprised of mitochondrial proteins. Both groups of proteins are, at least partly, under transcriptional control of CAR, therefore it is likely that CAR upregulation will inhibit the dedifferentiation of PHHs.
The mechanistic processes downstream of CAR in regulating energy homeostasis are still under investigation. These studies are complicated by species-specific differences between rodent and human CAR for their gene targets and mode of activity (43, 44). Highly intriguing, however, is the new finding that the signaling of the mammalian target of rapamycin complex 1 (mTORC1) is downregulated in HepaRG-CAR vs HepaRG cells, as well as the Myc target genesets (Table 2). The mTORC1 complex is a nutrient sensor that, through activation of c-myc, drives cancer progression and metabolic reprogramming, characterized by upregulation of glycolysis, despite the presence of sufficient oxygen, and simultaneously limiting OxPhos, a phenomenon known as the Warburg effect (45-47). In line with this, our study shows that CAR overexpression is associated with the reversion of the Warburg effect in HepaRG cells, with downregulation of glycolysis and upregulation of OxPhos genesets, which may be mediated by the mTORC1 pathway through c-myc. Interestingly, Parent et al. showed that sustained mTOR activity induced a preneoplastic phenotype to HepaRG cells by altering the translation of genes vital for establishing normal hepatic energy homeostasis and moderating hepatocellular growth (48). Moreover, these neoplastic changes were reversible upon administration of the classic mTORC1 inhibitor rapamycin.

In parallel, mTORC1 signalling and c-myc, being well-known for their tumor-promoting effects, may play an important role in the transformation of HepaRG cells normally occurring at P20, and characterized by morphological changes, decreased ammonia elimination and bile acid synthesis and increased lactate production, which is inhibited in HepaRG-CAR cells. Changes in expression profile associated with the transformation in HepaRG cells included downregulation of genesets associated with hepatic functions and induction of epithelial mesenchymal transition and hypoxia. Moreover, several cell proliferation related genesets were induced, further confirming the epithelial mesenchymal transition. These manifestations, also described in (41, 42), as associated with the dedifferentiation of PHHs in culture, are characteristic for the Warburg-effect (46, 47).

Thus far, there are no studies about the mechanism governing the dedifferentiation process in HepaRG cells. However, several studies demonstrated the involvement of oxidative stress in pathological mechanisms of a vast range of liver pathologies (25), as well as mitochondrial dysfunction and the Warburg effect (46, 47). Oxidative stress is caused by high levels of ROS, the net result of production through oxidative phosphorylation and scavenging by antioxidant activity. The production of ROS may be increased in HepaRG-CAR cells, due to its increased mitochondrial energy metabolism. However, and possibly due to the induction of antioxidant enzymes, the net amount of ROS was reduced to 50% of the level of HepaRG cells. Given the pronounced effect on ROS production and the relatively limited upregulation of SOD1 and CAT mRNA, posttranslational modifications of these antioxidant enzymes or other antioxidant systems may also be involved (49-51). The relatively low ROS levels may, at least, partly attribute to HepaRG-CAR cells long-term stability. This finding seems in conflict with the downregulation of the ROS-related geneset in
HepaRG cells late vs early-passage, which was accompanied by the drastic loss of mitochondrial energy metabolism as shown by the suppression of genesets related to OxPhos and fatty acid metabolism and the increased production of lactate. The level and role of ROS may be dynamically regulated during passaging and may differ for both cell lines, which needs to be further analyzed. In addition, the role of mTORC1 signaling should be further analyzed. mTORC1 may play an essential role in the transformation of HepaRG cells, as inhibition of mTORC1 has been shown to delay age-related diseases, due to stimulation of autophagy and possibly modulation of immune responses (52, 53).

The transcriptional profile of HepaRG cells early-passage was dissimilar from that of the PHHs, in line with our previous study (54). The transcriptional profile of HepaRG-CAR cells, regardless of the passage number, clustered closely with HepaRG cells at early-passage and differed from that of PHHs. Interestingly, the cellular bioenergetics of HepaRG-CAR cells (regardless of passage number) was shifted towards the PHHs profile with the induction of OxPhos and inhibition of hypoxia and glycolysis Hallmark genesets. Yet, when related to PHHs, xenobiotic detoxification, bile acid metabolism and HSIAO liver specific-geneset were downregulated in HepaRG-CAR cells, indicating that there is still room for further improvement to advance their hepatic differentiation. However, the relatively high heterogeneity in the transcriptional profile of the PHH replicates, complicates the interpretation of the transcriptional data in relation to PHHs. This may be, in part, related to the optional dedifferentiation of the PHHs that involves down-regulation of important liver-enriched transcription factors and subsequently loss of hepatic differentiation (55). It is widely believed that the dedifferentiation process is initiated, as early as, during hepatocyte isolation phase and it progressively continues during their in vitro cultivation (55-57). To limit misinterpretation of transcriptional data, due to this optional PHH dedifferentiation, we used isolated PHHs without in vitro cultivation as reference material. Yet, the PHH replicates displayed substantial variations in their transcriptional profile, particularly for genesets related to OxPhos and liver-related functions, which may be related to biological variation between patients. The observed upregulation of hepatic functions, including albumin and bile acid synthesis, as well as a range of biotransformation activities in HepaRG-CAR cells vs HepaRG cells (11), seems in conflict with the lack of upregulated genesets associated with hepatic differentiation in the transcriptome analysis. This suggests that the effects of CAR overexpression most probably are mediated at posttranscriptional level. The shift in energy metabolism by CAR overexpression may modulate hepatic functions due to alteration in metabolite spectrum that governs the flux rates of many metabolic pathways (58) and to increased fluxes of energy-consuming processes, such as biotransformation. Moreover, the accumulation of metabolites normally eliminated by the mitochondria, such as lactate, will be inhibited, which may improve hepatic functionality (59). Furthermore, CAR overexpression enhances the mRNA expression of CYP450 oxidoreductase (POR) (11), which mediates the electron transfer required for P450 activity (60). Therefore, the upregulation
of POR may contribute at posttranscriptional level to the increased biotransformation activity of HepaRG-CAR. It will be, however, difficult to distinguish between the different players that may posttranscriptionally increase the hepatic functionality of HepaRG cells upon CAR overexpression, due to interconnections between the processes regulated by the concentration of metabolites and energy derivatives.

Given the advanced biotransformation properties, mitochondrial functions and the sustained stability, the HepaRG-CAR cell line provides an attractive alternative to PHHs for BAL application. For support of patients with end-stage liver failure it is estimated that a functional liver mass of 150 gram (1.10^9 cells) is required (61). This implies that at least 11 passages from the first single HepaRG cell are needed to load a single BAL. Therefore, the extended stability of the HepaRG-CAR cells upon passaging is essential for BAL application. Other applications requiring human liver cells, as pre-clinical studies in drug development and research on liver functions and infections, require substantial less cells, however, also for those applications, the highest hepatic functionality is required, and therefore also in those cases the HepaRG-CAR cells are the best available alternative to PHHs.

**Acknowledgement**

The authors would like to thank Erik Hendriks for cell culturing.
References


Supplementary information

Table S1: Details of the isolated PHHs and monolayer cultures used for the RNA-seq analysis. In this table the culture line and passage number of HepaRG and HepaRG-CAR cultures are given, together with the new ID of the replicates and total number of exonic reads per sample (after filtering).

<table>
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<tr>
<th>Sample</th>
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<th>Passage</th>
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<th>Exonic reads</th>
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NA; not applicable
Table S2: Primers used in PCR analyses and amplicon size.

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Overexpression of carbamoyl-phosphate synthase 1 significantly improves ureagenesis of human liver HepaRG cells only when cultured under shaking conditions


Aziza A.A. Adam
Vincent A. van der Mark
Jos P.N. Ruiter
Ronald J.A. Wanders
Ronald P.J. Oude Elferink
Robert A.F.M. Chamuleau
Ruurdtje Hoekstra
Abstract

Hyperammonemia is an important contributing factor to hepatic encephalopathy in end-stage liver failure patients. Therefore reducing hyperammonemia is a requisite of bioartificial liver support (BAL).

Ammonia elimination by human liver HepaRG cells occurs predominantly through reversible fixation into amino acids, whereas the irreversible conversion into urea is limited. Compared to human liver, the expression and activity of the three urea cycle (UC) enzymes carbamoyl-phosphate synthase1 \((CPS1)\), ornithine transcarbamoylase \((OTC)\) and arginase1, are low. To improve HepaRG cells as BAL biocomponent, its rate limiting factor of the UC was determined under two culture conditions: static and dynamic medium flow (DMF) achieved by shaking. HepaRG cells increasingly converted escalating arginine doses into urea, indicating that arginase activity is not limiting ureagenesis. Neither was OTC activity, as a stable HepaRG line overexpressing OTC exhibited a 90- and 15.7-fold upregulation of \(OTC\) transcript and activity levels, without improvement in ureagenesis. However, a stable HepaRG line overexpressing CPS1 showed increased mitochondrial stress and reduced hepatic differentiation without promotion of the \(CPS1\) transcript level or ureagenesis under static-culturing conditions, yet, it exhibited a 4.3-fold increased ureagenesis under DMF. This was associated with increased \(CPS1\) transcript and activity levels amounting to > 2-fold, increased mitochondrial abundance and hepatic differentiation. Unexpectedly, the transcript levels of several other UC genes increased up to 6.8-fold. We conclude that ureagenesis can be improved in HepaRG cells by CPS1 overexpression, however, only in combination with DMF-culturing, suggesting that both the low CPS1 level and static-culturing, possibly due to insufficient mitochondria, are limiting UC.
Introduction

The metabolism of ammonia, a toxic by-product of the breakdown of proteins, is one of the main functions of hepatocytes. Ammonia elimination is regarded as a critical feature of the Bioartificial Liver (BAL), which implies that the hepatocytes incorporated into the bioreactor must be capable of converting ammonia into urea in order to bridge the gap to liver transplantation or liver regeneration for patients with end-stage liver failure. This is all the more relevant since hyperammonemia is an important contributing factor to hepatic encephalopathy (1).

The two classic routes of hepatic ammonia metabolism include irreversible conversion into urea by means of the urea cycle (UC) and reversible fixation into amino acids, particularly the formation of glutamine from glutamate, through the activity of glutamine synthetase (GS), reviewed in (2). Recent updates revealed the intimate interaction between, on one hand, glutamine synthesis in peripheral tissues and pericentral hepatocytes, and, on the other hand, glutamine catabolism through glutaminase (GLS) activity in the periportal hepatocytes where UC activity is highest, thus ensuring efficient ammonia detoxification (3). Ammonia can also be released from glutamine to the circulation yielding glutamate by the activity of GLS enzymes. There are two major GLS isoforms; GLS1, which is mainly expressed in kidney cells, and the hepatic GLS2 isoform (4). The UC is the only metabolic pathway to definitively dispose excess ammonia, and consists of five enzymatic reactions, two catalyzed by mitochondrial enzymes: carbamyl phosphate synthase I (CPSI) and ornithine transcarbamylase (OTC), and three by cytosolic enzymes: argininosuccinate synthase (ASS), argininosuccinate lyase (ASL) and arginase1 (ARG1). ARG1 catalyzes the last step yielding urea and ornithine through hydrolytic breakdown of arginine. It should be noted however, that the same reaction can also be catalyzed by an extra-hepatic mitochondrial isoform, i.e. ARG2 (5, 6).

Several factors are involved in the regulation of the UC activity at different levels in healthy subjects. The regulation of UC takes largely place at the transcriptional level by the action of transcription factors, including CCAAT/enhancer-binding protein (CEBPa/β), members of the hepatic nuclear factor (HNF) family, (HNF1, HNF3, HNF4) and the glucocorticoid receptor. These regulators respond to the amino acid balance, insulin, glucocorticoids, as well as cAMP (7), and directly interact with the regulatory regions of UC genes (8, 9). In addition, CPS1 requires the presence of the allosteric activator N-acetylglutamate (NAG), a product of N-acetylglutamate synthase (NAGS) (10). Moreover, mitochondrial carriers, such as the ornithine transporter (ORNT), which internalizes ornithine into mitochondria in exchange for citrulline which is then exported to the cytosol, and the mitochondrial glutamate-aspartate antiporter, play an essential role in maintaining the fluxes between the mitochondrial and cytosolic parts of the UC (11, 12).
Although primary human hepatocytes (PHHs) display high UC activity, these cells are scarce and subjected to rapid dedifferentiation, limiting their in vitro applicability (13). Other sources of hepatocytes, including hepatoma-derived cell lines like HepG2 and C3A (a subclone of HepG2), are capable of ureagenesis, but since these cells produce ammonia instead of eliminating it the urea production is mainly related to ARG2 activity and it is not driven by the UC per se (14-16). Other proliferative sources of hepatocytes, as induced pluripotent hepatocytes or stem-cell derived hepatocytes vary in their maturation and the level of nitrogen metabolism, reviewed in (17). In some cases ammonia elimination is reported, as in stem-cell derived hepatocyte spheroids, however, these studies lack an assessment of the contribution of UC and fixation into amino acids to ammonia elimination (18). To our knowledge the proliferative cell source of hepatocytes with the highest ammonia elimination is the hepatoma cell line HepaRG. Indeed, the capacity of HepaRG cells to eliminate ammonia approaches that of PHHs (19). HepaRG cells progress within four weeks of culturing from liver progenitor cells into a mixed population of hepatocyte- and biliary-like cells (20). Also other hepatic functions of the HepaRG monolayers are in a wide range comparable to that of PHHs (21), and a vast proportion significantly further increases under mitochondria-stimulating conditions, such as in the AMC-BAL and in monolayers maintained under shaking conditions during the differentiation phase, referred to as dynamic medium flow (DMF) (22, 23).

Currently, the HepaRG cell line is used as the biocomponent of the AMC-BAL (24). For BAL application ammonia elimination should preferably occur irreversibly through ureagenesis, followed by urea excretion by the kidneys to limit the risk of consecutive ammonia production from glutamine by GLS activity. Yet, ammonia metabolism through UC is limited in HepaRG cells. Indeed, the transcript levels of three UC genes, i.e. CPS1, OTC and ARG1, are relatively low when compared to human livers amounting to 31%, 6% and 15% , respectively (19), which results in low enzyme activity levels of 3.4%, 9.6% and 4.6% of human liver homogenates, respectively (25), whereas the transcript levels of ASL and ASS are relatively high reaching 56% and 81% human livers, respectively (19). Moreover, NAGS activity is also insufficient, which can be circumvented by adding carbamoyl-glutamate (CAG), an analogue of NAG, to the medium to activate CPS1 (19, 26). By measuring $^{15}$N-urea production after loading with $^{15}$N-ammonia it was also established that only 3% of the urea was produced via UC (16), and the majority was produced independent of UC activity.

The aim of this study was to determine the limiting factor of UC in HepaRG cells, to restore the UC, and, this way, improve their potential for clinical BAL application. To this end, we studied the effect of arginine overloading and overexpression of OTC and CPS1 in HepaRG monolayers. Furthermore, the CPS-overexpressing HepaRG cell line and its parental HepaRG cell line were tested under static and DMF culture conditions.
Materials and Methods

HepaRG monolayer culture
HepaRG cells were kindly provided by Biopredic International (Rennes, France). HepaRG cells were maintained in William’s E–based culture medium (HepaRG medium), as described (20, 27). For testing, cells were seeded in 12-well plates (Corning) and kept for 4-5 weeks in an humidified atmosphere of 95% air and 5% CO₂. Static cultures were maintained statically for the whole culture period and DMF cultures were kept statically for the first two weeks (proliferation phase), followed by three weeks shaking at 60 revolution per minute.

Lentiviral Vector Production
For overexpression of CPS1 and OTC, 3rd generation, self-inactivating lentiviral plasmids pLV-CPS1 and pLV-OTC were produced and sequence-verified by Tebu-Bio/Genocopoeia (USA). These lentiviral plasmids encompassed either human CPS1, transcript variant 1, cDNA (NM_001122633.2) or OTC cDNA (NM_000531.5) under CMV promoter control with ampicillin- and puromycin-resistance cassettes for stable selection in bacterial or mammalian cells. For producing lentiviral vector particles, HEK 293T monolayers were transfected using polyethylenimine either with LV-CPS1 or LV-OTC together with packing elements (pVSVg, pRSV-rev, pMDLg/pRRE) DMEM medium (28-31). The medium was exchanged 4 hours after transfection. Medium containing viral particles was collected at 48 and 72 hours after transfection, filtered through 0.45-µm filters (Millipore) and stored at -80° C until use.

Transduction of HepaRG cultures
Low-passage (P12) HepaRG cells were seeded and 24 hours later transduced by overnight exposure to a 1:5 (LV-CPS1) or 1:1 (LV-OTC) mixture of viral-DMEM: HepaRG medium containing 10 mg/ml DEAE-dextran (Pharmacia). The polyclonal and stable OTC- or CPS1- overexpressing HepaRG lines were obtained by selection with 2.5 mg/ml puromycin (Sigma) for 2 weeks, starting 24 hours after transduction, when the cultures were 50%-60% confluent. The resulting HepaRG-OTC and HepaRG-CPS1 cell lines were cultured as described for the parental cell line.

Quantitative reverse transcription PCR (RT-qPCR).
Quantitative RT-PCR was performed as previously described (32, 33). Transcript levels were normalized for 18S ribosomal RNA and expressed as a % of the average of three human liver samples isolated from 40, 41 and 43 year-aged female patients that were diagnosed with liver adenoma without any sign of liver damage. The procedure by which the human liver samples were collected, was in accordance with the ethical standards of the institutional committee on human experimentation (protocol number 03/024) and the Helsinki Declaration of 1975. Primer sequences and amplicon sizes are depicted in S1 Table. The transcript levels of all genes tested in RT-qPCR in the liver samples are shown in S2 Table.
**Hepatic function tests**

The monolayer cultures were tested for their functionality as described (21). Briefly, monolayer cultures were exposed to 1.5 ml test medium, which was based on HepaRG medium supplemented with 1 mM CAG, 1.5 mM NH₄Cl, 2.27 mM D-galactose, 2 mM L-lactate and 125 μM testosterone (all compounds from Sigma Aldrich). During the function test, medium samples were taken at 0.75 h (0.5 mL) and at 24 h in which L-lactate, ammonia and urea were measured, as described (19, 22).

To assess nitrogen metabolism in relation to arginine concentration in the medium, we measured urea production and ammonia elimination in fully differentiated HepaRG monolayers, which were pre-conditioned with HepaRG medium supplemented with 0, 5 or 10 mM arginine (Sigma) three days prior to the exposure to test medium supplemented with similar escalating concentrations of arginine.

Metabolic activities were calculated on basis of the changes in ammonia and urea concentration in medium in time, normalized to the protein content per well.

**Determination CPS1 and OTC enzyme activity**

Fully differentiated HepaRG-Static, HepaRG-DMF, HepaRG-CPS1-Static, HepaRG-CPS1-DMF and HepaRG-OTC-Static monolayer cultures were supplemented with test medium for 24 hrs. Next, the cells were harvested using a mixture of Accutase, Accumax (both from Innovative Cell Technologies) and phosphate buffered solution (PBS) in ratio 2:1:1. The detached cells were then pelleted by centrifugation for 5 minutes at 50 g, washed twice with PBS and immediately stored at -80 °C until the determination of the enzymatic activity of CPS1 and OTC, as described (25).

**Mitochondrial vs nuclear DNA ratio**

Mitochondrial abundance was assessed on basis of the ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nucDNA), as described previously (23). Briefly, the mtDNA/nucDNA ratio was determined from the calculated starting input concentration of 20 ng DNA/sample established by quantitative PCR using primers of 2 nuclear genes, *i.e.* CCAAT/enhancer binding protein alpha (*CEBPa*) and N-acetyl transferase (*NAT*), and 2 mitochondria-encoded genes, *i.e.* mitochondrial-NADH dehydrogenase subunit1 and 5 (*MT-ND1* and *MT-ND5*).

**Statistical analysis**

We performed Student’s *t*-tests for the comparison between two groups and one-way ANOVA test for comparison between >2 groups with Tukey’s post hoc test to compare the mean of each group with the mean of every other group, using Prism version 7 (GraphPad Prism Inc). Data was represented as mean ± SD; *p* < 0.05 was considered as significant.
Results

**ARG activity is not limiting ureagenesis**

To investigate the factor limiting UC activity in HepaRG monolayers, we focused on the UC enzymes with relatively low transcript levels, i.e. CPS1, OTC and ARG1. Firstly, we assessed whether ARG activity may be limiting the ureagenesis, by measuring nitrogen metabolism in the presence of increasing arginine doses in HepaRG medium, already containing 0.3 mM arginine. Urea production increased dose-dependently with arginine concentration (Fig. 1A), without any change in ammonia elimination rate (Fig. 1B), indicating that the ureagenesis is mainly a result of direct arginine degradation without actual improvement of the UC activity. This finding, in combination with the high activity rate of ARG1&2 (1950 nmol/min/mg protein) in human liver homogenates, compared to that of other UC enzymes (45, 400, and 43 nmol/min/mg protein for CPS1, OTC, and ASL, respectively (34-38)) suggested that CPS1 and OTC, but not ARG activity, were potential limiting factors in UC of the HepaRG cells and represented appropriate targets for ectopic overexpression.

![Figure 1](image)

**Figure 1:** Arginase enzyme activity level is not limiting ureagenesis in HepaRG cells. (A-B) Urea production and ammonia elimination of HepaRG monolayers after 24h exposure to different supplementations of arginine to HepaRG test medium (n=6). Significance: # p<0.05 vs 0 mM arginine, & p<0.05 vs 5 mM arginine.
OTC overexpression does not improve UC activity

A stable cell line, HepaRG-OTC, was produced by lentiviral overexpression of OTC in HepaRG cells, using lentiviral vector LV-OTC. The transcript level of \( OTC \) was 90-fold increased in HepaRG-OTC, compared to the parental line, approximating 3.3-fold the level in human livers (Fig. 2A). The transcript levels of \( CPS1, ASL, ASS \) and \( ARG1 \) remained unchanged. Accordingly, OTC enzyme activity was significantly augmented to 15.7-fold the level in parental HepaRG cells (Fig. 2B), reaching 146% of human liver level. However, ammonia elimination and total urea production were unchanged (Fig. 2C-D). Therefore, improvement of OTC activity did not increase ureagenesis, implying that OTC was not limiting the UC in the HepaRG cells. Next, we investigated CPS1 as a potential limiting enzyme.

\[\text{Figure 2: OTC enzyme activity level is not limiting ureagenesis in HepaRG cells. A) Transcript levels of UC genes in HepaRG-OTC vs HepaRG cells, expressed as a \% of human livers (n=11/condition). Also shown is the level of 18S ribosomal RNA, which was not changed in HepaRG-OTC line vs HepaRG cells and was comparable to human liver level, and was used as reference gene for normalization of the RT-qPCR data (39). B) Enzymatic activity of CPS1 and OTC in HepaRG-OTC vs HepaRG cells (n=3). C-D) Ammonia elimination and urea production (n=9-10/condition). Significance: \# p<0.05 vs HepaRG}\]
CPS1 overexpression provokes mitochondrial stress that can be recovered by DMF-culturing

Similar to OTC, CPS1 was ectopically expressed in the HepaRG cells, generating stable cell line HepaRG-CPS1. Interestingly, unlike transduction with OTC-expressing lentivirus, the transduction of the HepaRG cells with CPS1-expressing lentivirus exerted morphological changes. In HepaRG-CPS1 monolayers, the hepatocyte islands were larger, but the hepatocytes had reduced cell size, suggesting less differentiation compared to parental HepaRG monolayers (Fig. 3 A-C). As CPS1 is a large mitochondrial protein of 160 kD, we hypothesized that this inhibitory effect of CPS1 overexpression on differentiation may be related to the overload of the limited mitochondrial system, as it is the case in HepaRG-Static, leading to cellular stress. This prompted us to culture the HepaRG-CPS1 line under a condition that stimulates mitochondrial biogenesis i.e. by DMF. We recently showed that DMF-culturing enhances mitochondrial biogenesis (23), mitochondrial energy metabolism and hepatic differentiation of the HepaRG cells (22). We therefore compared the parental HepaRG and HepaRG-CPS1 monolayer cultures under Static- and DMF-culturing conditions.

Culturing HepaRG-CPS1 monolayers under DMF reverted the negative morphological effects observed in HepaRG-CPS1-Static, as the size of hepatocytes was increased. Moreover, HepaRG-CPS1-DMF cultures displayed larger and diffuse hepatocyte islands compared to HepaRG-DMF (Fig. 3 B-D). Noteworthy, DMF-culturing changed the morphology of the hepatocyte clusters into a semi-3D appearance in both HepaRG and HepaRG-CPS1 lines (Fig.3 B-D).

To assess the plausibility of the hypothesis that CPS1 overexpression exerts mitochondrial stress in statically cultured HepaRG cells, which can be rescued by DMF-culturing, we assessed mitochondrial abundance and function. DMF-culturing increased mitochondrial abundance in HepaRG-CPS1 cells 2.0-fold, whereas, HepaRG cells exhibited only a positive trend (Fig. 3E). There was no significant difference in mitochondrial abundance between HepaRG and HepaRG-CPS1 cells, maintained either in Static- or DMF-conditions. However, the transcript level of Peroxisome Proliferator-Activated Receptor Gamma, Coactivator 1 Alpha (PGC1α), the master regulator of mitochondrial biogenesis (40), was 2.2-fold suppressed in HepaRG-CPS1-Static vs HepaRG-Static cultures. This negative effect was recovered by DMF-culturing (Fig. 3F). A similar pattern was observed for the transcript level of mitochondrially encoded NADH dehydrogenase 5 (MT-ND5) (Fig. 3G). Lactate production, as a measure of mitochondrial incompetence, was 2.2-fold increased in HepaRG-CPS1-Static vs HepaRG-Static. The lactate production of HepaRG-CPS1 was equaled to control level by DMF-culturing (Fig. 3H).

Collectively, these findings support the stressful effect of CPS1 overexpression on mitochondria under static culturing. DMF-culturing alleviates this stress, possibly through increasing mitochondrial abundance.
Figure 3: CPS1 overexpression exerts inhibitory effect on hepatic differentiation and mitochondria under conventional Static-culturing that is recovered by DMF-culturing. A-D) Morphology of HepaRG and HepaRG-CPS1 line under Static- and DMF-culturing. Arrows A and B indicate hepatocyte islands in HepaRG-Static (3A) and HepaRG-CPS1-Static (3C) respectively, scale bar = 100µm. (E) Mitochondrial abundance measured by mtDNA/NucDNA ratio. (F-G) Transcript levels of mitochondria-related genes, expressed as a % of human livers. (H) Lactate production, (n=6/condition). Significance: # p<0.05 vs HepaRG-Static, $ p<0.05 vs HepaRG-CPS Static.
Only under DMF, CPS1 overexpression positively modulates transcription of several UC genes. Only under DMF, the HepaRG-CPS1 line showed 2.4-fold upregulation of CPS1 transcript level. The transcript level of CPS1 was 2.1- and 1.8-fold increased compared to HepaRG-Static and HepaRG-DMF respectively, approaching 80% of human livers (Fig. 4B). Interestingly, the transcript levels of UC genes OTC and ARG1 were also positively modulated by CPS1 overexpression in combination with DMF-culturing; these were 8.4- and 7.5-fold higher in HepaRG-CPS1-DMF vs HepaRG-CPS1-Static, respectively, and 3.7- and 2.0-fold higher vs HepaRG-DMF controls, respectively (Fig. 4C-D). In addition, mitochondrial transporter ORNT was induced in HepaRG-CPS1-DMF compared to other groups (Fig. 4E). This synergistic positive effect of CPS-overexpression in combination with DMF on the transcript level was not found for the two UC genes with relatively high transcript levels, i.e. ASL and ASS (Fig. 4F-G), nor for a number of ammonia metabolism genes unrelated with UC, i.e. GS, GLS1, GLS2, ARG2 and for another liver-specific gene, Cytochrome P450 3A4 (CYP3A4) (Fig 4H-L).

To further investigate the unexpected upregulation of UC-related genes ARG1, OTC and ORNT by CPS1 overexpression in combination with DMF-culturing, we determined the transcript levels of two transcription regulators of UC genes, i.e. CEBPa and HNF4 (8, 9). However, for both genes there was no significant difference between the transcript levels in HepaRG-DMF and HepaRG-CPS-DMF, indicating no important role for these regulators under the specified conditions (Fig 4M-N).

Of the 13 investigated genes, 7 genes (ARG1, ASL, ASS, GS, GLS1, CYP3A4, CEBPa) showed a significant reduction in transcript level in HepaRG-CPS1-Static vs HepaRG-Static; except for ASL, this suppression was abolished by DMF-culturing. In conclusion, CPS1 overexpression in Static-cultures reduced the differentiation of HepaRG cells. CPS1 overexpression was only successful in combination with DMF-culturing, resulting in increased transcript levels of CPS1 and several other UC-related genes.
OVEREXPRESSION OF CARBAMOYL-PHOSPHATE SYNTHASE 1 IN HUMAN LIVER HEPARG CELLS

- **Figure 1:**
  - **A:** Transcript level of ASS (% of human livers)
  - **B:** Transcript level of GS (% of human livers)
  - **C:** Transcript level of G6P1 (% of human livers)
  - **D:** Transcript level of G6P2 (% of human livers)
  - **E:** Transcript level of ARG2 (% of human livers)
  - **F:** Transcript level of CYP4A4 (% of human livers)

- **Legend:**
  - HepG2-Static
  - HepG2-CPS1-Static
  - HepG2-DMF
  - HepG2-CPS1-DMF

- **Significance Levels:**
  - $p < 0.01$
  - $p < 0.001$
  - # $p < 0.05$
  - ### $p < 0.001$
Figure 4: Only under DMF, CPS1 overexpression positively modulates transcription of low-expressed UC genes. (A-N) Transcript levels of 18S ribosomal RNA and different UC-related and other genes, expressed as a % of human livers (n=6/condition). The 18S ribosomal RNA level was stable under different conditions (Fig. 4A), and therefore served as internal reference for normalization of the RT-qPCR data (39). Significance: # p<0.05 vs HepaRG-Static, $ p<0.05 vs HepaRG-CPS Static, * p<0.05 vs HepaRG-DMF.

CPS1 overexpression under DMF-culturing augments OTC enzymatic activity and improves ureagenesis.

In agreement with the transcript level data, CPS1 enzymatic activity was 2.4-fold increased in the HepaRG-CPS1-DMF cultures vs the other groups (Fig. 5A), and OTC enzymatic activity was 6.1-fold upregulated (Fig. 5B), reaching 11.7% and 57.1% the level of human livers, respectively (25). As a result, total urea production was 3.0-fold augmented in HepaRG-CPS1 line by DMF culturing. In addition, urea production was 4.3- and 1.9-fold increased compared to HepaRG-Static and HepaRG-DMF control cultures, respectively (Fig. 5C). Yet, the ammonia elimination was not increased, and was significantly reduced in HepaRG-CPS1-Static vs HepaRG-Static (Fig. 5D). The increase in ureagenesis is probably attributable to increased UC activity, as ARG2 transcript levels have remained unchanged in HepaRG-CPS1-DMF vs the other groups.
Figure 5: CPS1 overexpression under DMF-culturing augments OTC enzymatic activity and improves ureagenesis. (A-B) Enzymatic activity of CPS1 and OTC, (n=3/condition). (C) Total urea production, (n=6/condition). (D) Ammonia elimination, (n=6/condition). Significance: # p<0.05 vs HepaRG-Static, $ p<0.05$ vs HepaRG-CPS Static, * $p<0.05$ vs HepaRG-DMF.

Discussion

Ammonia metabolism through UC is highly functional in PHHs, however, rapid dedifferentiation and scarcity, limit the usage of these cells in BAL application. In addition, UC is substantially repressed in hepatocytes derived from stem cell and iPSCs differentiation, as well as in human liver cell lines including HepaRG cells (17). The present study shows that arginase and OTC activity do not limit the ureagenesis in HepaRG cells. The overexpression of CPS1 in conventional static HepaRG culture does not result in increased ureagenesis either, but on the contrary, inhibits...
differentiation. However, under DMF-culturing conditions, a positive phenotype is established, with increased \( \text{CPS1} \) transcript level, \( \text{CPS1} \) activity and urea production in HepaRG-CPS1 cells, while \( \text{ARG2} \) transcript levels remain unchanged, implying that both \( \text{CPS1} \) levels and static culturing limit UC activity. Of particular interest, \( \text{CPS1} \) overexpression under DMF, stimulates several other UC-related genes at the transcript level, including \( \text{OTC} \), \( \text{ARG1} \) and \( \text{ORNT1} \), and positively modulates OTC enzymatic activity. The consequences of \( \text{CPS1} \) overexpression under DMF-culturing on ammonia elimination is difficult to predict; however, the >4-fold increased ureagenesis and the induction of \( \text{ARG1} \), but not \( \text{ARG2} \) at transcript level, indicate towards an increased urea production through UC. Interestingly, total ammonia elimination was not changed, although it is likely that ammonia metabolism is slightly shifted towards UC and less towards fixation into amino acids. Yet, due to the relatively low contribution of UC to ammonia clearance in HepaRG cells (3%) (16), even a 4-fold increased ureagenesis, does not alter ammonia detoxification significantly.

The overexpression of \( \text{CPS1} \) under conventional Static-culturing induced severe cellular stress, as shown by the elevated lactate production, the suppression of mitochondrial and hepatic transcript levels and of ammonia elimination, and a less developed morphology (small sized hepatocytes) of the culture. This negative outcome was completely reversed by DMF-culturing, except for the transcript level of \( \text{ASL} \).

DMF-culturing stimulates hepatic differentiation and mitochondrial biogenesis of hepatoma cell lines, including HepaRG cells (22, 23), and primary mouse liver cells (41). Notably, the effect of DMF-culturing in this paper was, in general, less pronounced, potentially due to the unexpected high hepatic differentiation of HepaRG cells used as control. For example, the transcript level of \( \text{ARG1} \) in Adam et al 2018 (22) was limited to 2.7% in statically cultured HepaRG cells which was increased to 16.2% of human livers by DMF culturing. Whereas in this paper, in the statically maintained HepaRG cells, the transcript level of \( \text{ARG1} \) was relatively high, 12.3% of human livers, which was further augmented to 17.3% by DMF-culturing. Similarly, \( \text{CYP3A4} \) and \( \text{CEBP} \alpha \) were also expressed >3-fold higher than previously reported for statically cultivated HepaRG cells. The results presented in Adam et al (22), were derived from 3 different HepaRG cultures of various passage numbers, while in this study, to maintain similar background, the same HepaRG culture and passage number was used for control and generation of the HepaRG-CPS1 line. Ideally, this study would also have analyzed three different parenteral lines and three deriving HepaRG-CPS lines to compensate for biological variation between the different HepaRG cultures, however, that was too impractical and time demanding.

We tested the effect of DMF culturing, since we hypothesized that the overexpression of \( \text{CPS1} \) in static HepaRG cultures induced mitochondrial stress, possibly due to the accumulation of the sizable \( \text{CPS1} \) protein (160kD) in the mitochondria of cells with relatively low mitochondrial
content. In contrast and further supporting this hypothesis, the overexpression of OTC, a 36kD protein, resulted in substantial increase in both the transcript level and enzymatic activity of OTC without negative effects, neither on hepatic differentiation nor on mitochondrial function (data not shown). Both CPS1 and OTC are nuclear-encoded proteins that are synthesized from precursor molecules of 165kD and 39kD respectively, which are imported to mitochondrial matrix to yield the catalytically active forms of 160kD and 36kD (42). Interestingly, Cote et al reported that the efficiency of importing and processing of mitochondrial precursor proteins (subunits 2 (β) and 3 (γ) of the H+-ATPase and OTC were investigated) in mitochondria isolated from different tissues varies per combination of protein and tissue-origin of the mitochondria (43). Whether this variation in protein trafficking is related to mitochondrial factors (abundance/activity) or to tissue-specific mitochondrial proteome, however, was not investigated.

It is a considerable challenge to prove the causal link between mitochondrial biogenesis and successful CPS1 overexpression. Mechanistic studies by suppressing or inducing mitochondrial biogenesis under DMF- or Static-culturing, respectively, are difficult to establish. On one side, inhibiting mitochondrial biogenesis will probably exert diverse negative effects, because many energy-consuming processes including CPS1 function will be interrupted by ATP reduction (44). Furthermore, increased reactive oxygen species production upon mitochondrial inhibition (45), will suppress hepatic functionality, including UC and CPS1 as well. Therefore having reduced CPS1 activity following inhibition of mitochondrial functions will not be unexpected. On the other side, induction of mitochondrial biogenesis in HepaRG-Static cultures e.g. by upregulation of PGC1α, the master regulator of mitochondrial biogenesis (40), is an interesting way to overcome the limited mitochondrial abundance, yet, it is an added challenge to establish HepaRG cells overexpressing both PGC1α and CPS1.

Others have tried to improve the nitrogen metabolism in two other human liver cell lines, HepG2 and C3A. These studies suggested OTC and ARG1 levels, and not CPS1, to be limiting the UC (14, 15, 46). The transcript level of CPS1 markedly varied among different studies where Mavri-Damelin et al (15) reported a level comparable to that of PHHs, though, significantly low when related to human livers, in line with our previous finding that the transcript level of CPS1 in HepG2 was limited to 3.8% of human livers (47). This discrepancy may be explained by possible dedifferentiation of in vitro cultured PHHs. In addition to the low transcript level of CPS1 in HepG2 cells, we recently demonstrated that the C3A cell line, which is a subclone of HepG2, displays a low mitochondrial content compared to the HepaRG cell line (23), suggesting that CPS expression and mitochondrial content may also limit UC in HepG2/C3A cells.

Interestingly, CPS1 overexpression under DMF promoted the transcript level of other UC-related genes OTC, ARG1 and ORNT1. The mechanism(s) mediating this positive effect may be related to a
change in the amino acid spectrum when UC is operative, as in the HepaRG-CPS1-DMF cultures. Amino acids are increasingly recognized as critical regulators of fluxes through different metabolic pathways, such as UC and tri-carboxylic acid cycle (2, 48). Recently, another crucial role of amino acids in direct and indirect regulation of the mammalian target of rapamycin (mTOR), has been reported (49, 50). Activation of mTOR signaling and subsequent activation of eukaryotic initiation factor 4E is known to increase the expression of CEBPa, the master regulator of OTC and ARG1 at transcript level (49, 51). The transcript level of CEBPa was induced in the HepaRG-CPS1-DMF group compared to the HepaRG-Static and HepaRG-CPS1-Static groups, however, it only exhibited a positive trend when compared to the HepaRG-DMF control. Yet, to further evaluate the role CEBPa and other critical regulators of UC, such as HNF4, more investigations at protein and activity level are required.

This work represents the first successful attempt of CPS1 overexpression in a human liver cell line and indicates static-culturing as another limiting factor, and additionally shows that limited CPS1 expression may repress the expression of other UC-related genes. The resulting HepaRG-CPS1-DMF cells likely retain higher capacity of ammonia clearance through UC. Yet, the total ammonia elimination was not changed, and probably the contribution of the UC to the ammonia elimination is still negligible compared to fixation into amino acids, as in the parental HepaRG cells (16, 21). It is therefore still to be investigated whether the HepaRG-CPS1 cell line is currently more suitable as biocomponent for clinical BAL application as the parental HepaRG cell line, which exhibits an improved ammonia metabolism under AMC-BAL culturing with 2.3- and 2.6-fold upregulation of CPS1 and OTC enzyme activity, respectively, compared to monolayers (25). Interestingly, the mitochondrial abundance is 2.4-fold increased in HepaRG cells in BAL vs monolayer cultures (23), again linking CPS1 and OTC activity with mitochondrial biogenesis.

Still, HepaRG-CPS1-DMF cells may be interesting for in vitro studies on inborn errors disease related to CPS1 deficiency or other UC deficiencies. In addition, this study may prove valuable to achieve overexpression of other large mitochondrial proteins in cells with limiting mitochondrial content. Mitochondrial abundance can be enhanced by overexpressing PGC1α or using culturing conditions that stimulate mitochondrial biogenesis, such as DMF-culturing. Moreover, a favorable modification is the use of an inducible vector to avoid the inhibitory effects occurring under a constitutively active promoter.
References


Supplementary Material

Table S1: Primers used in PCR analyses and amplicon size.

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S2 Table: Transcript levels in human liver samples of all genes tested in RT-qPCR, expressed as a % of the average level of all liver samples.

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Chapter 8

Summary, conclusions and future perspectives
Summary

Bioartificial livers (BALs) have been developed to provide liver support to patients with end-stage liver failure (ESLF) and bridge them either to liver transplantation or native liver recovery (1). These devices consist of a bioreactor with a biocomponent displaying hepatic functionality connected extracorporeally to the patient’s circulation. The hepatic functions needed for effective BAL support are not fully elucidated, but the elimination of ammonia, preferably through urea cycle (UC) activity, biotransformation, mitochondrial energy metabolism leading to lactate elimination and the production of blood proteins are considered as crucial (2).

Primary human hepatocytes (PHHs) are most suitable as biocomponent, however, the low availability, limited proliferation and the rapid dedifferentiation during in vitro cultures limit their applicability. Human liver cell lines show almost unlimited proliferation capacity and therefore, represent an attractive substitute for PHHs, yet, most of these cell lines lack complex hepatic functions (2). Currently, human liver cell lines C3A and HepaRG are the biocomponents of choice for the extracorporeal liver assist device (ELAD) and the AMC-BAL, resp. (3, 4). The C3A cell line is a sub-clone of the human hepatoma cell line (HepG2) and exhibits an immature hepatocyte-like phenotype (5, 6). The HepaRG cell line, on the other hand, displays an increased hepatic differentiation and exerts a broad array of hepatic functions with levels up to that of PHHs. Ammonia is eliminated efficiently through conversion into amino acids, however, the UC activity is relatively low (3, 7). Moreover, xenobiotic detoxification properties are relatively low due to the limited expression of key regulatory genes, such as the constitutive androstane receptor (CAR). In addition, the cell line shows limited stability under repeated passaging and loses its capacity to fully differentiate after critical passage P20.

To further improve the performance of human liver cell lines as biocomponent of BALs, or for other applications requiring functional liver cells, as liver disease modelling and drug safety tests, critically low functions may be specifically corrected by overexpression of genes limiting the relevant pathways. Alternatively, an array of functions may be corrected by optimizing culture conditions or by overexpression of hepatic transcription factors. A first start with the latter strategy has recently yielded the HepaRG-CAR cell line overexpressing CAR, which showed increased xenobiotic metabolism and albumin production (8).

The aim of this thesis was to study the biocomponents applied in BALs, namely HepaRG and C3A cells, by determining critical factors regulating their maturation and further explore strategies for improvement of functionality with particular emphasis on the role of energy metabolism.

Part I provided an introduction to the thesis and gave the functional features of the HepaRG and C3A cells in monolayer and BAL-cultures and described the association between mitochondrial energy metabolism and differentiation.
Chapter 1 included a short introduction of ESLF, its causes and treatment modalities. A brief introduction of the concept of BAL treatment including the AMC-BAL and ELAD system was given. Furthermore, diverse potential sources of hepatocytes and criteria required for biocomponents in BALs have been addressed. Finally, the link between mitochondrial energy metabolism and hepatic differentiation has been introduced.

In Chapter 2 we compared the hepatic functionality of HepaRG and C3A cells in AMC-BAL and in conventional monolayer cultures. The performance of both cell lines was highly improved under BAL-culturing conditions compared to monolayer culturing. HepaRG cells showed higher hepatic functionality vs C3A cells, implying that HepaRG cells are more suitable for BAL application.

Chapter 3 further described the background of the improved functionality by BAL-cultivation of HepaRG cells using whole genome micro-array analysis. We found that culturing HepaRG cells in BAL significantly enhanced the transcription of genesets related to mitochondrial energy metabolism. The induced mitochondrial biogenesis was confirmed by increased mitochondrial abundance, expression of oxidative phosphorylation (OxPhos) complexes and mitochondrial membrane potential. The main differences between BAL and monolayer culturing are: three dimensional configuration, medium perfusion, as well as oxygenation. These three factors were found to contribute to the enhanced mitochondrial biogenesis. BAL culturing also increased mitochondrial biogenesis of C3A cells, implying that this is a general phenomenon of BAL culturing, independent of the applied biocomponent.

The effects of oxygen concentration and medium perfusion on hepatic functionality and energy metabolism were further studied in the following chapters.

We examined in Chapter 4 the critical role of pericellular oxygen concentration on the differentiation of human liver cell lines HepaRG and C3A. Culturing of HepaRG cells under hyperoxia (40%O\textsubscript{2}) instead of the regular 20%O\textsubscript{2} (normoxia) concentration, positively modulated hepatic differentiation and functionality. Hyperoxia induced the crucial hepatic transcription factor CCAAT/enhancer-binding protein α (CEBPα) and increased its translocation to the nucleus. The positive effects of hyperoxia on hepatic differentiation was shown in C3A cells as well. Microarray analysis of the transcriptome of HepaRG cells cultured under hyperoxia vs normoxia, revealed a narrow range of genes that were differentially expressed (only 1%), including, however, several well-recognized liver enriched transcription factors. This indicates that probably post-transcriptional processes play a major role in the improved functionality by hyperoxia. In contrast, HepaRG cultures maintained under hypoxia (5%O\textsubscript{2}) failed to acquire hepatic differentiation and remained in a progenitor phase. This progenitor phenotype was confirmed by predominant expression and nuclear translocation of the progenitor marker SOX9. However, keeping HepaRG cells under
hypoxia during the propagation (expansion) phase increased the conservation of their progenitor cell characteristics, which is lost under normoxia at passage P20. The effect of oxygen on hepatic differentiation is thought to be mediated by oxygen responsive factors, such as hypoxia-inducible-factor 1α (HIF1α). Indeed, the expression and nuclear translocation of HIF1α was augmented under hypoxia vs normoxia and hyperoxia.

In Chapter 5, the effect of dynamic medium flow (DMF) on hepatic differentiation and energy metabolism was extensively studied. DMF is one of the contributing factors to the enhanced mitochondrial biogenesis under BAL culturing (see Chapter 3). To further address the effect of DMF on hepatic functionality of the HepaRG and C3A cells, we simulated DMF of the BALs in monolayer cultures using a simple and easy applicable method based on shaking cultures at 60 rpm during the differentiation phase. This new practice-changing culturing technique proved superior to the conventional static-culturing of hepatocyte monolayers. DMF-culturing of HepaRG cells substantially upregulated hepatic differentiation by increasing transcription of hepatic genes and nuclear translocation of hepatic transcription factor CEBPα. In addition, hepatic functions were positively affected, including ammonia elimination, bile acid production, and cytochrome P450 3A4 (CYP3A4) activity. As found for BAL culturing, a strong association between hepatic differentiation and mitochondrial metabolism was found, as DMF-culturing shifted energy metabolism from aerobic glycolysis towards OxPhos, indicated by a decline in lactate production and glucose consumption, and an increase in oxygen consumption. Similarly, DMF-culturing increased mitochondrial energy metabolism and hepatic functionality of C3A cells.

In Part II, we focused on genetic modulation of HepaRG cells to promote limited hepatic functions including xenobiotic detoxification, mitochondrial energy metabolism and UC activity.

In Chapter 6, we further investigated the effect of the lentiviral overexpression of CAR, a well-recognized master regulator of xenobiotic detoxification and energy metabolism, in HepaRG cells. The newly developed HepaRG-CAR cells outperformed the parental HepaRG cells for bile acid synthesis and decreased lactate production. Interestingly, HepaRG-CAR cells were capable to sustain their hepatic differentiation for 10 extra passages above the critical P20, compared to HepaRG cells. RNA sequencing analysis identified changes in the transcriptome of HepaRG cells due to CAR overexpression, including upregulation of OxPhos and downregulation of glycolysis, hypoxia and proliferation related genesets. In addition, CAR overexpression downregulated the mTORC1 signalling pathway, which, as mediator of proliferation and metabolic reprogramming, may play an important role in the establishment of the HepaRG-CAR phenotype. RNA sequencing of early and late passages of HepaRG and HepaRG-CAR cells showed that limited transcriptional changes occur upon passaging of HepaRG-CAR, in contrast to the parental HepaRG line. Moreover, with serial passaging, the transcriptome of the HepaRG cells displayed changes in favor of the loss
of hepatic differentiation and epithelial mesenchymal transition. The stability of the HepaRG-CAR line may, in part, be linked to reduced generation of reactive oxygen species (ROS), which are reported to be associated with aging effects; the level of ROS was significantly lower in HepaRG-CAR cells vs HepaRG cells.

In Chapter 7 we unraveled the identity of the factors that limit the UC in HepaRG cells. Efficient ammonia removal through UC is a prerequisite for BALs to irreversibly dispose of ammonia, which is essential to treat hyperammonemia (9), frequently encountered in patients with ESLF. In HepaRG cells the expression and activity of three UC enzymes, including carbamoyl-phosphate synthase1 (CPS1-mitochondrial enzyme, heavy molecular weight), ornithine transcarbamoylase (OTC-mitochondrial enzyme, low molecular weight) and arginase 1 (ARG1-cytosolic enzyme), are relatively low compared to human liver. Probably, as a result > 95% of ammonia removal takes place through reversible fixation into amino acids and only a marginal fraction is eliminated through UC activity. HepaRG cells converted arginine in a dose-dependent manner into urea and OTC overexpression did not improve ureagenesis, demonstrating that neither OTC nor ARG1 were limiting ureagenesis. Unexpectedly, and in contrast to the overexpression of the mitochondrial enzyme OTC, the overexpression of CPS1 induced mitochondrial stress and suppressed hepatic differentiation in HepaRG cells maintained under conventional static-culturing. Yet, it promoted ureagenesis >4-fold, but only under DMF culture conditions, which are known to stimulate mitochondrial biogenesis (DMF, Chapter 5). Surprisingly, the transcript levels of several other UC genes also increased under these conditions. We conclude that both the low CPS1 level and static-culturing, possibly due to insufficient mitochondria, are limiting UC activity.

Conclusions

Our study to gain deeper insight in the applicability of human liver cell lines as biocomponent for BAL application has resulted in a number of strategies that improved their performance by simple and effective culture procedures, which were based on modifications of oxygen concentrations and on DMF. In addition, upregulation of liver-specific pathways improved the performance of HepaRG cells; overexpression of CAR resulted in counteracting in vitro aging effects and in improved xenobiotic detoxification and mitochondrial metabolism, and over expression of CPS1 under DMF resulted in improved UC activity. As an important common factor we found a positive correlation between mitochondrial biogenesis and hepatic differentiation.
Future perspectives

A) Improving hepatocyte functionality in monolayer

In this thesis the effects of hyperoxia and DMF were investigated on liver functions and energy metabolism of HepaRG monolayer cultures. DMF is most promising, due to the simplicity of the set-up and the substantial positive effects on a wide array of hepatic functions and lactate metabolism (10). The effect of hyperoxia was limited to hepatic differentiation whereas energy metabolism as measured by lactate metabolism and glucose consumption was not improved (11). The beneficial effect of DMF was confirmed in the C3A cell line as well as primary murine hepatocytes (10, 12, 13). Therefore, it is advisable that DMF culturing becomes the new standard culturing for hepatocyte, replacing the conventional static monoculturing. Once implemented in practice, it will improve the predictability of drug safety testing and the modeling of liver diseases and other fields requiring hepatocytes, which will advance basic and applied hepatology research. DMF culturing may even be extended to culture procedures of other cell types, particularly of those with high mitochondrial content.

Furthermore, given the intimate link between mitochondrial function and hepatic differentiation, it will be interesting to establish a new HepaRG cell line overexpressing PGC1α, the master regulator of mitochondrial biogenesis, using an inducible vector to allow its activation principally during the differentiation phase. This HepaRG-PGC1α liver cell line, likely, in addition to improving mitochondrial biogenesis, may automatically overcome, among others, the problem of limited urea cycle activity and OxPhos in HepaRG cells.

Finally, inhibition of the mTORC1 pathway may also prove beneficial. This pathway is downregulated by CAR overexpression in HepaRG cells and is associated with cancer growth and the Warburg effect (14-16).

B) Improving HepaRG cell line functionality for BAL application

Despite the fact that HepaRG cells are the most functional hepatocytes from proliferative sources, yet the dedifferentiation upon passaging and insufficiency in specific hepatic functions limit their value as biocomponent for BALs requiring huge quantities of cells (150 gram/BAL) and particular hepatic functions as xenobiotic and ammonia detoxification. Given its improved detoxification, albumin production, OxPhos, and stability, the HepaRG-CAR cell line may become the new biocomponent for BAL application.

In addition UC activity of HepaRG cells may be improved through inducible CPS1 overexpression rather than constitutive overexpression as we applied in Chapter 7. This way, we can activate CPS1 overexpression only for short duration when required, limiting the risk of CPS-induced mitochondrial stress, for instance during hepatic function test in mature cultures or during BAL
treatment sessions. Finally, it is an interesting option to overexpress CPS1 using an inducible vector in HepaRG-CAR cells, because in contrast to HepaRG, HepaRG-CAR cells possess more competent mitochondria that may provide optimal conditions to accommodate CPS1 functionality.

Alternatively, the hypothesized, HepaRG-PGC1a line, may be more attractive as a potential new biocomponent for BALs applications as it may substantiate the hepatic differentiation and upregulate critical functions, such as ureagenesis and OxPhos.

C) Improving HepaRG cell line stability upon passaging
The stability of HepaRG cells over passaging can be increased by expansion under hypoxia or by CAR overexpression. While the hypoxia-maintained HepaRG cells and HepaRG-CAR cells are highly different from each other regarding the differentiation grade, yet, the mechanisms underlying their increased stability upon passaging may partially be interrelated. The level of ROS, as an inducer of oxidative stress and concomitant aging effects, is reduced in HepaRG-CAR cells, probably due to an effective antioxidant system. In hypoxic cultures probably the ROS level is low due to a low OxPhos activity. However, the balance between ROS and antioxidant systems is highly regulated and this subtle balance is not easily restored in vitro. Simple addition of antioxidants such as N-acetyl cysteine with dopamine, during the expansion of HepaRG cells, inhibited their proliferative capacity (results not shown) and was yet, not successful.

Therefore, more mechanistic studies are required to address the full image of the factors behind HepaRG cell dedifferentiation upon passaging to interfere with the aging effect. Noteworthy, one limitation of the RNA-seq analysis (Chapter 6), was that we only investigated the early and late passages of HepaRG and HepaRG-CAR cultures, without including the intermediate passages. This resulted in a gap in the sequel of events prior to the full-establishment of the dedifferentiation and prevented the identification of on one side, the initial key players in the dedifferentiation of the HepaRG cells and on the other side, the critical regulators of HepaRG-CAR cells stability. Acquiring of more molecular background about the dedifferentiation may help to overcome the aging of HepaRG cells by genetic correction of disrupted critical hepatic regulators and by overexpression of PGC1a, the master regulator of mitochondrial biogenesis, to avoid the unwanted shift in energy metabolism from OxPhos towards glycolysis-dependent profile.

D) Adaptations to BAL-culturing protocol to promote its clinical application
The current culturing protocol of HepaRG cells in the AMC-BAL comprises a 1-2 weeks proliferation period and 1-2 weeks differentiation period. This relatively long duration adds a major hurdle to the application of BAL in clinic where immediate intervention is a matter of life and death to critically deteriorating ESLF patients. Therefore, shortening of the BAL culturing time is a crucial step to potentiate the clinical usage of the BAL and its marketing. It would be highly desirable if
AMC-BALs can be loaded with a sufficient quantity ~150 g of fully-differentiated HepaRG cells for immediate usage, omitting both the proliferation and differentiation phase. From this thesis two potential strategies can be extracted to be included into the BAL-culturing procedure. Firstly, the propagation of HepaRG cells needs to be carried out under hypoxia (5%O₂) to overcome or delay the dedifferentiation process and ensure sufficient cell supply. Secondly, HepaRG cells have to fully-differentiate under mitochondria-stimulating conditions as DMF-culturing before loading into BALs in high density to prevent the unwanted initiation of the proliferation phase. In case no BAL treatment is needed at the moment of cell harvesting, DMF-cultivated cells can be cryopreserved until usage. And, as outlined above, another way to advance the AMC-BAL towards the clinic is by establishing a new HepaRG line overexpressing both CAR (constitutive vector) and CPS1 (induced vector). The resulting HepaRG-CAR-CPS1 line is hypothesized to display an increased stability, detoxification capacity, enhanced mitochondrial functions and improved ammonia metabolism. For exploitation of this cell line as biocomponent for the AMC-BAL, the culturing, cell harvesting and cryopreservation protocol need to be optimized to warrant robust BAL treatment of ESLF patients.
References


Appendices

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I. Nederlandse samenvatting

Bioartificiële levers (BALs) zijn ontwikkeld voor de ondersteuning van leverfuncties van patiënten met eind-stadium leverfalen ter overbrugging tot levertransplantatie of tot herstel van de eigen lever (1). BAL systemen bestaan uit een bioreactor gevuld met een biocomponent met hoge leverfunctionaliteit die buiten het lichaam gekoppeld wordt aan de bloedcirculatie van de patiënt. De leverfuncties vereist voor effectieve BAL therapie zijn niet geheel opgehelderd, maar de ontgifting van ammoniak, bij voorkeur door ureum cyclus (UC) activiteit, en o.a. eiwitgebonden en vetoplosbare schadelijke stoffen, mitochondriale energie productie, die leidt tot melkzuur eliminatie, en de productie van belangrijke bloed eiwitten zijn waarschijnlijk cruciaal (2).

Primaire humane hepatocyten (PHHs) zijn het meest geschikt als BAL biocomponent, maar vanwege de slechte verkrijgbaarheid en snelle achteruitgang van functies in kweek is een alternatieve bron voor levercellen nodig. Humane lever cellijnen vertonen meestal onbeperkte celgroei en zijn daarom een aantrekkelijk alternatief voor PHHs, maar hebben als nadeel dat ze grotendeels tekort schieten in complexe leverfuncties, ook wel lage differentiatiegraad genoemd (2). Momenteel worden twee humane levercellijnen gebruikt als biocomponent voor BAL systemen: de C3A cellijn voor de Extracorporeal Liver Assist Device (ELAD) en de HepaRG cellijn voor de AMC-BAL (3, 4). De C3A cellijn is een derivaat van de humane hepatoma cellijn HepG2 en vertoont een zeer lage differentiatiegraad (5, 6). De HepaRG cellijn vertoont daarentegen een aanmerkelijk hogere differentiatiegraad, en benadert met een aantal functies het niveau van PHHs. Desondanks zijn er nog een aantal functies niet optimaal. Het toxische ammoniak wordt efficiënt ontgift door inbouw in aminozuren, maar de gewenste, onomkeerbare ammoniak eliminatie via UC activiteit is relatief laag (3, 7). Daarnaast zijn ook diverse andere ontgiftings activiteiten relatief laag, vanwege de lage expressie van een aantal essentiële regulatiegenen, waaronder de constitutive androstane receptor (CAR). Verder vertoont de cellijn een beperkte stabilitie tijdens celexpansie, het passeren van de cellen in kweek, waardoor de cellen hun capaciteit tot volledige uittijging verliezen vanaf de kritieke passage P20.

Teneinde humane lever cellijnen verder te verbeteren om te dienen als volwaardig biocomponent voor BAL toepassing, of voor andere toepassingen die functionele levercellen vereisen, zoals het modelleren van leverziektes en medicijn veiligheidsstudies, kunnen te lage functies specifiek opgereguleerd worden door genen die de relevante metabole routes limiteren tot overexpressie te brengen. Daarnaast kan een breder scala aan functies gecorrigeerd worden door kweekomstandigheden verder te optimaliseren of door lever transcriptie factoren tot overexpressie te brengen. Deze laatstgenoemde strategie heeft al een nieuwe cellijn opgeleverd, de HepaRG-CAR cellijn, die CAR overexpresseert, wat geleid heeft tot een verhoogde ontgiftingsactiviteit en albumine productie (8).
Het doel van dit proefschrift was om biocomponenten van BAL systemen, de HepaRG en C3A celllijnen, te bestuderen, de factoren te bepalen die hun uitrusting limiteren en strategieën te ontwikkelen om hun leverfuncties te verbeteren, met speciale aandacht voor de rol van de energie huishouding.

Deel I van dit proefschrift bevat een inleiding en beschrijft de functionaliteit van HepaRG en C3A cellen in monolaag en BAL kweek en de associatie tussen mitochondriale energie huishouding en differentiatie.


In Hoofdstuk 2 vergeleken we de leverfuncties van HepaRG en C3A cellen in AMC-BAL en conventionele monolaag kweken. De functionaliteit van beide lijnen was sterk verbeterd door BAL kweken vergeleken met monolaag kweken. De HepaRG cellen vertoonden daarnaast hogere functionaliteit ten opzichte van C3A cellen, wat maakt dat HepaRG cellen te verkiezen zijn als BAL biocomponent.

Hoofdstuk 3 beschrijft in meer detail de achtergrond van de verhoogde functionaliteit van HepaRG cellen veroorzaakt door BAL kweken. Microarray analyse toonde aan dat het kweken in de BAL de transcriptie van genen betrokken bij mitochondriale energie huishouding verhoogt. Daarnaast was er een opregulatie van de hoeveelheid mitochondriën, expressie van oxidatieve fosforylering (OxPhos) complexen en mitochondriële membraan potentiaal. De drie belangrijkste verschillen tussen BAL en monolaag kweken zijn de 3D vs 2D configuratie, de aan- vs afwezigheid van medium perfusie en de oxygenatiegraad (hoger in BAL). Alle drie factoren bleken bij te dragen aan verhoging van de hoeveelheid mitochondriën per cel in zowel HepaRG als C3A cellen, wat suggereert dat het stimuleren van mitochondriale biogenese een algemeen effect van BAL kweken is, onafhankelijk van de gebruikte biocomponent.

Het effect van zuurstofconcentratie en medium perfusie op leverfuncties en energiehuishouding werd verder bestudeerd en beschreven in de volgende hoofdstukken.

In Hoofdstuk 4 werd het effect van pericellulaire zuurstofconcentratie op de differentiatie van lever celllijnen HepaRG en C3A getest. Het kweken van HepaRG cellen onder hyperoxie (40% O₂), in plaats van de conventionele normoxie concentratie (20%O₂) verhoogde de lever differentiatie en functionaliteit. Hyperoxie induceerde de essentiële hepatische transcriptie factor CCAAT/
enhancer-binding protein α (CEBPα) en verhoogde de translocatie naar de kern. De positieve effecten van hyperoxie op hepatische functionaliteit werden bevestigd in C3A cellen. Microarray analyse van het transcriptoom van HepaRG cellen geëxplande onder hyperoxie vs normoxie toonde aan dat slechts een selecte hoeveelheid genen differentieel geëxpreisseerd werd (1%), waaronder een aantal bekende lever-verrijkte transcriptie factoren. Dit wijst erop dat mogelijk post-transcriptionele processen een belangrijke rol spelen bij de verbeterde functionaliteit onder hypoxie. Het kweken onder hypoxie (5% O₂) leidde in HepaRG cellen juist tot verlaging van de differentiatie graad, kenmerkend voor lever-stemcellen. Deze onvolwassen staat werd bevestigd door de relatief hoge expressie en nucleaire translocatie van stemcel eiwit SOX9. Daar staat tegenover dat hypoxie condities tijdens het expanderen van de HepaRG cellen hun stabiliteit verhoogde, waardoor het verlies van functionaliteit, onder normoxie geobserveerd na passage P20, uitgesteld werd. Het effect van zuurstof op hepatische differentiatie wordt waarschijnlijk veroorzaakt door zuurstof-gevoelige factoren, zoals de hypoxia-inducible factor 1α (HIF1α). De expressie en nucleaire translocatie van deze factor was inderdaad verhoogd in HepaRG cellen gekwekt onder hypoxie vs normoxie en hypoxie.

In Hoofdstuk 5 werd het effect van “dynamic medium flow“ (DMF), het bewegen van medium, op hepatische differentiatie en energie huishouding bestudeerd. Medium perfusie verhoogde de mitochondriale biogenese, zoals geobserveerd bij BAL kweken (zie Hoofdstuk 3). Om het effect van DMF op leverfunctionaliteit van HepaRG en C3A cellen verder te bestuderen simuleerden we DMF van de BAL door monolaag kweken te schudden bij 60rpm tijdens de differentiatie fase, wat een makkelijke methode is. Deze nieuwe kweek strategie leverde substantiële verbeteringen op ten opzichte van conventioneel statische kweken van monolagen bij HepaRG cellen; de transcriptie van hepatische genen was verhoogd alsmede de nucleaire translocatie van hepatische transcriptie factor CEBPα. Bovendien waren leverfuncties verbeterd, waaronder ammoniak eliminatie, galzout productie en cytochroom P450 3A4 (CYP3A4) activiteit. Zoals eerder gevonden voor BAL kweken was er een sterke associatie tussen hepatische differentiatie en mitochondriale energie huishouding; DMF kweken veroorzaakte een verschuiving van glycolyse naar OxPhos, blijkens een afname van melkzuur productie en glucose consumptie en een verhoging van zuurstof consumptie. De positieve effecten van DMF op hepatische functionaliteit en mitochondriale energiehuishouding werden ook gevonden in C3A cellen.

In deel II van het proefschrift worden strategieën beschreven om HepaRG cellen middels genetische modificatie te verbeteren in leverfuncties die relatief laag zijn, zoals ontgiftings capaciteit, mitochondriale energie huishouding en UC activiteit.

In Hoofdstuk 6 onderzochten we het effect van lentivirale overexpressie van CAR, een bekende regulator van ontgifting en energie huishouding in HepaRG cellen. De nieuw ontwikkelde HepaRG-
CAR cellen hadden een verbeterde galzout productie en verlaagde melkzuurproductie ten opzichte van de oorspronkelijke HepaRG cellen. Verder bleken HepaRG-CAR cellen in staat om hun capaciteit tot volledige differentiatie tien passages langer te behouden boven de kritische passage P20, ten opzichte van HepaRG cellen. Middels RNA sequencing vonden we dat het transcriptoom van HepaRG-CAR cellen verhoogde expressie vertoonde van OxPhos genen en verlaagde expressie van glycolyse, hypoxia en cel-deling-geassocieerde genen ten opzichte van HepaRG cellen. Verder bleek CAR overexpressie te leiden tot verlaging van de mTORC1 signalering, welke betrokken is bij verhoging van celdeling en glycolyse en reductie van mitochondriale energie huishouding, wat een belangrijke rol kan spelen bij de totstandkoming van het HepaRG-CAR fenotype.

Door RNA sequencing van vroege en late passages van HepaRG en HepaRG-CAR cellen vonden we dat het transcriptoom van HepaRG-CAR cellen weinig veranderde tijdens het passeren, in tegenstelling tot het transcriptoom van HepaRG cellen. Tijdens het passeren van HepaRG cellen verlaagde de expressie van hepatische genen en verhoogde de expressie van genen betrokken bij epitheliale-mesenchymale transitie. De stabiliteit van HepaRG-CAR cellen wordt mogelijk verklaard door een lager niveau van reactieve zuurstof radicalen (ROS), waarvan bekend is dat ze betrokken zijn bij verouderingsprocessen; het niveau van ROS was significant lager in HepaRG-CAR cellen vergeleken met HepaRG cellen.

In Hoofdstuk 7 hebben we onderzocht welke factoren de UC activiteit beperken in HepaRG cellen. Efficiënte ammoniak eliminatie via UC is zeer gewenst voor BAL toepassing, omdat op die manier ammoniak onomkeerbaar verwijderd wordt, wat essentieel is voor de behandeling van hyperammoniaemie (9), bij patiënten met leverfalen. In HepaRG cellen zijn de expressie en activiteit van drie UC enzymen laag ten opzichte van normale lever. Dit zijn: carbamoylphosphate synthase1 (CPS1-groot mitochondriaal enzym), ornithine transcarbamoylase (OTC-klein mitochondriaal enzym) en arginase 1 (ARG1-cytoplasmatisch enzym). Waarschijnlijk vindt daardoor >95% van de ammoniak eliminatie plaats door middel van inbouw in aminozuren, wat echter reversibel is, en slechts een marginale fractie van de ammoniak wordt definitief verwijderd uit de circulatie door UC activiteit. HepaRG cellen bleken arginine dosis-afhankelijk om te zetten in ureum en OTC overexpressie verbeterde niet de ureum productie, waaruit bleek dat OTC en ARG niet het lage niveau van de UC activiteit in HepaRG cellen bepaalden. Onverwacht bleek dat CPS overexpressie mitochondriale stress veroorzaakte en hepatische differentiatie onderdrukte in HepaRG cellen gekweekt in conventionele statische monolagen. Dit was met name onverwacht omdat dit niet het geval was met de overexpressie van OTC, ook een mitochondriaal enzym, maar van lager moleculair gewicht. Het negatieve effect van CPS overexpressie bleek gecorrigeerd te kunnen worden door de cellen te kweken onder DMF condities, waardoor ook de ureumproductie >4 maal verhoogde, mogelijk doordat DMF mitochondriale biogenese stimuleerde (zie Hoofdstuk 5). Daarnaast werden ook de transcript niveaus van een aantal andere UC genen opgereguleerd
onder deze condities. Op basis van deze gegevens concluderen we dat zowel het CPS niveau als statisch kweken, mogelijk door een te lage hoeveelheid mitochondriën, de UC activiteit beperken in HepaRG cellen.

**Conclusies**

Onze studie met het doel om meer inzicht te verkrijgen in de toepasbaarheid van humane levercelllijnen als biocomponent voor BAL toepassing heeft geresulteerd in een aantal strategieën die de celllijnen verbeteren middels simpele, maar effectieve, kweekprocedures, gebaseerd op aanpassingen van zuurstofconcentratie en op DMF. Daarnaast verhoogde opregulatie van lever-specifieke processen de toepasbaarheid van HepaRG cellen: overexpressie van CAR resulteerde in verhoging van stabiliteit tijdens expansie van de cellen en verbeterde ontgifting en mitochondriale energiehuishouding, terwijl CPS overexpressie in combinatie met DMF-kweken de UC activiteit verhoogde. Als belangrijke gemeenschappelijke factor vonden we een positieve correlatie tussen mitochondriale biogenese en hepatische differentiatie.
References


II. List of publications


III. List of contributing authors and affiliations

Robert AFM Chamuleau, Ruurdtje Hoekstra, Theodorus BM Hakvoort, Vincent A van der Mark, Ronald P Oude Elferink, Manon E Wildenberg, Joanne Donkers, Jung-Chin Chang
*Tytgat Institute for Liver and Intestinal Research, Amsterdam UMC, The Netherlands*

Thomas M van Gulik, Ruurdtje Hoekstra, Vincent A. van der Mark, Martien van Wenum, Erik J. Hendriks
*Department of Surgery, Amsterdam UMC, The Netherlands*

Aldo Jongejan, Perry D Moerland
Bioinformatics Laboratory, Department of Clinical Epidemiology, Biostatistics and Bioinformatics, Amsterdam UMC, The Netherlands

Jos PN Ruiter, Riekelt H Houtkooper, Ronald JA Wanders
*Laboratory Genetic and Metabolic Diseases, Amsterdam UMC, The Netherlands.*

Valery Shevchenko
*Biopredic international, Rennes, France*
# IV. PhD Portfolio

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<td>Biweekly-Differentiation meetings</td>
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<td>Thijjs Kuipers, Medical student, Liver disease course</td>
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<td>Afifa Amjad, vwo student, 1 week orientation about Biomedical Sciences</td>
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V. Dank woord

The most difficult part of writing a PhD thesis is how to express gratitude and appreciation to all the people who supported you throughout the whole journey of the research leading to this thesis. Without the tremendous support I received from all of you, this thesis would have never been a reality, so many thanks to all who contributed to this work, it was a great blessing and pleasure to work with you. I was deeply overwhelmed to work at both the Experimental Surgery Department and Tytgat Institute for Liver and Intestinal Research (Amsterdam-UMC). I would like to transmit my sincere gratitude to every one working in these departments. Here I will mention few names that enormously added to my PhD journey and made it possible.

I still remember the first time I came to the Tytgat Institute to conduct my first internship as part of my master program (Master of Biomedical Science-UvA). It was during the heaviest winter (of year 2010), I have ever seen in the 10 years I had spent till now in the Netherlands. As a medical doctor coming from Sudan, without any former research or lab experience, working in research field was totally a new adventure to me. My first supervisor to whom I’m extremely grateful is Dr. Ingrid Gaemers. Dear Ingrid, millions thanks for you for all the efforts you kindly spent to learn me how to start. Your task was quite challenging, but somehow that period was a great pleasure and fun with all crazy moments that happened during the experiments. Your warm-hearted personality overwhelmed me, your incredible knowledge and experience in liver metabolism triggered me to continue working in a quite similar direction. Although your interest is now shifted to a totally different era, but I’m happy to witness the progress you are making to advance the research quality in the lab.

Professor Dr. Ronald Oude Elferink, world-class expert in experimental hepatology. It is a great honor to defend my PhD thesis under your superb supervision. Despite the heavy duties, I deeply appreciate your contribution to all of the studies in this thesis through your critical and sharp remarks during the regular bi-monthly Liver Differentiation Meeting and during the writing phase. Having you as a promotor was very precious and no words of thanks would express my gratitude.

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BAL group: Martien, thanks for your contributions to my thesis, I hope you all the best finishing your MDL specialization. Erik, I’m so grateful to the great help I received from you particularly during the experiments and for preparation of the HepaRG medium and all needed culturing materials. Albert, the most funny and kindhearted colleague in the Experimental Surgery Department. Your friendly and lovely comments on my looking and the colors I wear every now and then were very delightful. Thanks for the thousands orderings you have perfectly organized so that we never run out of supplies. I hope to you more success in your career and personal life. Adrie, the exceptional bio-technician expert that one could have. Many thanks for the wonderful time we spent during the pilot phase of our regeneration experiment. It is a pity that we could not manage to finalize that important part of my PhD training, but we all recognized for many logistic reasons, that it was not realistic anymore. I have learned a lot from you about how to handle the rats and importantly how to spent the night shifts without dramatic events.

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To all my dear colleagues in Tytgat Institute without any exception, please accept my immense gratitude for making life so joyful, festive and cheerful during the whole journey.

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Dear Jurgen, Theo, Piter and Coen, I think I was so lucky to have your critical input and directing remarks in a lovely atmosphere on my research during our Bi-monthly Liver Differentiation Meeting throughout the whole PhD journey. Special thanks for your fundamental contributions to all phases of my research.

Manon, many thanks for helping me with the immune-staining experiments, your brilliant mind and smart tricks with the microscope worked super good to obtain images with high resolution even through the thick culturing plate. I have learned a lot from you and your company makes the long enduring experiments super great amusement.

Dear Kam, no words could express my gratitude to your endless assist during the experiments. You are always ready and around to help everyone finding the materials required. Esther, it was very nice to have you around in the lab, thanks a lot for all the help and technical support. Dear Wil, you were the heart of the lab, facilitating many technical and administrative tasks. I hope you will get through the current challenging circumstances and enjoy your retirees.

Jung-Chin and Simei, the best enthusiast team players in Tytgat institute, thank you very much for your support especially with the western blotting experiments. I’m expecting that Simei will be defending her wonderful SAC-thesis pretty soon and all the best wishes for the strongly coming principal investigator or shall I say professor Jung-Chin Chang.

Dagmar, Jyoti and Suzanne, the mama’s club, thanks for the joyful pregnancy and motherhood talks, I hope to all of you very wonderful and highly enlightened family and career life.

Mohammed and Fadi thanks for your cheerful company and the lovely Arabic conversations we had, I hope all the best to all of you in your family and your professional life.

Johan, many thanks for the pleasant funny talks. My deep gratitude extended to Stan’s group, with especial thanks to Joanne for her considerable assist in determining NTCP functionality in HepaRG cells.

Dear S1-176 companions, Derek, how can I thank you for solving all my pop-in computer problems with your magic hands. I still remember the philosophical and sometimes crazy conversations we had together, you absolutely made my days there very joyful.

Prof. dr. Wout Lamers, my room companion, the icon in embryology and expert in CPS1 and urea cycle regulation. I absolutely enjoyed your presence in our office, although it was only one day/week, but your incredible knowledge and gentle way of sharing thoughts and critical remarks were extremely useful. Many thanks for being also one of the member in the promotion committee.
Dear promotion committee members, Prof. dr. Bert A.K. Groen, Prof.dr. Riekelt H.L. Houtkooper, Prof. dr. Peter L.M. Jansen, Prof. dr. Wout H. Lammers, Dr. Fred A.J. Meijer and Prof. dr. Robert J. Porte; please accept my sincere gratitude and appreciation to all of you for the thorough revision of my PhD thesis and for giving me this magnificent chance to defend my thesis in your respectful presence.

Vincent and Lysbeth, my lovely paranymphs, Vincent, your great contributions almost to all the papers in this thesis were incredible. Your sharp, well-determined mind and your kind support with the performance of several experiments particularly those related to lenti-viral overexpression was tremendously big and unmeasurable. I enjoyed your company very well and all conversations and fun moments. Lysbeth, millions of thanks for all lovely talks about the kids and mama’s issues we had together. Many thanks for all the help you provided with cell culturing and with finding materials required for the experiments. Beside this all, both of you shared similar interest as former AMC-BAL colleagues, so having both of you beside me as paranymphs is just unbelievably wonderful and great honor.

To my beloved father, actually language fails to express my gratitude to you for your continuous encouragement and support that afforded me the energy to acquire this degree. To the soul of my darling Mum (RIP), I know how eager you were to witness this day, what so ever I did or said, I will never be able to thank you properly. This book is actually a result of your unlimited support and prayers. My gratitude expands to all my sisters and brothers, I was very lucky to be a part of this big and warm family.

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My precious Mohammed, the best man I have ever met. My husband, my intimate friend and the father of our darling kids. Without your unlimited support and endless love and care, I will not be able to be a mother of four children with two master degrees and a PhD. I love you so much.
VI. Curriculum vitae

Aziza Abdelrahman Abubakr Adam, born on July 28th, 1982 in Elgzira Abe te Sudan. She joined Khartoum University-Faculty of Medicine in October 2001 and graduated in April 2008. At the same year of graduation she got married to Mohammed Kara and moved to Netherlands to stay with her husband. In 2010, she started a full-time master study in Biomedical Sciences at the University of Amsterdam (UvA), simultaneously with a part-time master Evidence Based Practice study (UvA). In June 2013, she joined the Bioartificial Liver group at the Academic Medical Center of the UvA for a 4 years PhD program under the supervision of dr. RAFM Chamuleau, dr. R Hoekstra and prof. RP Oude Elferink, a combined effort between Surgical Laboratory and Tytgat institute for Liver and Intestinal Research, in which she studied the role of mitochondria in the hepatic differentiation of human liver cell lines in Bioartificial Liver applications. Subsequently, she worked from June 2017 to January 2018 as a post-doc, in the same group, to study the transcriptome of the newly developed HepaRG-CAR cell line.
