Preclinical development of the AMC-HepaRG-Bioartificial Liver

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Preclinical development of the AMC-HepaRG-Bioartificial Liver

Martinus van Wenum
Preclinical development of the
AMC-HepaRG-Bioartificial Liver

ACADEMISCH PROEFSCHRIFT

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aan de Universiteit van Amsterdam
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CHAPTER 1
General introduction and thesis outlines
GENERAL INTRODUCTION

This thesis revolves around the development of the AMC-bioartificial liver (BAL), an innovative device aimed at the treatment of patients suffering from acute liver failure (ALF) and Acute on chronic liver failure (ACLF).

Definition and prognosis of ALF and ACLF

Over 40 definitions of ALF can be found in literature [1]. The European Association for the Study of the Liver (EASL) defines ALF as severe acute liver injury, characterized by markers of liver damage (elevated serum transaminases) and impaired liver function (jaundice and International Normalized Ratio> 1.5) in combination with the clinical appearance of hepatic encephalopathy, in the absence of a pre-existing liver disease. Acute presentations of chronic auto-immune hepatitis, Wilson disease and Budd-Chiari syndrome are to be considered ALF when fulfilling the above criteria, despite the presence of pre-existing liver disease [2]. ALF is associated with high mortality rates, especially in those who are not eligible for liver transplantation. Due to technical advances in supportive care in specialized liver units and, most importantly, the availability of emergency liver transplantation in the last decades, survival of ALF patients increased from 16.7% in 1973–1978 to 62.2% in 2004-2008 [3]. As for ALF, the definitions of ACLF that is used in clinical practice and in the scientific literature is ununiformed. Working groups of the Asian, American and European scientific organizations, as well as the World Gastroenterology Organization all formulated their own definitions of ACLF. These definitions differ mainly in the criteria for organ failure and preexisting liver disease, as well as the requirement for the acute insult to be hepatic, all of them are associated with high mortalities [4]. The biggest problem that arises due to the non-uniformity of the term ACLF in the scientific literature is that results are not comparable and therefore very difficult to translate to the clinical context. Based on the results of the CANONIC study, ACLF was defined by the EASL chronic liver failure (CLIF) consortium as decompensated liver cirrhosis in the presence of at least one organ failure. In the case of single-organ failure, serum creatinine level must be ≥1.5 mg/dL, or hepatic encephalopathy present [5]. Based on the serial organ failure (SOFA) score, the CLIF-C ACLF score was developed and validated in an external cohort [6]. With this score, patients can be stratified from no ACLF to grade 3 ACLF with associated 180 days mortality rates ranging from 38% to 96%. This is currently the best defined scoring system available, with the restriction that is based on European data and may not be fully applicable to other geographic regions.
Etiology
ALF is triggered by acute liver injury, the cause of which varies geographically, but is most commonly an acute viral hepatitis (e.g. hepatitis A, B, D, E and, to a lesser extent, C), an intoxication (e.g. paracetamol or poisonous mushroom), or an idiosyncratic drug reaction. In a substantial proportion of cases, the cause remains unknown, although these may still be triggered by unrecognized infections, intoxications or idiosyncratic drug reactions. Less common causes of ALF include acute liver ischemia, Budd-Chiari syndrome, Wilson’s disease and acute fatty liver of pregnancy [7, 8].

Like ALF, the triggers for ACLF vary geographically: in Europe the most commonly identified triggers are bacterial infections (33%), active alcoholism (25%) and gastrointestinal hemorrhage (13%), while in China exacerbation of hepatitis B infection was reported as a trigger in 36% of cases [4].

Presentation
The time from liver insult to ALF varies from days to weeks. In general, the first symptoms are related to loss of liver synthetic and metabolic capacity, such as jaundice, coagulopathy, hypoglycemia, hyperlactatemia and accumulation of toxins, such as endogenous benzodiazepines and ammonia, leading to the development of hepatic encephalopathy. A more advanced disease feature is multi-organ failure, thought to be mediated by both the release of damage associated molecular patterns (DAMPs) from necrotic liver cells and pathogen associated molecular patterns (PAMPs) from secondary infections and bacterial translocation, leading to a self-reinforcing cytokine storm [9]. Another deadly complication of ALF is intracranial hypertension caused by cerebral edema, the precise pathogenesis of which is still not fully understood, but inflammation, hyperammonemia and increased cerebral glutamine levels appear to play key roles [10, 11].

ACLF, depending on the definition, generally develops within days, the current view is that preceding triggers, as described above, result in the release of DAMPs and/or PAMPs with a central role for bacterial translocation, leading to an escalation of the inflammatory response that cannot be tempered by the already compromised liver [4].

Treatment
Initial treatment of ALF and ACLF consists of supportive therapy of failing organs, with early endotracheal intubation and sedation when patients develop agitation, often invasive monitoring of intracranial pressure and measures to treat intracranial hypertension and special attention to the prevention of septic complications. Emergency liver transplantation is a treatment option for those cases that are considered to have a very poor prognosis without transplantation, and who have not yet suffered irreversible (brain) damage and are
fit enough to undergo surgery. The selection of these patients in an early stage remains challenging. The Kings College Criteria for this selection were first published in the 1980s and are still commonly used [12], with a reported sensitivity of 69% and specificity of 92% [13]. The 5-year survival after orthotopic liver transplantation for ALF in Europe is 72%, which is slightly lower compared to elective liver transplantation; a difference that is caused mainly by early septic and neurological complications [14]. In one randomized controlled trial, high volume plasma exchange in ALF has shown a survival benefit, suggesting this should also be considered as a treatment modality [15].

**Artificial liver therapy**

Artificial liver support systems, such as MARS and PROMETHEUS rely on albumin dialysis. These systems were tested in both ALF and ACLF patients and treatment resulted in a consistent improvement in clinical and biochemical parameters. However, despite multiple large randomized controlled trials, till date no study has shown a survival benefit in intention to treat analyses [16].

**Bioartificial liver therapy and the AMC-BAL**

BALs are envisioned to support patients suffering from ALF and ACLF in order to bridge them to either liver transplantation or recovery. BALs are extracorporeal devices, loaded with living cells (the biocomponent) and are generally connected to the patient via a plasmapheresis circuit.

The AMC-BAL was among the first generation of devices, conceived in the AMC in the 1990’s. It consists of a sheet of matrix to hold the biocomponent, that is wound around a core, and interleaved with gas-permeable capillaries for oxygenation [17]. The features that sets it apart from many other devices is that the biocomponent comes in direct contact with the patient’s plasma (Fig. 1), that the cells are in immediate contact with oxygen and are allowed to organize into three dimensions. Initially, the AMC-BAL was based on primary porcine hepatocytes. Efficacy was proven in a porcine model of ALF and a Phase I clinical trial was in progress [18, 19] when a change in regulation prohibited the further clinical use of xenogeneic cells in the European union [20]. The alternative biocomponent of first choice would have been primary human hepatocytes, however, these are not available in sufficient quantities. A quest for a human-derived proliferative cell source of sufficient quality eventually ended at the human progenitor cell line HepaRG [21]. In 2012, efficacy of the laboratory scale HepaRG-AMC-BAL was shown in a rat model of ALF [22].

The primary aim of this thesis was to further develop the HepaRG-AMC-BAL towards clinical application.
**THESIS OUTLINE**

**PART I** of this thesis focuses on the selection of a biocomponent for the AMC-BAL:

In **Chapter 2**, we first describe the demands posed to biocomponents of BALs applied clinically, as well as those applied in drug safety studies and other *in vitro* applications. We then proceed to review the literature on available proliferative cell sources to suit these demands and propose a benchmark set of tests to assess candidate biocomponents.

In **Chapter 3**, two such candidate biocomponents were compared head-to-head: the hepatic cell lines HepaRG and C3A. These were assessed in conventional monolayer cultures and

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**Figure 1. HepaRG-AMC-BAL.** Schematic representation of the HepaRG-AMC-BAL (A) with details (B-C) and hematoxylin/eosin-staining (D) showing medium/plasma inflow port (I), medium/plasma outflow port (II), gas inlet port (III) (gas outlet port at opposite position not visible), the non-woven matrix to which the cells attach (IV), gas capillaries (V) and inter-capillary space (VI) through which the culture medium/plasma flows). From van Wenum et. al. Biofabrication. 2017;9(3):035001
in laboratory-scale BALs. The results described led to the conclusion that both cell lines perform better in BALs than in monolayers, and that HepaRG is best suited for application as a BAL-biocomponent.

In **PART II**, HepaRG cells and their behavior as BAL-biocomponent under different culture conditions are further characterized:

**Chapter 4** continues on the observation that culturing in the AMC-BAL has a beneficial effect on the functionality of HepaRG cells compared to conventional monolayer culturing. We investigated the importance of three dimensional configuration, medium perfusion, as well as oxygenation to this effect, and investigated its association with increased mitochondrial biogenesis through whole-transcriptome microarray analysis, mitochondrial abundance, as well as mitochondrial membrane potential.

In **Chapter 5** we zoom in on the importance of pericellular oxygen concentration for the differentiation of HepaRG cells. We investigated the influence of oxygen concentration on their differentiation grade by functional and transcriptional assays, as well as immunostaining of immature and mature hepatocyte markers and of Hypoxia Inducible Factor 1α. We also applied hypoxia during the expansion phase of the cells in order to increase the proliferative capacity.

**Chapter 6** focusses on the occurrence and reversibility of the toxic effect of blood plasma on HepaRG cells in monolayer and BAL cultures. Cultures were exposed to different fractions of healthy human plasma in order to identify the fraction containing the detrimental factor(s). A whole-genome transcriptomic study on cells exposed to this plasma for different durations was performed to identify the underlying mechanisms. Finally, we also assessed the detrimental effect of healthy versus ALF-plasma of pigs on mitochondrial functions.

In **PART III** the HepaRG-AMC-BAL was prepared for pre-clinical and clinical testing:

In **Chapter 7**, we explored cryopreservation of both HepaRG cells prior to BAL-loading, as well as fully differentiated BALs. We determined the conditions for transport of cell-loaded BALs to the clinic and developed a clinically applicable BAL-transport system. We assessed tumorigenicity of HepaRG cells in immunodeficient mice, and we successfully scaled up the HepaRG-AMC-BAL from the 9 mL laboratory-model to a 540mL clinical size model. Finally, in **Chapter 8** the content of this thesis is summarized and future perspectives of the HepaRG-AMC-BAL are given.
REFERENCES


CHAPTER 2

Bioartificial livers in vitro and in vivo: tailoring biocomponents to the expanding variety of applications


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Chapter 2

ABSTRACT

Introduction: Bioartificial livers (BALs) were originally developed to treat patients suffering from severe liver failure, and relied on primary hepatocytes or on hepatoblastoma derived cell lines. Currently, new \textit{in vitro} BAL applications are emerging, including drug toxicity testing, disease modelling and basic clinical research, and in recent years, advances in the field of stem cell biology have resulted in potential alternative cell sources.

Methods: This review identifies the demands of clinical and \textit{in vitro} BAL applications to their biocomponent and summarizes the functionality and developmental state of BAL technology and cell types currently available. Relevant studies identified by searching the MEDLINE database until April 2014 were reviewed.

Results and conclusions: BALs have the potential to meet demands currently left unmet in both clinical and \textit{in vitro} applications. All the reviewed biocomponents show limitations towards one or more BAL applications. However, the generation of stem cell derived hepatocyte-like cells is progressing rapidly, so the criteria for patient-specific drug toxicity screening and disease modelling are probably met in the near future. HepaRG cells are the most promising biocomponent for clinical BAL application, based on their proliferative and differentiation capacity.
INTRODUCTION

Bioartificial livers (BALs) have originally been developed to treat patients suffering from acute liver failure (ALF) and acute-on-chronic liver failure (ACLF), both devastating syndromes with mortality rates of up to 80%[1]. A clinical BAL is a device that consists of a bioreactor filled with liver cells, the biocomponent, and is connected extracorporeally to the blood circulation of the patient. Clinical application is still a primary aim; clinical trials of several BAL systems are in preparation (AMC-BAL [2] and UCL Alginate encapsulated HepG2 BAL [3]) or recruiting at present (Extracorporeal Liver Assist Device, ELAD [4]).

BAL culture promotes hepatocyte differentiation, facilitates growth in 3D and sustains the differentiated hepatocyte phenotype over a longer time compared to monolayer cultures [2, 5]. In addition, perfused oxygenated systems can provide a high level of control over the cellular microenvironment and flexibility in cell-medium ratio, sampling volume and regime [5, 6]. For these reasons, new applications for BALs are emerging, including drug development and -toxicity screening, disease modelling and fundamental (stem) cell biology. These applications call for a much smaller device than their clinical counterparts. This can be accomplished by downsizing existing designs, but progression in the field of microfluidics has made ‘chip-sized organs’, including livers, a possibility [6-8].

Traditionally, BALs relied on primary hepatocytes or hepatoblastoma cell lines. In recent years many advancements in the field of regenerative and stem-cell medicine have been reported, especially on hepatocyte-like cells derived from stem cells. Our group has previously published an overview of liver cell culture devices[9] and of proliferative human cell sources applied as biocomponent in BALs [10]. An in depth review of clinical BAL systems has been published by Park et al [11]. Now, in the light of the expansion of in vitro BAL applications and new cell sources, we aim to provide an overview of BAL applications, their demands to bioreactor design and cell functionality in the first part, and in the second part to what extent these demands are met by the cell types currently available.

For this review we defined BALs as follows: perfused devices, loaded with living cells that exhibit liver specific functionality. We performed a Medline search with terms related to BALs, hepatocytes and hepatocyte-like cells, and selected papers until April 2014 on the basis of relevance for our aims and availability of full text in the English language.

Clinical BAL application

Clinical BALs are the largest BAL devices, intended to support liver function of patients who suffer from ALF or ACLF, two related syndromes with variable causes and courses, both characterized by acute massive necrosis of the hepatic cell compartment, leading to loss...
of liver functionality, accumulation of (neuro)toxins and to a strong immune response [1].

In this review we will use the term Severe Liver Failure (SLF) to indicate both. The majority of ALF patients in historic cohorts died due to intracranial hypertension [12, 13], but with modern intensive care management in specialized units, the proportion of these patients has dropped to 22% [13, 14]. This suggests that, next to intracranial hypertension and hepatic encephalopathy, other SLF components, such as inflammation, should be therapeutic targets. It is well-documented that SLF is associated with high plasma levels of several pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα) and pro-inflammatory interleukins, which contribute to the development of systemic inflammatory response syndrome (SIRS), and eventually will lead to multi-organ failure and death [15]. In a series of 887 ALF patients, SIRS was diagnosed in 57% of cases, and was associated with more severe disease outcome and a higher mortality [16].

To date, the only available curative therapy for SLF is liver transplantation, either orthotopic, in which the native liver is removed, or auxiliary, when the native liver is left in situ at least partially. After auxiliary liver transplantation, two thirds of surviving ALF patients undergo regeneration of their native liver [17, 18], indicating that temporary liver support, such as BAL therapy, is potentially a curative treatment for the majority of SLF patients.

On the basis of data from animal studies, Tsiaoussis et al. calculated that the minimum amount of high quality primary hepatocytes for effective liver support is 20-40% of liver-cell mass, which translates to approximately 15-10⁹ cells or 150 grams of hepatocytes[19]. Data from a hepatocyte transplantation study in a partial liver ischaemia rat model of ALF suggest that a biomass as little as 2% of native liver mass can have a beneficial effect through disinhibition of liver regeneration; the treated rats showed a marked increase in liver regeneration and survival time, but not in overall survival. This suggests that the newly regenerated livers (30% of original liver cells mass) were not able to sufficiently support the animals [20]. This suggests that the newly formed liver mass has not yet matured enough to support the animals, and underlines the importance of sufficient biomass to support the native liver during regeneration.

Besides cell mass, the efficacy of BAL therapy will be determined by the bioreactor configuration, as discussed in section 4, and by the functionality of the applied cells. The complex and largely non-elucidated cascade of events, as well as the variable nature of SLF, make specification of demands to liver cell functionality of clinical BALs challenging. SLF is a black box syndrome that can, at present, only be treated with a black box cure. Therefore, the phenotype of a clinical BAL biocomponent should approximate that of the most important epithelial cells in the liver: the primary human hepatocyte (PHH). We reviewed PHH functions
and their reported relevance in the setting of SLF treatment in Table 1 as a basis to screen potential clinical BAL biocomponents.

**In vitro applications**

Downscaled BALs were -and are still- used as tools to develop clinical BALs [21-23]. In addition, in recent years new applications for small scale *in vitro* BALs have emerged, such as drug development, disease modelling and, to a smaller extent, basic scientific research [6, 7].

**Drug development**

The most relevant *in vitro* BAL application in drug development is screening for hepatotoxicity, which is the main cause of late-stage failure and withdrawal of drugs from the clinical development and after introduction on the market [24]. Animal studies and *in vitro* studies with human hepatocytes and liver cell lines have limited predictive value, as illustrated by the fact that 38–51% of drug induced liver injuries (DILI) in humans are not detected preclinically [24].

The likelihood of a DILI differs between individuals, a phenomenon that can be explained by the inter-individual difference in expression of detoxification enzymes and corresponding metabolite profile, as well as the susceptibility to damage inducing molecules. It is thought that DILIs can occur through three pathways: direct cytotoxicity, direct mitochondrial impairment or specific immune reaction, as reviewed by Russmann *et al* [25]. The occurrence of DILIs therefore, depends on the abundance and activity of proteins involved in drug detoxification, as well as the metabolic state of the cells, the mitochondrial activity and the host immune system.

A drug toxicity screening-BAL should therefore exhibit full phase 1 and 2 detoxification functions, express the entire array of basolateral and apical transporters and, at the same time, have physiological energy metabolism. Furthermore, this BAL should show high viability, reproducibility and stability. An *in vitro* BAL from a single genetic background, exhibiting all of the properties listed above would be an extremely valuable tool. Preferably, however, these drug toxicity screening BALs should be supplied with biocomponents of diverse genetic backgrounds with corresponding phenotype, reflecting the variability within the normal population [5].

**Liver disease modelling**

For a number of human liver diseases, ranging from infectious to metabolic and auto-immune diseases, there are no suitable *in vitro* models. Here we will discuss three notable examples of fields that could benefit from an *in vitro* BAL system in the near future, namely: *Plasmodium vivax* malaria, viral hepatitis and metabolic diseases [45, 46]. *P. vivax* and *P. ovale* can reside quiescently in human hepatocytes, as hypnozoites. The mechanism of parasites reactivation...
## Table 1. Important hepatocyte functions for clinical BAL-biocomponents.

<table>
<thead>
<tr>
<th>Function</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ammonia detoxification</strong></td>
<td>There is a causal relationship between hyperammonaemia and intracranial hypertension in ALF. In vivo ammonia elimination occurs via two major routes: irreversible fixation into urea and reversible incorporation into amino acids, most notably glutamine. The latter route is thought to function as a temporary ammonia sink during SLF as in various cell types glutamine breakdown by glutaminase yields ammonia again. A clinical BAL must have the capacity to eliminate ammonia to the same extent as PHHs, preferably through urea cycle activity.</td>
</tr>
<tr>
<td><strong>Lactate elimination</strong></td>
<td>Hepatocytes, like many cell types, abundantly express lactate dehydrogenase (LDH), the enzyme that catalyses the reaction from lactic acid to pyruvate and back. In contrast to most cell types, PHHs eliminate excess lactate through this pathway. Lactate can effectively cross the blood-brain barrier and recent literature suggests a causal relation between increased cerebral lactate levels and the occurrence of cerebral edema in a rat. In ALF patients brain lactate concentration correlates closely with ICP, pointing out lactate elimination as a potentially important BAL function. However, it is also well-documented that lactate acts as a cerebral energy source under both physiological and pathophysiological conditions. Deeper insight is required before superphysiological lactate clearance can be a therapeutic objective. For clinical BAL application, lactate elimination up to the level of PHHs appears preferable over lactate production.</td>
</tr>
<tr>
<td><strong>Protein synthesis</strong></td>
<td>The liver produces a multitude of vital proteins, some of which may attenuate SLF. Albumin, clotting factors and apolipoprotein A1 (Apo-A1) are examples of such substances, although the first two can be administered exogenously in the ICU. Albumin is the most abundant plasma protein synthesized by the liver and acts as a carrier protein for soluble molecules. It is an antioxidant and an important determinant of blood-colloid osmotic pressure. Albumin has been suggested to modulate immune responses by buffering neutrophil derived reactive oxygen species. Studies in mice suggest that Apo-A1 can reduce inflammation by augmenting the effectiveness of the regulatory T-cell response. These proteins could, as such, play a role in SLF disease outcome. However there is no evidence that shortages of specific proteins contribute to SLF mortality under optimal ICU care. Protein synthesis should be a quality parameter for a clinical BAL, because the hepatocyte proteome is a biological aspect that remains largely non-elucidated and is therefore a prototypical “black box” property that BALs have been envisioned to accommodate.</td>
</tr>
<tr>
<td><strong>Carbohydrate and lipid metabolism</strong></td>
<td>In the clinical setting, plasma glucose supplementation is standard of care, therefore this is not an important therapeutic target on its own. Lipid metabolism is altered in SLF patients, high density lipoprotein (HDL) serum levels are decreased during SLF and reflect disease severity but are no independent risk factor for mortality. No specific part of carbohydrate or lipid metabolism other than lactate elimination has been described as a specific BAL target.</td>
</tr>
</tbody>
</table>
Xenobiotic detoxification

The xenobiotic detoxification system, also involved in metabolizing endobiotics, has been proposed to be an important function of BAL systems in clearing toxins that accumulate during ALF [39]. The system exists of phase 1 (e.g., cytochrome P450) and phase 2 metabolizing enzymes, as well as transport proteins. There is evidence that neurotoxins normally metabolized by the liver, such as neurosteroids, accumulate during ALF and synergistically contribute to the development of hepatic encephalopathy in rodents [40]. Very little is known, however, about the contribution of such toxins to the clinical outcome of human subjects and the impact of the xenobiotic detoxification system. One study covering the subject [39] reported a ~40% increase in survival time in a diazepam-induced ALF model in dogs when supported with a BAL-system filled with CYP3A4 and Glutamine Synthetase (GS) overexpressing HepG2 cells (HepG2-GS-Cyp3A4).

However, when the experiment was repeated several years later, the authors reported a comparable increase of survival in animals only treated with HepG2-GS loaded BALs [39, 41].

There are no data that xenobiotic detoxification-competence is a prerequisite for efficacious BAL therapy, but there is some evidence that toxic compounds may contribute to disease severity. Therefore a basal level of xenobiotic detoxification is desirable in a clinical BAL, for example the expression of several detoxification enzymes with a broad substrate range, such as CYP3A4, CYP2D6 and glycosyltransferases [42].

Immunomodulation

Immunomodulation is not a classic hepatic function, but is relevant to the treatment of SLF patients because SIRS is a determinant of disease outcome [16], and because an increased concentration of pro-inflammatory cytokines in the liver is associated with inhibition of regeneration [43].

The our knowledge there is only one experimental report on the support of liver regeneration by BAL-treatment. In a partial liver resection and liver-ischaemia-model in rat, PHH-BAL-support increased survival time and expression of liver-enriched and growth-induced transcription factors in the livers of treated animals. This led the authors to conclude that BAL-therapy has a positive effect on liver regeneration. Furthermore they reported a decrease in transforming growth factor β1 (TGFβ1) plasma levels and hypothesised this could be the mechanism of action [44]. TGFβ1 is a potent inhibitor of hepatocyte proliferation in vitro and in vivo, but hepatocytes are known to lose their TGFβ1 sensitivity during regeneration [43], indicating the importance of other mechanisms that are not yet fully understood.

Immunomodulation may be of added value for SLF treatment through attenuation of SIRS and in the stimulation of liver regeneration, but the limited information on the mechanisms does not allow a suggestion for critical parameters.

ICU: intensive care unit; SLF: severe liver failure; PHH: primary human hepatocyte; ALF: acute liver failure; BAL: bioartificial liver; SIRS: systemic inflammatory response syndrome.
is largely unknown. Diagnosis and treatment options are therefore limited, hindering the recently reaffirmed objective to eradicate malaria worldwide of especially \textit{P. vivax} \cite{47}. \textit{In vivo}, \textit{P. vivax} hypnozoites can only be studied in non-human primates, presumably because the parasite machinery requires highly differentiated primate hepatocytes. Until recently, a suitable \textit{in vitro} model was not available, as the required fresh primate liver material is not stable enough to support the life-cycle of \textit{P. vivax} \cite{45}. In a recent paper, Dembele \textit{et al.} reported on an advance towards the successful long-term infection of primary macaque hepatocytes with \textit{P. cynomolgi}, a recognized \textit{P. vivax}- model capable of infecting both humans and macaques \cite{48}. Primary macaque hepatocytes were co-cultured with the human hepatic progenitor cell line HepaRG to rapidly fill up any breaches in cell-cell contact and thereby attenuate hepatocyte dedifferentiation. This model supported the formation and maintenance of hypnozoites, with infection load decreasing gradually over the course of 40 days of culture. The authors state that limitations of their model include a limited cell mass and detrimental breach of cell-cell contact \cite{48}. An \textit{in vitro} BAL system would be a logical follow-up model, provided that it sustains a higher number of cells and aids cell-cell contact by the means of a 3D configuration. At present, there have been no reports published of cells other than primary primate hepatocytes that can facilitate \textit{Plasmodium} infection, illustrating the inherent difficulty in specifying biocomponent demands to model diseases that are not fully understood.

In Hepatitis B and C virus research, \textit{in vivo} models currently include chimpanzees and transgenic humanized immunodeficient mice. There is no established \textit{in vitro} system available that supports the entire life cycle of the viruses \cite{46, 49}. An \textit{in vitro} BAL system can be of value to this field by offering a stable liver model, provided that the biocomponent applied in such BALs is susceptible to infection by these viruses and able to sustain the entire viral lifecycle (\textbf{Table 2}).

The modelling of metabolic diseases relies on the ability of a biocomponent to express the specific hepatic phenotype associated with a disease, which is often genetically predisposed. This implies that the biocomponents applied in these models must be derived from different (epi)genetic backgrounds while retaining their phenotype (\textbf{Table 2}).

\textit{Basic research}

In basic research there are several applications for \textit{in vitro} BAL devices, including the study of cell-cell interaction in a 3D setting, and the influence of perfusion rate, nutrients and oxygenation on hepatocyte differentiation \cite{6, 7, 50}. Especially microfluidic \textit{in vitro} BALs have the potential to become valuable tools to study hepatocyte differentiation because of the high level of control over the micro-environment they offer, including nutrient and oxygen gradients \cite{6}. The demands to the applied cell type or types for these applications are dependent on the research question and will therefore not be extensively explored in this review.
### Table 2. Different sizes of BALs, their applications and cell source requirements.

<table>
<thead>
<tr>
<th>BAL size</th>
<th>Biomass required</th>
<th>Design</th>
<th>Limitations</th>
<th>Applications</th>
<th>Biomass functional requirements</th>
<th>Current developmental stage</th>
</tr>
</thead>
</table>
| Clinical  | ~1.5×10^10^ cells (~150g) [19] | -Radial flow  
- Hollow fibre  
- Direct perfusion  
- Suspension | -High operational costs due to large size  
- Lower level of control of the cellular (micro) environment compared to chip sized BALs | Treatment of patients suffering from SLF  
- Basal level xenobiotic detoxification  
- Protein synthesis  
- Urea cycle activity  
- Stability in plasma environment  
- Financial feasibility of large scale production | Up to stage 3 clinical trial (HepatAssist, AMC-BAL and ELAD) [4, 55] |                                                                  |
| Intermediate size | 10^6-10^9 cells [56, 57] | -Radial flow  
- Hollow fibre  
- Direct perfusion  
- Suspension  
- Microfluidic  
- High throughput systems are favourable or required for most applications. | Lower level of control of the cellular (micro) environment compared to chip sized BALs | Developmental tool for clinical BALs  
- Predichnical hepatotoxicity screening  
- Balanced expression of xenobiotic detoxification proteins  
- High viability  
- Stable phenotype during long-term culture  
- Physiological energy metabolism and mitochondrial function  
- Diverse genetic backgrounds, preferably including donors who suffered from DILI. | To be used with the biomass applied in the clinical counterpart | In use                                                                                           |
| Inherited disease modelling |  |  |  |  |  | Under development                                                                 |
| Infectious disease modelling |  |  |  |  |  | Under development                                                                 |
| Chip size | 10^4-10^6 cells [7, 51] | Microfluidic | Limited sample volume | Research requiring strict control of the cellular microenvironment such as (stem) cell differentiation, experiments that involve very costly compounds and high throughput applications, not requiring large sample volumes. | Dependent on the type of research. | First commercial models entered end-user beta test phase. |

BAL: bioartificial liver; DILI: drug induced liver failure; SLF: severe liver failure
Chapter 2

**BAL hardware**

BAL-bioreactor models vary to large extent in size and configuration. Roughly, the bioreactors can be divided into 3 groups on the basis of their size, from chip-sized in vitro BALs loaded with less than one million cells, via intermediate sized in vitro BALs to clinical size BALs supporting up to $10^{11}$ cells [19, 51] (Table 2).

The configuration of the bioreactor has a direct impact on the functionality of the cells cultured in it. Characteristics with a large impact on functionality include: 3D vs 2D configuration, extracellular matrix composition, oxygenation, shear stress and exchange of nutrients and metabolites between cells and culture medium. The configuration will determine the level of control over the microenvironment, scalability and financial feasibility, often conflicting with one another. Therefore, the configuration will influence the applicability of a certain bioreactor model to different fields of research, as summarized in Table 2.

Cells reach a higher differentiation grade when cultured in 3D compared to 2D, a phenomenon thought to be mediated by improved cell-cell interaction and direct activation of differentiation pathways [2, 52]. 3D configuration can be achieved through a 3D scaffold or the use of spheroids: cell-aggregates in a 3D sphere-configuration. Spheroids can be deployed in BAL systems based on suspension culture [53], nano-patterned microfluidic chips [7] or encapsulated in a supportive matrix such as a hydrogel [54]. 3D cell configuration has proven feasible in BAL bioreactors of all sizes [5, 7, 51] and for this reason, should be considered state of the art.

The precise role of oxygenation tension during the different stages of hepatocyte development is not known. Terminally differentiated hepatocytes, however, require high oxygen tensions. Therefore, the general consensus is that sufficient oxygenation is a main challenge when designing a BAL-bioreactor [55].

*Clinical and intermediate size BAL hardware*

Small pore capillaries are regularly used to compartmentalize cells, medium and/or gas [11]. This technology is easily scalable between intermediate and clinical size BALs, capable of supporting cell numbers in the $10^7$-$10^{11}$ range, but not less. A disadvantage of compartmentalization of medium and cells is the hindrance of nutrient and metabolite exchange, a problem solved in the AMC-BAL by growing cells in a non-woven polyester matrix in direct contact with the perfusate, whilst securing oxygenation at site through gas-capillaries [21]. Other currently used clinical BALs are based on suspension cultures for spheroids or hydrogel encapsulated cells [3, 53]. The different options for clinical and in vitro BAL-bioreactor configuration have been reviewed in more detail in articles by Park et al. [11] and Godoy et al. [5].
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**Chip sized BAL hardware**

A highly controlled microenvironment and small cell numbers are feasible in microfluidic BAL systems. Much as in the physiological situation, medium perfusion and substrate availability can be precisely controlled and quantified, aiding reproducibility and allowing the set-up of high throughput systems. The microfluidic design comes at the expense, however, of limited sample volume and limited and costly scalability. Still there is a group pioneering larger scale microfluidic devices, showing proof of principle that the microfluidic approach could work for a clinical BAL [58]. There are several chip sized systems in advanced stages of commercialization [5, 59]. The state of the art of chip sized organs has been concisely described in a paper by van de Stolpe *et al* [8].

**Primary hepatocytes**

PHHs are generally regarded as the gold standard for clinical BAL application and drug toxicity testing, being the main epithelial cell type responsible for the functions of interest in the physiological situation [5, 19]. There are, however, two major drawbacks to the use of primary hepatocytes for all BAL applications: availability and phenotypic instability.

**Availability**

Since PHHs undergo maximally 1-2 population doublings in vitro, and human livers are scarce, PHH’s are not available in large numbers and batch sizes are limited. Although the availability of cells from diverse genetic backgrounds is favourable for many applications, for the reproducibility of both clinical and in vitro applications the limitation of hepatocyte batch size poses a challenge.

Xenogeneic primary hepatocytes have been suggested and applied as an alternative source of biomass for clinical BAL systems [11, 19] as well as for in vitro applications. Concerns about zoonosis have led to extremely strict regulations on the clinical application of xenogeneic material, de facto ruling out animal hepatocytes as a clinical BAL biocomponent. Xenogeneic primary hepatocytes also differ phenotypically from PHHs, resulting in different susceptibility to pathogens, drug metabolism and transporter activity, and are therefore regarded as unsuitable for many in vitro applications [48, 49, 60, 61].

PHHs can proliferate in vivo and repopulate animal livers. By transplantation of PHHs in immunodeficient mice, human hepatocytes are commercially produced [62]. Theoretically this technique could be expanded by using large animals. However, this does not solve the issues of zoonosis and phenotypic instability.

*In vitro* transduction with pro-proliferative genes to obtain expandable hepatocytes is propagated by Burkard *et al* [63]. PHHs transduced with a set of non-disclosed ‘proliferation
inducing genes’ are commercially available. However, their functionality does not approach that of PHHs in xenobiotic drug metabolism or ammonia detoxification [63].

Phenotypic instability
Hepatocytes are most commonly isolated through 2 step collagenase perfusion as first described by Seglen et al. in 1976 [64], which involves perfusion of liver tissue with a Ca$^{2+}$ free medium to disrupt adhesion molecules and collagenase to free the hepatocytes from the extracellular matrix. Directly after isolation, hepatocytes start to dedifferentiate. Dedifferentiation is now thought to be an active process, rather than a gradual loss of cell viability, and is characterized by the upregulation of structural- and extracellular matrix proteins and an activation of a mechanism similar to epithelial-to-mesenchymal transition, driven by MAP-kinase and AKT signalling, leading to the loss of liver enriched transcription factors (LETFs) expression [65]. Over the course of as little as 24 hours, hepatocytes loose the majority of LETFs transcription, leading to a rapid decrease in transcription and synthesis of hepatic proteins. Several phase 1 and 2 detoxification enzymes and anti-oxidative proteins are amongst the most rapidly down-regulated proteins [60, 61]. A review covering strategies to counter hepatocyte dedifferentiation through interventions in cell-cell contact, paracrine signals, cell- extracellular matrix interaction, epigenetics, gene transcriptional and post-transcriptional processes was written by Fraczek et al. [65].

Regulatory authorities regard data gathered from fresh and cryopreserved PHHs to be of equal value [66], and thus these are frequently used as gold standard control in papers describing PHH alternatives. One should keep in mind, however, that reference PHHs constituting of cryopreserved PHHs or of PHHs at more than 1-2 days after isolation have a lower functionality compared to that of freshly isolated PHHs, leading to a major overestimation of the tested cell functionality.

Hepatocyte cell lines
The main advantages of hepatocyte cell lines are their almost unlimited proliferative capacity and the relatively cheap culture process. There are two general approaches to acquire proliferative hepatocyte cell lines: through genetic engineering of PHHs, or by isolating cells from liver tumours that have already acquired the necessary mutations to render the cells immortal [67]. Most of the available cell lines only exhibit marginal hepatocyte specific functionality, which can improve to some extent when cultured in a BAL [2, 5]. We and others have published extensive reviews on immortalized cell lines for application in clinical BAL and toxicology systems [10, 19, 68, 69]. Briefly, the most differentiated and most frequently applied cell lines are the hepatoblastoma derived HepG2 especially its subclone HepG2/C3A- and the hepatocellular carcinoma derived progenitor cell line HepaRG.
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HepG2 cells are robust and proliferate quickly, making them easy and economical to expand and to select or engineer sub-clones. Especially sub-clone HepG2/C3A exhibits some liver-specific functionality, most notably synthesis of albumin and low expression -but not inducibility- of several major CYPs. Carbohydrate metabolism and ammonia detoxification are not comparable to that of PHHs at all, since they do not detoxify ammonia and produce large amounts of lactate [27, 67, 70]. Some of the genes necessary for clinical BAL application have been introduced into HepG2 cells, including CYP3A4 and glutamine synthetase, to improve xenobiotic metabolism and ammonia elimination by glutamine synthesis, respectively. As discussed in Table 1, these cells have produced encouraging results in animal models of ALF and there is a theoretical ground to believe that treatment of SLF-cases with a pronounced hyperammonaemia component can be beneficial, but none of the HepG2-derived cells meet the clinical BAL criteria summarized in Table 1.

HepaRG is a bipotent hepatic progenitor cell line that differentiates into a mixed culture of hepatocyte- and cholangiocyte-like cells, and is generally regarded to be the most differentiated proliferation-competent alternative for PHHs currently available [10, 67]. HepaRG exhibit substantial phase1- and 2 drug metabolism, ammonia elimination, contact-inhibition, protein synthesis and lactate consumption when cultured in 3D [2]. A disadvantage of HepaRG is the slow growth rate: cells can be split in a 1:5 ratio only once every 2 weeks, and maximum differentiation is reached after approximately 4 weeks, making expansion time-consuming and relatively costly. Also, in our experience, the cells have the tendency to lose their hepatocyte phenotype as the passage number increases or if the culture protocol is slightly violated (unpublished data). HepaRG is the only cell line with the intrinsic ability to support the entire hepatitis B life cycle, indicating that this cell line is appropriate for infectious liver disease modelling.

In conclusion, the HepaRG line is superior to HepG2 in terms of hepatic functionality, but not in flexibility and costs. HepaRG meets the criteria for clinical and most in vitro BAL applications, while HepG2 only meets part of the criteria for clinical BAL application after genetic manipulation. A major disadvantage inherent to all cell lines is that they only represent the phenotype of one single donor.

Stem cell derived hepatocytes

Both pluripotent and multipotent stem cells can be directed towards differentiation into hepatocytes in vitro by defined chemical signals and/or by transduction with specific transcription factors. As of yet, nobody has reported the successful differentiation of stem cells into fully mature hepatocytes, therefore stem cell derived cells with hepatocyte characteristics are commonly referred to as hepatocyte-like cells (HLCs) [71]. Stem cells and derived HLCs require growth factor- and small molecule enriched culture media, and often
extracellular matrix substrate coating. These requirements make stem cells too costly to be applied in clinical BALs in the near future. The available human stem cells include: human adult stem cells, induced pluripotent stem cells (iPSC) and embryonic stem cells.

**Human adult stem cells**

In adults, there are several sources of stem cells that can potentially be harvested to create HLCs, including mesenchymal stem cells and human liver stem cells. Several reports have been published on mesenchymal stem cell-derived HLCs, although, as of yet, no evidence of a high differentiation grade or a comparison with PHHs has been provided [72, 73].

The existence of the resident human liver stem cell or progenitor cell remains the subject of debate, but several reports have been published on the isolation of cells from the human liver that exhibit proliferative capacity and the ability to differentiate into HLCs [74-76]. Fonsato et al. isolated human liver cells, named ‘human liver stem cells’, which stained positive for embryonic stem cell- and mesenchymal markers, and negative for hematopoietic markers [76]. When cultured in a BAL system, hepatic differentiation was induced. Functions exhibited by these cells include activity of several major CYPs, uptake and excretion of indocyanine green and production of albumin and urea. No further analyses of carbohydrate metabolism, ammonia detoxification or CYP inducibility were published.

Duret et al. have isolated nonparenchymal epithelial cells from liver tissue of patients who underwent a partial liver resection and proposed these to be the human liver progenitor cells [75]. These cells were negative for most classic hepatocyte- and stem cell markers, but had a high proliferation potential and could be differentiated into HLCs under the influence of the growth factors HGF, EGF and FGF. Liver specific gene transcription was induced, as well as production of albumin and alpha 1-antitrypsin. No comparison with PHHs or further functional characterisation was included.

**Induced pluripotent stem cells.**

In 2007 Takahashi et al. succeeded in generating pluripotent stem cells from human dermal fibroblasts by overexpressing four transcription factors (Oct 3/4, Sox2, c-Myc, and Klf4) [77]. This ground breaking work has led to a rapidly developing field with private and public hiPSC banks emerging worldwide, aiming to produce thousands of human derived cell lines over the coming years from donors with different genetic backgrounds, both healthy and diseased [78]. Thus, theoretically providing the ideal source of BAL biocomponents if iPSCs can be efficiently differentiated into functional hepatocytes.

iPSCs can be differentiated into HLCs in a heterogeneous culture with different grades of differentiation [79]. IPS-HLCs are capable of supporting the entire life cycle of the hepatitis C
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virus [80], and iPS-HLCs derived from subjects suffering from alpha 1-antitrypsin deficiency, familial hypercholesterolemia or glycogen storage disease type 1a showed preservation of the donors’ disease phenotype [81].

We have summarized the papers that report on the generation of HLCs from human iPSCs and that included at least functional data and a PHH control group in Table 3. Interpretation of the reported functionality is, however, often hampered by the limited or lacking information on PHH source and culture procedure, therefore the reported data may be significantly overestimated. Also, the available literature focuses on HLCs cultured in monolayer, but there are reports available indicating that HLC differentiation is promoted by 3D configuration [52, 82]. Activity and inducibility of major CYPs are reported which implicates usefulness to drug toxicity and -metabolism studies. However, variation in expression between the drug detoxification genes and transporters in combination with the mixed cell phenotype could impair their applicability. No information on respiratory status or mitochondrial function are available. Only one study reported on urea cycle activity as measured by heavy ammonia conversion, in which case no urea cycle activity was found [83]. All iPSC-HLCs expressed the foetal marker alpha-fetoprotein (AFP), although, noticeably, the co-cultured liver organoids of Takebe et al. expressed AFP only in 50% of the albumin positive cells [84]. AFP expression indicates that iPSC derived HLCs are of an immature hepatocyte phenotype, or in fact represent foetal liver cells rather than hepatocytes as proposed by Schwartz et al. and Hannan et al. [79, 94]. The authors propose this to be due to our lack of understanding of the physiological process of fetal liver maturation [79]. Takayama et al. and Yu et al. conclude that a better understanding of the physiological hepatic microenvironment and differentiation stimuli is required to produce cells with a higher differentiation grade [83, 89]. According to our best knowledge, confirmed by others [71], no data have been published on the long term phenotypic stability of stem cell derived HLCs in vitro, information that is necessary to assess the applicability of iPS-HLC in disease modelling and drug development.

**Lineage reprogramming**

Lineage reprogramming is an approach adapted from iPSC technology; a selected set of transcription factors is introduced into primary cells to directly reprogram them into a specific cell type, bypassing the state of pluripotency. Zhu et al. [91] directly reprogrammed fibroblasts into a multipotent state, comparable to endoderm, and subsequently differentiated the cells towards HLCs. These cells did not resemble terminally differentiated hepatocytes in their expression pattern of 1299 genes, and showed a very limited capacity to repopulate mouse livers compared to PHHs. The expression patterns of the cells changed drastically into that of PHHs after transplantation into mice, showing that the cells have the potency to fully differentiate under the right conditions (in vivo) into hepatocytes, but that, again, our knowledge to establish those conditions in vitro is still insufficient.
Table 3. Summary of generated IPSs and lineage reprogramming-derived HLCs.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Method</th>
<th>Efficiency</th>
<th>Hepatic functionality</th>
<th>Control PHHs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Song 2009 [85]</td>
<td>-iPSCs, chemically mediated differentiation -Mtomicyn-C-treated feeder layer*</td>
<td>60% Albumin positive cells</td>
<td>-AFP positive -Urea synthesis ~10% of PHH -Albumin synthesis ~10% of PHH -Glycogen positive, PAS (periodic acid shift) staining -CYP3A4 activity ~3% of PHH</td>
<td>Cryopreserved PHHs from discarded donor organs, cultured for several days, AFP positive</td>
</tr>
<tr>
<td>Si-Tayeb 2010 [86]</td>
<td>-iPSCs, chemically mediated differentiation -Matrigel coated plates</td>
<td>~80% Albumin positive cells</td>
<td>-AFP positive -Urea synthesis ~equal to HepG2 -ICG uptake and excretion -LDL accumulation -Glycogen positive, PAS staining -Lipid droplets uptake -Transcript levels CYPs &lt;1%</td>
<td>Human liver tissue for normalization of assayed transcript levels, source not discussed</td>
</tr>
<tr>
<td>Jozefczuk 2011 [87]</td>
<td>-iPSCs, 3-step cytokine driven differentiation -Matrigel coated plates</td>
<td>Heterologous population</td>
<td>-AFP positive -Urea synthesis ~80% of PHH -ICG uptake and excretion -Glycogen positive, PAS staining -Low CYP transcript levels</td>
<td>Commercial fresh PHHs, 1 donor, culture method not discussed, AFP negative</td>
</tr>
<tr>
<td>Chen 2012 [88]</td>
<td>-iPSCs, 3-step cytokine/growth factor driven differentiation -Matrigel coated plates</td>
<td>70% cells positive for FOXA2 and Sox17</td>
<td>-AFP positive -Urea synthesis ~100% of PHH -CYP3A4 activity ~100% of PHH -Low CYP transcript levels</td>
<td>Age, source and culture conditions PHHs not specified, AFP positivity suggesting low quality</td>
</tr>
<tr>
<td>Yu 2012 [83]</td>
<td>-iPSCs, 3-step cytokine/growth factor driven differentiation -Matrigel coated plates</td>
<td>80% Albumin positive cells</td>
<td>-AFP positive -Urea synthesis ~25% of PHH, 0% -Conversion of heavy ammonia into urea (41.7% in PHH) -Albumin production ~25% of PHH -Diazepam hydroxylation ~30% of PHH (CYP3A4/2C19 activity) -Glycogen positive, PAS staining</td>
<td>Fresh PHHs, expanded in repopulated mice livers, AFP negative</td>
</tr>
<tr>
<td>Takayama 2012 (1) [89]</td>
<td>-iPSCs, differentiated in 3 stages, driven by growth factors cytokines and transduction with SOX17, HEX and HNF4a consecutively -Matrigel coated plates</td>
<td>~80% E-cadherin positive cells</td>
<td>-AFP positive -Sensitive to hepatotoxic compounds -Expression and inducibility of several major CYPs -Glycogen positive, PAS staining -ICG uptake and excretion</td>
<td>Cryopreserved PHHs, single donor, tested 48 hr after plating on collagen coated plates, AFP negative</td>
</tr>
<tr>
<td>Takayama 2012 (2) [90]</td>
<td>-iPSCs, combination of growth factors, complemented with LacZ, FOXA2 and HNF1α transduction -Matrigel coated plates</td>
<td>80% Albumin positive cells</td>
<td>-AFP positive -Urea synthesis ~50% of PHH -Albumin synthesis ~60% of PHH -CYP inducibility 1.5-50% of PHH -Basal activity panel of 8 CYPs: 1.7% -24% of PHH -LDL uptake -ICG uptake and excretion</td>
<td>Cryopreserved PHHs, single donor, tested 48 hr after plating on collagen coated plates</td>
</tr>
<tr>
<td>Reference</td>
<td>Method</td>
<td>Efficiency</td>
<td>Hepatic functionality</td>
<td>Control PHHs</td>
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<tr>
<td>Takayama 2013 [52]</td>
<td>iPSCs, combination growth factors, complemented with LacZ, FOXA2 and HNF1a transduction - 3D spheroids, in matrigel sandwich</td>
<td>Not reported</td>
<td>-AFP positive</td>
<td>Cryopreserved PHHs, 3 batches, 48 hr after plating</td>
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<td></td>
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<td>-Albumin synthesis ~70% of PHH</td>
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<td>-Urea synthesis ~50% of PHH</td>
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<td>-Baseline CYP3A4 activity comparable to PHH, inducibility &lt;25%</td>
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<td></td>
<td>-Acetaminophen toxicity &lt;40% compared to &gt;80% in PHH</td>
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<td></td>
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<td>71.9% Albumin positive cells</td>
<td>-Albumin synthesis ~100% of PHH</td>
<td>Cryopreserved PHHs, 24 hours after plating</td>
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<td></td>
<td>-&gt;50% of Albumin positive cells are AFP negative</td>
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<td></td>
<td></td>
<td>-no further testing of in vitro-construct.</td>
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<tr>
<td>Takebe 2013 [84]</td>
<td>iPSCs, 3-step growth factor-mediated differentiation, followed by co-culture with mesenchymal stem cells and HUVECS, upon which liver buds formed - Matrigel coated plates</td>
<td>~65% Albumin positive cells</td>
<td>-Albumin synthesis ~50% of PHH</td>
<td></td>
</tr>
<tr>
<td>Zhu 2014 [91]</td>
<td>iMPCs (induced endoderm), followed by a 2-step cytokine/growth factor driven differentiation - Matrigel coated plates</td>
<td>36% Albumin positive cells</td>
<td>-AFP undetectable by IHC</td>
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<td></td>
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<td>-Albumin and AAT syntheses comparable to PHH</td>
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<td></td>
<td>-Biliary markers absent</td>
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<td></td>
<td>-Glycogen positive</td>
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<td>-Inducible expression of several CYPs at the level of PHH</td>
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<td>-Activity of tested CYPs at ~1-6% of PHHs</td>
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<td>-Biliary transporter activity in range of PHHs for several compounds</td>
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<td></td>
<td></td>
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<td>-IGC uptake and excretion</td>
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<tr>
<td>Huang 2014 [92]</td>
<td>iHEPs, HNF4a, FOXA3 and HNF1a transduction and growth factor containing medium - Collagen sandwich culture.</td>
<td>91.7% Albumin positive cells</td>
<td>-AFP negative IHC</td>
<td>Cryopreserved PHHs from 3 donors, culture period not specified for all assays, directly used after thawing for functional detoxification tests. Function reported to be consistent with previous reports by others</td>
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<td></td>
<td></td>
<td>-99.8% AAT positive cells</td>
<td>-Albumin synthesis ~70% of PHH</td>
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<td>-Several major CYPs: Expression and activity ~25 to &gt;100% of PHH</td>
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<td>-Transcription of major drug transporters and phase 2 detoxification enzymes ~20 to &gt;100% of PHH</td>
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<td>-ICG uptake and excretion</td>
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<td></td>
<td>-Glycogen positive</td>
<td></td>
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<tr>
<td>Du 2014 [93]</td>
<td>iHeps, 6 transcription factors - Matrigel coated plates</td>
<td>91.7% Albumin positive cells</td>
<td>-AFP negative IHC</td>
<td>Freshly isolated PHH control from 2 discarded donor livers, cultured for a non-specified duration. (reason to discard organs not mentioned.)</td>
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<tr>
<td></td>
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<td>-99.8% AAT positive cells</td>
<td>-Albumin synthesis ~70% of PHH</td>
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<td>-Several major CYPs: Expression and activity ~25 to &gt;100% of PHH</td>
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<td>-ICG uptake and excretion</td>
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<td>-Glycogen positive</td>
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</table>

*IPS cells were grown on a layer of embryonic fibroblast derived cells of which proliferation was halted with Mitomycin-C-treatment.

AAT: alpha 1-antitrypsin; APF: alpha fetoprotein; CYP: cytochrome p450; ICG: indocyanine green; iPSC: induced pluripotent stem cell; LDL: low-density lipoprotein; PAS: Periodic acid–Schiff; PHH: Primary human hepatocyte
Very recently, two papers were published on directly induced hepatocytes, generated from human fibroblasts through transfections with a combination of LETFs. Huang et al. produced highly differentiated HLCs by transducing adipocytes and fibroblasts with a set of 3 LETFs (HNF4α, FOXA3 and HNF1α) and using a defined growth factor-containing medium. These HLCs, named induced hepatocytes (iHEPs), exhibited protein synthesis, biliary excretion indices and inducible expression of a substantial proportion of CYPs at the level of cryopreserved control PHHs, and did not express foetal liver markers, indicating they may represent a more differentiated hepatocyte phenotype compared to previously described HLCs. However, not all the parameters that we identified as important in Table 1 and section 2 have been tested, most importantly nitrogen- and carbohydrate metabolism were not. CYP activity ranged between ~1% and ~6% of freshly thawed PHHs. iHEPs were restricted in their proliferation and formed a heterogeneous population with approximately 20% well-differentiated cells. Through transduction with simian virus 40 large T-antigen (Tag) the cells could regain their proliferation potential for at least up to 10 passages, however AFP expression was induced and hepatic functionality was substantially lost. Tag is a recognized oncogene and is therefore not suitable for clinical application.

The second paper, by Du et al. reported a similar approach, using a combination of six transcription factors to induce the hepatocyte phenotype (HNF1A, HNF4A and HNF6, ATF5, PROX1 and C/EBPa) and conditional MYC and P53 siRNA expression to accommodate proliferation. This resulted in the efficient generation of HLCs, but these cells were not characterized extensively.

**Embryonic stem cells**
Embryonic stem cells can be similarly differentiated into HLCs as iPSCs. There are, however, two disadvantages to HLCs derived from embryonic stem cells compared to iPSCs. Firstly, for applications that require cells from a range of genetic backgrounds with established phenotypes, embryonic stem cells are unpractical because their donors’ mature phenotype is unknown. Secondly, they are the subject of ethical controversy, as they originate from human embryos. Because of these issues we believe that embryonic stem cells are not the biocomponent of choice for clinical and in vitro BAL application.

In conclusion, human adult stem cell derived HLCs do not offer benefits over other stem cell sources in terms of availability or differentiation potential, and are therefore not the most promising candidate biocomponent. Embryonic stem cells share these disadvantages and additional ethical concerns make application of this biocomponent unrealistic. iPSC derived HLC technology has not yet developed into the stage that it is suitable for any BAL applications, but the ability to host hepatitis C virus and maintain donor phenotype characteristics are promising. iHEPS generated through lineage reprogramming are a
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promising source of hepatocytes for in vitro BAL systems preferably based on patient-specific cell sources, but several difficulties need to be overcome before they can be successfully applied. The main problems are the lack of proliferative capacity and the heterogeneity of the cultures. In addition, although the cells exhibit substantial drug metabolism capacities, they are still not on par with PHHs, and nitrogen and carbohydrate metabolism data are still lacking. The use of BAL technology could aid in further differentiation of these cells [6]. Due to their limited proliferative capacity and complex composition of culture media these cells are more suitable for in vitro BAL application than for clinical BALs.

Co-culture

Next to hepatocytes, which represent the major proportion of human liver cells, there are other cell types that may be valuable to use in a BAL to support hepatocyte differentiation and add the dimension of cell-cell interaction to in vitro applications. Hepatocyte functionality and phenotypic stability can be improved in BAL systems by direct co-culture with mesenchymal stem cells, hepatic stellate cells and endothelial cells [96, 97], as well as through paracrine action of stellate cells [7]. These are, however, all primary cells, posing similar challenges in terms of availability as PHHs, making them highly unpractical for BAL application, with the exception of mesenchymal stem cells, which still have in vitro proliferative capacity [98].

Takebe et al. have reported that co-cultured iPSC derived mesoderm cells, human vein umbilical cord cells (HUVEC) and mesenchymal stem cells spontaneously differentiate into vascularized liver buds, with the most differentiated phenotype of IPS-HLCs reported as of yet [84]. Kupffer cells, the resident liver macrophages, as well as circulating immune cells have been proposed to play an important role in some forms of DILI [25], a hypothesis strengthened by the observation that pro-inflammatory cytokines and drugs known to cause idiosyncratic liver toxicity, synergistically induced hepatocyte damage in vitro [99]. However, at present, to our knowledge there are no suitable in vitro models available for these and other immune reactions in the liver.

CONCLUSION

BAL devices hold the promise to meet demands currently left unmet by alternative systems for both clinical and in vitro application. The choice of a BAL biocomponent depends on the intended application. The cell line HepaRG is currently the most promising biocomponent for clinical BAL application. For in vitro applications, there is no cell type that meets all demands, since PHHs are instable and have a low availability, HepaRG cells reach a high differentiation grade, but come from a uniform genetic background, and stem cell derived
HLCs show promising results, but their limited differentiation grade and proliferation capacity need to be overcome.

**EXPERT OPINION**

Until now, a major problem with the generation of fully matured hepatocytes from expandable cell sources suitable for any BAL application is the incompatibility between cellular processes relating to proliferation and hepatic differentiation. The development of HLCs has deepened our insight into these processes. iHEPs generated by Huang et al. did not express biliary or foetal markers, but induction of proliferation by transduction with an oncogene impaired differentiation grade [92]. Du et al. circumvented this issue of apparent mutual exclusiveness of proliferation and terminal differentiation by conditional RNAi expression [93]. In human developmental physiology, hepatocytes go through many intermediate stages from blastocyst to hepatocyte. Each stage is characterized by up- and downregulation of different transcription factors. We believe that in a similar way controlled phased expansion and differentiation can be the next step to produce highly differentiated hepatocytes now that the molecular mechanisms driving hepatic lineage development are progressively being unravelled. Advanced conditional expression systems are becoming increasingly available and can be exploited to temporarily overexpress transcription factors or inhibit their expression by siRNA. In addition, the role of growth and differentiation promoting factors becomes progressively clear. As their production increases, the costs will decrease, allowing their temporary addition to culture media to drive or inhibit specific phases of the cell production process.

No gold standard for the hepatic differentiation status exists, and the degree of characterization required depends on the aim of the study. Many reports on newly developed HLCs and hepatocyte cell lines only include a limited characterization, making it difficult to fully appreciate the differentiation grade and applicability in BALs. We have therefore summarized which parameters are, in our opinion, necessary to evaluate a biocomponent for BAL applicability in Table 4.

Two points that are especially relevant, although often neglected, are ammonia detoxification and the use of proper reference PHHs. Urea production is often used as a parameter for ammonia detoxification. Sole urea production is, however, of limited value because urea can be a product of arginase activity without involving the complete urea cycle that detoxifies ammonia in an energy dependent way [27]. Heavy isotope ammonia tracing is required to assess urea cycle activity. As described in section 5, the use of high quality reference PHHs is paramount. However, dedifferentiated PHHs have often been used as a reference material or even no PHHs at all, as illustrated by the last column of Table 3.
Table 4. Proposed hepatocyte benchmark for clinical and in vitro BAL-application.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Transcript level</th>
<th>Functional level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature hepatic markers</td>
<td>Low levels of Alpha fetoprotein, Glutathion synthetase pi, Cytokeraatin 19</td>
<td>At least one activity assay to confirm transcript level data</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>Panel of proteins, such as Albumin, apolipoproteins and coagulation factors</td>
<td>At least one activity assay to confirm transcript level data</td>
</tr>
<tr>
<td>Detoxification</td>
<td>-Phase 1 and phase 2 enzymes</td>
<td>-At least one phase 1 and phase 2 activity assay to confirm transcript level data</td>
</tr>
<tr>
<td></td>
<td>-Apical and basolateral transporters</td>
<td>-Indocyanine green/Mebrofenin/radio-isotope labelled ligand transport assay</td>
</tr>
<tr>
<td>Nitrogen metabolism</td>
<td>-Urea cycle enzymes</td>
<td>-Ammonia elimination rate to be tested at pathophysiological concentrations. The starting concentration should be in high micromolar to low millimolar range and not higher.</td>
</tr>
<tr>
<td></td>
<td>-Glutamine synthetase</td>
<td>-Urea production preferably tested by measurement of mass enriched ammonia conversion into urea (for clinical BAL application)</td>
</tr>
<tr>
<td></td>
<td>-Arginase 2; high levels are indicative for urea production through arginine degradation, rather than through urea cycle activity</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate metabolism</td>
<td>Glucose and lactate consumption/production and oxidative phosphorylation</td>
<td></td>
</tr>
<tr>
<td>Differentiation factors</td>
<td>LETFs and nuclear receptors, such as Hnf4a, Hnf1a, Pxr, Car</td>
<td></td>
</tr>
</tbody>
</table>

CAR: Constitutive adrostane receptor; HNF: Hepatocyte nuclear factor; LETF: Liver enriched transcription factor; PHH: Primary human hepatocyte; PXR: Pregnane X receptor.

In this review we defined the relevant hepatic parameters for the various BAL applications, and gave an overview of performances of the available biocomponents. There lies a risk in focusing merely on hepatocyte specific functionality, since other cell types or surrogates may be of added value for both clinical and in vitro applications. Immunomodulation for example, mentioned in section 2 as a potential important therapeutic strategy in the treatment of SLF, is not likely to be achieved using only hepatocytes. An interesting series of experiments in a galactosamine-induced model of ALF in rats, provided evidence that BAL treatment using mesenchymal stem cells, either alone or in combination with rat hepatocytes drastically improved survival as compared to treatment with hepatocytes only or with hepatocyte-
fibroblast co-cultures [96, 100]. In addition, interleukin-2 receptor antagonist (IL-1RA) treatment in galactosamine-treated mice lowered the pro-inflammatory cytokine load and accelerated early phase regeneration after partial hepatectomy [101, 102]. These results should be validated in an additional model of SLF, since it is known that galactosamine-induced liver injury is, at least in part, dependent on cytokine-induced apoptosis. If pro-inflammatory cytokine load indeed is an important contributor to SLF disease severity, BAL therapy should be supplemented accordingly, either through the use of an additional biocomponent such as mesenchymal stem cells, or through the addition of an artificial cytokine scavenger [103].

Taken together, our findings show that BAL systems are evolving to meet requirements posed by different applications. Innovations in stem cell technology will provide a profound understanding of liver development and insight in mechanisms behind liver damage that can be utilized to further optimize the biocomponents for BALs, both for clinical and in vitro applications.
REFERENCES


CHAPTER 3

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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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 tumour-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 \textit{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 tumour-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 aetiology 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, foetal 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.
A functional comparison between the HepaRG and C3A cell lines

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 $^{15}$NH$_4$Cl, 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

Figure 1. AMC-BAL Culture. (A) 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.
L-lactate, ammonia, urea, $^{15}$N-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 Tables 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.

**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
A functional comparison between the HepaRG and C3A cell lines

...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 S1. An overview of the transcript data is given in 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 $^{15}$N-ammonia into $^{15}$N-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, 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 (Table 1).

In C3A-BALs, we assessed hepatic functionality (Table 1) and transcript levels (Fig. 3A, 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

Figure 2. C3A culture medium selection. Comparison of C3A cells in monolayers cultured in MEM+ or WE+ medium for 7 days. (A) Hepatic functions and total protein content normalized to MEM+ cultured cells. (B) Gene transcript levels relative to MEM+ cultured cells. N.D. = not detectable. *p<0.05 compared to MEM+.
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 [8]. 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>7 days</th>
<th>14 days</th>
<th>21 days</th>
<th>Unit</th>
<th>3 days</th>
<th>7 days</th>
<th>14days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia elimination</td>
<td>µmol⋅h⁻¹⋅g⁻¹⋅protein⁻¹</td>
<td>-33.54 ± 9.59</td>
<td>-55.93 ± 9.18</td>
<td>-37.57 ± 6.99</td>
<td>µmol⋅h⁻¹</td>
<td>-2.06 ± 0.40</td>
<td>1.19 ± 0.60</td>
<td>±0.60</td>
</tr>
<tr>
<td>Urea production</td>
<td>µmol⋅h⁻¹⋅g⁻¹⋅protein⁻¹</td>
<td>1.42 ± 0.34</td>
<td>1.19 ± 0.27</td>
<td>0.86 ± 0.14</td>
<td>µmol⋅h⁻¹</td>
<td>0.042 ± 0.053</td>
<td>0.37 ± 0.06</td>
<td>0.44 ± 0.08</td>
</tr>
<tr>
<td>Albumin Synthesis</td>
<td>mg⋅h⁻¹⋅g⁻¹⋅protein⁻¹</td>
<td>35.65 ± 3.12</td>
<td>35.34 ± 6.34</td>
<td>NM</td>
<td>mg⋅h⁻¹</td>
<td>NM</td>
<td>12.17 ± 0.82</td>
<td>15.52 ± 1.5</td>
</tr>
<tr>
<td>CYP3A4 activity</td>
<td>nmol⋅h⁻¹⋅g⁻¹⋅protein⁻¹</td>
<td>2.27 ± 0.12</td>
<td>4.26 ± 0.78</td>
<td>NM</td>
<td>nmol⋅h⁻¹</td>
<td>NM</td>
<td>87.33 ± 15.59</td>
<td>95.66 ± 15.89</td>
</tr>
<tr>
<td>Lactate elimination</td>
<td>µmol⋅h⁻¹</td>
<td>17.10 ± 0.66</td>
<td>4.03 ± 1.66</td>
<td>1.24</td>
<td>µmol⋅h⁻¹</td>
<td>0.16 ± 0.12</td>
<td>0.62 ± 0.13</td>
<td>1.38 ± 0.07</td>
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<tr>
<td>AST leakage</td>
<td>IU⋅h⁻¹</td>
<td>0.78 ± 0.06</td>
<td>1.14 ± 0.07</td>
<td>2.00</td>
<td>IU⋅h⁻¹</td>
<td>0.78 ± 0.06</td>
<td>1.14 ± 0.07</td>
<td>2.00 ± 0.20</td>
</tr>
</tbody>
</table>

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.

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

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**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.
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.
Chapter 3

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.

**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).
A functional comparison between the HepaRG and C3A cell lines

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 analysed reliably for all amino acids due to overlapping peaks; these data points were excluded.

**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 analysed 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, Table S6).

Figure 7. Amino acid and carbohydrate metabolism. HepaRG and C3A cells, cultured in monolayers and BALs, were analysed for metabolism of (A) amino acids, several amino acids could not be analysed 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.
A functional comparison between the HepaRG and C3A cell lines

**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.

**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 (ALB, TF and 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 ALB, TF and FVII between the four groups (Fig. 8B-D). The ALB transcript level was higher in HepaRG-BALs compared to C3A-BALs, FVII transcript levels were upregulated in C3A-BALs compared to all other groups and transcript levels of 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.

**Drug detoxification is enhanced in HepaRG vs C3A and in BALs vs monolayers**
Transcript levels of CYP3A4 and 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 6B-hydroxylation, was tested in BAL cultures only and was 6-fold higher in HepaRG-BALs compared to C3A-BALs (Fig. 9A).

### 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 favourable 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.
A functional comparison between the HepaRG and C3A cell lines

**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.

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,
A functional comparison between the HepaRG and C3A cell lines

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 (Elkayam et al. 2006; Rebelo et al. 2015; Selden et al. 2013)[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


A functional comparison between the HepaRG and C3A cell lines


### SUPPLEMENTAL MATERIAL

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Template dilution</th>
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Primer sequences used in the RT-PCR analyses with resulting amplicon sizes. Primers are indicated from 5’ → 3’. bp, base pairs; rRNA, ribosomal RNA; AFP, Alpha fetoprotein; ALB, Albumin; ARG1, arginase 1; ARG2, arginase 2; CEBPA, constitutive androstane receptor; CPS1, carbamoyl phosphate synthetase; CYP2B6, cytochrome p450 2B6; CYP3A4, cytochrome p450 3A4; CYP3A7, cytochrome p450 3A7; FVII, factor seven; GS, glutaminase; GLS2, glutaminase 2; GS, glutamine synthetase; HNF4A, hepatic nuclear factor alpha; NTCP, sodium tauroine co-transporting peptide; SLCO1B1, Solute carrier organic anion transporter family member 1B1; OTC, ornithine transcarbamylase; PXR, pregnane x receptor; TF, transferrin.
A functional comparison between the HepaRG and C3A cell lines

Table S2. C3A hepatocyte functions and gene transcript levels in MEM+ and WE+ culture medium

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Transcript levels, normalized to MEM+

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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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<td><strong>NTCP</strong></td>
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<td>0.32</td>
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<td><strong>AFP</strong></td>
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<td><strong>Cyp3A7</strong></td>
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<td>0.09</td>
<td>0.03</td>
<td>***</td>
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A functional comparison between the HepaRG and C3A cell lines

### Table S3b. Total protein in monolayers

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<td><strong>C3A</strong></td>
<td><strong>HepaRG</strong></td>
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<tr>
<td>T=3 d</td>
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<td>T=7 d</td>
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<td>T=14 d</td>
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<td>T=21 d</td>
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<tr>
<td>T=29 d</td>
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* = P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001
Table S4. HepaRG and C3A hepatocyte functions in monolayer and in BALs

<table>
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<tr>
<th>Function</th>
<th>C3A monolayer</th>
<th>C3A BAL</th>
<th>HepaRG monolayer</th>
<th>HepaRG BAL</th>
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<tr>
<td></td>
<td>mean</td>
<td>SD</td>
<td>mean</td>
<td>SD</td>
</tr>
<tr>
<td>LDH leakage % of total ∙ 24 hours^{-1}</td>
<td>12.99</td>
<td>1.32</td>
<td>10.38</td>
<td>0.59</td>
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<tr>
<td>AST leakage % of total ∙ 24 hours^{-1}</td>
<td>13.95</td>
<td>0.73</td>
<td>9.88</td>
<td>0.66</td>
</tr>
<tr>
<td>Ammonia elimination μmol ∙ hour^{-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 μmol ∙ hour^{-1} ∙ g protein^{-1}</td>
<td>1.42</td>
<td>0.34</td>
<td>0.79</td>
<td>0.14</td>
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<tr>
<td>15N urea enrichment % of urea produced</td>
<td>0.40</td>
<td>0.15</td>
<td>0.90</td>
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<tr>
<td>Glutamine balance mmol ∙ g protein^{-1} ∙ 24 hours^{-1}</td>
<td>-75.54</td>
<td>5.47</td>
<td>-10.81</td>
<td>0.47</td>
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<tr>
<td>Glutamate balance mmol ∙ g protein^{-1} ∙ 24 hours^{-1}</td>
<td>28.81</td>
<td>6.41</td>
<td>8.87</td>
<td>2.42</td>
</tr>
<tr>
<td>Ammonia balance mmol ∙ g protein^{-1} ∙ 24 hours^{-1}</td>
<td>55.93</td>
<td>9.18</td>
<td>3.20</td>
<td>0.60</td>
</tr>
<tr>
<td>Lactate production μmol ∙ h^{-1} ∙ g protein^{-1}</td>
<td>156.55</td>
<td>42.70</td>
<td>-2.22</td>
<td>3.26</td>
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<tr>
<td>Glucose consumption μmol ∙ h^{-1} ∙ g protein^{-1}</td>
<td>88.94</td>
<td>3.88</td>
<td>121.71</td>
<td>8.04</td>
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<tr>
<td>Albumin synthesis mg ∙ h^{-1} ∙ g protein^{-1}</td>
<td>35.65</td>
<td>3.12</td>
<td>23.67</td>
<td>2.31</td>
</tr>
<tr>
<td>Testosterone 6B hydroxylation nmol ∙ h^{-1} ∙ g protein^{-1}</td>
<td>95.66</td>
<td>15.89</td>
<td>620.58</td>
<td>96.06</td>
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Table S5. HepaRG and C3A gene transcript levels in monolayer and in BALs

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<th>C3A BAL</th>
<th>HepaRG monolayer</th>
<th>HepaRG BAL</th>
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<tr>
<td></td>
<td>MEAN</td>
<td>SD</td>
<td>MEAN</td>
<td>SD</td>
</tr>
<tr>
<td><strong>AFP</strong></td>
<td>% of human liver</td>
<td>558069.20</td>
<td>250756.80</td>
<td>484317.80</td>
</tr>
<tr>
<td><strong>CYP3A7</strong></td>
<td>% of human liver</td>
<td>154.49</td>
<td>26.27</td>
<td>99.54</td>
</tr>
<tr>
<td><strong>HNF4A</strong></td>
<td>% of human liver</td>
<td>310.94</td>
<td>86.84</td>
<td>373.24</td>
</tr>
<tr>
<td><strong>CEBPA</strong></td>
<td>% of human liver</td>
<td>202.44</td>
<td>54.64</td>
<td>165.32</td>
</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>
<td>0.06</td>
<td>0.78</td>
</tr>
<tr>
<td><strong>OTC</strong></td>
<td>% of human liver</td>
<td>0.18</td>
<td>0.08</td>
<td>1.26</td>
</tr>
<tr>
<td><strong>ARG2</strong></td>
<td>% of human liver</td>
<td>6198.66</td>
<td>2016.26</td>
<td>19411.21</td>
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<tr>
<td><strong>GLS</strong></td>
<td>% of human liver</td>
<td>1492.10</td>
<td>480.30</td>
<td>1326.90</td>
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<tr>
<td><strong>GLS2</strong></td>
<td>% of human liver</td>
<td>10.00</td>
<td>3.60</td>
<td>23.40</td>
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<tr>
<td><strong>GS</strong></td>
<td>% of human liver</td>
<td>225.69</td>
<td>127.07</td>
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<td><strong>FVII</strong></td>
<td>% of human liver</td>
<td>16.38</td>
<td>7.12</td>
<td>129.83</td>
</tr>
<tr>
<td><strong>TF</strong></td>
<td>% of human liver</td>
<td>276.47</td>
<td>168.49</td>
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<td><strong>ALB</strong></td>
<td>% of human liver</td>
<td>25.76</td>
<td>10.26</td>
<td>33.12</td>
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<tr>
<td><strong>CYP3A4</strong></td>
<td>% of human liver</td>
<td>N.D</td>
<td>0.45</td>
<td>0.36</td>
</tr>
<tr>
<td><strong>CYP2B6</strong></td>
<td>% of human liver</td>
<td>N.D</td>
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<td>0.12</td>
</tr>
<tr>
<td><strong>OATP1B1</strong></td>
<td>% of human liver</td>
<td>N.D</td>
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<td>0.01</td>
</tr>
<tr>
<td><strong>NTCP</strong></td>
<td>% of human liver</td>
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<td>0.07</td>
<td>0.56</td>
</tr>
<tr>
<td><strong>CAR</strong></td>
<td>% of human liver</td>
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<tr>
<td><strong>PXR</strong></td>
<td>% of human liver</td>
<td>52.85</td>
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N.D. = Not determined
Table S6. HepaRG and C3A amino acid metabolism rates in millimole $\cdot h^{-1} \cdot g$ protein$^{-1}$

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<th>HepaRG mono3</th>
<th>HepaRG BAL 1</th>
<th>HepaRG BAL 2</th>
<th>HepaRG BAL 3</th>
<th>C3A mono1</th>
<th>C3A mono2</th>
<th>C3A mono3</th>
<th>C3A BAL 1</th>
<th>C3A BAL 2</th>
<th>C3A BAL 3</th>
</tr>
</thead>
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<td>-17.45</td>
<td>-10.21</td>
<td>-4.98</td>
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<td>52.94</td>
<td>33.41</td>
<td>44.42</td>
<td>-9.71</td>
<td>10.64</td>
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</table>
CHAPTER 4

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

Mitochondrion 2017, in press

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Ronald J.A Wanders
Ronald P. Oude Elferink
Robert A.F.M. Chamuleau
Ruurdijke Hoekstra
ABSTRACT

Introduction: 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: 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 Bioartificial 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%O₂ 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.

**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,
The role of oxygen, medium perfusion and 3D configuration

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%O₂, 5%CO₂ and 55%N₂) 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.
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*10⁵ cells/cm² in Primaria 6-well plates (BD Falcon). Cells were kept for 2 days at 37°C in a humidified atmosphere (95% air, 5%CO₂) before harvesting the total RNA. The purity of the isolation was confirmed by the measurement of transcript level of immature hepatic-markers including GSTπ, 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, 1μM dexamethasone, 20 mU/mL insulin (Novo Nordisk), 2mM ornithine (Sigma-Aldrich), 100U/mL penicillin, 100ug/mL streptomycin. Cells
The role of oxygen, medium perfusion and 3D configuration

were kept for 2 days at 37°C in a humidified atmosphere (95% air, 5%CO₂) 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.
Chapter 4

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, BILIAD 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 (CEBPα) and N-acetyl transferase (NAT), and 2 mitochondria-encoded genes, i.e. mitochondrial-NADH dehydrogenase subunit1 (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

Table 1. Primers used in the qPCR and RT-qPCR and amplicon size

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense sequence</th>
<th>Anti-sense sequence</th>
<th>Size bp</th>
</tr>
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<tr>
<td>18S rRNA</td>
<td>TTCGGAACCTGAGGCCATGAT</td>
<td>GGAACCTCGACTTTCTTCTGAT</td>
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<tr>
<td>CEBPa</td>
<td>CGGCGGGCGGGCGACTTTTG</td>
<td>GGGCGGGCGGGCGCTTTGTA</td>
<td>254</td>
</tr>
<tr>
<td>MT-ATP6</td>
<td>GCTTCATTCATGGCCCAAC</td>
<td>GGTAAAGAAGTGGGCTAGGAC</td>
<td>371</td>
</tr>
<tr>
<td>MT-ATP8</td>
<td>ATGGCCCATCATATATTACCC</td>
<td>GGCTTTTGGTGAAGGAGG</td>
<td>100</td>
</tr>
<tr>
<td>MT-CO3</td>
<td>ATGACCCACACATCATACG</td>
<td>ATCACATGGCTAGGCCGAGG</td>
<td>103</td>
</tr>
<tr>
<td>MT-CYB</td>
<td>AACTCGGCTCACCTCCGGA</td>
<td>CCGATGTGAGAAGAGCGG</td>
<td>204</td>
</tr>
<tr>
<td>MT-ND1</td>
<td>ATACCCATGGCCCAACCTGG</td>
<td>GGGGCTTTGCGTATTGATTG</td>
<td>110</td>
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<tr>
<td>MT-ND5</td>
<td>CGGAAGCCCTATTCGGAGG</td>
<td>TGGAGGGAGGATGTGGTGG</td>
<td>347</td>
</tr>
<tr>
<td>NAT</td>
<td>TCAGGTCATGGGAGAGCCA</td>
<td>ATGGGTAGGGGTGAAGC</td>
<td>298</td>
</tr>
</tbody>
</table>
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 home-made 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.
Chapter 4

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).

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 2), 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 3).
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.
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 β-oxidation. In contrast, the down-regulated gene SLC25A14 (known also as UCP5) is a mitochondrial uncoupling protein that functions to separate OxPhos from ATP.

### Table 2. Top 20 up-regulated genes in HepaRG-BAL vs HepaRG-MONO

<table>
<thead>
<tr>
<th>ILMN Probe ID</th>
<th>Symbol</th>
<th>Full name</th>
<th>Log2 fold change</th>
<th>Adjusted P value</th>
</tr>
</thead>
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<tr>
<td>ILMN_1688543</td>
<td>APOA2</td>
<td>Apolipoprotein A2</td>
<td>+3.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>ILMN_1801205</td>
<td>GPNMB</td>
<td>Glycoprotein nmb</td>
<td>+3.2</td>
<td>0.006</td>
</tr>
<tr>
<td>ILMN_1772206</td>
<td>CYP3A4</td>
<td>CytochromeP450 member 3A4</td>
<td>+2.8</td>
<td>0.014</td>
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<tr>
<td>ILMN_1666924</td>
<td>BEGAIN</td>
<td>Brain enriched guanylate kinase-associated</td>
<td>+2.4</td>
<td>0.028</td>
</tr>
<tr>
<td>ILMN_1656333</td>
<td>ECM1</td>
<td>Extra-cellular matrix 1</td>
<td>+2.4</td>
<td>0.002</td>
</tr>
<tr>
<td>ILMN_1791647</td>
<td>ASIP</td>
<td>Agouti signaling protein</td>
<td>+1.9</td>
<td>0.021</td>
</tr>
<tr>
<td>ILMN_1816342</td>
<td>MTRNR2L1</td>
<td>MT-RNR2-like 1</td>
<td>+1.8</td>
<td>0.019</td>
</tr>
<tr>
<td>ILMN_1662587</td>
<td>PNPLA7</td>
<td>Patatin like phospholipase domain containing 7</td>
<td>+1.6</td>
<td>0.032</td>
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<tr>
<td>ILMN_1767129</td>
<td>ABC8</td>
<td>ATP binding cassette subfamily C member 8</td>
<td>+1.6</td>
<td>0.038</td>
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<tr>
<td>ILMN_1737298</td>
<td>MAT2A</td>
<td>Methionine adenosyltransferase 2A</td>
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<tr>
<td>ILMN_2198859</td>
<td>NAT 16</td>
<td>N-acetyltransferase 16</td>
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<tr>
<td>ILMN_2366212</td>
<td>CD79B</td>
<td>Cluster of Differentiation 79B</td>
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<td>ILMN_1784948</td>
<td>SPOCD1</td>
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<tr>
<td>ILMN_1695759</td>
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<td>Amidohydrolase domain containing 2</td>
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<tr>
<td>ILMN_1705297</td>
<td>MYBH</td>
<td>Myosin binding protein H</td>
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<tr>
<td>ILMN_1721732</td>
<td>GSDMC</td>
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<tr>
<td>ILMN_2319588</td>
<td>OSGIN1</td>
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<td>ILMN_1733851</td>
<td>DACT3</td>
<td>Dishevelled binding antagonist of beta catenin 3</td>
<td>+1.4</td>
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<td>ILMN_1666893</td>
<td>TRIML2</td>
<td>Tripartite motif family like 2</td>
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<tr>
<td>ILMN_1787576</td>
<td>CLCNK4</td>
<td>Chloride channel K4</td>
<td>+1.3</td>
<td>0.029</td>
</tr>
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</table>
The role of oxygen, medium perfusion and 3D configuration 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 upregulation of genes involved in mitochondrial energy metabolism by BAL culturing prompted us to further investigate mitochondrial biogenesis in the context of the BAL culturing.

<table>
<thead>
<tr>
<th>ILMN Probe ID</th>
<th>Symbol</th>
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<th>Log2 fold change</th>
<th>Adjusted P value</th>
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<tr>
<td>ILMN_2148527</td>
<td>H19</td>
<td>Imprinted maternally expressed transcript (non-protein coding)</td>
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<tr>
<td>ILMN_2313672</td>
<td>IL1RL1</td>
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<td>-2.4</td>
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<td>ILMN_2132982</td>
<td>IGFBP5</td>
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<td>ILMN-1752750</td>
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<td>ILMN_1694548</td>
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<td>ILMN_1671123</td>
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<td>ILMN_2078975</td>
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Figure 3. AMC-BAL culture positively regulates the expression of genes involved in energy metabolism and mitochondria in HepaRG cells. A-B) Top 20 up- and down-regulated Gene-sets between HepaRG-BAL and HepaRG-MONO (FDR: Benjamini-Hochberg adjusted p value). C) Volcano plot displaying gene-set “Mitochondrial part” that contains 839 genes and was significantly up-regulated (HepaRG-BAL vs HepaRG-MONO) in the CAMERA analysis (FDR=0.006). Genes included in the indicated gene-set are marked in red, selected examples of up and down-regulated genes discussed in the text are marked in blue and the other genes are marked in grey.
AMC-BAL culturing enhances mitochondrial biogenesis in the HepaRG cells, an effect driven by the 3D configuration, dynamic medium perfusion and higher oxygenation (40%O$_2$)

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%O$_2$ in the AMC-BAL vs 20%O$_2$ 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%O$_2$ (HepaRG-40%O$_2$) and the effect of dynamic medium flow on monolayers cultured under 60 rpm supplied with 20%O$_2$ (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%O$_2$ 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%O$_2$ (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%O$_2$, 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%O$_2$, 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%O$_2$, 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%O$_2$ and HepaRG-DMF cultures. Noteworthy, no significant differences was observed in the level of MIM complexes when HepaRG-BAL was compared to HepaRG-40%O$_2$, HepaRG-DMF or HepaRG-BALIAD, (Fig. 4C-D).
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).
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Figure 4. Continued. D) quantification of the immunoblots using ImageJ software. E) Transcript level of mitochondria-encoded genes (n=6-8/group).
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.
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 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. 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 AND 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
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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
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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 \cite{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 \cite{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.
REFERENCES


The role of oxygen, medium perfusion and 3D configuration


CHAPTER 5

Oxygen drives hepatocyte differentiation and phenotype stability in liver cell lines

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Chapter 5

ABSTRACT

**Background and 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 analysed 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 analysed 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 signalling. 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], signalling pathways [3, 4], mechanical forces and paracrine stimuli [4], and of ways to influence these in vitro through coculturing [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 of 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 5

MATERIALS 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% O₂ (=hypoxic; 5% O₂, 5%CO₂ and 90%N₂), 21% O₂ (=normoxic; 5%CO₂, 21% O₂, 75%N₂) and 40% O₂ (=hyperoxic; 40% O₂, 5%CO₂, 55%N₂) (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
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\(^{15}\text{NH}_4\text{Cl}, 2.27 \text{ mM D-galactose, 2 mM L-lactate and 125 \mu M 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}\text{N urea and total bile acids. Next, the accumulation or disappearance rates could be calculated. The accumulation of }^{15}\text{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 \mu\text{M 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 \text{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 (RNeasy; 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 \(\text{P-value} > 0.05\) (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 \(\text{P-values} \) 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 \( \mathrm{H}_2\mathrm{O}_2 \)) 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).

After four weeks in culture, normoxic cultures differentiated into patches of polygonal hepatocytes, surrounded by flat cholangiocyte-like cells, as described [19] (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.

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

To confirm that hyperoxia increases hepatic differentiation, we analysed 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),
Oxygen drives hepatocyte differentiation and phenotype stability in liver cell lines

Figure 2. Nuclear SOX9 expression is downregulated, while CEBPα 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 CEBPα (red). Arrow A indicates positive nuclear staining. Scale bar = 50 µM

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).

Figure 3. Ambient hypoxia 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.
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).

**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). 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).

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 \( (P=1.14 \times 10^{-12}) \), whereas V-myc avian myelocytomatosis viral oncogene homolog (MYC) was predicted to be inhibited \( (P=4.45 \times 10^{-3}) \) in PHH and in HepaRG under hyperoxia compared to HepaRG under normoxia. HNF1α 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 signalling.

**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 HNF4A and PXR were downregulated to 22% - 24% of normoxic cultures, while CEBPA transcript levels did not change significantly (Fig. 5D). These data confirm
Oxygen drives hepatocyte differentiation and phenotype stability in liver cell lines

### Table 1. Top-10 upregulated genes in hyperoxic versus normoxic HepaRG cultures.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change</th>
<th>Adj. P-value</th>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2E1</td>
<td>5.37</td>
<td>8.98·10⁻⁵</td>
<td>Cytochrome p450 2E1</td>
<td>Detoxification</td>
</tr>
<tr>
<td>ASNS</td>
<td>3.65</td>
<td>9.00·10⁻³</td>
<td>Asparagine Synthetase</td>
<td>Amino acid metabolism</td>
</tr>
<tr>
<td>F9</td>
<td>3.58</td>
<td>2.36·10⁻³</td>
<td>Coagulation Factor IX</td>
<td>Hemostasis</td>
</tr>
<tr>
<td>PLG</td>
<td>3.19</td>
<td>3.37·10⁻⁴</td>
<td>Plasminogen</td>
<td>Hemostasis</td>
</tr>
<tr>
<td>TRIB3</td>
<td>3.10</td>
<td>3.37·10⁻³</td>
<td>Tribbles Pseudokinase 3</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>PPP1R1A</td>
<td>3.07</td>
<td>2.19·10⁻³</td>
<td>Protein phosphatase 1 regulatory subunit 1A</td>
<td>Carbohydrate metabolism</td>
</tr>
<tr>
<td>RASD1</td>
<td>2.67</td>
<td>2.19·10⁻³</td>
<td>RAS, Dexamethasone-Induced 1</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>HABP2</td>
<td>2.65</td>
<td>2.36·10⁻³</td>
<td>Hyaluronan Binding Protein 2</td>
<td>Hemostasis</td>
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<tr>
<td>PSAT1</td>
<td>2.58</td>
<td>1.82·10⁻²</td>
<td>Phosphoserine Aminotransferase 1</td>
<td>Amino acid metabolism</td>
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<tr>
<td>FGL1</td>
<td>2.56</td>
<td>2.19·10⁻³</td>
<td>Fibrinogen-Like 1</td>
<td>Hemostasis</td>
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### Table 2. Top-10 downregulated genes in hyperoxic versus normoxic HepaRG cultures.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change</th>
<th>Adj. P-value</th>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL28A1</td>
<td>-7.14</td>
<td>3.73·10⁻⁵</td>
<td>Collagen, type XXVIII, alpha 1</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>SLC10A1</td>
<td>-5.17</td>
<td>1.44·10⁻³</td>
<td>Sodium/bile acid cotransporter</td>
<td>Transport</td>
</tr>
<tr>
<td>SPINT3</td>
<td>-4.96</td>
<td>1.60·10⁻⁴</td>
<td>Serine Peptidase Inhibitor, Kunitz Type, 3</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>KRT6A</td>
<td>-4.70</td>
<td>3.73·10⁻⁶</td>
<td>Keratin 6 alpha</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ANKRD30A</td>
<td>-4.27</td>
<td>5.24·10⁻⁴</td>
<td>Ankyrin repeat domain 30A</td>
<td>Anchorage</td>
</tr>
<tr>
<td>REG3G</td>
<td>-3.98</td>
<td>2.04·10⁻⁵</td>
<td>Regenerating islet-derived protein 3 gamma</td>
<td>Innate immune response</td>
</tr>
<tr>
<td>CYP4B1</td>
<td>-3.79</td>
<td>3.92·10⁻²</td>
<td>CYP4B1</td>
<td>Detoxification</td>
</tr>
<tr>
<td>LDHB</td>
<td>-3.73</td>
<td>2.28·10⁻⁴</td>
<td>Lactate dehydrogenase B</td>
<td>Carbohydrate metabolism</td>
</tr>
<tr>
<td>FOLR1</td>
<td>-3.42</td>
<td>8.98·10⁻⁵</td>
<td>Folate Receptor 1</td>
<td>Transport</td>
</tr>
<tr>
<td>PDZD3</td>
<td>-3.28</td>
<td>8.98·10⁻⁵</td>
<td>PDZ Domain Containing 3</td>
<td>Innate immune response</td>
</tr>
</tbody>
</table>
the opposite effects of hyperoxia and hypoxia on hepatic differentiation of HepaRG cells.
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 hyperoxic 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

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.
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.

**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.

**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
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.
Chapter 5

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.
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REFERENCES


Oxygen drives hepatocyte differentiation and phenotype stability in liver cell lines


SUPPLEMENTAL MATERIAL

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. CEBPa) and Fig 6A (C HIF1a).
CHAPTER 6

HepaRG-progenitor cell derived hepatocytes cultured in Bioartificial Livers are protected from healthy- and acute liver failure-plasma induced toxicity

Submitted

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ABSTRACT

Background and Aims: For applicability of cell-based therapies aimed at the treatment of liver failure, such as bioartificial livers (BALs) and hepatocyte transplantation, it is essential that the applied hepatocytes tolerate exposure to the patient plasma. However, plasma from both healthy donors and acute liver failure (ALF) patients is detrimental to hepatocytes and hepatic cell lines, such as HepaRG. We aimed to elucidate the underlying mechanisms of plasma-induced toxicity against HepaRG cells in order to ultimately develop methods to reduce this toxicity and render HepaRG-BAL treatment more effective.

Methods: Differentiated HepaRG cells cultured in monolayers and laboratory-scale BALs were exposed to culture medium, healthy human plasma, healthy porcine plasma and ALF porcine plasma. Healthy human plasma was fractionated based on size and polarity, albumin depleted and heat treated to characterize the toxic fraction. The cells were assessed for viability by total protein content and trypan blue staining. Their hepatic differentiation was assessed on transcript level through qRT-PCR and microarray analysis, and on functional level for Cytochrome P450 3A4 activity and ammonia elimination. Mitochondrial damage was assessed by JC-1 staining and mitochondrial gene transcription.

Results: Sixteen hours of healthy human plasma exposure did not affect viability, however, hepatic gene-transcript levels decreased dramatically within four hours of exposure. These changes were associated with early NF-κB signaling and a shift from mitochondrial energy metabolism towards glycolysis. Healthy human plasma-toxicity was associated with the dose-dependent presence of heat-resistant, albumin-bound and (partly) hydrophobic toxic compound(s).

HepaRG cells cultured in BALs were partially protected from plasma-toxicity, which was mainly attributable to medium perfusion and/or 3D configuration applied during BAL culturing. The detrimental human plasma effects were reversible in BAL-cultured cells. Porcine ALF-plasma elicited mitotoxicity additional to the basal detrimental effect of porcine healthy plasma, which were only partially reversible.

Conclusion: A specific fraction of human plasma reduces hepatic differentiation of HepaRG cultures, in association with early NF-κB activation. In addition, ALF-plasma elicits mitotoxic effects. These findings allow for a targeted approach in preventing plasma-induced cell damage.
INTRODUCTION

Acute liver failure (ALF) and acute-on-chronic liver failure are syndromes associated with substantial mortality, for which there is an unmet need of therapeutic options [1], as orthotopic liver transplantation is limited by donor organ shortage. Two types of cell-based treatment modalities are under development: Bioartificial Liver (BAL) therapy [2-4] and hepatocyte transplantation [5, 6]. The performance of the applied cells in the presence of human plasma is of paramount importance, especially in BALs, that are designed as extracorporeal hepatocyte-bioreactors perfused with patient plasma through plasmapheresis. Healthy-donor human plasma (hplasma) is known to induce intracellular lipid accumulation, stress and a decrease in hepatic functionality of primary hepatocytes and hepatic cell lines, through unclarified mechanisms [7, 8]. In addition, plasma from liver failure patients contains not only detrimental compounds which are normally detoxified by the liver, such as ammonia, bile acids, and lactate, but also compounds associated with inflammation and infection, such as endogenous damage-associated molecular patterns released from necrotic cells, cytokines and chemokines, as well as lipopolysaccharides and other stimulators of innate immune response due to bacterial translocation [9-11].

The AMC-BAL is a bioreactor that holds liver cells in 3D-configuration, in a spirally wound, non-woven matrix, interwoven with capillaries which supply oxygen-enriched gas [12, 13]. Liver cells come in direct contact with patient plasma during treatment. Currently, the device is loaded with the human liver progenitor cell line HepaRG [14, 15] and was proven efficacious in prolonging survival time of rats with ischemic ALF [4]. Previously we described that healthy- and ALF-rat plasma induced toxicity in HepaRG cells cultured in monolayers and in BALs [16, 17].

In this study we show that hplasma has a rapid detrimental effect on hepatic differentiation and functionality of HepaRG cells. In order to develop protective strategies, we studied the underlying mechanism of plasma-induced toxicity and the toxic fraction of hplasma. We further studied whether culture procedures or different culture platforms could inhibit or reverse the plasma-induced toxicity. Finally we assessed whether ALF plasma induced additional toxicity compared to plasma of healthy subjects, using pig plasma.
MATERIALS AND METHODS

Cell culture and plasma exposure procedures
HepaRG cells (Biopredic International) were cultured in supplemented Williams’ E medium without dimethyl sulfoxide (DMSO), with 10% fetal bovine serum, as described [14, 18]. Differentiated monolayers were acquired after seeding 1:5 in 12-well plates and culturing for 28 days under control conditions, i.e. statically under an atmosphere of 5% CO₂, 75%N₂ and 20% of O₂. Monolayers cultured under hyperoxia were kept under normoxia until reaching confluence at day 14 and then transferred to 5% CO₂, 55%N₂ and 40% of O₂ until day 28.

3D ‘Bal-in-a-dish’ (BALIAD) cultures were seeded into 28.2 mm² pieces of non-woven matrix and kept in medium in 12-well plates under control conditions until day fourteen, after which they were transferred to an orbital-shaker incubator under normoxia and shaken at 60 RPM until day 28. Laboratory-scale BALs (9 mL volume) were loaded with 0.6g cryopreserved HepaRG cells and cultured for 21 days as described previously [19] before commencing plasma-exposure-experiments.

To test the effect of plasma exposure, culture medium was replaced by undiluted plasma, except for the experiment with serial dilutions, for which plasma was diluted with fresh culture medium as indicated. As a negative control, cells were kept in fresh culture medium. Plasma exposure times varied between experiments, as indicated.

To test whether HepaRG-BAL cultures could recover from plasma-toxicity, plasma was removed from the BALs after 16h exposure by a single-pass flush with fresh culture-medium, after which the system was filled entirely with fresh culture medium and allowed to recover for 24 hours. Matrix samples for RNA isolation were obtained before plasma exposure (control) and immediately after plasma exposure, as described previously [20].

Plasma
Healthy human plasma (hplasma) derived from mixed-gender pooled donors (Sera Laboratories International Ltd.). Healthy porcine plasma (pplasma) derived from landrace pigs. For porcine ALF plasma (pALFplasma), ALF was induced as described [21] in a 31 kg female landrace pig by intravenous administration of paracetamol 1.5 g/kg body weight over 12 hours. After 21 hours the animal went into cardiovascular failure, and subsequently plasma was acquired by exsanguination. Liver failure was confirmed by biochemical analysis (Table 1). All procedures involving animals were conducted in agreement with the Animals (Scientific Procedures) Act 1986 under (UK Home Office) Project License 60/4557, and after approval by the Roslin Institute’s Animal Welfare and Ethical Review Board.
All plasmas were anticoagulated with Lithium-Heparin, filter-sterilized and supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL) before exposure to HepaRG cells.

**Plasma fractionation or treatment**

To characterize the toxic component in plasma, we fractioned or treated this plasma with different techniques. Fractionation by size was performed by filtering with 100kDa Molecular weight cut-off filters (Amicon Ultra-4, Merck), which are specified to retain molecules of over 100kDa, including albumin, which we confirmed.

Fractionation by polarity was performed by Bligh and Dyer extraction [22], after which the hydrophobic fraction was dried under a stream of nitrogen, resuspended and sonicated in DMSO, which was then diluted in culture medium to equal volume as the original plasma sample (final concentration of DMSO was 1%). Vehicle control treatment was performed with culture medium that was treated the same way (n=6, 2 independent experiments).

Albumin depletion was done by concentrating 3mL of plasma with 100kDa Molecular weight cut-off filters until 40 uL retentate, which was then resuspended in a 20 mM sodium phosphate binding-buffer (pH 7.0), as recommended by manufacturer, and exposed to 7 mL albumin-binding Blue Sepharose 6 Fast Flow beads (GE Healthcare) for 3 hours at 4°C and 30 min at room temperature on an orbital shaker at 100 rpm. Subsequently, the mix was concentrated over 100kDa Molecular weight cut-off filters and the retentate was diluted in culture medium to a final volume of 3 mL. Control plasma was treated with the same protocol, except for the exposure to Blue Sepharose beads (n=6, 2 independent experiments). Albumin was under the level of detection in the albumin-depleted plasma, as confirmed by the clinical chemistry laboratory (data not shown).

Heat treatment of 100% plasma consisted of a 45-minutes exposure to 58°C in a block heater (n=6, 2 independent experiments).

### Table 1. Biochemical profile of pALFplasma.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Time of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
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<td>243</td>
</tr>
<tr>
<td>Ammonia (uM)</td>
<td>58</td>
<td>316</td>
</tr>
<tr>
<td>Mir-122 (Fold change vs baseline)</td>
<td>1</td>
<td>160</td>
</tr>
<tr>
<td>FV/FVIII (ratio)</td>
<td>1.07</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Baseline value is before paracetamol administration. AST = Aspartate Aminotransferase; Mir-122 = MicroRNA-122; FV = clotting factor five activity; FIII = clotting factor eight activity.
Chapter 6

Cell viability assessment

HepaRG monolayer cultures were exposed to plasma or culture medium for 16 hours, washed 2x with PBS and then assessed for toxicity by two methods (n=4, 2 independent experiments). Firstly the cultures were incubated with trypan blue 0.4% solution (Sigma) diluted 1:4, after which the cultures were immediately assessed for viability by microscopy. Secondly, the total protein content/well was determined as described [18].

Function tests

Hepatic function tests were performed as previously described [4]. Briefly, test medium was prepared from HepaRG medium supplemented with NH₄Cl (1.5 mM), L-lactate (2.5 mM), D-galactose (2.27 mM) and testosterone (125 μM). Monolayers and BALs were exposed to 1.5 and 110 mL of test medium respectively and samples taken after 0, 6 and 24 hours for monolayers and 0, 30, 60, 120, 240, 480 and 1440 min for BALs. Concentrations of ammonia were quantified using the Megazyme Ammonia Assay kit (Megazyme International). Cytochrome P450 3A4 (CYP3A4) activity was measured in BALs by the quantification of 6b-hydroxytestosterone as described [4], and in monolayers using the P450-Glo™ CYP3A4 with Luciferin-IPA assay according to the manufacturer’s instruction (Promega). All metabolic rates were normalized to total protein as described [18]. Synthetic properties could not be assessed adequately, due to some retention of plasma proteins after plasma exposure.

RNA isolation, qRT-PCR and microarray analysis

Cells in monolayers or representative samples of BAL-matrix were lysed in 600 μL RLT buffer (RNeasy minikit, Qiagen) from which RNA was isolated according to manufacturer’s instructions. Transcript levels were determined by qRT-PCR using gene-specific reverse transcriptase (RT)-primers and touchdown qPCR protocol and normalized to 18S ribosomal RNA, as previously described [23]. Where expressed as % of control, the data were normalized to the average of non-treated control cells within each independent experiment. For the microarray experiment RNA was isolated after 0, 1, 2, 4 or 8 hours exposure to plasma (n=3 independent ~1cm² monolayer cultures per group) RNA was biotinylated with the cRNA labeling kit (Ambion) and hybridized to Illumina HumanHT-12 v4 arrays (Illumina) after randomization of the samples. Scanning was performed on the Illumina iScan (Illumina). 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).

The microarray data were analyzed with Bioconductor packages (version 3.0) using the statistical software environment R (version 3.1.3). 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 3.22.7).
The arrayQualityMetrics package (version 3.22.1) was used to assess that the microarray data was of good quality. Probes with a detection P-value > 0.05 (non-expressed) on all arrays (16,560 of 47,323 probes) were filtered out. Differential expression between time points 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. Corrected P-values ≤ 0.05 were considered statistically significant. Probes were reannotated using the illuminaHumanv4.db package (version 1.24.0). Gene sets from the hallmark collection and two liver-specific gene sets from the C2 collection, HSIAO_LIVER_SPECIFIC_GENES and SU_LIVER [24, 25], were retrieved from the Molecular Signatures Database (MSigDB) v5.1 (Entrez Gene ID version). Gene set enrichment analysis was performed using CAMERA (limma package) and gene set variation analysis (GSVA) was performed using the GSVA package (version 1.14.1). Sample-specific gene set enrichment scores calculated by GSVA were clustered using Pearson correlation as distance measure and complete linkage as agglomeration method (function hclust). Top-10 upregulated genes were cross-referenced against the Boston University Gilmore Lab NF-κB target gene set (http://www.bu.edu/nf-kb/gene-resources/target-genes/).

**Mitochondrial membrane potential and mitochondrial abundance**

Mitochondrial membrane potential was determined using JC-1 staining. This cationic dye emits green fluorescence in the cytosol of the cells (monomeric form) and red fluorescence when aggregates are formed (dimeric form) in active mitochondrial membrane [26]. BAL matrix samples (6mmx6mm) were incubated with 0.5 mL 4 µM JC-1 (Invitrogen) for 30 min at 37°C. Simultaneously, the cells were incubated with 1uM verapamil (Sigma) to inhibit the efflux of JC-1 through the activity of ATP binding cassette subfamily B member 1. Red to green fluorescence (Fλ585/Fλ510) was quantified on a NOVOstar microplate reader (BMG Labtech).

The ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nucDNA) was assessed as a measure of mitochondrial abundance expressed as the weighted mean. Total DNA was isolated from AMC-BAL matrix samples (6 mmx6 mm) using the QIAamp DNA kit (QIAGEN) according to the manufacturer’s specifications. 2 nuclear encoded genes, *i.e.* CCAAT/enhancer binding protein alpha (*CEBPa*) and N-acetyl transferase (*NAT*), and 2 mitochondrial-encoded genes, *i.e.* mitochondrial-NADH dehydrogenase subunit1 (*MT-ND1*) and mitochondrial cytochrome c oxidase subunit 3 (*MT-CO3*) were quantified by qPCR.

**Statistical analyses**

Data were analyzed and processed for graphical representation in Prism 7.01 (GraphPad). Values are expressed as mean ± standard deviation (SD). Student’s t-tests were used when comparing two groups and 1-way ANOVA when comparing multiple groups. All results were corrected for multiple testing according to the Holm-Sidak method. Significance was reached...
if \( P<0.05 \). The data produced in BALs were \( n=3 \), 3 independent experiments, compared to monolayer cultures \( n=8 \), 3 independent experiments. Plasma-treatment experiments were performed as indicated in text.

## RESULTS

**Healthy-donor human plasma has a rapid, dose-dependent detrimental effect on the hepatic differentiation of HepaRG-derived hepatocytes**

We determined the effects of plasma exposure on HepaRG monolayer cultures on total protein content, morphology, ammonia elimination rate, CYP3A4 activity and the transcript levels of the hepatic genes CYP3A4, hepatic nuclear factor 4 Alpha (HNF4A) and arginase 1 (ARG1), which were previously established to be highly responsive to plasma exposure [16, 17]. HepaRG monolayers showed a rapid decrease in transcript levels of these hepatic genes when exposed to 100% plasma (Fig. 1A). After 4 hours exposure, HNF4A transcript level was decreased to 17±2% of the level in control cultures, while CYP3A4 and ARG1 transcript levels decreased more gradually to 5±2% and 7±2% respectively after 24 hours. Decrease in transcript levels of these genes was accompanied by profound morphological changes. The clusters of hepatocyte-like polygonal cells in HepaRG cultures disappeared (Fig. 1B) and cell-cell contact was lost after 16 hours exposure to plasma. There was no significant cell death as determined by total protein content (Fig. 1C) or trypan blue staining (Fig S1). However, rates of ammonia elimination and CYP3A4 activity had decreased to 48±21% and 29±7% of levels in control cultures respectively (Fig. 1D).

A 16 hours exposure of monolayers to plasma in escalating concentrations revealed a plasma-concentration-dependent decrease of hepatic gene transcript levels of CYP3A4, HNF4 and ARG1 (Fig. 1E). The transcript levels of the control (0%, plasma-exposure) group were significantly higher (\( P<0.05 \)) compared to all other groups, with the exception of ARG1 versus 25% plasma. The gene transcript levels were also significantly lower after exposure to 100% compared to 25%, plasma for all three genes (\( P<0.05 \)). Together, these data indicate that plasma has a rapid and dose-dependent toxic effect on the differentiation of HepaRG monolayers, although their viability is still unaffected.

**Detrimental plasma fraction is hydrophobic, albumin-bound, and heat-stable**

Next, we determined whether the detrimental effect of plasma was due to a lack of indispensable culture medium components or to the presence of a toxic fraction, by exposing monolayers to different fractions of plasma for 16h and testing the effect on transcript levels of ARG1, HNF4A, and CYP3A4.
Healthy- and acute liver failure-plasma induced toxicity

Figure 1. Effect of plasma on differentiated HepaRG cells. A.) Transcript levels of the hepatic genes ARG1, CYP3A4 and HNF4A in HepaRG cells after 0.5, 1, 2, 4, 8, 12 and 24 hours of exposure to plasma, expressed as a % of the transcript levels in non-exposed cells. B.) Morphology of HepaRG cells before and after 16 hours exposure to plasma, bar= 50µm. C.) Total protein content per well after 16 hours exposure to plasma or culture medium. D.) Hepatic functions ammonia elimination and CYP3A4 activity before and after 16 hours exposure to plasma. E.) Hepatic gene transcript levels after 16 hours exposure to plasma in different concentrations. * = P<0.05 versus control.
Plasma filtered through 100kDa molecular weight cut-off filters (which also depleted albumin, Mw 67kDa), did not negatively affect transcript levels of ARG1 or HNF4A, while CYP3A4 was even significantly upregulated compared to control cultures (Fig. 2A). This supported the presence of a toxic fraction rather than depletion of essential culture medium components by 100% plasma incubation. Plasma that was albumin-depleted induced a significantly smaller decrease in transcript levels of ARG1 (P<0.01) and HNF4A (P<0.001) and an increase in CYP3A4 transcript levels (P<0.001) compared to control-treated plasma (Fig. 2B). Heat treated plasma (58°C 45 min) yielded similar effects as untreated plasma (Fig. 2C) and finally, the hydrophobic plasma fraction also reduced transcript levels of ARG1 and HNF4A, but not of CYP3A4 (Fig. 2D).

These data show that the cytotoxic effect of plasma is due to an albumin-bound, heat-stable fraction, that is, at least partly, hydrophobic, and not due to a lack of essential culture medium factors.

Figure 2. Toxicity of plasma fractions. Transcript levels of the hepatic genes ARG1, CYP3A4 and HNF4A in differentiated HepaRG monolayers exposed to different fractions of plasma for 16 hours, expressed as a % of the transcript levels in non-exposed cells. A.) Plasma filtered through a molecular weight cut-off filter compared to untreated plasma. B.) Albumin depleted plasma compared to control-treated plasma. C.) Heat treated plasma compared to untreated plasma. D.) The hydrophobic plasma fraction compared to treatment vehicle control. *= P<0.05 versus monolayers exposed to untreated plasma. &= P<0.05 versus monolayers exposed to control-treated plasma. $= P<0.05 versus monolayers exposed to vehicle-treated control.
Plasma-induced damage is associated with rapid activation of NF-κB target genes

To investigate the pathways involved in plasma-induced toxicity we compared the whole-genome transcriptomes of HepaRG monolayers exposed to plasma for different durations by microarray analysis. Since loss of hepatic gene transcription commenced within eight hours (Fig. 1A), we compared monolayers after 1, 2, 4 and 8 hours of plasma exposure with control monolayers. There were profound and rapid changes in transcriptomes; after 1, 2, 4 and 8 hours there were 134, 1154, 5548 and 6584 genes differentially expressed, respectively, compared to control cultures (Fig. 3A).

**Figure 3. Differentially expressed genes and gene sets.** A.) Venn diagram of differentially expressed (adjusted \( P<0.05 \)) genes of the monolayer cultures exposed to plasma for 1 to 8 hours compared to unexposed cultures. B.) Heatmap of the sample-specific gene set enrichment scores calculated by gene set variation analysis (GSVA) on the hallmark gene sets and two previously described human liver-specific gene sets. A high detail full-resolution version is included in Fig. S1, the results of the CAMERA analysis are summarized in Table S1. EMT = Epithelial to mesenchymal transition, Ox. phosphorylation = Oxidative phosphorylation.
Of the top-10 upregulated genes at the different time points versus control cultures (Table 2), 11 out of 26 unique genes were NF-κB targets (depicted in bold).

**Table 2.** Top-10 upregulated genes after different durations of plasma exposure compared to non-exposed controls

<table>
<thead>
<tr>
<th>Genes</th>
<th>1h exposure</th>
<th>2h exposure</th>
<th>4h exposure</th>
<th>8h exposure</th>
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<td>CYR61</td>
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<td></td>
<td></td>
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<tr>
<td>EGR1</td>
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<td></td>
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</table>

Values represent fold change versus control cultures. Genes written in **bold** are recognized targets of NF-κB according to the Boston university Gilmore Lab NF-κB target gene set.
Gene set enrichment analysis on the hallmark collection of the Molecular Signatures Database (MSigDB) v5.1 showed that genes regulated by NF-κB in response to TNF were the most upregulated gene set after two hours exposure (Fig. 3B, Fig. S2). Energy metabolism changed drastically, as oxidative phosphorylation was strongly downregulated at later time points. In line with the qRT-PCR data (Fig. 1A), enrichment analysis of two previously described gene sets of liver-specific genes (1: [25] and 2:[24]) showed a profound decrease of hepatic gene transcript levels at 4 and 8 hours after exposure (Fig. 3B, Fig. S2).

BAL-culturing protects against plasma-induced deterioration, which may be explained by 3D configuration and medium perfusion

HepaRG cultures in BALs were less sensitive to plasma-induced damage compared to monolayers. After 16h of exposure, ammonia elimination was significantly decreased in monolayer cultures, but not in BAL cultures (Fig. 4A), while CYP3A4 activity was significantly decreased in both cultures, although significantly less in BAL cultures than in monolayers (2.2-fold versus 3.4-fold reduction) (Fig 4B). After exposure, transcript levels of ARG1, CYP3A4 and HNF4A were declined 6.9-, 5.4- and 4.0-fold, respectively, in monolayer cultures, while in BAL cultures CYP3A4 transcript levels were unchanged after plasma exposure and ARG1 and HNF4A transcript levels were less reduced, (2.2- and 1.8-fold) (Fig. 4C-E).

Three main differences between the monolayer and BAL culture systems are the level of oxygenation (20% vs 40% O2, respectively), the configuration (2D vs 3D, respectively) and medium perfusion (absent or present, respectively). To assess which of these factors contribute to the relative resistance to plasma toxicity in BAL cultures versus monolayer cultures, we exposed medium-perfused 3D cultures under ambient normoxia (BALIAD cultures) and static monolayer cultures under 40% of ambient oxygen to plasma and compared the decrease in transcript levels of ARG1, CYP3A4 and HNF4A between standard monolayer and BAL cultures (Fig. 4C-E). The effect of plasma on transcript levels did not differ between normoxic or hyperoxic monolayers. In BALIAD cultures, the transcript levels of ARG1 and CYP3A4, but not of HNF4A, were less decreased by plasma exposure compared to monolayer cultures. In addition, the transcript levels of all three tested genes were not significantly different after plasma exposure in BAL- and BALIAD-cultures, indicating that medium perfusion and 3D culture may be important contributing factors to the protective effect of BAL-culturing.

Loss of hepatic gene transcription is reversible in BALs after exposure to healthy human plasma

The previous experiments were performed with healthy human plasma, because of the scarcity of human ALF plasma. However, when applied clinically, the BAL-system will be exposed to plasma from patients suffering from ALF, which has a different composition
Figure 4. Effect of culture platform on plasma toxicity. 

A-B.) Functionality of HepaRG monolayer or BALs cultures in culture medium (control) or after exposure to plasma for 16h, expressed as a % of unexposed cultures in the same culture-platform: A.) Ammonia elimination, B.) CYP3A4 activity. 

C-E.) Transcript levels of hepatic genes in HepaRG cells cultured in monolayers under normoxia or hyperoxia, in BALIADs or in BALs, exposed to plasma for 16h, expressed as a % of unexposed cultures in the same culture-platform: C.) ARG1, D.) CYP3A4, E.) HNF4A. *= P< 0.05 versus plasma exposed monolayer. #= P< 0.05 versus plasma exposed monolayer. $= P< 0.05 versus unexposed cultures in the same culture platform.
Healthy- and acute liver failure-plasma induced toxicity

from healthy plasma. The effects of the ALF contribution on plasma toxicity were therefore analyzed by investigating hepatic transcript levels of BALs exposed to plasmas from: healthy pigs (pplasma) and pigs with acetaminophen-induced ALF (pALFplasma). As a control for species differences, pplasma-exposed BALs were also included. After plasma exposure, all BALs were allowed to recover in culture medium for 24 hours and transcript levels were again measured to assess the reversibility of the plasma-induced toxicity.

HepaRG BALs exposed to pplasma, pplasma and pALFplasma all showed a significant decrease in transcript levels of ARG1 and HNF4A (Fig. 5), while only the latter two groups showed a decrease in CYP3A4 transcript levels. The level of recovery from the plasma exposure varied with the origin of the plasma. After recovery from pplasma exposure, transcript levels of all three genes were higher compared to post-exposure levels, up to or exceeding control levels. After recovery from pplasma exposure, only CYP3A4 transcript levels increased significantly.

Figure 5. Effect of pplasma, pplasma and pALFplasma on BAL-cultured HepaRG cells and recovery procedure. Transcript levels of hepatic genes ARG1, CYP3A4 and HNF4A in BAL-cultured HepaRG cells before exposure (control), directly after 16 hours of plasma-exposure and after 24 hours recovery in culture medium, expressed as a % of the transcript levels in non-exposed BAL-cultured HepaRG cells. Three types of plasma were applied: A) pplasma, B) pplasma, C) pALFplasma. *=P<0.05 versus control, #= P<0.05 versus post-plasma.
although \( HNF4A \) transcript levels no longer differed significantly from the pre-exposure levels. After recovery from \( \text{pALF} \) plasma exposure, there was no significant upregulation of any of the three genes compared to post-exposure levels, although there was no significant difference anymore to pre-exposure level for \( HNF4A \) transcripts. Thus we conclude that the decrease of gene transcript levels after 16 hours plasma exposure is reversible, although the degree of reversibility varies with the origin of plasma.

Porcine ALF plasma induces additional mitochondrial damage compared to healthy plasma

Since hepatocytes are dependent on mitochondrial activity for their high energy demanding functions, and microarray analysis indicated a shift in energy metabolism from oxidative phosphorylation towards glycolysis upon plasma exposure (Fig. 3B), we assessed

![Graph A](image1.png)

**Figure 6. Effect of \( \text{pPlasma} \) and \( \text{pALF Plasma} \) and recovery procedure on mitochondria of BAL-cultured HepaRG cells.** A-B.) Transcript levels of mitochondrially encoded genes \( MT-CYTB \) and \( MT-ND5 \) in BAL-cultured HepaRG cells before exposure, directly after 16 hours of plasma-exposure and after 24 hours recovery in culture medium, expressed as a % of the transcript levels in non-exposed BAL-cultured HepaRG cells. C.) Membrane potential as determined by JC-1 staining after exposure to \( \text{pPlasma} \), or \( \text{pALF Plasma} \) (geometric mean ± geometric SD). D.) Mitochondrial abundance as reflected by the ratio between DNA encoding the mitochondrial genes \( MT-CO \) or \( MT-ND1 \) and DNA encoding the nuclear gene \( CEBPA \) (geometric mean ± geometric SD). *=\( P<0.05 \) versus control. #= \( P<0.05 \) versus post-plasma.
mitochondrial abundance, membrane potential and mitochondrial gene transcript levels on the plasma and ALF plasma exposed BALs.

After exposure to ALF plasma, transcript levels of the mitochondrially encoded genes MT-CYTB and MT-ND5 decreased by 3.2 and 3.6 fold respectively, after recovery they increased 1.9 and 1.7 fold respectively compared to control BALs, that were not exposed to any plasma (Fig. 6A-B). BALs exposed to plasma showed no change in transcript levels, suggesting an additional mitotoxic effect of the ALF plasma. This was confirmed by JC1-staining on samples of BAL matrix after recovery (Fig. 6C), which indicated a significant loss of mitochondrial membrane potential for ALF plasma exposed BALs, but not plasma exposed BALs. However, the ratios between DNA of the mitochondrial genes MT-CO and MT-ND1 and the nuclear gene CEBPA, were unaffected in both groups (Fig. 6D), indicating that the abundance of mitochondria had not changed.

These results indicate that ALF plasma, but not plasma impairs mitochondrial gene transcription, which is reversible after 16h plasma exposure, and also that mitochondrial functionality is negatively affected by ALF plasma.

DISCUSSION

It is of paramount importance for BALs and other cell-based therapies that the applied cell source is resistant to human blood plasma. In order to improve this resistance it is vital to unravel the underlying mechanism. In this study we showed that plasma did not affect viability after 16 hours of exposure, but had a dose-dependent detrimental effect on the hepatic functionality and differentiation grade of HepaRG-derived hepatocytes, in close association with NF-κB signaling. Cells were partly protected from this basic plasma-induced damage when cultured in BALs and partial restoration of hepatic gene transcription could be achieved by a 24-hour regeneration period on standard culture medium after plasma exposure. In addition we found that toxicity can be attributed to an albumin-bound, and heat-resistant plasma fraction that is, at least partly, hydrophobic. In plasma that was filtered or albumin depleted, CYP3A4 transcript levels were found to be increased, which is likely to reflect preservation of the detoxification system’s capability to be induced by exposure to ligands.

The 3D configuration and/or medium perfusion contribute in a large part to the increased plasma-resistance observed in BALs compared to monolayer cultures. This is in accordance with a previous report that primary rat hepatocytes are protected from plasma-induced toxicity by 3D-culturing [27]. The applied 3D culture platform provides a flexible culture
surface, which may be essential for maintaining cell-cell contact. Loss of adhesion is one of the first events in plasma-induced toxicity, and anchorage proteins, such as E-cadherin and the beta1-integrin receptor are known to protect against hepatocyte dedifferentiation and apoptosis [28-31]. In addition, matrix rigidity is known to limit hepatocyte differentiation, in part through transcription factor HNF4A [32].

Plasma was more toxic compared to hplasma, and pALF plasma exerted additional mitochondrial toxicity, underlining that ALF plasma is a hostile environment for both the patient and cell-based medicinal therapies. Due to the scarcity of human ALF plasma there was no opportunity to assess the effect of plasma from ALF patients. In this study we used a model of paracetamol-induced liver failure which is commonly associated with mitotoxicity and disruption of cell tight junctions [33], although also ALF-plasma of other origins has been reported to impair mitochondrial activity to varying degrees [31, 34]. However, the detrimental effects of ALF-plasma are likely to vary between etiologies, patients and clinical status, implicating the necessity of close monitoring of biocomponent functionality during therapy. This way, the BAL can be replaced after reaching critical plasma-induced toxicity of its biocomponent. Yet, it would be advantageous to inhibit at least the basic plasma-induced toxicity caused by toxic components already present in healthy plasma, which likely varies to lesser extent between individuals.

Hepatic gene transcript levels started to decline as early as four hours after hplasma exposure, preceded by upregulation of pro-inflammatory genes within one hour, suggesting a rapid mechanism, probably mediated through the activity of the pro-inflammatory NF-κB signaling-pathway. NF-κB-associated pro-inflammatory cytokines, such as IL-6, are known to have a dedifferentiating effect on primary hepatocytes [35, 36]. Especially for drug-detoxification enzymes, it is well documented that pro-inflammatory cytokines, and particularly IL-6, downregulate functionality through inhibition of transcription [37, 38]. These mechanisms were confirmed in HepaRG cells by others: pro-inflammatory cytokines suppressed transcription of phase 1 and phase 2 detoxification enzymes and drug transporters, as well as CYP450-enzyme activities [39].

There are several isoforms of NF-κB, with partially overlapping signaling cascades, exerting a multitude of effects. Pan-inhibition of NF-κB is known to induce and exacerbate hepatocyte apoptosis [40, 41], therefore this is not an attractive strategy to counter plasma toxicity. It has been described that the RELA/p65 isoform leads to inflammatory cytokine production, posing a more specific target. However, genetic disruption of this pathway is also known to sensitize hepatocytes to apoptosis [42, 43].
Ideally, plasma toxicity should be inhibited by targeting more upstream processes. We have established that the detrimental compound(s) reside(s) in a plasma fraction that is albumin bound, non-polar and heat resistant. NF-κB can be activated through extracellular receptor signaling, such as Toll-Like Receptors, which are classically stimulated by bacterial and viral particles, mitochondrial reactive oxygen species (ROS)-production and hypoxia [44, 45]. We found that antioxidant treatment of HepaRG cells with N-acetyl cysteine did not decrease the detrimental plasma effects (data not shown), indicating that ROS is unlikely to be a main contributor to plasma toxicity [45]. NF-κB activation through hypoxia-induced pathways might be an option, as our microarray data showed that oxidative phosphorylation is severely affected. This does not necessarily imply a direct association with hypoxia; culturing the HepaRG cells and exposing them to plasma under hyperoxic conditions did not yield any protection against plasma toxicity.

In case of direct receptor stimulation, there is the possibility to inhibit plasma toxicity by molecular interference. Plasmapheresis filters with high affinity for Toll-like receptor 2 ligands ameliorated clinical symptoms of ALF in pigs with paracetamol overdose [46]. However, for specific targeting, further studies into the nature of the toxic component in plasma are required. Specific detoxification modules could easily be integrated into the plasmapheresis set-up, potentially protecting both the biocomponent and the native liver. A hybrid system, combining BAL-treatment with albumin dialysis may reduce damage of plasma to the biocomponent. One study, performed with human ALF plasma, addressed this option and showed, however, that albumin dialysis of the plasma did not reduce its pro-apoptotic effect on hepatocytes [31]. This may indicate that the toxic fraction has a very high affinity for albumin, since dialysis only removes the unbound fraction, and compounds with high binding affinities are therefore not removed efficiently.

Alternatively, the basic plasma-induced toxicity can be prevented by filtration for molecular size; plasma passed through a molecular weight cut-off filter of 100 kDa was no longer toxic. A disadvantage of small-pore plasma filters is that the mass-transfer of compounds during BAL therapy may be limited, which will decrease its efficacy. Others have previously studied the optimal plasmapheresis filter pore size, and concluded that a high-convection 400 kDa cut-off membrane offers the optimal balance between protection of the biocomponent and mass-transfer of toxins [47].

As an alternative strategy, the negative effects of basic plasma on hepatocytes can also be reversed by limiting the exposure-time to plasma so that damage to the biocomponent is still reversible. Our data show that gene transcription of HepaRG-BAL cultures can be restored after 16 hours of plasma exposure by recirculating culture medium through the...
device, indicating that alternated treatment- and restoration runs are also a viable strategy to increase the life-span of BALs, as proposed by others previously [7].

In conclusion, plasma has a detrimental effect on differentiated HepaRG cultures, which is associated with early NF-κB activation. Strategies to extend functional time in BAL set-up include intermitted exposure- and recovery runs, the use of small-pore size and/or cytokine scavenging plasmapheresis filters and direct inhibition of involved pathways such as NF-κB signaling. ALF plasma may elicit additional toxic effects, which may vary between patients. Therefore, close monitoring of the functionality will be required for optimal BAL therapy of ALF patients.
REFERENCES


SUPPLEMENTAL INFORMATION

Figure S1. Cell viability evaluated by trypan blue exclusion test (stained cells have lost membrane integrity and are not viable), of differentiated HepaRG monolayers exposed for 16 hours to: A.) culture medium control, or B.) plasma. Red arrows indicate trypan blue positive cells.
Healthy- and acute liver failure-plasma induced toxicity

<table>
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<th>1hr</th>
<th>2hr</th>
<th>4hr</th>
<th>8hr</th>
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**Figure S2. Heatmap of sample-specific gene set enrichment scores.** A high detail full-resolution version of Fig. 3B.
CHAPTER 7

Scaling-up of a HepaRG progenitor cell based bioartificial liver; optimization for clinical application and transport

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ABSTRACT

Background and aims: A new generation of bioartificial livers, based on differentiated proliferative hepatocyte sources has been developed. Several practicable and regulatory demands have to be addressed before these can be clinically evaluated. We identified three main hurdles: 1.) expansion and preservation of the biocomponent, 2.) development of scaled-up culture conditions, and 3.) transport of the device to the bedside. In this study we address these three issues for the HepaRG- progenitor cell line-loaded AMC-Bioartificial Liver.

Methods and results: HepaRG cells were expanded in large quantities and then cryopreserved or loaded directly into bioreactors. After 3 weeks of culture, key hepatic functions (ammonia/lactate elimination, apolipoprotein A1 synthesis and cytochrome P450 3A4 activity) did not differ significantly between the two groups.

Bioartificial livers were scaled up from 9mL to 540mL priming volume, with preservation of normalized hepatic functionality. Quantification of amino acid consumption revealed rapid depletion of several amino acids.

Whole-device cryopreservation and cooled preservation induced significant loss of hepatic functionality, whereas simulated transport from culture-facility to the bedside in a clinical-grade transport unit with controlled temperature maintenance, medium perfusion and gas supply, did not affect functionality.

In addition, we assessed tumorigenicity of HepaRG cells in immune-incompetent mice and found no tumor formation of HepaRG cells (n=12), while HeLa cells induced formation of carcinomas in eight out of twelve mice in 140 days.

Conclusion: The HepaRG AMC-Bioartificial Liver was scaled-up to a clinical size, and large-scale HepaRG expansion and cryopreservation, as well as transport to the bedside proved feasible without loss of functionality. For the future, the culture medium regimen can be modified by supplementing the critically depleted amino acids in a fed-batch set-up.
INTRODUCTION

Bioartificial livers (BALs) were conceived to improve the condition of patients suffering from Acute Liver Failure (ALF) and serve as a bridging-therapy to liver transplantation or spontaneous regeneration. They consist of extracorporeal bioreactors, loaded with liver cells and usually interfaced with the patient by means of a plasmapheresis circuit.

The AMC-BAL is a cartridge-based system with the distinct feature of direct contact between patient plasma and liver cells, in order to maximize mass-transfer (Fig. 1). The system was originally based on primary porcine hepatocytes and successfully applied in a series of 12 patients suffering from ALF [1, 2]. However, further clinical evaluation was abandoned upon the enforcement of a moratorium on xenotransplantation in the European Union, which includes primary-porcine hepatocyte-based BAL therapy. Furthermore, concerns about xenotransplantation and the negative outcome of a phase 3 clinical trial with a BAL system based on cryopreserved porcine hepatocytes have led to a set-back in the implementation of this technology [3, 4].

Figure 1. AMC-BAL. Schematic representation of the HepaRG BAL (A), with details (B-C) and hematoxylin/eosin-staining (D) showing medium/plasma inflow port (I), medium/plasma outflow port (II), gas inlet port (III) (gas outlet port at opposite position not visible), the non-woven matrix to which the cells attach (IV), gas capillaries (V) and inter-capillary space (VI) through which the culture medium/plasma flows)
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Alternatives to xenogeneic hepatocytes are human primary hepatocytes or proliferative cells of human origin. The latter are the most attractive option, since primary human hepatocytes are scarcely available. In recent years, a new generation of BAL systems based on proliferative human cells has been developed, of which several are under or near clinical evaluation [5-7]. The new generation AMC-BAL, now based on the human hepatic progenitor cell line HepaRG, is one of these systems [8-10].

Before these BALs can be clinically applied, several important challenges have to be addressed. First, the biocomponent has to be expanded and preserved in large amounts: estimations of the required cell mass to support ALF-patients range from 15g-300g for a 70 kg patient, amounting to ~2.5-50 m² of monolayer culture surface [11-13]. Once expanded, it is of paramount importance that cells can be preserved, for example by cryopreservation, to facilitate large-scale batch-wise production, characterization and validation, as required by the regulatory authorities for approval of the BAL as an advanced therapy medicinal product. Secondly, hardware and culture conditions have to be scaled-up and modified to meet clinical standards. Thirdly, BALs need to be preserved and shipped to the bedside without loss of functionality.

Previously, a laboratory-scale model of the HepaRG loaded- AMC-BAL was proven efficacious in prolonging survival time of rats after total liver ischemia [6]. In this study we address the abovementioned challenges for the HepaRG-AMC-BAL and provide additional safety data in preparation of clinical evaluation.

MATERIAL AND METHODS

HepaRG cell culture
HepaRG cells (Biopredic International, Rennes, France) were expanded as previously described [6]. Briefly, cells were cultured in supplemented Williams’ Medium E (HepaRG medium) in stacked monolayer culture vessels for two weeks and split in a 1:5 ratio. Where applicable, cells were cryopreserved in HepaRG medium, supplemented with 10% DMSO to prevent ice-crystal formation [14], in liquid nitrogen. Every cell-batch loaded in the 540mL-BALs passed a set of quality control tests: viability after thawing (trypan blue exclusion test >85%), microbial sterility, transcript levels of three hepatic genes (urea cycle enzyme arginase1 ARG1; detoxification enzyme cytochrome P450 3A4 CYP3A4 and hepatic transcription factor HNF4A), tested against a HepaRG reference batch) and morphology (>50% of culture surface covered by well delineated, granular hepatocyte clusters with bright canalicular structures).
**BAL hardware**

The 9mL- and 540mL-BAL cartridges were custom-made to specification (RanD, Medolla, Italy). The configuration of 9mL-BALs was described previously [6] (Table 1), and 540mL-BALs had a similar configuration, but with a priming volume of 540mL and 6254cm² of DuPont™ Spunlaced Nonwoven Fabric- matrix (DuPont, Wilmington, DE, USA), interlaced with 3800 gas capillaries for aeration (Fig. 1, Table 1).

The 540mL-BALs were produced and tested for biological and chemical safety in accordance with ISO10993-1 and ISO10993-4 quality standards and in compliance with Good Manufacturing Practice (GMP) (RanD, Medolla, Italy).

<table>
<thead>
<tr>
<th>Table 1A. Characteristics of the 9mL- and 540mL-BAL</th>
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<tr>
<td><strong>BAL type</strong></td>
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<td>Internal volume (mL)</td>
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<td>Matrix surface (cm²)</td>
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<td>Gas capillaries (cm per cm² matrix)</td>
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<td>Certified for clinical testing</td>
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<td>Cell mass loaded (g)</td>
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<td>Total protein loaded (mg)*</td>
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<td>Total protein day 22 (mg)*</td>
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* data from cryopreserved 9mL-BALs from figure 2F

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<tr>
<th>Table 1B. Function and damage parameters of 9mL-BALs loaded with non-preserved cells (n=3) and 540mL-BALs loaded with cryopreserved cells, (n=9).</th>
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<tr>
<td><strong>9mL-BAL</strong></td>
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<tr>
<td><strong>MEAN</strong></td>
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<tr>
<td>Ammonia elimination (µmoles·h⁻¹·g protein⁻¹)</td>
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<td>Urea production (µmoles·h⁻¹·g protein⁻¹)</td>
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<td>ApoA1 synthesis (mg·h⁻¹·g protein⁻¹)</td>
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<td>Albumin synthesis (mg·h⁻¹·g protein⁻¹)</td>
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<td>AST leakage (U·h⁻¹·g protein⁻¹)</td>
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<td>LDH leakage (U·h⁻¹·g protein⁻¹)</td>
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<td>Lactate elimination (µmoles·h⁻¹·g protein⁻¹)</td>
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<tr>
<td>Glucose consumption (µmoles·h⁻¹·g protein⁻¹)</td>
</tr>
<tr>
<td>CYP3A4 activity * (µmoles·h⁻¹·g protein⁻¹)</td>
</tr>
</tbody>
</table>

* (Testosterone 6β-hydroxylation)
Chapter 7

**BAL culture**

BAL culturing was performed in HepaRG medium as described previously [6]. Briefly, cells were brought into suspension in culture medium and loaded into the bioreactors, allowing 4 hours for attachment to the matrix before commencing perfusion. Medium flow inside the bioreactors was kept at 3 ml·cm⁻²·min⁻¹ and a mixture of 40% O₂, 5% CO₂ and 55% N₂ was perfused through the gas capillaries. The temperature was maintained at 36°C. Culture medium volume was 500ml for 9mL-BALs, and 90% of medium volume was replenished twice weekly. The recirculating culture medium volume for 540mL-BALs was 7L, of which 5L was refreshed daily.

The fetal bovine serum (FBS) percentage was reduced from 10% to 5% for 540mL-BAL cultures after we confirmed that the FBS reduction did not affect functionality or growth rate (Fig. S1).

**BAL loading optimization and biomass assessment**

Laboratory scaled 9mL-BALs (n=3-5 per group) were loaded with HepaRG cells that were freshly isolated (non-preserved, 2g cells) or cryopreserved (2g, 1g or 0.5g cells). Hepatic functions were tested after 7, 14 and 21 days of culture. Aliquots of the loading suspensions and BALs, harvested after the last function test, were lysed in 0.2 M sodium hydroxide for total protein content determination, as described previously [6]. 540-mL BALs were loaded similarly, with 40 grams of cryopreserved HepaRG cells.

**BAL cryopreservation**

Four 9mL-BALs loaded with 0.5g of cryopreserved cells, were cultured for 21 days and tested for hepatic functionality and hepatic transcript levels. Subsequently, the BALs were flushed with HepaRG medium (cooled until 4°C) supplemented with 10% DMSO to prevent ice-crystal formation [14], sealed off and cooled down to -80°C at a rate of ~1°C/minute. After one week of cryogenic storage, BALs were thawed at room temperature under aeration, reperfused with culture medium (cooled until 4°C), and then maintained at 37°C. The medium perfusion rate was gradually normalized from 1/5th to normal speed over the course of 5 hours.

**Tumorigenicity evaluation in mice**

The welfare of the animals was maintained in accordance with the general principles governing the use of animals in experiments of the European Communities (Directive 2010/63/EU) and Dutch legislation (The Experiments on Animals Act, 1997). This included approval of the study by the local animal experimental committee (DEC-number 3517). Animal studies were performed under Good Laboratory Practice license and in adherence to the applicable EMEA and FDA guidelines.
Athymic Nude-Foxn1nu mice were randomized into three groups (n=12/group): subcutaneous injection with a suspension of 10⁷ HepaRG cells (test group), 10⁷ HeLa cells (positive control) or HepaRG medium only (negative control group). Tumor sizes were measured weekly with calibrated digital calipers, tumor volume was estimated using the formula: Volume = (length x width²)/2. Follow-up was 140 days or until a humane endpoint, after which animals were sacrificed and examined macroscopic and microscopic for pathological changes. Microscopic evaluation included hematoxylin and eosin staining (4µm sections) of brain, kidneys, liver, spleen, lungs, axillary and brachial lymph nodes, gross lesions (including any remote nodules) and the injection site.

**Hepatic function tests**

Function tests were performed as previously described [15]. Briefly, BALs were exposed to HepaRG medium supplemented with 1.5 mM ammonia, 125µM testosterone, 2.27 mM D-galactose and 2 mM L-lactate. Medium volume was 7.8-9.1 mL/g biomass/hour test time (120 mL for 9mL-BALs and 1250 mL for 540mL-BALs) and samples were taken at 0, 30, 60, 120, 240, 480 and 1440 min. (9mL-BALs) or at 0, 15, 30, 60, 120 and 240 min. (540mL-BALs). Concentrations of L-lactate, ammonia, apolipoprotein A1 (ApoA1), 6b-hydroxytestosterone (as the metabolite through CYP3A4 activity), urea and glucose, as well as activities of aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) were quantified, and the metabolic or cell leakage rates were calculated as described previously [15].

**qRT-PCR**

Cells were harvested from 9mL-BALs at different time points as previously described [16], and RNA was isolated using the RNeasy mini-kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany). Transcript levels of hepatic genes ARG1, CYP3A4 and HNF4A were determined by qRT-PCR using gene-specific RT primers and a touchdown qPCR protocol as described previously [17].

**Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry**

Amino acids (AAs) were quantified in HepaRG medium of 540mL-BALs at 0 and 24 hours after medium refreshment at days 0, 2, 4, 7, 10, 14 and 21 of culturing. A mix of stable isotope-labelled internal standards was added to 50µl medium after which 500µl of acetonitrile was added while vortexing. The mixture was centrifuged for 10 min at 12,000 x g and the supernatant was taken to dryness under a nitrogen flow. The residue was resuspended in 200µl 0.01% heptafluorobutyric acid and 10µl was injected into the Ultra Performance Liquid Chromatography-Tandem Mass Spectrometer (Acquity-Quattro Premier XE, Waters, Milford, Massachusetts, USA) operated in the positive electrospray ionization mode using multiple reaction monitoring for the preselected analytes. The AAs were separated from interfering compounds by a linear gradient between solution B (acetonitrile/water, 4:1, v/v) and solution
A (0.1% heptafluorobutyric acid). The gradient (0.4ml/min) was as follows: 0-2min 100% A, 2-5 min 50% A–50% B, 5-6min 100% B and 6-9min, equilibration with 100% A. All gradient steps were linear, and the total analysis time, including the equilibration, was 9min. Data processing was performed using Masslynx 4.1 software.

**Hypothermic culture**

9mL-BALs loaded with 0.5g of cryopreserved cells, were tested for hepatic functionality at day 21. Then temperature was reduced to 20°C or 30°C (n=4/group) for 24 hours. Next, the temperature was brought back to 37°C and hepatic functions were immediately tested.

**BAL transport unit**

The system for transportation of the 540mL-BAL cultures was custom designed and produced in an ISO 9100-certified facility (AMC medical innovations unit, Amsterdam, the Netherlands) (Fig. 4A). The design was evaluated through Failure Mode and Effects Analysis. The transport system utilizes disposable clinical-grade closed-circuit tubing-sets (RanD, Medolla, Italy). For the transport simulation, a 540mL-BAL cultured for 21 days was tested for hepatic functionality and then kept in the transport unit for 24 hours. In this time, the unit was loaded into a vehicle several times, driven over uneven terrain and left at 4°C for 16 hours. After this, hepatic functions were tested again and expressed as a percentage of the pre-transport tests.

**Statistics**

Data were collected, graphically represented and statistically analyzed using Prism 6.0 (GraphPad, La Jolla, CA, USA). Data are expressed as means ± standard deviations (SD). Multiple groups with repeated measures were compared with mixed-ANOVA analysis (alpha=0.05) and corrected for multiple testing using the Holm-Sidak method. Hepatic functions and cell leakage rates of 9mL- and 540mL-BALs were compared by repeated T-tests and corrected for multiple testing using the Holm-Sidak method without assuming consistent SD (alpha=0.05).

**RESULTS**

**HepaRG cells can be loaded into BALs directly from cryogenic storage without loss of function and undergo two population doublings inside the AMC-BAL**

Laboratory-scale 9mL-BALs were loaded with HepaRG cells; one group with freshly isolated cells (non-preserved), and one group with cryopreserved cells (cryopreserved). Hepatic functionality was similar between cryopreserved cells and non-preserved cells after 21 days.
of BAL culturing (Fig. 2A-D). Limited differences in functionality were measured at earlier time points: at days 7 and 14; non-preserved cells produced more of the hepatocyte-specific ApoA1 (p<0.01 and p <0.05, respectively) and had higher CYP3A4 detoxification enzyme activity at day 14 (p<0.05). Ammonia and lactate elimination did not differ significantly at any time point. The cell damage, as quantified by leakage of the intracellular enzyme AST, was also similar between both groups at all three time points (Fig. 2E).

Next, we compared BALs loaded with the original cryopreserved cell amount (2g) to BALs with half or one quarter of the loading mass (Fig. 2A-E). Strikingly, in both lower-cell mass groups, ammonia elimination capacities were at the level of control BALs from day 7, and lactate elimination was only significantly lower in the quarter-mass group at day 7. ApoA1 production was lower in both lower-cell mass groups at days 7 and 14, but not at day 21, and CYP3A4 activity was not different between the groups at day 21 (not measured at days 7 and 14) (Fig. 2A-D). AST release remained lower at all three time points in the lower-cell-mass groups (Fig. 2E). Total protein analysis revealed that the cell mass loaded at half and one quarter of the original mass underwent approximately one and two population doublings respectively, while total protein content of control BALs remained stable (Fig. 2F).

Combined, these results indicate that BALs can be loaded with a cryopreserved HepaRG cell mass corresponding to 25% of the final cell mass obtained after 21 days of BAL culturing, which is the optimal culture period. These conditions were applied to the HepaRG cultures in 540mL-bioreactors.

**HepaRG cells in 540mL-BALs exhibit all tested functions, but do not proliferate**

To accommodate the amount of cells required for clinical application, a scaled-up version of the AMC-BAL with a priming volume of 540mL was produced and tested after 21 days (Fig. 1, Table 1A). The tested functions were normalized to total protein content (Table 1A) and compared to those of the 9mL-non-preserved BALs at day 14 (Table 1B). Total protein-corrected hepatic functions, including ammonia and lactate elimination, urea, albumin, and ApoA1 synthesis and Cyp3A4 activity, did not differ between the two BALs. In addition, glucose metabolism remained comparable. The only difference found was that the leakage of AST and LDH into the culture medium was 3.5 and 7.1 fold decreased respectively, in the 540mL-BALs compared to the 9mL-BALs. Together, these results indicate that the performance of the 540mL-BAL compared to 9mL-BALs did not differ significantly (p=0.6312). Unexpectedly, total protein content after 23 days of culture was not increased relative to the starting amount, meaning that no net proliferation had occurred (Table 1A).
**HepaRG-BAL substrate consumption changes over time**

To assess culture medium depletion, we quantified the net change in glucose and AA concentrations in culture medium of 540mL-BALs under a regime of once-daily 5L medium change for 22 days. Glucose consumption increased over the culture period, but never exceeded 27% of the starting concentration (Table 2). After one week of culture, leucine and aspartate were consumed >50% of their starting concentration. Towards the end of the culture period, seven AAs had become depleted >50% in 24 hours: branched chain AAs leucine, isoleucine and valine, as well as aspartate, tryptophan and methionine (Table 2).

**Figure 2. Culture time and biocomponent loading and preservation conditions in 9mL-BALs.**

Hepatic functions of BALs loaded with non-preserved HepaRG cells and cryopreserved HepaRG cells at different loading masses, measured at day 7, 14 and 21 (A-D). Testosterone 6β-hydroxylation as a measure of CYP3A4 activity, was not measured at day 14 for the half and quarter mass-loaded BALs (E). Total protein content of BALs loaded with different amounts of cryopreserved HepaRG cells, measured at 0 and 21 days (F).

\$ p<0.05 \text{ vs non-preserved at same time-point;} \& \ p<0.05 \text{ vs previous time-point in same group;} \#\ p<0.05 \text{ vs cryopreserved}
Table 2. Glucose, lactate and amino acid concentration in culture medium (given as % of start concentration after 24 hours of culture)

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Cryopreserved and hypothermic transport conditions compromise HepaRG-BAL functionality

Ideally, once differentiated, BALs would be cryopreserved and stored until required and shipped via existing cold-chain solutions to be thawed at the bedside. To explore this possibility, we adapted the protocol for cryopreservation of suspended HepaRG cells and stored fully differentiated 9mL-BALs at -80°C for one week. Unfortunately, after thawing, ammonia elimination and ApoA1 elimination capacity were lost almost completely and lactate elimination reversed into production (Fig 3A-C). Over the course of one week of BAL culturing after cryopreservation, ammonia- and lactate elimination capacity were partly restored. Transcript levels of the hepatic genes ARG1, Albumin (ALB) and CYP3A4 decreased to 0.7±0.26%, 5.2±4.1% and 1.3±0.6% of their pre-preservation levels, respectively. Transcript levels remained significantly decreased for up to seven days, indicating profound loss of phenotype (Fig. 3D).

Next to cryopreservation we explored preservation conditions at 4°C with and without active aeration, but these also led to unacceptable loss of function (data not shown). Therefore, we

Figure 3. Whole-BAL cryopreservation. A comparison of hepatic functions (A-C) and hepatic transcript levels (D) of 9mL-BALs tested at baseline (21 days of culture) and after cryopreservation. $ p<0.05$ vs baseline.
concluded that the HepaRG-BAL should be transported at (sub-) normothermic temperature, which also requires gas supply- and culture medium perfusion.

Next, we assessed the effect of 24 hours medium-perfused and oxygenated preservation at 20°C and 30°C on hepatic functions of differentiated 9mL HepaRG-BALs. The 20°C group
showed a significantly decreased lactate elimination (72%), ammonia elimination (40%) and ApoA1 synthesis (41%), as well as an increased AST leakage (265%), indicating cell death (Fig 4A-D), compared to baseline BAL cultures maintained at 36°C. BALs incubated at 30°C maintained hepatic functionality, including lactate elimination, ammonia elimination, ApoA1 synthesis and CYP3A4 activity, as measured by testosterone 6β-hydroxylation (Fig. 4E). However, AST leakage was significantly higher (38%) than the baseline value for this group as well, indicating that although functionality is not impaired, there is more cell death compared to 36°C and thus transport at temperatures lower than 30°C is not opportune.

These data indicate that existing temperature-controlled infrastructure at 20°C does not suffice, and that HepaRG-BALs should be transported at 30-36°C.

The AMC-BAL can be transported safely in a novel transport unit, fit for clinical use
A dedicated transport unit was developed for the 540mL-BAL to maintain gas- and culture medium perfusion, as well as a temperature at 30-36°C during transport. To function autonomously, it requires an independent power source, the capacity of which is largely determined by the energy-consumption of the temperature-control system.

To meet the required transport conditions in the clinical setting, the transport system can operate autonomously for at least 48 hours, while logging culture conditions (Fig. 5A-C). As a proof of principle, one BAL was tested on hepatic functions before and after a 24-hour transport simulation, including a period of 12 hours at 4°C and exposure to realistic handling strain. Tested hepatic functions did not differ before or after the transport simulation (Fig 5B).

HepaRG cells are not tumorigenic in immunocompromised mice
Mice were injected subdermally with HepaRG cells (test group), HeLa cells (positive control) or vehicle (negative control group). After 6 days, nodules were observed in 0/12 vehicle control-injected animals, 8/12 HepaRG-injected animals and in 12/12 HeLa-injected animals (Table S1). After 14 days, all nodules had resolved in HepaRG-injected animals, indicating that the initial swelling reflects a temporary effect. In seven HeLa-injected mice the initial nodules regressed completely within 28 days. In the other five mice the initial nodules reduced in size, but still persisted at 28 days and increased later onwards.

After 140 days, nodules were observed in 0/12 negative controls and HepaRG-injected mice and in 8/12 positive controls. Four positive-control mice were sacrificed before the end of the study because of large nodule size or ulceration of the skin at the site of the nodule.
Upon pathological examination, the nodules in positive controls were confirmed to be carcinomas, as characterized by pleomorphic cells with a high mitotic activity, invading the surrounding tissue. In contrast, microscopic examination of negative control and HepaRG-injected mice did not reveal any treatment-related findings.

**DISCUSSION**

In this study we addressed three important hurdles that need to be overcome for clinical application of the HepaRG AMC-BAL system, and that largely apply to other (future) BAL systems and advanced cell-based therapies as well: expansion and preservation of the biocomponent, development of scaled-up culture conditions that are feasible in the clinical setting and transport of the BAL to the bedside.

Large-scale biocomponent culture and preservation are crucial for two reasons. On the one hand it is common practice and a requirement of regulatory authorities to facilitate sound characterization and safety testing on each batch of cells without time constrains. Secondly, long-term preservation significantly reduces complexity and costs of the production process, because cells can be expanded in large quantities at any site in the world, without the need to synchronize with the production of the final product. In the case of the AMC-BAL we successfully applied a scalable monolayer culture platform in combination with
cryopreservation after cell expansion, to generate cell mass that could be loaded into the bioreactor directly from cryogenic storage.

Once the expansion and preservation of the biocomponent is established, the culture process of the BALs has to be scaled-up in a way that is both practically and financially feasible. The scaled-up culture conditions were designed to be compatible with GMP standards, and the scaled-up BALs had protein-normalized functionality as predicted. We found that the amount of cryopreserved cells, a factor that contributes substantially to total costs, can be reduced at least four-fold without loss of functionality in 9mL-BALs, owing to significant proliferative activity (~2 population doublings). However, HepaRG cells did not proliferate inside the 540mL-BALs. Based on animal experiments and experience from liver resections, the estimations of required BAL-biomass vary between 2% and 40% of functional hepatocyte mass [11, 13], corresponding to approximately 15g-300g for a 70 kg patient [11]. On the basis of its matrix capacity, the 540mL-AMC-BAL can theoretically facilitate 900g of cell mass. Yet, the lack of proliferation of cell mass in the 540mL-bioreactor in the current study limited the cell mass to 40g, or 5% of functional liver cell mass. Although this is within the spectrum that is considered acceptable, 20% of functional hepatocyte mass would be more appropriate, since this was the amount successfully applied in the proof-of-principle study of the HepaRG AMC-BAL in rats [6]. We speculate that the difference in facilitating cell expansion in the 9mL- and 540mL-bioreactors may relate to the difference in dimension between the two bioreactor designs. Since cells have to spread over a larger distance after loading into the 540mL-BAL, the distribution is likely to be more heterogeneous, with a higher cell density at the periphery of the bioreactor. Because HepaRG cells exhibit contact inhibition [9], this may lead to clusters of cells in the periphery and empty areas in the center of the bioreactor. One possible way of resolving the inhomogeneous distribution of cell mass in the large bioreactor, is to carve-out additional loading channels from the matrix.

In addition to the required cell mass, the feeding regimen is also a major determinant for practical and financial feasibility of the upscaling process of BAL cultures. We explored glucose and AA depletion in the 540mL-BAL under the currently applied regimen. All AAs were increasingly consumed, except for glycine and ornithine, indicating change of the energy metabolism of the BAL in time, which are unrelated to cell mass changes, as the protein content remained stable. As a result, substrate requirements changed in time. Since hepatic functions were expressed at their expected levels, we conclude that the abovementioned culture regime suffices, and that the depletion of several AAs was not yet critical. AAs can be used for protein synthesis or energy metabolism. Seven AAs were depleted for more than 50% within 24 hours towards the end of the culture period: tryptophan, methionine, valine, leucine, isoleucine aspartate and alanine. All of these are considered essential, except for aspartate and alanine. Aspartate can be deaminated into oxaloacetate, which can in
turn be used as an energy source in the citric acid cycle and alanine can be deaminated, yielding pyruvate as a source of energy. Tryptophan, leucine and isoleucine can in turn function as substrates to produce alanine. As these AAs get depleted, glucose consumption increases. This analysis of medium requirements enables the development of a fed-batch culture protocol [18], in which limiting supplements can be replenished into the system (semi-) continuously, thus reducing the total amount of culture medium and handling required and possibly at the same time, improving functionality.

HepaRG cells, like other highly functional proliferative hepatocytes, such as stem cell derived hepatocytes, require time to differentiate and once fully differentiated, can be kept in culture for several weeks until needed [7, 15]. The implication of this culture scheme is that differentiated BALs need to be transported to the clinic from the production site under conditions warranting safe and reproducible preservation. To facilitate transport of the BAL system to the bedside, we successfully developed and tested a dedicated transport unit that can autonomously support a pre-validated and functional BAL for 48 hours. In addition, we explored hypothermic and subnormothermic conditions and cryopreservation of entire BALs. In this study, cryopreservation of entire differentiated 9mL-BALs led to major loss of functionality, and even after 7 days post-thawing, functions were not restored. The fact that transcript levels of hepatic genes had not restored indicates that cryopreservation and thawing did not only induce cell death, but also dedifferentiation. Others have described that control over the rate of cooling is of major importance for the successful cryopreservation of alginate-encapsulated cells of the proliferative HepG2 liver cell line [19]. To gain precise control over the rate of cooling in the entire bioreactor is challenging, due to the dimensions and relative solidity of the device. Conceivably, a higher level of control over the rate of cooling can be achieved by perfusion of the aeration-capillaries with a cooling-agent. This would require the development of new technologies and still, the cells furthest away from the capillaries may be affected by freezing or thawing injury. We believe it is uncertain that this issue can be overcome in the foreseeable future. An alternative strategy would be to focus on pre-differentiation of HepaRG cells before cryopreservation. This would eliminate differentiation time, so the BAL could be loaded with cells whenever required, just as the classical primary hepatocyte based systems.

Finally, the safety of BAL application should be extensively scrutinized prior to clinical evaluation. Despite the cancer-related origin of HepaRG cells [9], we found no signs of in vivo tumorigenicity of HepaRG cells, which is in accordance with previous findings [20]. We chose for a 140 days follow-up to balance between the increased sensitivity that might be obtained using a longer test, against the likelihood of false-positive results due to spontaneous tumor formation, in accordance with the appropriate FDA guidance [21]. When HepaRG cells are applied in a BAL in combination with a double filter setup of 0.45 uM and 0.2 uM pore
size the risk of primary tumor initiation is negligible. Furthermore, materials and production procedures for the 540mL-BALs comply with GMP standards, a requirement for clinical application. HepaRG culture medium contains the xenogeneic compound FBS, however, this does not prohibit clinical application when it is certified free of prion diseases. Although not required by law before clinical application, we believe it is preferable to gain experience with the device at its final scale in a large-animal model and to generate additional safety data to maximize safety and prospects of clinical testing.

In summary, we have successfully overcome three hurdles towards clinical application of the new generation HepaRG-AMC-BAL: the preservation of HepaRG cells, upscaling of BAL cultures, and transport of BALs to the bedside. The optimization of bioreactor culture still calls for further research, particularly focusing on expansion and preservation of the biomass inside the bioreactor.
REFERENCES

Figure S1. Reduction of FBS in culture medium of 9mL-BALs to 5%. Hepatic functions (A-D) and enzyme leakage (E) of 9mL-BALs loaded with cryopreserved HepaRG cells, cultured in medium with 5% or 10% FBS. N.m. not measured.
CHAPTER 8

Summary, conclusions and future perspectives
SUMMARY

Acute liver failure (ALF) and acute on chronic liver failure (ACLF) are syndromes that are both associated with high mortalities, ranging from approximately 30% up to 96%, depending on disease severity and eligibility for emergency liver transplantation [1, 2]. Liver transplantation is a curative treatment option, but not every patient is eligible and a liver transplant is not available in time for every patient. Even when available, survival after emergency liver transplantation is lower compared to elective transplantation, which is likely due to a worse neurological and inflammatory status of patients in the emergency setting. This means that there is an unmet need for liver support, a need that has been attempted to be met by several means, including high volume plasma exchange therapy, artificial liver support and bioartificial liver (BAL) support. Of these therapies, only high volume plasma exchange has so far proven a benefit on survival in one study: approximately 10% absolute risk reduction of in-hospital mortality in ALF patients in the intention-to-treat analysis [3].

The Academic Medical Center (AMC)-BAL has been under development since the 1990’s. The system was originally based on primary porcine hepatocytes and was under evaluation in a Phase I clinical trial when the group was forced to find an alternative cell source, due to altered xenotransplantation regulation in the early 2000’s. Eventually this alternative was found in the human progenitor cell line HepaRG, and efficacy of the laboratory scale HepaRG-AMC-BAL was proven in a rat model of ALF, due to total liver ischemia [4]. The primary aim for this thesis was to further develop the HepaRG-AMC-BAL towards clinical application.

PART I of this thesis gives an introduction of the AMC-BAL and the selection of its biocomponent for BAL systems.

Chapter 1 starts with a concise introduction on ALF and ACLF, including the definitions, causes and current therapeutic options. Then, the AMC-BAL is introduced with a short history of its development and the current choice of biocomponent: HepaRG.

Chapter 2 is a narrative review on available BAL biocomponents. First, we described the demands posed to cell sources applied in BAL systems when utilized clinically or for in vitro applications, such as drug-toxicity testing. We concluded that a high hepatocyte differentiation grade is important for both applications and that the main differences in demand between in vitro and clinical application are the necessity of a diverse genetic backgrounds in the former and more emphasis on the possibility of economically feasible large-scale production in the latter application. Second, we discussed the available cell sources: primary hepatocytes, liver cell lines and stem-cell-derived hepatocytes. We then systematically reviewed the available literature on stem-cell-derived hepatocyte-like cells and their differentiation grade.
and functional capacity. We found that many studies do not provide sufficient data to fully assess the functional capabilities of hepatocyte-like cells. In the cases where these data were adequately reported, the functional level was several orders of magnitude lower compared to the most functional hepatic cell line available: HepaRG.

Finally, we concluded that for clinical BAL application, HepaRG cells are currently the most suitable cell source, and that there is not yet an all-round superior alternative to primary human hepatocytes for \textit{in vitro} applications.

In \textbf{PART II} of the thesis, HepaRG cells and their behavior as a BAL-biocomponent under different culture conditions are further characterized.

In \textbf{Chapter 3}, we performed a head-to-head comparison between HepaRG cells and the HepG2 clone C3A that is applied in a BAL that is currently under evaluation in a Phase III clinical trial, but of which little functional data were publicly available. Both cell lines were tested in monolayers and in laboratory scale BALs. HepaRG cells outperformed C3A cells in protein synthesis, ammonia detoxification and xenobiotic metabolism, regardless of culture modality. However, like HepaRG cells, C3A cell performance markedly increased when cultured in BALs. For example, lactate production turned into consumption, and ammonia partly eliminated through the urea cycle by C3A cells only when cultured in BALs. The main conclusion from the comparison was that HepaRG cells are superior to C3A cells for application in a BAL.

\textbf{Chapter 4} continues on the observation in Chapter 3 that both HepaRG and C3A cells reach higher hepatocyte differentiation grades when cultured in BALs compared to monolayers. We performed whole-genome microarray analysis on HepaRG cells cultured in monolayers and BALs and found that genes related to mitochondrial biogenesis differed most prominently in their expression, being upregulated in BAL cultures. Increased mitochondrial biogenesis was confirmed by an increase in mitochondrial abundance, mitochondrial membrane potential, protein expression of mitochondrial oxidative phosphorylation complexes as well as transcript levels of mitochondria-encoded genes in BAL- versus monolayer cultures. The three main differences between monolayer and BAL culture are: pericellular oxygenation concentrations, medium perfusion and 2D versus 3D cell configuration. We showed that all three factors contribute to an increase in mitochondrial biogenesis. Finally, we confirmed the stimulatory effect of BAL culture on mitochondrial biogenesis in C3A cells, in addition to HepaRG cells.

\textbf{Chapter 5} focusses on the effect of oxygen concentration on HepaRG hepatocyte differentiation in monolayer cultures. We showed that HepaRG cells cultured under hypoxia (5% ambient oxygen) did not acquire a hepatocyte phenotype, but rather represented hepatic
progenitor cells, as indicated by protein expression of progenitor cell marker SOX 9. These effects are likely to be related to oxygen-responsive factors; we confirmed the protein level of one of these factors, Hypoxia-inducible factor 1-alpha (HIF1α), to be higher under hypoxia versus normoxia. HepaRG cells are known to lose their ability to differentiate into hepatocytes around passage 20 from isolation. We showed that HepaRG cells expanded under hypoxia maintained their differentiation potential well beyond 20 passages.

In contrast to hypoxic cultures, cells that were differentiated under hyperoxia (40% ambient oxygen) were morphologically more hepatocyte-like, exhibited increased hepatic functions and nuclear expression of the hepatocyte transcription factor CCAAT/enhancer-binding protein alpha (CEBPα) compared to normoxic cultures. We confirmed these positive effects of hyperoxic culture in C3A cells. Next, we analyzed the transcriptome of HepaRG cells cultured under normoxia or hyperoxia by microarray analysis. Only 240 (of 23,223) probes were differentially expressed between normoxic and hyperoxic HepaRG cultures, including several well-known liver enriched transcription factors. The discrepancy between increased hepatocyte-specific functions and largely unaffected transcriptional activity indicates that the effects of hyperoxic cultures on hepatocyte functions is largely post-transcriptionally.

In Chapter 6, the effects of blood plasma on HepaRG cells is studied. First, we showed that human blood plasma from healthy donors had a dose-dependent toxic effect on HepaRG cells. Within several hours, hepatic gene transcript levels were dramatically downregulated, followed by hepatic functions. Notably, exposure of up to 16 hours did not affect viability. Exposure to different plasma fractions revealed that the effects were mediated by heat-resistant, albumin-bound and (partly) hydrophobic compound(s). Whole-transcriptome microarray analysis of cells exposed to human blood plasma for 30 minutes up to eight hours revealed that the toxic effects of plasma exposure on hepatic gene transcription was within hours and closely correlated with activation of nuclear factor kappa-light-chain-enhancer of activated B cells NF-κB activation, a master regulator of pro-inflammatory cytokine response.

HepaRG cells cultured in BALs were less affected by plasma exposure compared to HepaRG monolayers, and in BALs the negative plasma-effect proved to be largely reversible after 16 hours of exposure. The protective effect of BAL culturing was found to be mainly attributable to medium perfusion and 3D configuration. Because of the unavailability of human ALF plasma, we exposed HepaRG cultures to porcine ALF plasma, which elicited a mitotoxic effect, in addition to the detrimental effect of healthy porcine plasma.

In PART III of the thesis, the HepaRG-AMC-BAL was prepared for pre-clinical and clinical testing.
In Chapter 7 we describe the improvement of the logistics and the scaling-up of the HepaRG-AMC-BAL for clinical application. First, the process of large-scale expansion and preservation of the biocomponent needed to be developed. We showed that HepaRG cells can be expanded in monolayer cultures in the Hyperflask™ platform, cryopreserved in large quantities, and seeded directly from cryo-storage into the BAL-cartridge where they underwent two additional population doublings. Second, the BAL cultures were scaled-up from 9mL to 540mL priming volume without loss of functionality, when corrected for total biomass. In addition, we quantified amino-acid depletion during culture to set-up a fed-batch medium supply during BAL culture. Third, the transport conditions of the BAL from production site to the bedside were addressed. Initially, we attempted whole-device cryopreservation, hypothermic and subnormothermic preservation, however, all three strategies resulted in an unacceptable loss of function. To facilitate transport, we therefore successfully developed and tested a clinical-grade transport unit that can maintain temperature, as well as culture medium- and gas perfusion for at least 24 hours. Lastly, tumorigenicity of HepaRG cells was assessed in immunodeficient mice; no animals injected with HepaRG cells developed tumors, while in the positive control group 67% of animals did.

**CONCLUSIONS**

The objective of this thesis was to better understand and further develop the HepaRG-based AMC-BAL. We gained insight in the roles of 3D configuration, oxygenation, temperature, medium-starvation and plasma exposure on the cells and successfully used this knowledge to develop a clinical size BAL and ditto transport module.

**FUTURE PERSPECTIVES**

**Application of the current generation HepaRG-AMC-BAL**

Now that the device is scaled-up, the logical next steps in the application would be a large-animal study and then a Phase I clinical trial. In fact, we have already invested substantial amounts of funds and effort in the development of a paracetamol induced ALF model in pigs to gain data on the efficacy of the HepaRG-AMC-BAL. However, no consistent data could be generated during these studies due to variations in time to occurrence and degree of liver failure, as well as the unexpected lower yield of functional cells inside the bioreactors. Currently, we are looking into the best way to use and represent the data that were collected, although not suitable to judge efficacy of treatment, they may still prove valuable as safety data. The reality of modern market-driven development of innovative medical therapies dictates that in order to gather funds to further develop a product, it must have been applied
in humans first. The HepaRG AMC-BAL has previously proven efficacious in an ALF model in rat [4], and the question arises whether proof of concept in a large animal experimental model is necessary before a Phase I clinical trial is performed.

As discussed in the Introduction, ideally a BAL does not only bridge liver failure patients to transplantation, but to regeneration of the native liver, to avoid a transplantation. A proof-of-principle study for this aim is currently missing for the HepaRG-AMC-BAL, and a survival study should therefore be performed. Several animal models are suitable: subtotal liver resection with/without liver ischemia or toxic models, such as galactosamine + lipopolysaccharide induced liver failure. Ideally, the study set-up should allow treated animals to fully recover from liver injury for a substantial amount of time.

**Further development of the AMC-BAL**

**Hardware**

The AMC-BAL features separate circuits for oxygenation and plasma perfusion, however it lacks a separate drainage system for bile, leading to an accumulation bile components in the patient plasma. The integration of an extra dimension of an artificial biliary tree would involve a high degree of microstructures and cell polarity, a highly complex task that is not likely to be solved in the near future. An achievable alternative is the addition of an albumin dialysis circuit to the BAL set-up to cleanse out (part of) the bile salts and other hydrophobic toxins.

Currently, the design of the AMC-BAL allows treatment using one dosage: one loaded cartridge. However, it has been known for a long time that in humans there is a linear correlation between body surface area and liver size [5], and the degree of remnant native liver function also differs per liver failure patient. It is therefore rational to adapt the amount of biomass to the body surface area and maybe even to the degree of liver damage, although, at this moment, no data are available on how to titrate the latter. Modular BAL cartridges of different sizes and contents that can be combined as needed, would allow for a patient-tailored approach, and could prevent both the use of BALs of insufficient strength, as well as the use of excess amounts of costly biomass.

As described in Chapter 6, the biocomponent is affected by the exposure to patient plasma and, therefore, exposure time should be limited to the functional window of the biocomponent. As this may be variable depending on etiology as well as the individual patient and the moment in disease course, integration of sensor technology into the hardware that can monitor key functions, such as ammonia elimination, markers of cell decay, such as aminotransferases, and/or impedance as a derivative for membrane- or tight-junction integrity [6], will be valuable future additions to the design.
Summary, conclusions and future perspectives

HepaRG
As described in Chapter 2, the main detoxification mechanism of ammonia in HepaRG cells is through fixation in the amino acid glutamine through activity of the enzyme Glutamine Synthetase (GS), which has as a disadvantage that the process can be reversed at multiple sites in the human body, including muscles and brain, leading ammonia production. Preferably, ammonia would be converted into urea through urea cycle activity, since this process cannot be reversed in human cells. In the human liver, the majority of hepatocytes exhibits urea cycle activity, a high capacity low-affinity system. Peri-central hepatocytes, however, express, like HepaRG cells, GS, a low capacity, high affinity system that can scavenge residual ammonia not processed peri-portal, located more upstream along the liver sinusoid. The expression of GS and the inhibition of urea cycle rate-limiting enzyme carbamoyl phosphate synthetase 1 (CPS1) are regulated through the WNT/β-catenin pathway [7].

One possible strategy to induce urea cycle enzyme activity in HepaRG cells is by overexpression of CPS1, and possibly other enzymes of the urea cycle. Another way to induce urea cycle activity is through downregulation of β-catenin expression. The difficulty with this approach lies in the broad functional spectrum of the WNT/β-catenin pathway. Many liver tumors have activating mutations in the β-catenin gene, which gives them a growth advantage [8], and, although whole exome sequencing of the (tumor-derived) HepaRG cell line did not reveal mutations in β-catenin gene CTNNB1 (data not shown), it is probable that inactivating β-catenin will have undesired side-effects, such as a decrease in proliferative capacity and, possibly, viability. In fact, in our hands, β-catenin-inhibition appeared to strongly inhibit proliferation in HepaRG cells (data not shown). Possible solutions to this problems include chemical inhibition and transfection with an inducible promoter, although both will require thorough additional safety tests before clinical application.

Once HepaRG cells with increased urea cycle activity have been established, an interesting strategy would be to strive for a zonally designed BAL in a modular bioreactor placed in series to most efficiently eliminate ammonia, as executed along the liver sinusoid. Cells with the current phenotype would be loaded into the distal cartridge, while the proximal cartridge would be loaded with cells that exhibit increased urea cycle activity. Not only ammonia detoxification, but also other functions, such as glucose metabolism and xenobiotic detoxification, are expressed zonally in the liver. Liver zonation enables interaction between specialized cells that is thought to enable the liver to rapidly respond to efficiently maintain homeostasis in, for example, glucose-, nitrogen- and xenobiotic metabolism, while preventing metabolic loops that could appear when opposite functions are expressed simultaneously. Not all of these functions are divided binary into liver zones, nor are they all linked to β-catenin, and in fact, HepaRG cells do not represent peri-central hepatocytes in all respects. However,
despite the difficulties that are to be anticipated, the road to a zonated BAL can eventually lead to a more efficient device with a broader palette of hepatic functions.

Another strategy to improve HepaRG hepatocyte-specific functions is by overexpression of liver-enriched transcription factors that are low compared to human liver. For example, ONECUT1 (4-fold lower in HepaRG versus human liver, data not shown), which forms a transcriptional network with HNF1A and HNF4A, and that is pivotal to hepatocyte development [9]. Finally, the transcription factor Constitutive Androstane Receptor (CAR) is a worthwhile target to explore. Recently, our group produced a HepaRG line that stably overexpressed CAR, which unexpectedly not only induced known CAR-target cytochrome p450 genes, but also induced activity of non-CAR target cytochrome p450 family members and albumin synthesis, as well as a more hepatocyte-like polygonal morphology [10].

**Alternative hepatocyte cell sources**

As discussed in Chapter 2, HepaRG cells are currently the most suitable biocomponent for clinical BAL application. Cell sources with higher differentiation grades are likely to become available in the future, as hepatocyte differentiation protocols keep improving over time. Since publication of the review, several highly interesting papers on novel developments have been published, including the creation of a BAL loaded with Induced pluripotent stem cells (iPSC)-derived hepatocytes [11], and the generation of genomically stable bipotent liver organoids from discarded human liver transplants [12].

Co-culturing of HepaRG cells with other cell types is another method that can potentially improve BALs; co-culturing of primary hepatocytes and non-parenchymal cells has been long known to improve hepatocyte phenotype stability [13]. In C3A cells co-culturing with human umbilical vein derived endothelial cells (HUVECs) resulted in increased protection from acetaminophen toxicity [14], and a combination of iPSC-derived progenitor cells with HUVECs and mesenchymal stem cells resulted in vascularized liver buds that formed viable mini-transplants in mice [15]. Therefore, co-culturing is an interesting option to explore in pursuit of both higher hepatocyte differentiation grade as well as resistance to exogenous factors, such as (ALF-) blood plasma.

**Other novel treatments for liver failure**

This thesis focusses on BAL support as a treatment for liver failure patients, however, others are working on different treatment modalities. Artificial liver support systems envisioned to aid detoxification through albumin dialysis were mentioned previously in this Chapter. Another form of detoxification is aimed at dampening the pro-inflammatory state in liver failure through the use of cytokine scavengers. This method has recently been successfully evaluated in an animal study in combination with albumin dialysis, and a clinical trial is anticipated [16].
Others, yet, are targeting inflammation through macrophage modulation [17], transplantation with mesenchymal stem cells [18], or stimulation of hematopoietic stem cells by injection with granulocyte colony-stimulating factor [19-21]. At this point it is impossible to predict which strategy will be most successful. The treatment may well prove to be most efficacious when multiple approaches are combined.
REFERENCES


APPENDICES

Nederlandse samenvatting
List of contributing authors
List of publications
PhD Portfolio
Dankwoord
Curriculum vitae
Acuut leverfalen (ALF) en acuut op chronisch leverfalen (ACLF) zijn syndromen die beiden een hoge mortaliteit kennen, variërend van 30% tot 96% afhankelijk van de ernst van de ziekte en of een patiënt in aanmerking komt voor levertransplantatie [1, 2]. Levertransplantatie is een in opzet curatieve behandeling voor leverfalen, echter niet iedere patiënt komt hiervoor in aanmerking, en ook is er niet altijd op tijd een donorlever beschikbaar. Zelfs als een donorlever wel beschikbaar is, dan blijkt dat de overleving na een spoed levertransplantatie lager is dan na een electieve ingreep; dit heeft waarschijnlijk te maken met een slechtere neurologische en ontstekingsstatus van patiënten in de acute setting. Uit bovenstaande volgt dat er een noodzaak bestaat voor leverondersteunende therapie. Er is op verschillende manieren getracht om tegemoet te komen aan deze behoefte, onder andere middels hoog-volume plasma-wisseltransfusie, artificiële leverondersteuning en bioartificiële lever (BAL)-ondersteuning. Van deze therapieën is alleen van de eerste in een gerandomiseerde studie een positief effect op de overleving aangetoond: ongeveer 10% absoluut risicoreductie van ziekenhuis mortaliteit in ALF patiënten in de intention-to-treat analyse [3].

Sinds het begin van de jaren 1990 wordt in het Academisch Medisch Centrum (AMC) te Amsterdam gewerkt aan de AMC-BAL. Het systeem was oorspronkelijk was gebaseerd op primaire varkenslevercellen en had het stadium van klinische toepassing in een Fase 1 studie bereikt, toen er een Europees moratorium op xenotransplantatie van kracht werd dat een einde maakte aan het verdere gebruik van dierlijke cellen in BALs. Dit luidde het begin in van een zoektocht naar een geschikte proliferatieve humane celbron, welke uiteindelijk werd gevonden in de bipotente levervoorlopercellijn HepaRG. Met een studie op een rattenmodel van ALF middels totale leverischemie werd effectiviteit van een laboratorium-schaal HepaRG-AMC-BAL op overlevingsduur bewezen [4]. Het hoofddoel van dit proefschrift was om de HepaRG-AMC-BAL verder te ontwikkelen in de richting van klinische toepasbaarheid.

DEEL I van dit proefschrift geeft een inleiding over het AMC-BAL systeem en de selectie van celbronnen voorBAL systemen.

Hoofdstuk 1 begint met een introductie over de definities, oorzaken en huidige therapeutische opties voor ALF en ACLF. Daarna wordt de AMC-BAL geïntroduceerd met een korte geschiedenis van de ontwikkeling en de selectie van de huidige celbron: HepaRG.

Hoofdstuk 2 betreft een literatuuronderzoek naar beschikbare celbronnen voor BALs. Eerst beschreven we welke eisen er worden gesteld aan cellen voor gebruik in een BAL bedoeld voor klinische toepassing of in vitro toepassingen, zoals medicijnveiligheidstests.
We concludeerden dat voor beiden een hoge graad van levercel-differentiatie onontbeerlijk is, en dat de voornaamste verschillen in eisen tussen de twee toepassingsgebieden liggen op de economische haalbaarheid van celmassa productie voor klinische toepassing en de heterogene genetische achtergrond voor *in vitro* toepassingen.

Daarna behandelden we de beschikbare levercelbronnen: primaire levercellen, lever cellijnen en uit stamcellen opgekweekte levercellen. We hebben een literatuur search gedaan op ‘hepatocyte like cells’, voortgekomen uit geïnduceerd pluripotente stamcellen (iPSCs), en hun gerapporteerde differentiatiegraad en functionele capaciteit vergeleken. Bij een substantieel deel van de publicaties ontbraken essentiële gegevens, en in de gevallen waar deze data wel gerapporteerd werden, bleek dat de functionele capaciteit enkele ordes van grootte lager uitviel dan in het geval van primaire levercellen en HepaRG cellen.

We concludeerden dat HepaRG cellen momenteel de meest geschikte celbron vormen voor toepassing in een klinische BAL, en dat er geen proliferatieve celtypen beschikbaar zijn die primaire levercellen op alle eigenschappen volwaardig kunnen vervangen.

In DEEL II van dit proefschrift karakteriseerden we HepaRG cellen als BAL-biocomponent onder uiteenlopende kweekomstandigheden.

In Hoofdstuk 3 vergeleken we de cellijn HepaRG met de HepG2 kloon C3A, welke momenteel wordt toegepast in een BAL systeem in een Fase 3 klinische studie, maar waarvan weinig openbare data beschikbaar waren.

Beide cellijnen werden in monolaag en laboratoriumschaal BALs opgekweekt en vergeleken met betrekking tot leverfunctionaliteit en transcriptniveaus van lever-specifieke genen. HepaRG cellen presteerden beter dan CA cellen op eiwitsynthese, ammoniakdetoxificatie en xenobiotisch metabolisme in beide kweekmodaliteiten. Opvallend was dat zowel HepaRG- als C3A cellen aanzienlijk beter presteerden in BALs vergeleken met monolagen. Zo was er in C3A BAL-kweken bijvoorbeeld sprake van melkzuurconsumptie in plaats van -productie, zoals gevonden in monolagen, en alleen in BAL-kweken was er in C3A cellen ureumcyclusactiviteit aantoonbaar. De hoofdconclusie uit deze vergelijking was dat HepaRG cellen superieur zijn aan C3A cellen als biocomponent voor een BAL.

**Hoofdstuk 4** gaat in op de observatie dat zowel HepaRG als C3A cellen een hogeren differentiatiegraad bereiken in BALs vergeleken met monolagen. We voerden een ‘whole genome microarray’ analyse uit op HepaRG cellen gekweekt in tweedimensionale monolagen en in BALs, en vonden dat expressie van genen gerelateerd aan mitochondriale biogenese het meest verschilden tussen de groepen, met een hogere expressie in de BAL-gekweekte
HepaRG cellen. Toegenomen mitochondriale biogenese werd bevestigd door een toename van mitochondriën, mitochondriale membraanpotentiaal, eiwitexpressie van mitochondriale oxidatieve fosforylering complexen en transcriptniveaus van mitochondriaal gecodeerde genen in BAL versus monolaag kweken.

De drie meest voornamme verschillen tussen BAL- en monolaagkweken zijn: pericellulaire zuurstofconcentratie, mediumperfusion en 3D versus 2D configuratie. We toonden aan dat deze drie factoren afzonderlijk bijdroegen aan een toename van mitochondriale biogenese. Tenslotte vonden we dat BAL-kweken ook de mitochondriale biogenese van C3A cellen stimuleerde, naast dat van HepaRG cellen, wat aangeeft dat het een breder voorkomend fenomeen betreft.

In Hoofdstuk 5 gingen we dieper in op het effect van zuurstofvoorziening op HepaRG levercel differentiatiegraad in monolaag kweken. We lieten zien dat HepaRG cellen die onder zuurstofarme (5% zuurstof) werden gekweekt zich niet ontwikkelden tot levercel, maar meer op leervoorlopercellen leken, een observatie die wordt ondersteund door expressie van stamcel marker SOX9. Het is aannemelijk dat dit fenomeen wordt gereguleerd door zuurstof-responsieve factoren; we toonden voor één van deze factoren, Hypoxia-inducible factor 1-alpha (HIF1α), aan dat het eiwit niveau hoger was in de zuurstofarme kweken vergeleken met de kweken onder normale zuurstofspanning.

HepaRG cellen verliezen het vermogen om in levercellen te differentiëren rond passage 20 vanaf isolatie. We toonden aan dat HepaRG cellen die worden gekweekt onder hypoxie hun differentiatiepotentiaal behouden tot in ieder geval enkele passages na de 20e.

HepaRG cellen die onder hoge zuurstofrijke omstandigheden (40% zuurstof) werden gedifferentieerd oogden morfologisch meer als levercellen, vertoonden toegenomen levercel functionaliteit en een toename van nucleaire expressie van de liver transcription factor ‘CCAAT/enhancer-binding protein alpha’ (CEBPα) vergeleken met kweken onder normale zuurstofvoorziening. Deze bevindingen werden ook in C3A cellen gereproduceerd. We analyseerden de transcriptomen van HepaRG cellen die werden gedifferentieerd onder normale of hoge zuurstofvoorziening middels ‘whole genome microarray’ analyse. Slechts 240 (van 23.223) probes kwamen differentieel tot expressie, waaronder enkele bekende lever-specifieke transcriptie factoren. De discrepantie tussen de toename in levercel-specifieke functies en de grotendeels onveranderde transcriptionele activiteit duidt erop dat de effecten van hyperoxie op levercel differentiatie grotendeels post-transcriptioneel van aard zijn.

In Hoofdstuk 6 bestudeerden we het effect van bloedplasma op HepaRG cellen. Eerst toonden we aan dat bloedplasma van gezonde donoren een dosisafhankelijk toxisch effect op HepaRG cellen in monolagen had. Binnen enkele uren decimeerden transcript
niveaus van diverse hepatische genen, later gevolgd door levercel-specifieke functies. Noemenswaardig is dat na blootstelling tot en met 16 uur geen verlies van viabiliteit optrad. Blootstelling van de cellen aan verschillende fracties van humaan bloedplasma toonde aan dat de toxische effecten werden gemediëerd door warmte-resistente albumine-gebonden en (deels) hydrofobische bestandsdeels. ‘Whole genome microarray’ analyse op HepaRG monolagen die werden blootgesteld aan humaan plasma gedurende 30 minuten tot 8 uur toonde aan dat er binnen enkele uren een dramatisch verlies van hepatische gen-transcriptie optrad. Deze daling correleerde met opregulatie van target genen van nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), een regulator van onder andere pro-inflammatoire cytokinerespons. HepaRG cellen die in BALs werden geweekt bleken minder gevoelig voor het toxische effect van humaan plasma, en de repressie van hepatische gen transcriptie bleek grotendeels reversibel na 16 uur blootstelling. Het beschermende effect van BAL kweek bleek grotendeels toe te schrijven te zijn aan medium perfusie en 3D configuratie. Aangezien er geen humaan ALF plasma beschikbaar was, werden HepaRG kweken ook nog blootgesteld aan ALF-varkensplasma, wat een mitotoxisch effect sorteerd bovenop het toxische effect van gezond varkensplasma.

In DEEL III van dit proefschrift werd de HepaRG-AMC-BAL klaargemaakt voor preklinische en klinische toepassing.

In Hoofstuk 7 beschreven we hoe de HepaRG-AMC-BAL werd opgeschaald en hoe het logistieke proces rondom kweek en transport werd gestroomlijnd. Ten eerste diende het efficiënt proces voor expansie en preservatie van HepaRG cellen te worden ontwikkeld. We toonden aan dat HepaRG cellen kunnen worden geëxpandeerd middels het Hyperflask™ kweekplatform, om vervolgens in grote hoeveelheden gecryopreserveerd te worden, waarna de cellen vanuit cryo-opslag direct in de BAL geladen kunnen worden. In de BAL ondergingen de cellen vervolgens nog twee populatieverdubbelingen.

Ten tweede werden de BAL kweken opgeschaald van 9mL naar 540mL volume-inhoud, zonder verlies van functionaliteit, wanneer er werd gecorrigeerd voor biomassa. Daarnaast kwantificeerden we aminozuurdepletie gedurende de kweekperiode om een ‘fed-batch medium toedienings systeem tijdens BAL kweek op te zetten.

Ten derde werd onderzocht onder welke condities de BALs van productieplaats naar het bed van de patiënt konden worden vervoerd zonder verlies van functionaliteit. In eerste instantie onderzochten we cryopreservatie en zowel hypotherme als subnormotherme preservatie van de geladen BAL. Helaas leidden alle drie deze strategieën tot een onacceptabel verlies van functionaliteit. Om toch transport zonder functieverlies te kunnen faciliteren ontwikkelden
wij een transport unit die tenminste 24 uur lang in een constante temperatuur en continue gas- en mediumperfusion kan voorzien.

Tenslotte werd de tumorigeniciteit van HepaRG cellen beoordeeld in immuundeficiënte muizen; geen van de dieren die werden geïnjecteerd met HepaRG cellen ontwikkelden tumoren tegenover 67% van de dieren in de positieve controlegroep.

Conclusies
Het doel van dit proefschrift was om de HepaRG-AMC-BAL beter te leren begrijpen en verder te ontwikkelen richting klinische toepassing. We hebben inzichten verkregen in de invloed van 3D configuratie, zuurstofvoorziening, temperatuur, mediumdepletie en plasmablootstelling op de cellen en we hebben deze kennis succesvol ingezet om de HepaRG-AMC-BAL op te schalen naar klinisch formaat en om een transport unit te ontwikkelen.
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LIST OF CONTRIBUTING AUTHORS

Thomas M van Gulik, Ruurdtje Hoekstra, Erik J. Hendriks, Vincent A. van der Mark, Chung-Yin Tang, Esme J. Coppens, Koen Jansen
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Robert AFM Chamuleau, Adriaan Siliakus, Jurgen Seppen, Ruurdtje Hoekstra, Aziza A.A. Adam, Theodorus B.M. Hakvoort, Vincent A. van der Mark, Ronald P. Oude Elferink, Manon E. Wildenberg
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Philipp Treskes
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LIST OF PUBLICATIONS


Appendices

PHD PORTFOLIO

Name PhD student: Martien van Wenum
PhD period: 2012-2017
PhD supervisors: Prof. dr. T.M. van Gulik, dr. R.A.F.M. Chamuleau and dr. R. Hoekstra

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**Oral presentations**

Cryopreserved HepaRG cells preserve functionality in the AMC-Bioartificial liver.

*ACM MDL, Lunteren*

How to make a better BAL (Bioartificial Liver)

*NVH Dutch Liver Retreat, Spier*

Human plasma toxicity in the AMC-BAL

*Meeting of the European Society for Artificial Organs, Rome*

Application of the scaled-up AMC-BAL in a porcine model of paracetamol-induced liver damage.

*NVH Dutch Liver Retreat, Spier*

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**Poster presentations**

Comparison of the C3A and HepaRG liver cell lines in monolayer and 3D for Bioartificial Liver application.

*EASL International Liver Congress, Vienna*

Human plasma toxicity on HepaRG cells in the AMC-bioartificial liver.

*EASL International Liver Congress, Vienna*

Effects of healthy- and acute liver failure plasma on differentiated human HepaRG progenitor cells in monolayers and bioartificial livers

*EASL International Liver Congress, Barcelona*

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DANKWOORD

Een promotietraject moet je zelf trekken, maar een proefschrift kan alleen tot stand komen met de bijdragen van (vele) anderen, zo ook het mijne. Een aantal van die mensen wil ik hier graag persoonlijk noemen.

Mijn promotor Prof. dr. T.M. van Gulik. Beste Thomas, ik heb veel bewondering voor hoe je uit een ogenschijnlijk oneindige hoeveelheid energie put om klinische, wetenschappelijke en bestuurlijke rollen met verve te vervullen. Je geeft je promovendi alle ruimte om zelf hun weg te vinden in zowel hun wetenschappelijke als verdere klinische loopbaan, maar je deur staat altijd open. Als je een manuscript of abstracts ter revisie kreeg (al dan niet krap gepland) volgde je commentaar vaak nog dezelfde dag, of anders nacht.

Mijn co-promotor dr. R.A.F.M Chamuleau. Beste Rob, capo dei capi van de kunstlevergroep. Het was een waar genoegen om met je samen te werken, zowel op het lab als tijdens de memorabele uitstapjes naar onder andere Rome, Medolla en Edinburgh. Je bent een onuitputtelijke bron van kennis en ervaring gebleken, en als dingen niet gingen zoals het moest zorgde je altijd voor een oplossing die voor iedereen goed uitpakte, ook al heeft het je waarschijnlijk de nodige nachten slap gekost. Op naar nog eens 5 jaar AMC-BAL onderzoek?


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Appendices

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CURRICULUM VITAE

Martinus van Wenum (1986, Rotterdam) graduated high-school at the ‘Rotterdams Montessori Lyceum’ in Rotterdam. In 2004 he started Pharmacy at the University of Utrecht, in 2005 he switched to medical school at the Academic Medical Center (AMC) of the University of Amsterdam. He performed his Master’s thesis in the hepatology laboratory of Klinikum Großhadern of the University of Munich, under supervision of Prof. dr. U.H.W. Beuers, which sparked his interest in both research and gastroenterology and hepatology.

From 2012 he worked as a PhD student in the Bioartificial Liver Group at the Department of Experimental Surgery of the AMC under supervision of prof. dr. T.M. van Gulik, dr. R.A.F.M. Chamuleau and dr. R. Hoekstra. He worked on the preclinical development of the AMC-bioartificial liver. The work presented in this thesis was presented at several international conferences.

From May 2016 to April 2017, Martien worked at the MC Slotervaart in Amsterdam, and in April 2017 he started Internal Medicine training in the St. Antonius Hospital in Nieuwegein as a part of the Gastroenterology and Hepatology residency program of the University of Utrecht.

Martien lives in Amsterdam with Sylvia van Rooij and is the proud father of Philou Maria van Wenum.