

Research Paper

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

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

In 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.

Key words: HepaRG, C3A, BAL, Liver support, Hepatocytes, Bioartificial liver.

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 *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.

Hepatic function tests

The function tests were performed as described [10]. Briefly, monolayer cultures (n=8 per cell line) were exposed to 1 ml, and BAL cultures (C3A n=3, HepaRG n=4) to 120 ml of test medium for 24 h. Test medium was WE+ with 1 mM N-carbamoyl-L-glutamate, 1.5 mM ¹⁵NH₄Cl, 2.27 mM D-galactose, 2 mM L-lactate and 125 μM testosterone (all compounds from Sigma Aldrich). During the

function test, medium samples were taken at 0.75, 6 and 24 h (monolayers) or at 0.5, 1, 2, 8 and 24h (BALs). In the obtained samples we quantified 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 supplemental table S2 and S4.

High-performance liquid chromatography (HPLC)

Cytochrome P450 3A4 (CYP3A4) activity was determined in function test samples taken after 45 minutes by quantification of 6B-hydroxylation of testosterone (6B-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, 1.5 μm 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.

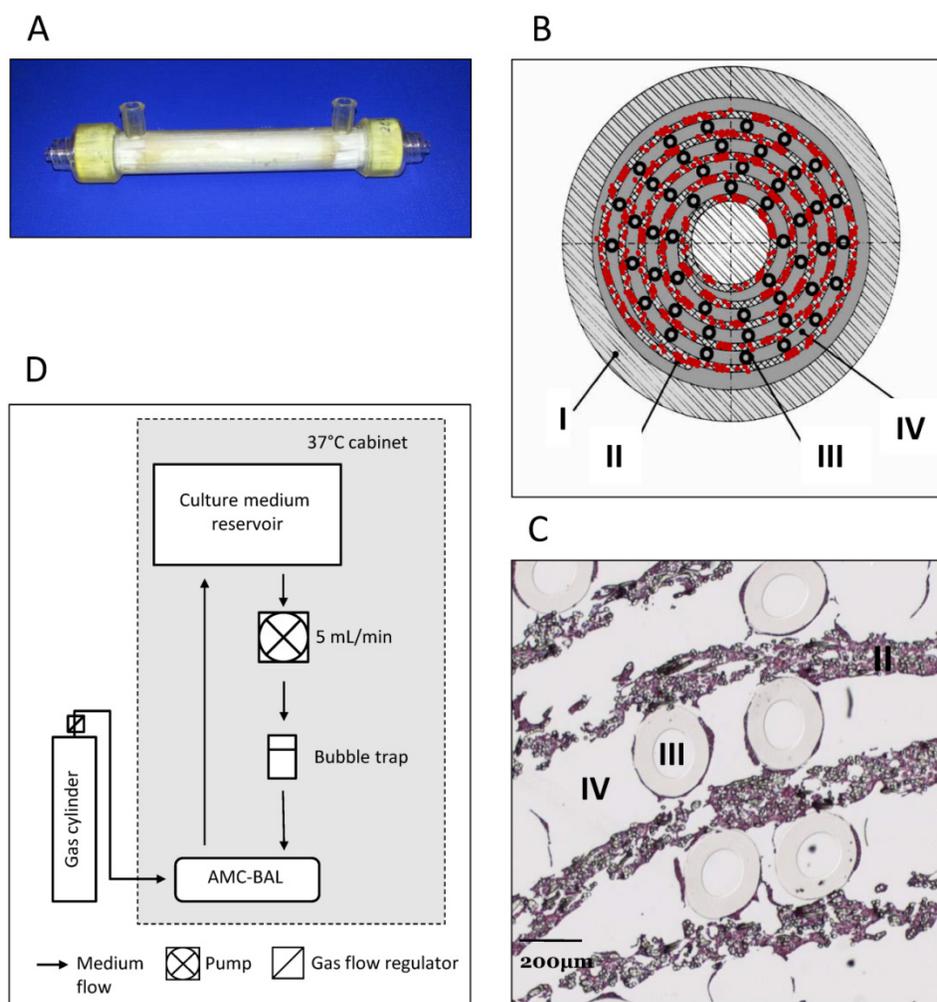


Figure 1. AMC-BAL Culture. (A) AMC-BAL cartridge. (B-C) Schematic cross-section and detail of a HE-staining of a paraffin embedded HepaRG-AMC BAL. Depicted are: I, The outer shell of the cartridge; II, the matrix and attached cells; III, gas capillaries and IV, the intercapillary space through which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

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 $20,000\times g$ at 4°C . For the separation of the *o*-phthalaldehyde-derivatized amino acids, two serial coupled BDS Hypersil C18 columns (150 \times 4.8 mm, 3 μm 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 methylarginine (respectively IS-1 and IS-2 in the chromatogram, Fig. S1). A typical chromatogram is depicted in supplemental figure S1 and an overview of the data is given in supplemental table S6.

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

For gene expression analyses we obtained matrix samples (T-bags) from the BALs at different time points as described [14]. RNA from BALs (C3A: $n=3$, HepaRG: $n=4$) and monolayers (C3A: $n=6$, HepaRG: $n=4$) were isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). qRT-PCR was performed using a touchdown annealing temperature protocol, as previously described [10]. Transcript levels were normalized for 18S ribosomal RNA and are expressed as % of the average level of two human liver samples isolated from the healthy parenchyma in liver resection material from female patients, aged 40 and 41, with liver adenomas and no signs of liver damage. The transcript levels of the tested genes differed no

more than 1.45 fold between these samples, except for *CEBPA*, *AFP* and *CYP3A7* which differed 1.88, 2.19 and 4.24 fold respectively. The procedure was in accordance with the ethical standards of the institutional committee on human experimentation (protocol number 03/024) and the Helsinki Declaration of 1975. Primer sequences and amplicon sizes are listed in supplemental table S1. An overview of the transcript data is given in supplemental tables S2, S3 and S5.

Statistical analyses

We performed one-way ANOVA and Holm Sidak's multiple comparisons test ($\alpha = 0.05$) to assess differences between more than 2 groups, and multiple t-tests with Sidak-Bonferroni's multiple testing correction for the comparison between two groups on multiple parameters. Statistical analyses were performed in Prism version 6 (GraphPad Prism Inc. San Diego, CA, USA). Data are presented as mean \pm 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).

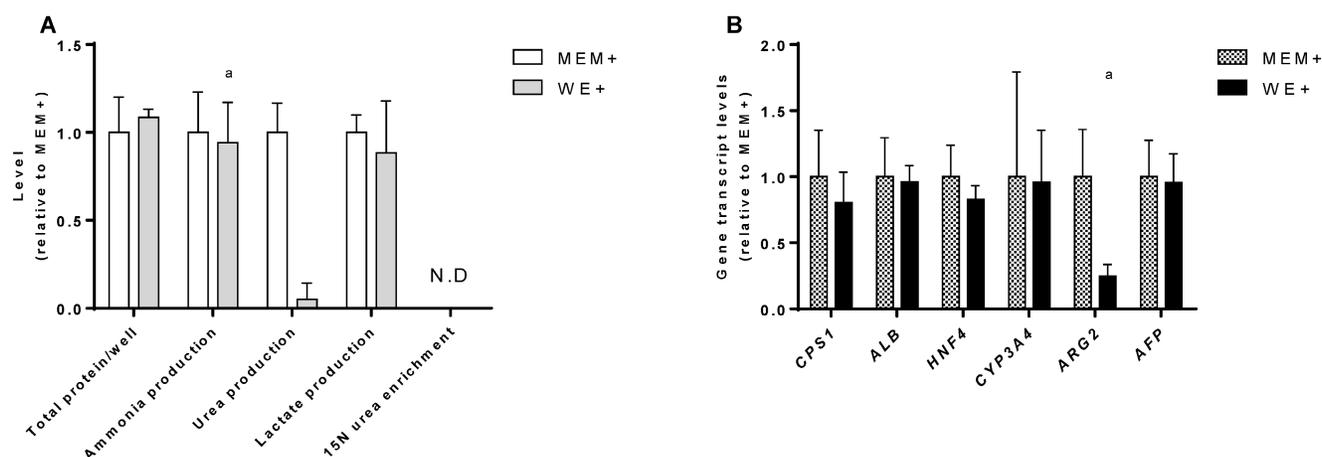


Figure 2. C3A culture medium selection. Comparison of C3A cells in monolayers cultured in MEM+ or WE+ medium for 7 days. (A) Hepatic functions and total protein content normalized to MEM+ cultured cells. (B) Gene transcript levels relative to MEM+ cultured cells. N.D.= not detectable. a= $p < 0.05$ compared to MEM+.

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, supplemental table S3). However, the transcript levels of eight out of sixteen tested mature hepatic genes were also downregulated >2-fold, and thus hepatic differentiation could not be confirmed. These genes encoded for hepatic transcription factors (*HNF4A*, *CEBPA*, *PXR*, *CAR*), as well as for proteins involved in the urea cycle (*OTC*, *CPS1*), plasma protein synthesis (*TF*) and transport (*NTCP*). The other tested mature hepatic genes that remained stable in their expression (<2-fold up/down) were involved in plasma protein synthesis (*ALB*, *FVII*), nitrogen metabolism (*GLUL*, *ARG1*) biotransformation (*CYP3A4*, *CYP2B6*) and transport (*SLCO1B1*). Moreover, the panel of tested hepatic functions (ammonia elimination, urea production, albumin synthesis) did not increase after day 7 (Table 1).

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

In the following part of the study we compared HepaRG cultures with C3A cultures at their optimal maturation times, being 28 days and 7 days for the HepaRG and C3A monolayers respectively, and 14 days for both HepaRG and C3A BALs.

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

As previously reported, HepaRG monolayers followed a distinct proliferation- and differentiation pattern [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.

Function	C3A monolayer			C3A BAL				
	Unit	7 days	14 days	21 days	Unit	3 days	7 days	14 days
Ammonia elimination	$\mu\text{mol h}^{-1} \cdot \text{g protein}^{-1}$	-33.54 ± 9.59	-55.93 ± 9.18	-37.57 ± 6.99	$\mu\text{mol h}^{-1}$ per BAL	-2.06 ± 0.40	-1.66 ± 1.19	-2.02 ± 0.60
Urea production	$\mu\text{mol h}^{-1} \cdot \text{g protein}^{-1}$	1.42 ± 0.34	1.19 ± 0.27	0.86 ± 0.14	$\mu\text{mol h}^{-1}$ per BAL	0.042 ± 0.053	0.37 ± 0.06	0.44 ± 0.08
Albumin Synthesis	$\text{mg h}^{-1} \cdot \text{g protein}^{-1}$	35.65 ± 3.12	35.34 ± 6.34	NM	mg h^{-1} per BAL	NM	12.17 ± 0.82	15.52 ± 1.5
<i>CYP3A4</i> activity	$\text{nmol h}^{-1} \cdot \text{g protein}^{-1}$	2.27 ± 0.12	4.26 ± 0.78	NM	nmol h^{-1} per BAL	NM	87.33 ± 75.5	95.66 ± 15.89
Lactate elimination					$\mu\text{mol h}^{-1}$ per BAL	-17.10 ± 0.66	4.03 ± 1.66	1.24 ± 1.8
AST leakage					IU h^{-1} per BAL	0.16 ± 0.12	0.62 ± 0.13	1.38 ± 0.07
LDH leakage					IU h^{-1} per BAL	0.78 ± 0.06	1.14 ± 0.07	2.00 ± 0.20

NM=not measured.

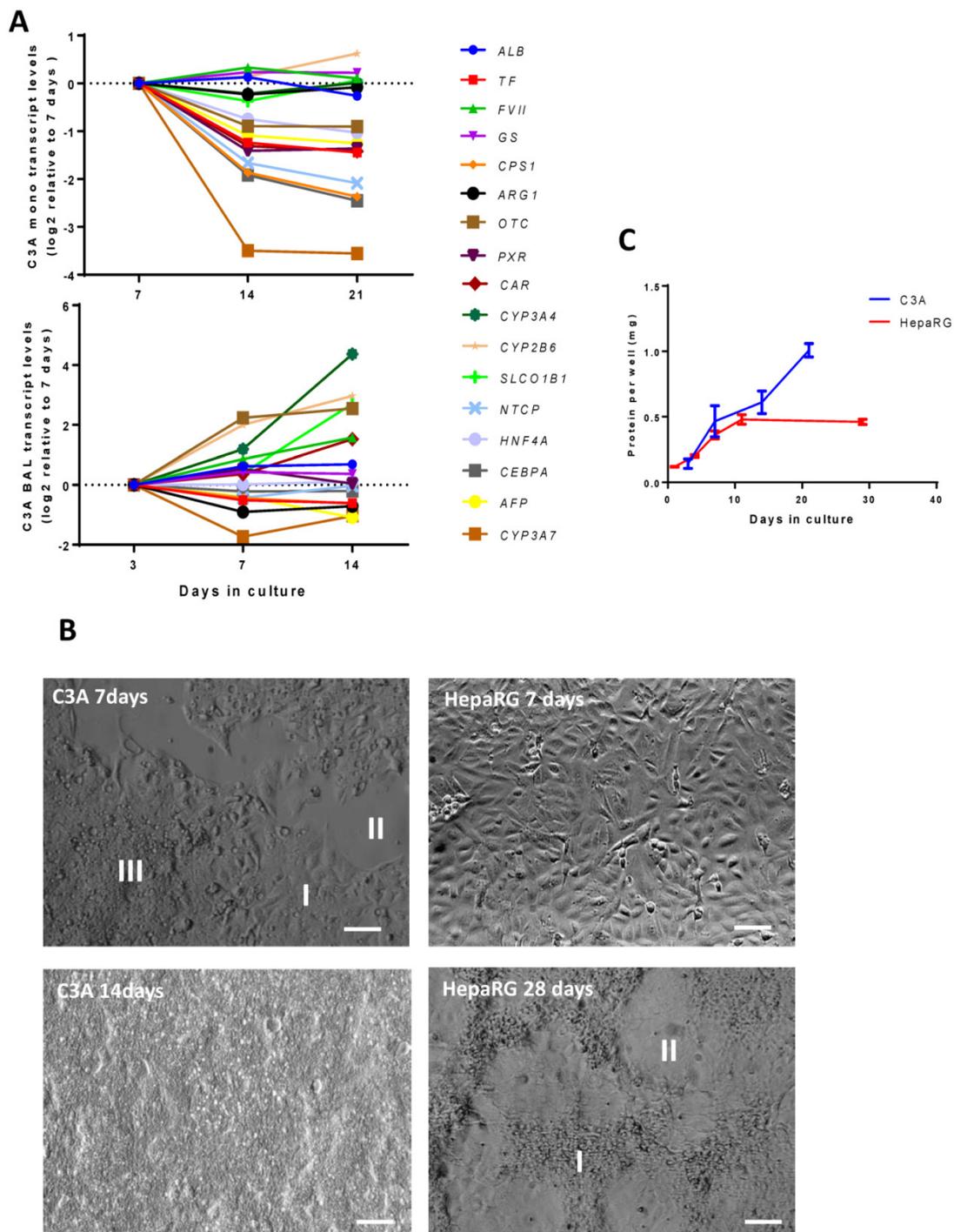


Figure 3. Time course of differentiation, morphology and proliferation. (A) Transcript levels of hepatic in time in C3A cells cultured in monolayer and BALs (B) Morphology of monolayer cultures at days 7 and 14 for C3A and days 7 and 28 for HepaRG. C3A cells form confluent monolayer patches (I), flanked 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).

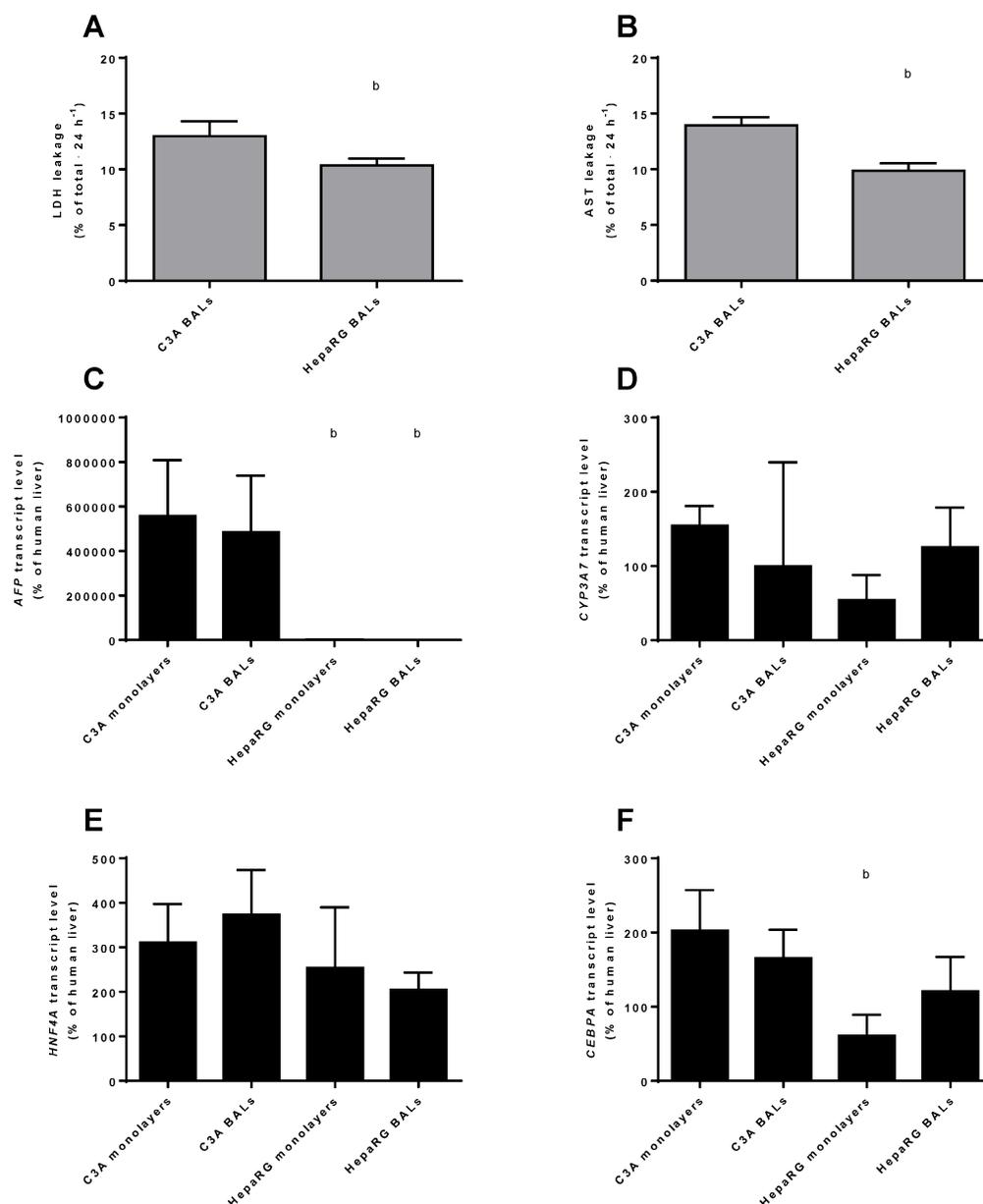
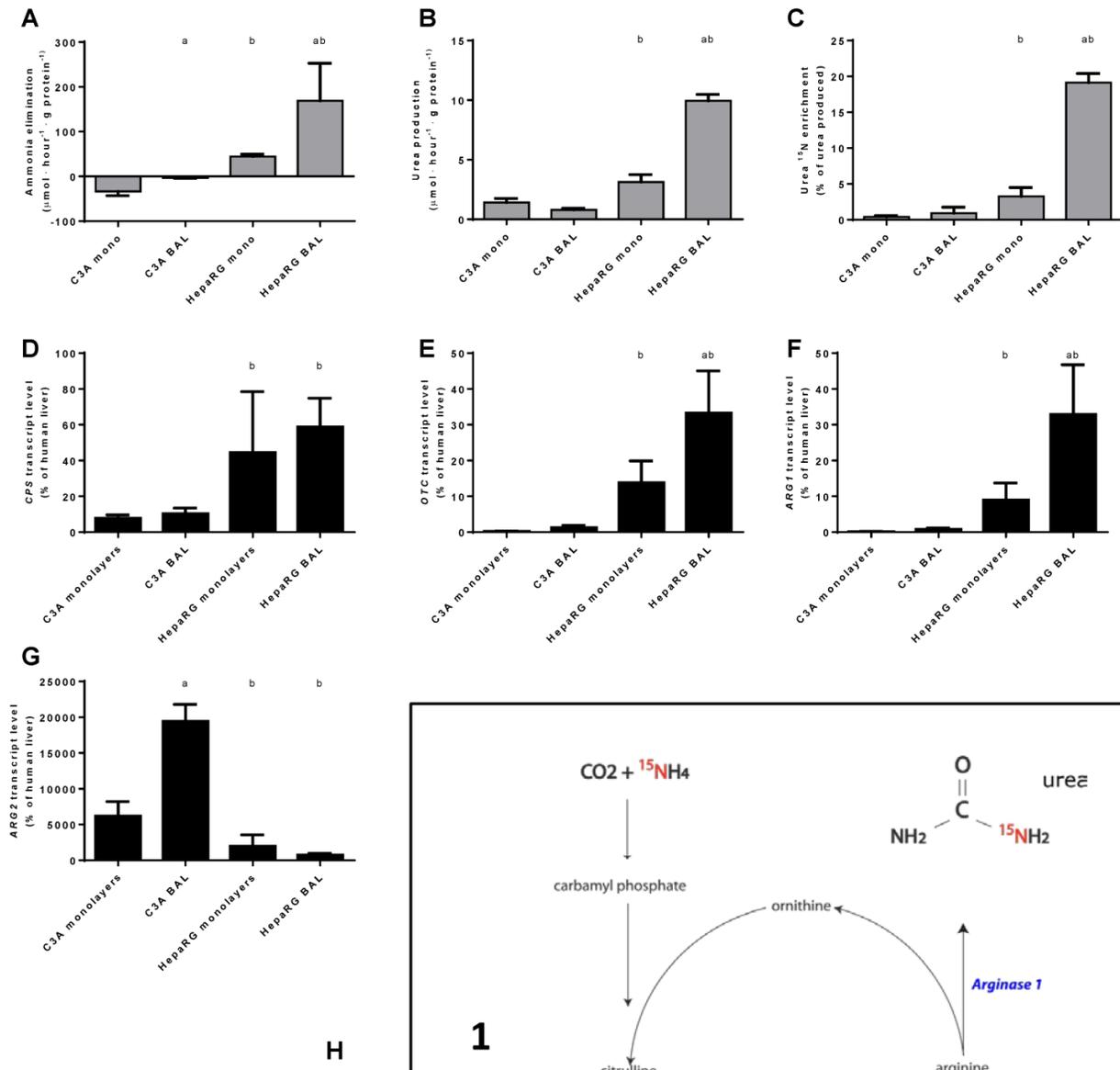


Figure 4. Differentiation and integrity. Comparison of HepaRG and C3A cultures in monolayers and BALs for (A-B) enzyme leakage and (C-D) transcript levels of fetal hepatic genes and of (E-F) hepatic transcription factors. b=p<0.05 compared to C3A cells in the same culture platform.



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 ^{15}N 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= $p \leq 0.05$ compared to same cell line in monolayer, b= $p \leq 0.05$ compared to C3A cells in the same culture platform.

Next, we investigated the contribution of the urea cycle (UC)-activity to ammonia elimination. Urea production alone is not an adequate measure of UC-activity, since urea can also be formed by the degradation of arginine into ornithine and urea through (non-hepatic) Arginase 2 activity [15] (Fig. 5H). The production of ^{15}N -urea after a challenge with ^{15}N -ammonia is a measure of the contribution of UC-activity to urea production (Fig. 5H).

Urea production in BALs compared to monolayers was ~3-fold higher for HepaRG cultures and not different in C3A cultures. In HepaRG cells compared to C3A cells, urea production was ~2 and 13-fold higher in monolayers and BALs respectively (Fig. 5B). In line with a previous report, we found that ^{15}N -urea enrichment in C3A monolayers was around the detection limit (Fig. 5C) [15]. However, BAL culturing induced a trend towards higher ^{15}N -urea enrichment (~2-fold) and higher expression of urea cycle genes *CPS1*, *ARG1* and *OTC* (2- to 5-fold) (Fig. 5B-F). For HepaRG cells, BAL culturing increased the transcript levels of two of the three tested urea cycle genes (*OTC* and *ARG1*, up to 4-fold) and the ^{15}N -urea enrichment (6-fold) (Fig. 5C-F). ^{15}N -urea enrichment was 10- to 20-fold higher compared to C3A cultures and the transcript levels of the tested urea cycle genes were 5- to 77-fold higher (Fig. 5C-F). *ARG2* expression was highest in C3A cultures and lowest in HepaRG BALs, explaining the high production of non- ^{15}N enriched urea (Fig. 5G-H). Because, even in HepaRG BALs, the estimated urea cycle activity amounts to <10% of total ammonia elimination, we conclude that the urea cycle does not play a significant role in ammonia detoxification in either of the cell lines.

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).

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, supplemental table S6).

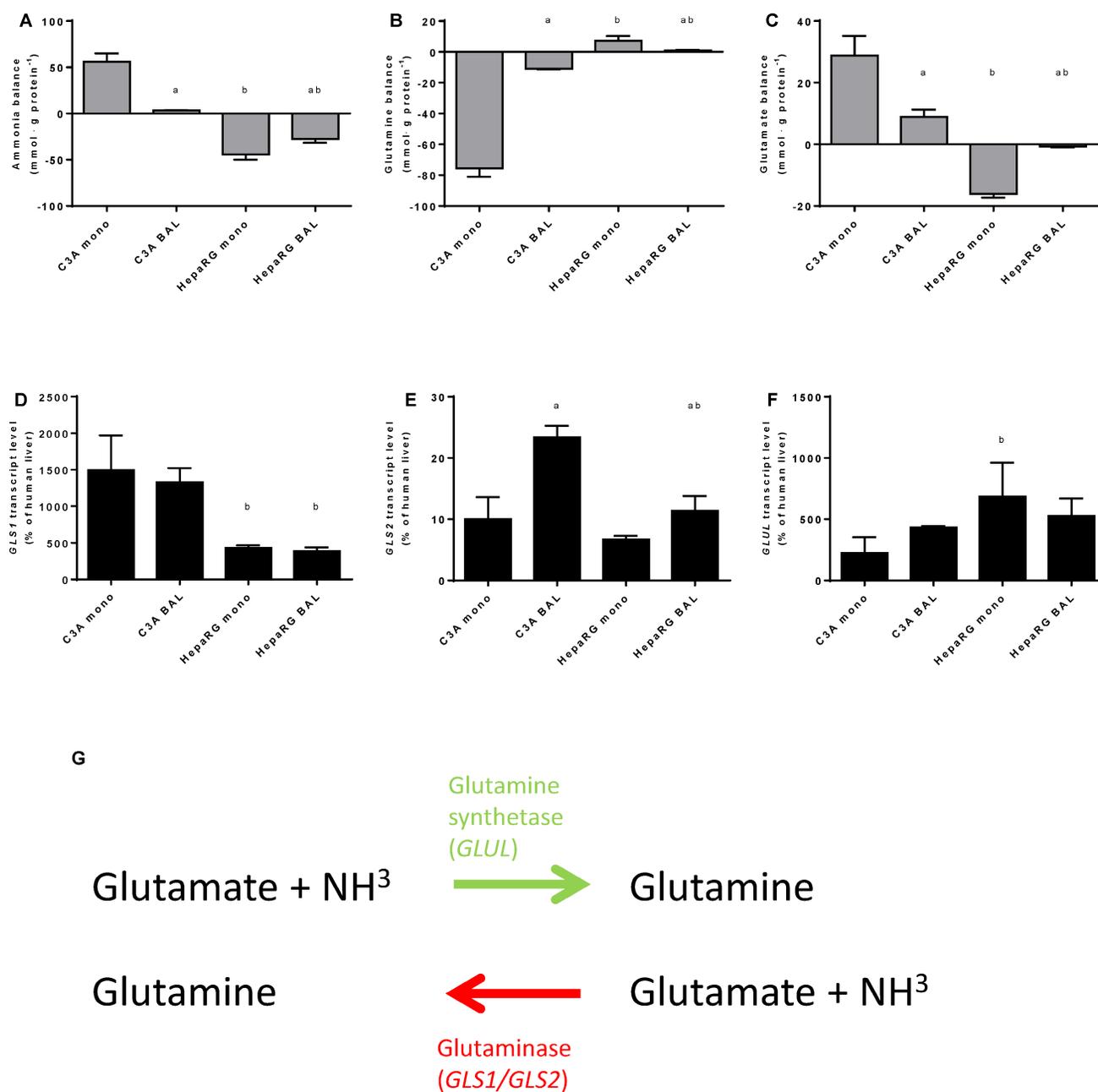


Figure 6. Nitrogen balance. Comparison of HepaRG and C3A cultures in monolayers and BALs after 24 hours exposure to culture medium containing 1,5 mM of ammonia and 2 mM lactate for (A-C) ammonia, glutamine and glutamate balance, and for (D-F) transcript levels of *GLS*, *GLS1* and *GLUL*. Diagram G illustrates the reversible conversion of glutamine into glutamate. a= $p \leq 0.05$ compared to same cell line in monolayer, b= $p \leq 0.05$ compared to C3A cells in the same culture platform.

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).

Amino acid metabolism (umole/mg protein/hour)

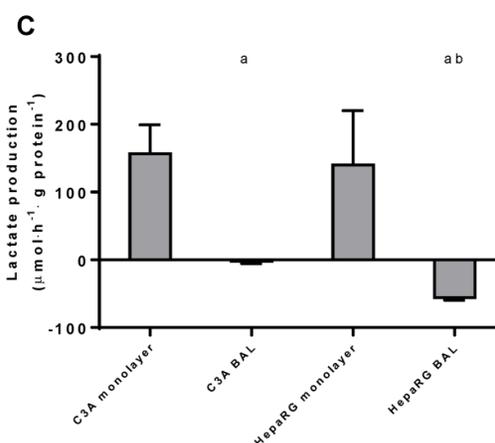
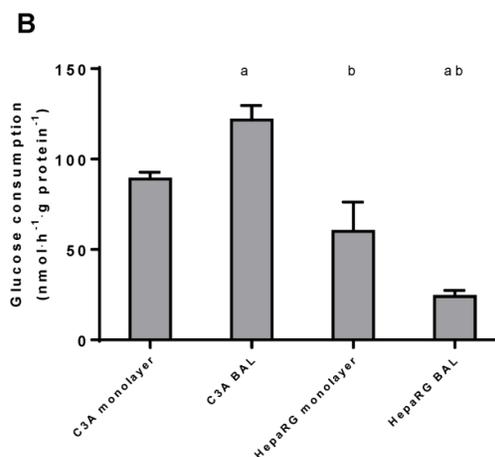
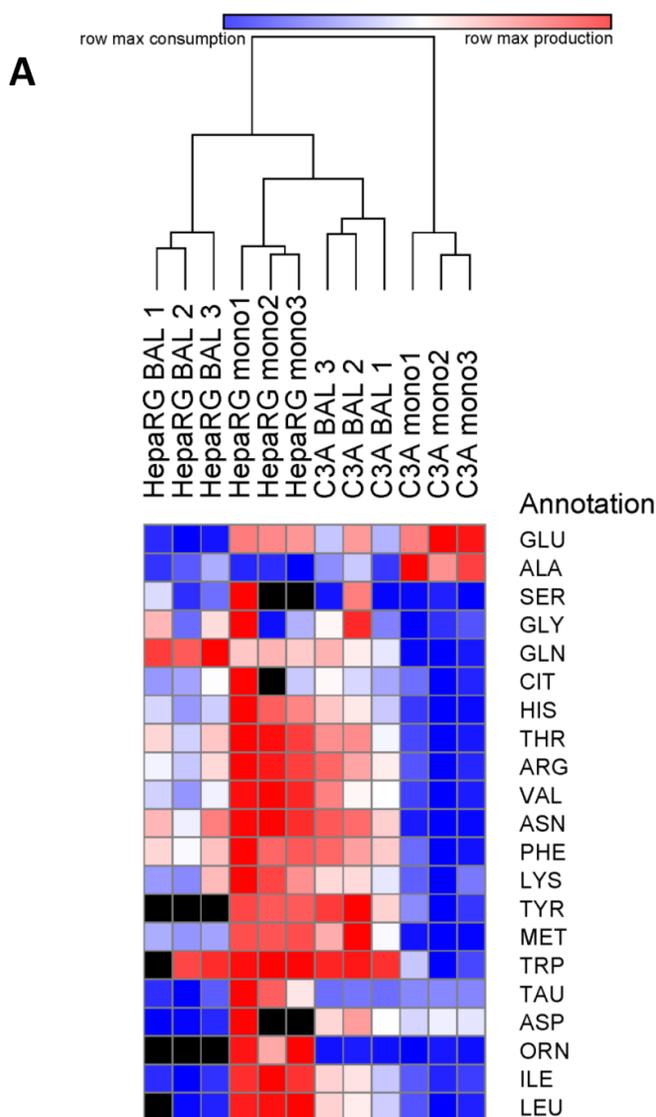


Figure 7. Amino acid and carbohydrate metabolism. HepaRG and C3A cells, cultured in monolayers and BALs, were analysed for metabolism of (A) amino acids, several amino acids could not be analysed in a number of samples due to overlapping peaks, these data points were excluded from analysis and depicted 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.

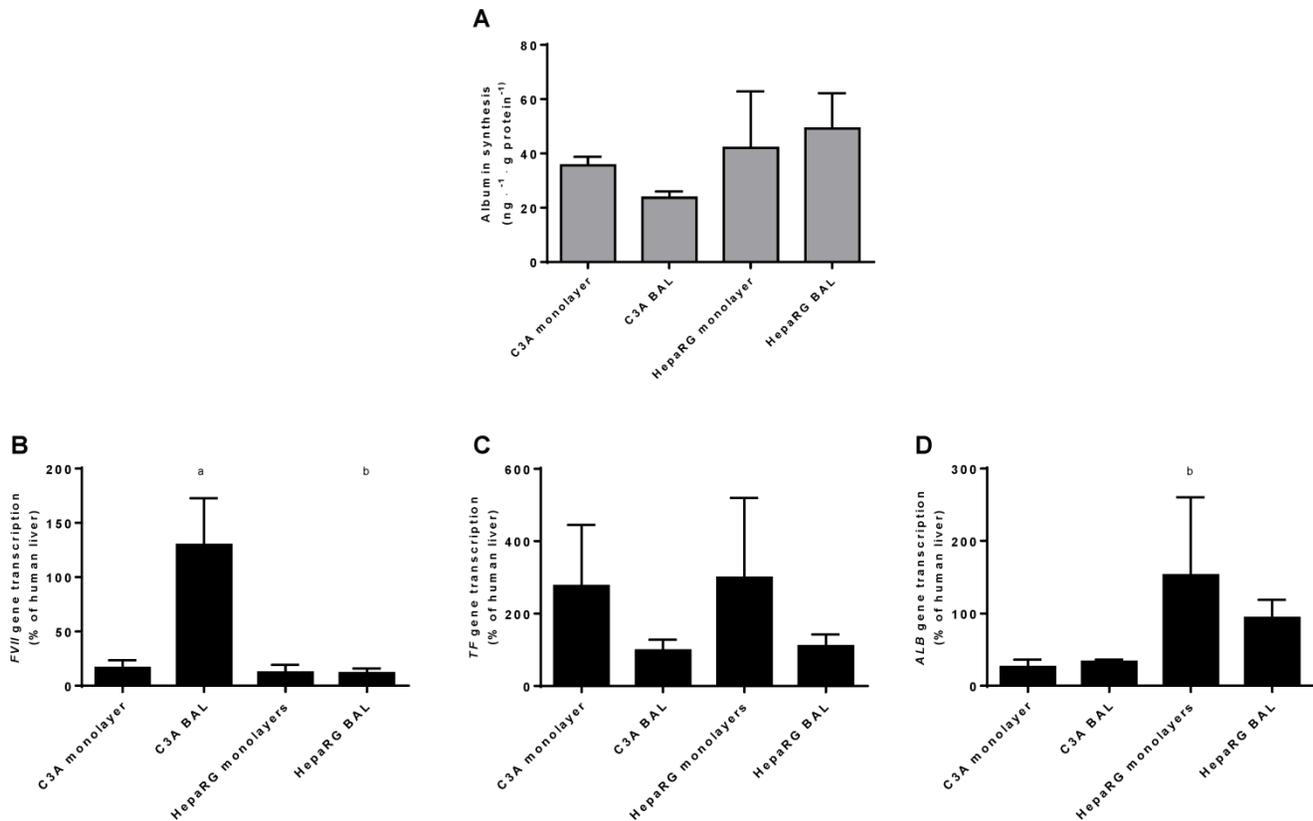


Figure 8. Protein synthesis in culture medium. Comparison of HepaRG and C3A cultures in monolayers and BALs for (A) albumin synthesis and (B) transcript levels of genes encoding proteins related to hepatic protein synthesis. a= $p \leq 0.05$ compared to same cell line in monolayer, b= $p \leq 0.05$ compared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

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.

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.

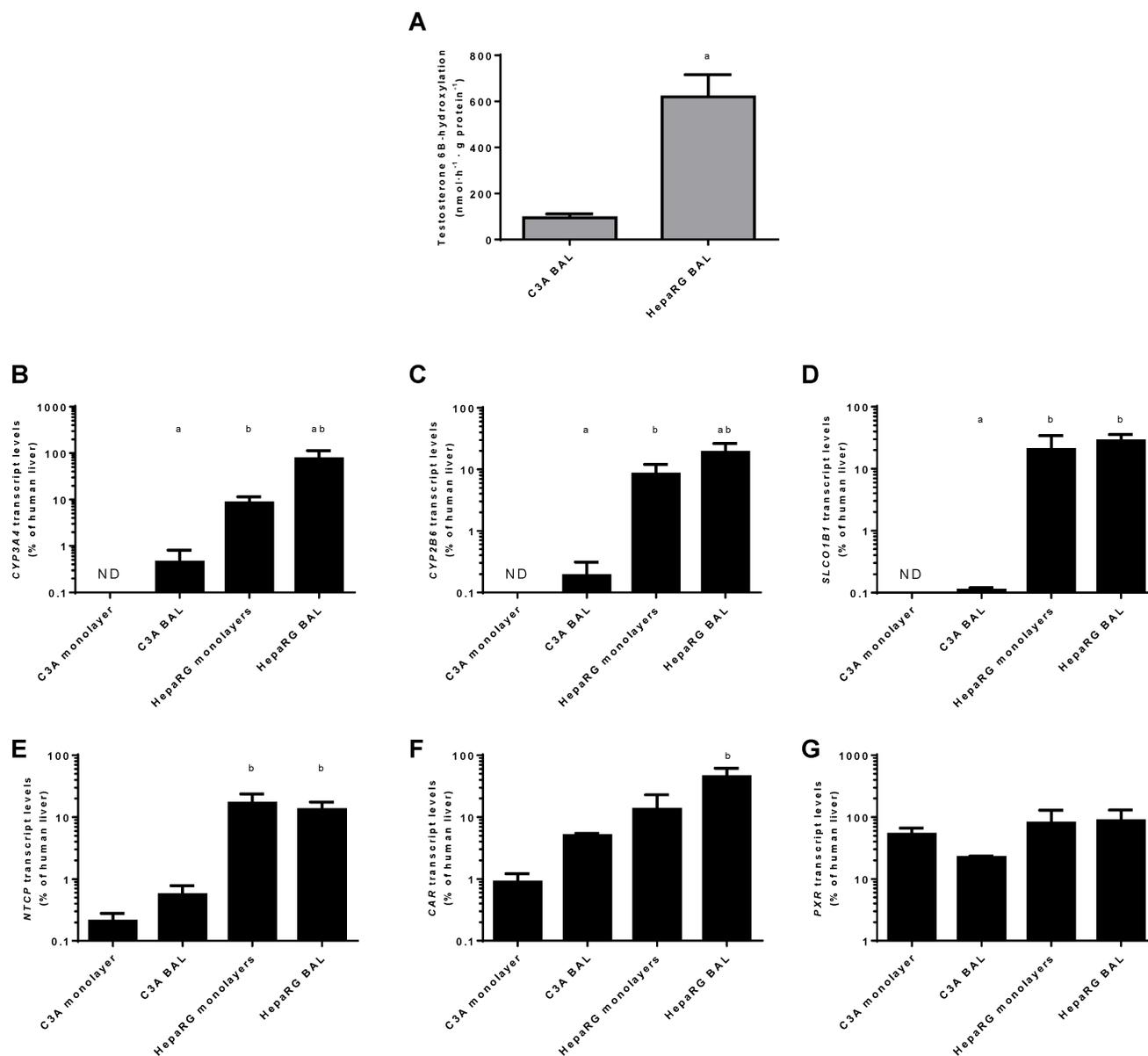


Figure 9. Xenobiotic metabolism. Comparison of HepaRG and C3A cultures in monolayers and BALs for (A) CYP3A4 activity and transcript levels of genes encoding (B-C) enzymes related to xenobiotic metabolism (D-E) basolateral transporter proteins, and (F-G) nuclear hormone receptors. ND= not detectable.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

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

C3A cells have been studied in the context of BAL application by others in the past, although one should exercise caution in comparing outcomes due to differences in test- conditions, culture time, culture

platforms and normalization methods [20-24]. One study reported conditions similar to this study, except for a difference in culture time. In this study, urea and albumin synthesis were 2 fold lower and 3-fold higher respectively, indicating external validity of the data in our current research [24].

C3A monolayers did not exhibit contact inhibition, in contrast to HepaRG monolayers. This gives rise to the concern that C3A cells are tumorigenic and may compromise safety. From studies in immunocompromised mice, HepaRG cells are known to have some remnant tumorigenicity when undifferentiated, but not when differentiated [25]. Data on tumorigenicity of C3A are not available to the best of our knowledge. The parental cell line of

C3A, HepG2, is known to be highly tumorigenic [26]. Since HepaRG cells become strictly non-tumorigenic only when differentiated, it is conceivable that tumorigenicity in C3A cells decreases and contact inhibition increases with increased differentiation upon BAL-culture. Due to technical limitations, contact inhibition could only be assessed in monolayers and therefore we feel we cannot draw firm conclusions on the tumorigenicity of C3A cells in BAL cultures. To eliminate the risk of tumorigenicity in patients, plasma should be passed over multiple barriers before re-entering the body in order to prevent cells from entering the circulation [27].

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

Another way to improve ammonia detoxification would be to increase UC-activity up to the level of PHs. *In vivo*, PHs exhibit a specific functional phenotype depending on their distance from the portal- and central vein [34]. The GS activity is restricted to the pericentral region, whereas UC-activity takes place in the intermediate and portal zones. Recently it was described how this metabolic zonation is driven by the Wnt signalling pathway:

proliferative pericentral hepatocytes are high in β -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 through 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.

Supplementary Material

Additional File 1:

Figure S1, Table S1.

<http://www.ijbs.com/v12p0964s1.pdf>

Additional File 2:

Table S2-S6. <http://www.ijbs.com/v12p0964s2.xlsx>

Abbreviations

AAA: aromatic amino acids; ADP: adenosine diphosphate; AFP: alpha fetoprotein; ALB: albumin; ALF: Acute liver failure; AMC: Academic medical center; ARG1: Arginase 1; AST: aspartate aminotransferase; BAL: bioartificial liver; BCAA: branched chain amino acids; CAR: constitutive androstane receptor; CEBPA: CCAAT/enhancer-binding protein alpha; CPS1: carbamoyl phosphate synthetase 1; CYP2B6: cytochrome P450 2B6; CYP3A4: cytochrome P450 3A4; CYP3A7: cytochrome P450 3A7; FVII: blood coagulation factor VII; GLS1: Glutaminase1; GLS2: Glutaminase2; GLUL: glutamine synthetase; HNF4A: hepatic nuclear factor four-alpha; HPLC: High-performance liquid chromatography; LDH: lactate dehydrogenase; NTCP: Sodium/bile acid cotransporter; OTC: Ornithine transcarbamylase; PPH: primary porcine hepatocyte; PXR: Pregnane X receptor; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; SLC01B1: solute carrier organic anion transporter family member 1B1; TF: transferrin; UC: urea cycle.

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Conflicts of Interest

Robert Chamuleau, is Chief Scientific Officer of the university spin-off company Hep-Art Medical Devices B.V that holds the exclusive licence to the AMC-Bioartificial liver. Ruurdte Hoekstra was previously employed part-time by Hep-Art Medical Devices B.V.

Valery Shevchenko is an employee of Biopredic International, which currently holds the exclusive license to the HepaRG cell line. The contribution of Biopredic Int. was strictly limited to the technical execution of tests, as proposed by MW and RH. Biopredic Int. had no influence on the content of this article, nor on the decision to publish.

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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