Human liver cell lines for the AMC-bioartificial liver
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CHAPTER 6

Increased hepatic functionality of the human hepatoma cell line 
HepaRG cultured in the AMC bioreactor

Submitted

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ABSTRACT

Background: The clinical application of a bioartificial liver (BAL) depends on the availability of a human cell source with high hepatic functionality, such as the human hepatoma cell line HepaRG. This cell line has demonstrated high hepatic functionality, but the effect of BAL culture on its functionality in time is not known.

Aim: To study the characteristics of the HepaRG-AMC-BAL over time, and compare the functionality of the HepaRG-AMC-BAL with monolayer cultures of HepaRG cells.

Methods & Results: Histological analysis of 14-day-old BALs demonstrated functional heterogeneity similar to that of monolayer cultures. Hepatic functionality of the HepaRG-AMC-BALs increased during 2-3 weeks of culture. The majority of the measured protein-normalized hepatic functions were already higher in day 14 BAL cultures compared to monolayer cultures, including ammonia elimination (3.2-fold), urea production (1.5-fold), conversion of $^{15}$N-ammonia into $^{15}$N-urea (1.4-fold), and cytochrome P450 3A4 activity (7.9-fold). Moreover, lactate production in monolayer cultures switched into lactate consumption in the BAL cultures, a hallmark of primary hepatocytes, and protein-normalized cell damage was 4-fold lower in day 14 BAL cultures compared to monolayer cultures. Transcript levels of cytochrome P450 genes and of regulatory genes hepatic nuclear factor 4α and pregnane X receptor increased in time in BAL cultures and reached higher levels than in monolayer cultures. Lastly, metabolism of amino acids, particularly the alanine and ornithine production of HepaRG-AMC-BALs more resembled that of primary hepatocytes than monolayer HepaRG cultures.

Conclusions: We conclude therefore that BAL culture of HepaRG cells increases its hepatic functionality, both over time, and compared to monolayer, and this is associated with a reduction in cell damage, upregulation of both regulatory and structural hepatic genes, and changes in amino-acid metabolism.
**INTRODUCTION**

Bioartificial livers (BALs) have been developed to bridge patients with acute and acute-on-chronic liver failure to liver transplantation or liver regeneration. BALs typically comprise a bioreactor loaded with a biocomponent (e.g., hepatocytes) and are able to replace the failing liver when connected to the patient’s circulation.

The AMC-BAL that was developed in our laboratory is based on a bioreactor with an internal oxygenator and a spirally wound, non-woven polyester matrix, yielding a three-dimensional culture environment that provides direct cell-plasma contact and optimal oxygenation of the biocomponent.

BAL therapy should compensate for the loss of liver function that gives rise to hepatic encephalopathy, inflammation, and multi-organ failure. Functionally, this entails, among others, the elimination of ammonia, drug-metabolizing activity (e.g., cytochrome p450 (CYP) activity), lactate elimination, and the synthesis of blood proteins, such as clotting factors and apolipoprotein A-1.

The human hepatoma cell line HepaRG is a promising candidate in this respect. HepaRG is a bipotent liver progenitor cell line that, in monolayer, differentiates into two distinct cell populations in a 28-day period. It differentiates upon reaching confluence, optionally followed by treatment with 2% dimethyl sulfoxide (DMSO) into: 1) hepatocyte-like cells that self-organize into clusters; and 2) cluster-neighboring cells that express biliary duct cell markers.

We confirmed that DMSO treatment increased the expression of CYP genes 4- to 20-fold in HepaRG monolayers, leading to e.g. a final CYP3A4 activity of 19% of primary human hepatocytes. However in the absence of DMSO a high level of ureagenesis, ammonia elimination, and hepatic protein synthesis was reached, partly due to a higher cell mass. Moreover, when cultured in the laboratory model of the AMC-BAL without DMSO for 14 days, HepaRG cells not only demonstrated a high level of ammonia elimination and hepatic protein synthesis, but also a relatively high CYP3A4 activity (6β-hydroxylation of testosterone), reaching 31% of primary human hepatocytes. The high functionality of this HepaRG-AMC-BAL was proven by significantly improving survival time during treatment of rats with acute liver failure.

These results led to the hypothesis that culture of HepaRG cells in the AMC-BAL (in the absence of DMSO) increases their functionality. To investigate this hypothesis, we cultured HepaRG in laboratory scale AMC-BALs for 21 days and assessed hepatic functions at different time points. Subsequently, we compared the state of differentiation of 14 day-old HepaRG-AMC-BALs and HepaRG monolayers, both cultured in absence of DMSO, on basis of their protein-normalized functionality. Finally, we investigated the underlying factors for the upregulated hepatic functionality in the BAL related to cell damage, the expression of both structural and regulatory genes, and amino acid metabolism, the last playing an important role in the regulation of metabolic energy and nitrogen metabolism.
Chapter 6

METHODS

Monolayer cell culture
HepaRG cells were kindly provided by prof. C. Guguen-Guillouzo (INSERM, Rennes, France). HepaRG cells were cultured in 24-well culture plates (Corning, New York, U.S.) on HepaRG culture medium without DMSO but supplemented with 1 mM N-carbamoyl-L-glutamate (Sigma Aldrich, St. Louis, U.S.) to increase urea production. Analyses were conducted on monolayer HepaRG cultures of 28 days post-seeding.

AMC-BAL culture
For BAL cultures, the HepaRG cells were large-scale expanded in Hyperflasks (Corning, New York, U.S.) in HepaRG medium without DMSO. Cultures were maintained at 37°C in a humidified atmosphere (95% air, 5% CO2). Culture medium was refreshed every 3 to 4 days. 14-Day-old Hyperflask cultures were harvested using an Accutase (Innovative Cell Technologies, San Diego, U.S.), Accumax (Innovative Cell Technologies), and phosphate-buffered saline (PBS, Fresenius Kabi GmbH, Bad Homburg vor der Höhe, Germany) mix of 2:1:1 (v/v/v). Prior to loading the AMC-BAL, the isolated cells were centrifuged at 50x g for 5 min and washed twice with HepaRG medium.

A suspension of 2 mL cell pellet of HepaRG cells (~750 million cells) was loaded and cultured in laboratory-scale versions (9 mL) of the third generation AMC-BALs (RanD S.r.l. Medolla, Italy) (HepaRG-AMC-BALs) for maximally 21 days on HepaRG culture medium without DMSO and also supplemented with 1 mM N-carbamoyl-L-glutamate as described.

Immunohistochemical analysis
Day 14 BALs were formaline-fixed, paraplast-embedded, and cut in complete transverse 8 µm sections. Sections were stained for glutamine synthetase (GS), carbamoyl phosphate synthetase (CPS) and albumin (ALB) as described. In short, sections were incubated with primary antibodies, followed by incubation with alkaline-phosphate labeled secondary antibodies, and visualized using 5-bromo-4-chloro-3'-indolyphosphate p-toluidine and nitroblue. Per staining, all sections were treated similarly and negative controls did not include the primary antibody incubation.

Determination hepatic functions and protein content
Hepatocyte function tests were performed on 28-day-old monolayer cultures (monolayer, n = 6-20) and HepaRG-AMC-BALs after 7 days (d7 BAL, n = 4-11), 14 days (d14 BAL, n = 4-12), and 21 days of culture (d21 BAL, n = 4-6).

For monolayer function tests, cultures were washed twice with phosphate buffered saline. Subsequently, HepaRG culture medium was replaced by 1 mL of HepaRG test medium (HepaRG
Increased hepatic functionality of the human hepatoma cell line HepaRG cultured in the AMC bioreactor

medium with 1 mM N-carbamoyl-L-glutamate, 1.5 mM $^{15}$NH$_4$Cl, 2.27 mM D-galactose, 2 mM L-lactate, 125 µM testosterone, and 2 mM ornithine hydrochloride (all compounds from Sigma Aldrich)). Medium samples were taken at different time points of incubation, maximally after 24 hours. Subsequently, cultures were washed twice with PBS and stored at -20°C for protein determination.

For HepaRG-AMC-BAL function tests, bioreactors were first flushed with 30 mL test medium (see above), followed by a 24-hour period of recirculation with 100 mL of test medium. Samples were taken from the test medium prior to connection and after 0.5, 1, 2, 4 and 24 hours of testing.

Test medium samples of monolayer and d7, d14 and d21 BAL cultures were analyzed for concentrations of ammonia, urea, $^{15}$N-urea, 6β-hydroxytestosterone (6β-OHT), apolipoprotein A-1, and lactate as described. In addition, amino acids concentrations were determined in monolayer and d14 BAL test medium samples. Notably, concentrations of ammonia, 6β-OHT and lactate (other compounds not tested) did not change after 24 hours of perfusion through an acellular bioreactor under the same culture conditions (data not shown). Function-parameter rates were determined by calculating the changes in concentration in medium per hour as described. Of note, the ammonia elimination rates in the HepaRG-AMC-BAL were calculated over the first two hours of testing in HepaRG-AMC-BALS, during which the elimination rate was not reduced by substrate limitation, as occurring at later time points. Likewise, the increase in 6β-OHT concentration in time was not linear at later time points, probably related to subsequent phase-2 detoxification of 6β-OHT. Therefore, the rate of 6β-OHT production was calculated over the first 30 minutes of testing. All other function rates, including metabolism of the different amino acids, were constant over 24 hours of testing.

To study proliferation of HepaRG cells in the AMC-BAL and normalize function parameters for total protein, we determined the total protein content of monolayer cultures (n = 6-20), of cell suspensions at the time of BAL loading (n = 5) and of d14 BALs (n = 7). We lysed these cells using 0.2 M NaOH (Merck, Darmstadt, Germany), as described. Subsequently, protein analysis was performed using the Coomassie Brilliant Blue G-250 staining kit (Bio-Rad, Hercules, California, U.S.). We normalized hepatic function parameters of monolayer d28 cultures and d14 BAL cultures for protein content.

Determination of cell leakage
To determine cell leakage in monolayer and AMC-BAL cultures of HepaRG, we determined the aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) activity during function tests of monolayer and d14 BAL cultures over a 24-hour period, as described. To relate the measured activities to their total cell contents, the AST and LDH activity per g of protein in total lysates of monolayer cultures (n = 3) and of pieces of cell-filled-matrix extracted from d14 BALs (T-bags; n = 4) were determined as described.
Quantitative reverse transcription-polymerase chain reaction
For gene expression analyses, we isolated RNA samples from (d28) monolayer cultures (n = 3) and from HepaRG-AMC-BAL matrix samples (T-bags) after 7 (n = 3-4), 14 (n = 3-5), and 21 (n = 3) days of BAL culture, using the RNeasy mini kit (Qiagen, Venlo, The Netherlands). First strand cDNA was generated as described using gene-specific reverse transcription primers. The quantitative reverse transcription polymerase chain reaction was performed as described. Transcript levels were normalized for 18S ribosomal RNA and calculated as the mean levels of two human liver samples that were included in the analysis as described. These liver samples derived from two female patients, aged 40 and 41 years, with liver adenoma and no elevated liver damage, after obtaining written informed consent. The patients were not on medication and had no history of drug/alcohol abuse. 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 depicted in Table 1.

Statistical analysis
We compared differences between two experimental groups using unpaired Student’s t-tests. Differences between three or more experimental groups were compared using one-way analysis of variance (ANOVA) with Tukey post-hoc analysis. SPSS 16.0.1 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Prism version 4.01 (GraphPad Prism Inc, San Diego, CA, USA) was used for graphical presentation of the data. Data are expressed as means ± standard deviations. Significance was reached if $p < 0.05$. 

Table 1. Primer sequences used in the RT-PCR analyses with resulting amplicon sizes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>RT primer</th>
<th>Sense primer</th>
<th>Antisense primer</th>
<th>Amplicon size (bp)</th>
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<tr>
<td>18S rRNA</td>
<td>CGAACCTCCGACTTTCTTTTT</td>
<td>TTCGGAACTGAGGCCATGAT</td>
<td>CGAACCTCCGACTTTCTTTCTTTT</td>
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<tr>
<td>ALB</td>
<td>ACTTCCAGAGCTGAAAAGCAGG</td>
<td>GTCAGCTTGGAGAGGATCA</td>
<td>GTTCAGGACACGGGATAGAT</td>
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<td>ARG1</td>
<td>TGTGATTACCTCCGAGCAAGTC</td>
<td>TTTGCGCAAGGTAGGGAAGAACCTG</td>
<td>CGCCCCAGCAAGGAGAACAGAAGAAGA</td>
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<td>CPS</td>
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<td>GAAACGGGAGCCACAGTGAAGAA</td>
<td>CTCAACCAGGGGAGGAAAC</td>
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<tr>
<td>CYP1A2</td>
<td>TCAGTGCCACTTTCACGCC</td>
<td>GGAGGCTTCATCTGCTGAGA</td>
<td>TCTCCACACTGGCCGAGAGA</td>
<td>299</td>
</tr>
<tr>
<td>CYP2B6</td>
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<td>CCGCCCTCTGCGCCCTTCTTG</td>
<td>TCCCACCTCCGCTTCCATCC</td>
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<td>CYP3A4</td>
<td>CCCGTGAGAAGCAGAGGA</td>
<td>AGCTTAGAGGACTTCTTCACCC</td>
<td>AGCCATATCTACTCCCTCCTACACT</td>
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<tr>
<td>CYP3A5</td>
<td>GGATCCTGCGTTTGACCCG</td>
<td>TGAACCAAGTACTGAGCACG</td>
<td>TGGAGAATCTCGCTCGGAGTGC</td>
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<tr>
<td>CYP3A7</td>
<td>AGCCAAATCTACTTCCCGCAGC</td>
<td>ATTACGCTTTGAGGCTACTTCCTCTTCT</td>
<td>CGTCTTTGATTCCAGGGTCTTTATTT</td>
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<td>GS</td>
<td>TTGGCAGAGGGGGAGGAGAT</td>
<td>GCCGTTGTGTATGGAGGATGCT</td>
<td>GGCCTACGATGGGCTACAC</td>
<td>420</td>
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<tr>
<td>HNF4A</td>
<td>CACTCCACCGGCCCCTC</td>
<td>CGGGGTGTTCTAGCGACTCCCT</td>
<td>CAGGTGTTCTACCTTGGCC</td>
<td>321</td>
</tr>
<tr>
<td>PXR</td>
<td>TCTGGCTCTGAGTGGGACAAGTC</td>
<td>GAGAGCGCATGAAGGAGAGA</td>
<td>CATGTGGGGCAGCAGGAGAAGAG</td>
<td>420</td>
</tr>
</tbody>
</table>

Primers are indicated from 5’ → 3’.
RESULTS

Heterogeneous distribution of hepatic proteins of HepaRG-AMC-BALs
We stained transverse sections of d14 BALs for the hepatic proteins GS, CPS, and ALB. The HepaRG cells resided in the matrix of the BALs, were all vital, and the three cytosolic proteins were heterogeneously distributed among the cells (Fig. 1A-F), as found previously for -DMSO monolayer cultures. Notably, the distribution of ALB concentrated at the rims of cell-clusters, which possibly reflects an accumulation of this protein at the secretion site.

Figure 1. Immunostainings of cross-sections of d14 HepaRG-AMC-BALs. Proteins stained were GS (A) with its negative control (B), CPS (C) with its negative control (D), and ALB (E) with its negative control (F). Fibers of the AMC-BAL’s matrix are indicated with arrows. Bars: 10 µm. For color figure, see page 219.

Hepatic functionality of HepaRG-AMC-BALs increases with time
We compared various hepatic functions of HepaRG-AMC-BALs during three weeks of culture (Fig. 2). From d7 to d21 the urea production (Fig. 2B), 15N-urea production (Fig. 2C), 6β-OHT production (Fig. 2D), and apolipoprotein A-1 production (Fig. 2E) increased (in most cases gradually) 3.2-, 2.2-, 5.1-, and 2.2-fold, respectively, whereas ammonia (Fig. 2A) and lactate production (Fig. 2F) remained stable.
The increased functionality was (at least for the first 14 days) not due to an increase in proliferation in the AMC-BAL, as the total protein content of the HepaRG-AMC BAL at the time of loading (149 ± 18 mg) was not significantly different from the total protein content after 14 days of BAL culture (163 ± 16 mg) ($p = 0.20$). This is in line with the finding that the total DNA content neither changes during the first 14 days of BAL culture.\(^9\)

HepaRG-AMC-BALs reach higher hepatic functionality than HepaRG monolayer cultures

We compared protein normalized functional parameters of HepaRG-AMC-BAL after 14 days of culture with 28-day-old monolayer HepaRG cultures (Fig. 3A-F). All functions assessed were substantially higher in HepaRG-AMC-BALs than in HepaRG monolayer cultures, except for the...
apolipoprotein A-1 production (Fig. 3E), which was equal. The relative increases were 3.2-fold for ammonia elimination, 1.5-fold for urea production, 1.4-fold for conversion of $^{15}$N-ammonia into $^{15}$N-urea, and 7.9-fold for CYP3A4 activity. Moreover, lactate production (Fig. 3F) in monolayer cultures had switched into lactate consumption in HepaRG-AMC-BALs, a hallmark of BAL cultures of primary hepatocytes.$^{17}$

**Figure 3.** Hepatic functions of 28-day monolayer cultures of HepaRG cells and d14 HepaRG-AMC-BALs. Values are expressed as means ± standard deviations. Significance: *(p < 0.05 versus monolayer cultures.

**Cell leakage in HepaRG-AMC-BALs is lower than in monolayer cultures of HepaRG**

As one of factors potentially related to the BAL-culture induced increase in hepatic functionality of HepaRG cells, we compared cell damage in monolayer cultures and d14 HepaRG-AMC-BALs. As markers for cell damage, we measured the amounts of AST and LDH leakage per hour, expressed as a fraction of the total amount of AST and LDH content in the cell cultures. Monolayer cultures and HepaRG-AMC-BALs contained $318 \pm 28$ and $896 \pm 442$ U AST/g
Increased hepatic functionality of the human hepatoma cell line HepaRG cultured in the AMC bioreactor

protein, respectively, and 789 ± 442 and 755 ± 181 U LDH/g protein, respectively. The AST and LDH leakage of the monolayer and d14 BAL cultures corrected for total cellular contents was between 0.1 and 1% per hour, with d14 BAL cultures displaying a 4-fold lower leakage compared to the monolayer cultures (Fig. 4).

![Figure 4](image)

**Figure 4.** AST leakage (A) and LDH leakage (B) of monolayer cultures of HepaRG cells and d14 HepaRG-AMC-BALs. Cell leakage is expressed as the percentage of the total AST and LDH content of the entire culture per hour ± standard deviations. Significance: *p < 0.05 versus 28-day monolayer cultures.

Transcript levels of both structural and regulatory hepatic genes increase upon BAL culture of HepaRG cells.

In addition, we investigated whether the observed differences between monolayer and BAL culture, as well as the increase in functionality during BAL culture, are regulated on a transcriptional level.

Transcript levels of genes encoding rate-limiting urea cycle enzymes CPS (Fig. 5A) and arginase 1 (ARG1) (Fig. 5B) were not significantly different between monolayer and BAL cultures, and remained stable during BAL culture, although we observed a trend towards an increase for ARG1 in d21 HepaRG-AMC-BALs (*p = 0.14 vs monolayer). The transcript level of the gene encoding GS, the enzyme catalyzing the conversion of glutamate into glutamine thereby eliminating ammonia, was higher during the whole period of BAL culture compared to monolayer, being the highest in d7 HepaRG-AMC-BALs (Fig. 5C). In general, the transcript levels of the measured CYP genes showed a trend towards an increase during BAL culture, with maximal levels being higher than those in monolayer (Fig. 5 D-G). Most importantly, the transcript level of *CYP3A4*, the gene encoding the most dominant CYP in human liver, increased strongly (10.4-fold) upon BAL culture to reach a level at day 21 comparable to human liver (Fig. 5F).
Figure 5. Transcript levels of various structural hepatic genes of 28-day-old monolayer cultures of HepaRG cells and 7-, 21-, and d14 HepaRG-AMC-BALs. Values are expressed as means ± standard deviations. Abbreviations: ARG1, arginase 1; CPS, carbamoylphosphate synthetase; CYP, cytochrome P450; GS, glutamine synthetase; UD, undetectable. Significance: * above accolade indicates \( p < 0.05 \) between all groups, \( ^a p < 0.05 \) versus monolayer cultures, \( ^b p < 0.05 \) versus d7 HepaRG-AMC-BALs.

We determined the transcript levels of two hepatic transcription factors: hepatic nuclear factor 4α (HNF4α; nuclear receptor subfamily 2, group A, member 1), an important regulator of morphological and functional liver development (including drug metabolism) and a repressor
of dedifferentiation, and pregnane X receptor (PXR; nuclear receptor subfamily 1, group I, member 2), a master regulator of drug metabolism.\textsuperscript{18,19} Both \textit{HNF4\textalpha} and \textit{PXR} transcript levels increased upon BAL culture (Fig. 6A, B) from 50\% and 41\% the level in human liver to 245\% and 142\% the level of human liver, respectively, and they may therefore contribute to the observed increase in hepatic functionality, including drug metabolism.\textsuperscript{20}

\textbf{Figure 6.} Transcript levels of regulatory hepatic genes of 28-day-old monolayer cultures of HepaRG cells and 7-, 21-, and d14 HepaRG-AMC-BALs. Values are expressed as means ± standard deviations. Abbreviations: \textit{HNF4\textalpha}, hepatic nuclear factor 4A; \textit{PXR}, pregnane X receptor. Significance: * above accolade indicates $p < 0.05$ between all groups, \textit{a} $p < 0.05$ versus monolayer cultures.

\textbf{Amino-acid metabolism}

Total amino-acid consumption was similar in monolayer and d14 HepaRG-AMC-BALs (Table 2). However, the net metabolism of individual amino-acids varied between both cultures. Net glutamate consumption in BAL cultures was more than 2-fold lower than in monolayer cultures, while glutamine production remained constant. In contrast, alanine was \textit{produced} at a net rate of 20.5 ± 4.75 \textmu mol/h in monolayer cultures, whereas it was \textit{consumed} at a net rate of 5.85 ± 2.51 \textmu mol/h in HepaRG-AMC-BALs. This might reflect a BAL-culture induced shift in the transamination reaction catalyzed by alanine transaminase, resulting in the increased production of pyruvate and glutamate (see Discussion section).

Ornithine was produced in BAL cultures, whereas it was consumed in monolayer cultures, and we also observed a trend towards an increase in arginine consumption in BAL cultures compared to monolayer cultures ($p = 0.05$). These findings are in accordance with the observed increase in urea production by arginase 1 upon BAL culture. Lastly, leucine was consumed at a lower rate in BAL cultures compared to monolayer cultures, while the metabolism of the other branched chain amino acids did not differ upon BAL culture.
Table 2. Amino acid metabolism of HepaRG monolayer cultures and HepaRG-AMC-BALs.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>HepaRG Monolayer (µmol h⁻¹ g of protein⁻¹)</th>
<th>HepaRG-AMC-BAL 14d (µmol h⁻¹ g of protein⁻¹)</th>
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<tr>
<td>ALA</td>
<td>20.5 ± 4.75</td>
<td>-5.85 ± 2.51 *</td>
</tr>
<tr>
<td>ARG</td>
<td>-1.64 ± 0.90</td>
<td>-3.66 ± 1.26</td>
</tr>
<tr>
<td>ASN</td>
<td>-1.31 ± 0.86</td>
<td>-0.77 ± 0.44</td>
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<tr>
<td>ASP</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CIT</td>
<td>-0.15 ± 0.33</td>
<td>-0.24 ± 0.60</td>
</tr>
<tr>
<td>GLN</td>
<td>7.15 ± 3.19</td>
<td>9.72 ± 2.90</td>
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<tr>
<td>GLU</td>
<td>-16.14 ± 1.19</td>
<td>-7.13 ± 2.17 *</td>
</tr>
<tr>
<td>GLY</td>
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<td>N/A</td>
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<tr>
<td>HIS</td>
<td>-1.71 ± 0.91</td>
<td>-1.78 ± 0.73</td>
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<td>ILE</td>
<td>-7.88 ± 1.28</td>
<td>-6.84 ± 2.02</td>
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<td>LEU</td>
<td>-17.55 ± 1.30</td>
<td>-10.66 ± 2.59 *</td>
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<tr>
<td>LYS</td>
<td>-10.34 ± 6.13</td>
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<tr>
<td>MET</td>
<td>-2.06 ± 1.20</td>
<td>-1.68 ± 0.57</td>
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<tr>
<td>ORN</td>
<td>-8.57 ± 7.98</td>
<td>1.69 ± 1.30 *</td>
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<tr>
<td>PHE</td>
<td>-2.82 ± 0.61</td>
<td>-1.25 ± 0.46 *</td>
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<tr>
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<td>-4.30 ± 3.44</td>
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<tr>
<td>VAL</td>
<td>-4.35 ± 1.16</td>
<td>-5.06 ± 1.62</td>
</tr>
<tr>
<td>Total</td>
<td>-62.36 ± 57.04</td>
<td>-54.05 ± 22.35</td>
</tr>
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</table>

Amino acid production and consumption are indicated as positive and negative values, respectively. Abbreviations: AMC-BAL, Academic Medical Center-bioartificial liver; N/A, not available. Values are given as averages ± standard deviations (n = 3 to 5). *p < 0.05 versus HepaRG monolayer cultures.

DISCUSSION

In this study, we demonstrate that culturing of the human hepatoma cell line HepaRG in the AMC-BAL increases its hepatic functionality, both over time, and compared to monolayer. The increase in functionality was most prominent after 14 and 21 days of BAL culture and was associated with a reduction in cell damage, upregulation of both regulatory and structural hepatic genes, and changes in amino-acid metabolism. In addition, the HepaRG cells maintain their functional heterogeneity upon BAL culture, as demonstrated by histological analysis.

The cause of this BAL-culture induced increase in hepatic functionality is most likely multi-factorial. Firstly, the AMC-BAL provides a three-dimensional culture environment whereas monolayer is two-dimensional. Three-dimensional cultures of hepatocytes more resemble the in vivo cellular organization in terms of cell-cell contacts, polarity, morphology, and the composition of the extracellular matrix. In addition, the AMC-BAL has an internal
Increased hepatic functionality of the human hepatoma cell line HepaRG cultured in the AMC bioreactor

oxygenator, providing superior oxygenation of the cells compared to monolayer, known to increase functionality.\textsuperscript{23-25} Lastly, the AMC-BAL is perfused with culture medium in contrast to the static culture environment of the monolayer cultures, also known to increase functionality.\textsuperscript{26}

Gene expression analysis revealed that the difference in hepatic functionality in d14 HepaRG-AMC-BALs \textit{vs} HepaRG monolayer cultures may be partially explained by regulation on a transcriptional level. For instance, the 7.9-fold increase in CYP3A4 activity (6β-OHT production) coincided with a comparable 10.4-fold increase in \textit{CYP3A4} transcript levels, suggesting a transcriptionally regulated increase of CYP3A4. This may well be caused by the observed upregulation of both \textit{PXR} and \textit{HNF4A} expression, both important transcriptional regulators of \textit{CYP3A4}.\textsuperscript{20} The increase in ammonia elimination may likewise be regulated on a transcriptional level, as GS transcript levels were significantly higher in the d14 BAL cultures compared to the monolayer cultures. Notably, ammonia elimination in HepaRG cells is mainly realized by fixation into amino acids (initially glutamate and glutamine), and not by conversion into urea. Both \textit{15N}-urea production and total urea production increased significantly upon BAL culture of HepaRG cells, notwithstanding the lack of a significant increase in the transcription level of CPS and arginase 1, suggesting regulation at a posttranscriptional level, or regulation by other factors or enzymes involved in the urea cycle.

The amino acid metabolism in HepaRG-AMC-BAL cultures more resembled the metabolism of primary hepatocytes than that of HepaRG monolayer cultures.\textsuperscript{2} In contrast to monolayer HepaRG cultures, both primary porcine hepatocyte and HepaRG BAL cultures produced alanine and ornithine. However, glutamate metabolism of HepaRG-AMC-BALs (consumption) differs with primary-porcine-hepatocyte-AMC-BALs (production). This difference might be related to the high GS activity in HepaRG-AMC-BALs compared to primary porcine hepatocyte AMC-BALs.\textsuperscript{2} Furthermore, we observed a substantial decrease in glutamate consumption upon BAL culture of HepaRG cells and a transition from alanine production into consumption. This suggests that the transamination reaction catalyzed by alanine aminotransferase in which glutamate and pyruvate are converted into alanine and α-ketoglutarate and \textit{visa versa} shifts towards the production of glutamate and pyruvate. This may, in part, explain the discrepancy between a high increase in elimination of ammonia and the limited change in glutamate/glutamine metabolism in HepaRG cells (< 10% of the change in ammonia elimination) of 14d BALs versus monolayers. The higher \textit{15N}-urea production in 14d BALs relative to monolayers coincided with a switch from ornithine consumption into production, and a strong trend towards an increase in arginine consumption (\(p = 0.05\)), the respective product and substrate of the ARG1 reaction in the urea cycle.

In addition, the amino acid, ammonia, and urea data provide interesting insights in the nitrogen metabolism of the HepaRG-AMC-BAL. The rate of ammonia elimination is 168 µmol per hour per gram of protein. The net total amino acid consumption is 54 µmol per hour per gram of protein. This equates to a total influx of at least 222 µmol of nitrogen per hour per
gram of protein – at least, as several amino acids also contain nitrogen atoms in the side chain next to the amine group. However, the rate of total urea production is only 13 µmol per hour per gram of protein, covering the elimination of only 26 µmol of ammonia per hour. The majority of the nitrogen that is taken up by the HepaRG cells must therefore be used for protein synthesis, and as the total protein content in the HepaRG-AMC-BAL does not change during two weeks of culture, this suggests a substantial protein secretion. This would also explain the lack of stoichiometry in the changes in ammonia elimination and urea production by quantitative amino acid analysis in our set-up.9

In monolayer cultures, we observed lactate production, whereas BAL cultures of HepaRG cells consumed lactate. Lactate consumption is a liver-specific function and part of the Cory cycle, in which lactate that is produced by anaerobic glycolysis in the muscles and subsequently transported to the liver where it is converted into pyruvate and later glucose. Lactate consumption is also a hallmark of freshly isolated hepatocytes.17 In contrast, liver cell cultures produce lactate under anaerobic conditions or when the cells have dedifferentiated. The latter effect, known as the Warburg effect, is also a feature of tumor cells as a means of rapid cytosolic energy production.27, 28 Based on the increased hepatic functionality upon culturing the HepaRG cells in the BAL, the shift from lactate production into consumption can probably be attributed to the higher differentiation state of the HepaRG cells rather than to the increased oxygenation in the BAL versus monolayer, however the causal relation between oxygenation, lactate metabolism and hepatic differentiation needs to be further established.

In conclusion, the results of this study contribute to the clinical implementation of a BAL based on the HepaRG cells, as it shows that BAL culture improves its hepatic functionality. Notably, the functionality of a HepaRG-AMC-BAL is higher and more all round than that of a DMSO-treated HepaRG monolayer culture, without the DMSO-associated negative effects as cell damage and repression of particular hepatic functions.8, 9 The d21 BALs functionally outweigh d14 BALs, raising the question whether further extension of the BAL culture period, i.e. 4 weeks and longer, would further increase the hepatic functionality of the HepaRG-AMC-BAL. This also offers high perspectives for BAL therapy, as the d14 HepaRG-AMC-BAL was already effective in the treatment of rats with ALF.9 Finally, this study further encourages clinical studies with the HepaRG-AMC-BAL to evaluate its safety and efficacy in patients with ALF.

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