Preclinical development of the AMC-HepaRG-Bioartificial Liver

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CHAPTER 4

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

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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%O$_2$ supply, 2) monolayer cultures with dynamic medium flow and 3) 3D cultures with dynamic medium flow, in a system called BAL-In-A-Dish (BALIAD).

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

**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%O$_2$ under regular conditions, i.e. static in an humidized atmosphere of 95% air and 5% CO$_2$ (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%O$_2$, 5%CO$_2$ and 55%N$_2$ (HepaRG-40%O$_2$). 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%O$_2$,
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 GSTα, AFP and CYP3A7, for more details refer to [11].

MHHs were isolated from tumor-free liver tissue of three patients undergoing partial hepatectomy, because of metastatic carcinoma. The procedure was approved by the Medical Ethical Committee of the Academic Medical Center subjected to informed patient consent. The hepatocyte isolation method was adapted from the protocol described by Seglen [13], as previously described [11]. The MHHs were cultured in William’s E culture medium containing 4% heat-inactivated FBS, 2mM L-glutamine, 1uM dexamethasone, 20 mU/mL insulin (Novo Nordisk), 2mM ornithine (Sigma-Aldrich), 100U/mL penicillin, 100ug/mL streptomycin. Cells
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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_SPECIFICGENES (C2), for more details see Hsiao et al 2001 [14]. Gene set enrichment analysis was performed using CAMERA (limma package) with preset value of 0.01 for the inter-gene correlation using the same linear model as above. In case multiple probes mapped to the same Entrez Gene ID according to the updated probe annotation, the probe with highest standard deviation of its expression values was chosen. \( P \) values were calculated for each gene set for two alternative hypotheses (up or down). Hierarchical clustering of the whole-genome expression profiles and the profiles of the set of liver-specific genes was performed using Pearson correlation as distance measure and complete linkage as agglomeration method.
Quantitative reverse transcription PCR (RT-qPCR)

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

Mitochondrial vs nuclear DNA ratio

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

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

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

Mitochondrial abundance was further determined by Western blot analysis of different mitochondrial proteins. Cell lysates of the different HepaRG cultures were obtained after incubation at 4°C in RIPA buffer (25 mM Tris (Bio-Rad), 150 mM NaCl (Merck), 0.1% SDS (Merck), 0.5% sodium deoxycholate (Sigma), 1% Triton X-100 (Bio-Rad), pH 7.4) containing 1% of protease inhibitor cocktail (Roche) for 30 min. The protein concentration of the

<table>
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<th>Gene</th>
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<th>Anti-sense sequence</th>
<th>Size bp</th>
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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.
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

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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 3. Top 20 down-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>
<tbody>
<tr>
<td>ILMN_2148527</td>
<td>H19</td>
<td>Imprinted maternally expressed transcript (non-protein coding)</td>
<td>-2.7</td>
<td>0.022</td>
</tr>
<tr>
<td>ILMN_2313672</td>
<td>IL1RL1</td>
<td>Interleukin 1 receptor like 1</td>
<td>-2.4</td>
<td>0.006</td>
</tr>
<tr>
<td>ILMN_2132982</td>
<td>IGFBP5</td>
<td>Insulin like growth factor binding protein 5</td>
<td>-2.1</td>
<td>0.027</td>
</tr>
<tr>
<td>ILMN_1682176</td>
<td>CLEC3B</td>
<td>C-type lectin domain family 3 member B</td>
<td>-2.1</td>
<td>0.005</td>
</tr>
<tr>
<td>ILMN-1752750</td>
<td>MCF2</td>
<td>MCF 2 cell line derived transforming sequence</td>
<td>-2.0</td>
<td>0.043</td>
</tr>
<tr>
<td>ILMN_1694548</td>
<td>ANXA3</td>
<td>Annexin A3</td>
<td>-1.9</td>
<td>0.019</td>
</tr>
<tr>
<td>ILMN_2346339</td>
<td>FLOR1</td>
<td>Leucine-rich repeat (LRR) family protein</td>
<td>-1.9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>ILMN_1671123</td>
<td>LOC285419</td>
<td>Long intergenic non-protein coding RNA</td>
<td>-1.8</td>
<td>0.005</td>
</tr>
<tr>
<td>ILMN_2078975</td>
<td>GRM3</td>
<td>Glutamate metabotropic receptor 3</td>
<td>-1.8</td>
<td>0.001</td>
</tr>
<tr>
<td>ILMN_1693270</td>
<td>SUSD2</td>
<td>Sushi domain containing 2</td>
<td>-1.8</td>
<td>0.003</td>
</tr>
<tr>
<td>ILMN_2233314</td>
<td>SPANXA1</td>
<td>Sperm protein associated with the nucleus, X-linked, family member A1</td>
<td>-1.7</td>
<td>0.006</td>
</tr>
<tr>
<td>ILMN_3200140</td>
<td>LOC645638</td>
<td>WAP four-disulfide core domain 21, pseudogene</td>
<td>-1.7</td>
<td>0.028</td>
</tr>
<tr>
<td>ILMN_1799243</td>
<td>ANXA13</td>
<td>Annexin A13</td>
<td>-1.7</td>
<td>0.006</td>
</tr>
<tr>
<td>ILMN_1800091</td>
<td>PARRES1</td>
<td>Uncharacterized</td>
<td>-1.7</td>
<td>0.035</td>
</tr>
<tr>
<td>ILMN_1713125</td>
<td>SPANXE</td>
<td>Sperm protein associated with the nucleus, X-linked, family member E</td>
<td>-1.7</td>
<td>0.005</td>
</tr>
<tr>
<td>ILMN_3272441</td>
<td>PTPRQ</td>
<td>Protein tyrosine phosphatase, receptor type Q</td>
<td>-1.7</td>
<td>0.003</td>
</tr>
<tr>
<td>ILMN_2334359</td>
<td>GFRA1</td>
<td>Glial cell line-derived neurotrophic factor receptor (GDNFR) A1</td>
<td>-1.6</td>
<td>0.002</td>
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<tr>
<td>ILMN_1767281</td>
<td>PPBP</td>
<td>Pro-platelet basic protein</td>
<td>-1.6</td>
<td>0.013</td>
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<tr>
<td>ILMN_1878728</td>
<td>DB222718</td>
<td>Uncharacterized</td>
<td>-1.6</td>
<td>0.028</td>
</tr>
<tr>
<td>ILMN_1732781</td>
<td>SPANXD</td>
<td>Sperm protein associated with the nucleus, X-linked, family member D</td>
<td>-1.5</td>
<td>0.003</td>
</tr>
</tbody>
</table>
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%O₂)

Mitochondria provide the cell with ATP through oxidative phosphorylation (OxPhos) activity [27], and therefore the abundance of mitochondria is finely tuned to meet cell-and state-specific energy needs [28]. When compared to other subcellular organelles, the mitochondrion is unique in possessing a separate autonomously replicating genome and its own translation machinery [29].

We investigated the effect of BAL culturing on mitochondrial biogenesis. As outlined in the introduction, the three major differences between the AMC-BAL culture and the regular monolayer comprise the oxygenation (40%O₂ in the AMC-BAL vs 20%O₂ in monolayer), presence (AMC-BAL) or absence (monolayer) of medium perfusion and culture configuration (3D in the AMC-BAL vs 2D in monolayer). To study the effect of the different factors, we applied different culture conditions to the differentiation phase. We tested the effect of oxygenation in HepaRG monolayers cultured under 40%O₂ (HepaRG-40%O₂) and the effect of dynamic medium flow on monolayers cultured under 60 rpm supplied with 20%O₂ (HepaRG-dynamic medium flow (HepaRG-DMF). To study the joint effect of 3D configuration and DMF on mitochondrial biogenesis, we developed the BAL-in-a-dish culture platform (HepaRG-BALIAD). The BALIAD system consists of 6 mm diameter discs of BAL matrix that are placed in cell culture plates under continuous shaking with 20%O₂ supply, a graphic representation of different culture conditions is shown in (Fig. 4A). Parameters of mitochondrial biogenesis of these 3 different culture conditions and HepaRG-BAL cultures were compared to those of control static HepaRG monolayer cultures supplied with 20%O₂ (HepaRG-MONO).

The relative mitochondrial abundance was determined by measuring the ratio between mitochondrial DNA to nuclear DNA copies (mtDNA/NucDNA ratio). The relative mitochondrial abundance was significantly increased up to 2.4-fold in the HepaRG-BAL compared to the HepaRG-MONO group (Fig. 4B). Culturing of the HepaRG cells with 40%O₂, DMF or BALIAD, positively affected the abundance of the mitochondria as indicated by a 2.1-, 3.3- and 5.1-fold increase, respectively. Noteworthy, mitochondrial abundance was not significantly different between HepaRG-BAL, HepaRG-40%O₂, HepaRG-DMF and HepaRG-BALIAD. In line with this, the expression of the mitochondrial inner membrane (MIM) complex II was 2.4-, 1.9-2.3- and 2.1-fold increased in HepaRG-BAL, HepaRG-40%O₂, HepaRG-DMF and HepaRG-BALIAD, resp., while, MIM complex IV was 2.2- and 2.5-fold increased in HepaRG-BAL and HepaRG-DMF, compared to HepaRG-MONO cultures. The expression of MIM complex V was 2.3-, 1.9 and 2.3-fold induced in HepaRG-BAL, HepaRG-40%O₂ and HepaRG-DMF cultures. Noteworthy, no significant differences was observed in the level of MIM complexes when HepaRG-BAL was compared to HepaRG-40%O₂, HepaRG-DMF or HepaRG-BALIAD, (Fig. 4C-D).
Figure 4. Mitochondrial biogenesis in HepaRG cells cultured in monolayer and BAL; the role of higher oxygenation (40% O₂), DMF and 3D configuration. A) Graphic representation of different culturing conditions. B) Mitochondrial abundance measured by mtDNA/NucDNA ratio (n=4 for HepaRG-BAL and n=8-11/other groups). C) Mitochondrial abundance measured by the expression level of mitochondrial inner membrane complexes (MIM complexes) complex II, IV and V, as determined by immune-blotting (n=4-9/group).
Figure 4. Continued. D) quantification of the immunoblots using ImageJ software. E) Transcript level of mitochondria-encoded genes (n=6-8/group).
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

**Figure 5.** Mitochondrial biogenesis in human liver cell line C3A cultured in monolayer and BAL. A) Mitochondrial abundance measured by mtDNA/NucDNA ratio. B) Transcript level of mitochondria-encoded genes (n=3/group).
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 [63]. Yet, when compared to the whole-transcriptome profile of MHHs, the profile of the HepaRG cells clearly deviates, indicating that there is still room for further optimization of HepaRG cells. New culture platforms that are capable to enhance mitochondrial metabolism and subsequently the functionality through 3D culture and medium perfusion, such as the BALIAD system, could provide in this need. Besides optimizing the culture platform, interfering with the mitochondrial system by over-expression of master regulators of mitochondrial biogenesis, such as Peroxisome proliferator-activated receptor gamma coactivator 1-alpha or addition of chemical compounds that stimulate mitochondrial biogenesis, e.g. S-nitrosoacetylpenicillamine or metformin may boost the differentiation process [64-66].

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


Chapter 4


