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AMC-Bio-Artificial Liver culturing enhances mitochondrial biogenesis in human liver cell lines: The role of oxygen, medium perfusion and 3D configuration

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ABSTRACT

Background: Human liver cell lines, like HepaRG and C3A, acquire higher functionality when cultured in the AMC-Bio-Artificial Liver (AMC-BAL). The three main differences between BAL and monolayer culture are the oxygenation (40% *vs* 20%O_2), 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% O_2, dynamic medium flow and/or in 3D increased the mitochondrial abundance and expression of mitochondrial complexes *vs* standard monolayer culturing. The stimulatory effect of the BAL culture on mitochondrial biogenesis was confirmed in C3A cells in which mitochondrial abundance increased 2.2-fold with induction of mitochondria-encoded genes.

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

Abbreviations: ABCA12, ATP binding cassette subfamily A member 12; ACO2, aconitase 2; AMC, Amsterdam Medical Center; BAL, bio-artificial liver; BALIAD, bio-artificial liver-in-a-dish; CEBPa, CCAAT/enhancer-binding protein alpha; CPT1A, carnitine palmitoyltransferase 1A; CS, citrate synthase; CYP3A4, cytochrome P450 3A4; ELAD, extracorporeal liver assist device; FDR, false discovery rate; GO, gene ontology; HepaRG-MONO, HepaRG monolayer; HFLCs, human fetal liver cells; MDR1, multi-drugs resistant transporter 1; MHHs, mature human hepatocytes; MIM complexes, mitochondrial inner membrane complexes; MMPA, mitochondrial membrane activity; MT-CO3, mitochondrial cytochrome *c* oxidase subunit 3; mtDNA/NucDNA, mitochondrial deoxynucleic acids/nuclear deoxynucleic acids ratio; MT-ATP6, mitochondrial ATP synthase subunit 6; MT-CYP, mitochondrial cytochrome *c* oxidase; MT-ND1, mitochondrial-NADH dehydrogenase subunit1; MT-ND5, mitochondrial-NADH dehydrogenase subunit 5; NAD, nicotinamide adenine dinucleotide; NAT, *N*-acetyl transferase; NDUFB9, NADH-ubiquinone oxidoreductase subunit B9; OxPhos, oxidative phosphorylation; ROS, reactive oxygen species; SLC25A14, solute carrier family 25 member 14; TCA cycle, tricarboxylic acids cycle; UQRC1, ubiquinol-cytochrome *c* reductase core protein; 2D, 2 dimensional; 3D, 3 dimensional

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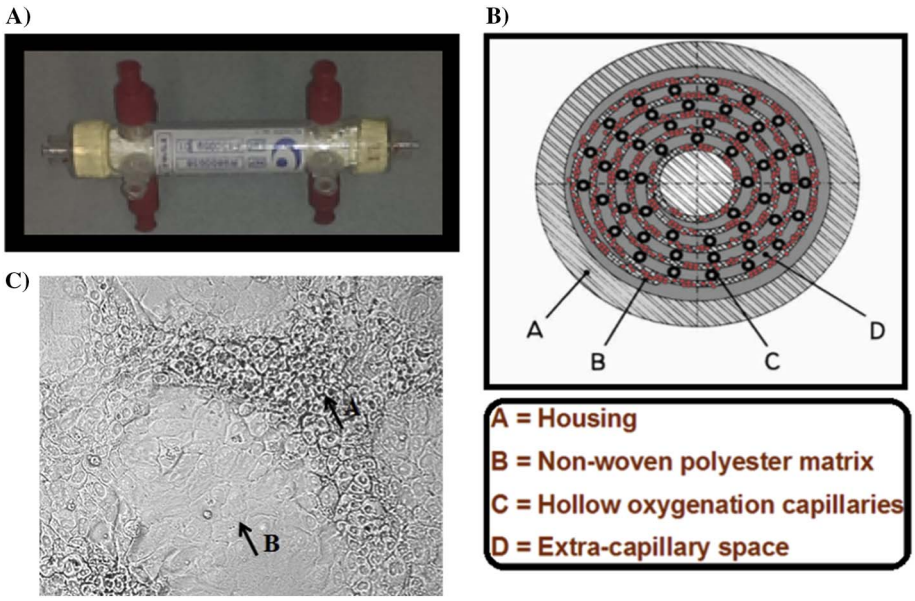

Fig. 1. The AMC-Bio-Artificial Liver and HepaRG cells. A) Laboratory model of the AMC-BAL. B) Schematic cross section of the AMC-BAL showing the spirally wound non-woven polyester matrix in which the HepaRG cells attach in a 3D configuration, positioned between oxygen capillaries for continuous gas (40%O_2, 5%CO_2 and 55%N_2) supply. The cells inside the BAL receive continuous medium perfusion at a rate of 5 mL/min. **C)** HepaRG monolayer morphology, arrow A indicates the hepatocytes islands, arrow B indicates the bile duct-like cells.

1. Introduction

There is an unmet need for highly differentiated human hepatocytes from proliferative sources to serve as predictive *in vitro* hepatocyte models and as biocomponents for Bio-Artificial Livers (BALs) (van Wenum et al., 2014). BALs are bioreactors housing functional hepatocytes, developed to support patients with end-stage liver failure (Flendrig et al., 1997). 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) (Gripon et al., 2002; Nibourg et al., 2012a) (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 (Gripon et al., 2002; Hoekstra et al., 2011). 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 (van Wenum et al., 2017). 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 *p*450 (CYP) 3A4 activity are 3.2-, 1.4- and 7.9-fold higher, respectively (Nibourg et al., 2012a; Nibourg et al., 2013; van Wenum et al., 2016). Moreover, HepaRG-BAL cultures eliminate lactate, whereas HepaRG monolayers produce lactate and consume more glucose (van Wenum et al., 2016). Notably, lactate elimination is a hallmark of highly differentiated hepatocytes, and is absent in currently available proliferative sources of hepatocytes (van Wenum et al., 2016). 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 (Knowles et al., 1980). 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 (van Wenum et al., 2016).

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%O_2 in the AMC-BAL *vs* 20%O_2 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.

2. Materials and methods

2.1. 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 (Gripon et al., 2002; Hoekstra et al., 2011). 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