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Functional properties of hepatocytes *in vitro* are correlated with cell polarity maintenance

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ABSTRACT

Exploring the cell biology of hepatocytes *in vitro* could be a powerful strategy to dissect the molecular mechanisms underlying the structure and function of the liver *in vivo*. However, this approach relies on appropriate *in vitro* cell culture systems that can recapitulate the cell biological and metabolic features of the hepatocytes in the liver whilst being accessible to experimental manipulations. Here, we adapted protocols for high-resolution fluorescence microscopy and quantitative image analysis to compare two primary hepatocyte culture systems, monolayer and collagen sandwich, with respect to the distribution of two distinct populations of early endosomes (APPL1 and EEA1-positive), endocytic capacity, metabolic and signaling activities. In addition to the re-acquisition of hepatocellular polarity, primary hepatocytes grown in collagen sandwich but not in monolayer culture recapitulated the apico-basal distribution of EEA1 endosomes observed in liver tissue. We found that such distribution correlated with the organization of the actin cytoskeleton *in vitro* and, surprisingly, was dependent on the nutritional state *in vivo*. Hepatocytes in collagen sandwich also exhibited faster kinetics of low-density lipoprotein (LDL) and epidermal growth factor (EGF) internalization, showed improved insulin sensitivity and preserved their ability for glucose production, compared to hepatocytes in monolayer cultures. Although no *in vitro* culture system can reproduce the exquisite structural features of liver tissue, our data nevertheless highlight the ability of the collagen sandwich system to recapitulate key structural and functional properties of the hepatocytes in the liver and, therefore, support the usage of this system to study aspects of hepatocellular biology *in vitro*.

1. Introduction

The liver performs a wide number of metabolic functions such as regulation of glucose and lipid metabolism, glycogen storage, plasma protein synthesis, detoxification and bile acid production, which are essential for maintaining whole body physiology. Dysfunction of these processes is associated with common human diseases, *e.g.* drug-induced liver diseases, cholestasis, type-2 diabetes and non-alcoholic fatty liver disease (NAFLD) [1–4]. Type-2 diabetes and liver disease are amongst the 10 most common causes of death worldwide. Therefore, alterations of liver physiology have major consequences for general health.

The central cells responsible for liver metabolic function are the hepatocytes. Hepatocytes are polarized cells, whose apical membranes collectively form a continuous network of bile canaliculi (BC) throughout the liver. Their basal membranes are in contact with the sinusoidal endothelial network where the blood flows. From the blood, hepatocytes take up nutrients, signaling molecules (*e.g.* hormones) and metabolites *via* a process called endocytosis. Endocytosis is an essential cellular function, not only for the uptake of nutrients but also for mediating signal transduction and metabolic processes [5–8]. Defects in endocytosis have severe pathological consequences at the organism level. For example, mutations causing dysfunctions in low-density lipoprotein (LDL) uptake in familiar hypercholesterolemia

Abbreviation: AKT/PKB, protein kinase B; APPL1, adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1; EEA1, early endosomal antigen 1; EGF, epidermal growth factor;; G6Pase, glucose-6-phosphatase;; LDL, low-density lipoprotein;; LDLR, LDL-Receptor; MRP2, multidrug resistance-associated protein 2; NAFLD, non-alcoholic fatty liver disease;; Pepck, Phosphoenolpyruvate-Carboxykinase; ZO-1, zona occuldens protein 1

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result in cardiovascular defects leading to heart attack [9]. In addition, endocytosis is mandatory for maintaining apical and basal membrane integrity. Junctional components and polarized cargo have to be sorted to the correct plasma membrane domain *via* specialized endocytic and recycling membrane trafficking pathways [10]. Defects in trafficking cause diseases in organs in which epithelial cell polarity is crucial, such as kidney, intestine and liver [2,11]. Loss of cell polarity in the liver results in a redistribution of apical bile acid transporters and the appearance of intracellular pseudocanaliculi [2,12,13]. This has severe pathological consequences leading to the development of various diseases, *e.g.*, bile secretory failure (cholestasis). The impaired integrity of tight junctions causes an increased permeability between blood and bile [14], resulting in liver damage [15].

Studying the molecular mechanisms responsible for hepatocyte polarity is therefore essential for the understanding of liver tissue structure and function. The understanding of such mechanisms has been pioneered by A. Hubbard and G. Palade, who described apical and basolateral trafficking pathways in the liver *in vivo* using subcellular fractionation techniques [16–18]. Progress was later enormously facilitated by the use of *in vitro* culture systems amenable to cell biological manipulation. For example, the vesicular trafficking pathways of hepatocytes have been explored due to the original work by Hubbard and colleagues on hepatic cell lines [19]. However, with very few exceptions [20], the conventional cell lines used to address this problem are either derived from various tumors and, thus not polarized, or do not recapitulate all *in vivo* functions of hepatocytes [21]. On the other hand, primary culture models that reconstitute cell polarity *in vitro* similar to hepatocytes in liver tissue *in vivo* are hard to develop and manipulate. When hepatocytes are grown conventionally as monolayers they fail to re-establish cell polarity and can preferable be used in the first 24 h after isolation [22–25]. They exhibit a rapid loss of liver-specific functions [26] and even revert their metabolic pattern to that of non-polarized cells within 2 days [27,28]. Despite these limitations, the culture of hepatocytes as 2D monolayers is employed by many laboratories as the system of choice to investigate hepatocyte functions *in vitro*[29,30]. However, the loss of cell polarity questions the use of the 2D system for studying mechanisms that are relevant to the structure and function of hepatocytes as in the liver. An *in vitro* system should ideally recapitulate hepatocellular polarity, including the expression, endocytosis and intracellular transport of junctional components and apical transporters to the bile canalicular membrane while preserving metabolic and signaling functions.

A well-established system that reproduces hepatocellular polarity is the “collagen sandwich”, where primary hepatocytes are cultured between two layers of collagen. Here, the cells are able to re-establish cell polarity and maintain hepatic functions [31–35]. These properties depend on LKB1 mediated activation of AMP-activated protein kinase (AMPK), which is necessary for BC network formation and maintenance [36]. Interestingly, these cellular features are associated with the modulation of metabolism specifically occurring in the collagen sandwich system. Activation of AMPK induces mitochondrial activity to switch from glycolysis to oxidative phosphorylation [36]. Concomitantly, the expression of metabolic genes is much increased in this system [37]. Nevertheless, how such as system compares with the 2D culture system and to what extent it recapitulates additional features of hepatocytes *in vivo* is unclear. What are the consequences of loss of cell polarity for hepatocyte function *in vitro*? Which culture method preserves the most native functional properties besides hepatocellular polarity of the hepatocytes in the liver and by which criteria? Here, we performed a cell biological characterization of the most commonly used culture methods of monolayer and collagen sandwich cultures. We focused on cell polarity, endosomal distribution and endocytosis, metabolic activity and signaling responses as diagnostic features of hepatocytes under physiological conditions.

2. Material and methods

2.1. Animals

All animal studies were conducted in accordance with German animal welfare legislation and in strict pathogen-free conditions in the animal facility of the Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany. Protocols were approved by the Institutional Animal Welfare Officer (Tierschutzbeauftragter), and necessary licenses were obtained from the regional Ethical Commission for Animal Experimentation of Dresden, Germany (Tierversuchskommission, Landesdirektion Dresden).

2.2. Antibodies and reagents

Rabbit anti-EEA1 and rabbit anti-APPL1 were developed in the Zerial lab. Other rabbit polyclonal antibodies were as follows: anti-ZO1 from Invitrogen (Darmstadt, Germany), anti-AKT and phospho-AKT (Ser473) from Cell Signaling Technology (New England Biolabs GmbH Frankfurt/Main, Germany). Rat anti-CD13 was obtained from Novus Biologicals Europe (Cambridge, UK). Rabbit anti-MRP2 was a generous gift from Bruno Stieger, University hospital Zuerich, Switzerland [38]. Secondary antibodies labeled with Alexa fluorophores and Alexa-488-phalloidin were purchased from Molecular Probes, Europe. Fluorescently labeled EGF was purchased from Invitrogen. LDL was purified from human serum and labeled as previously described [39]. All other chemical were from Sigma unless otherwise stated.

2.3. RT-PCR

Quantitative RT-PCR was carried out as described before [12]. The primers and reference genes used are listed in Table S1.

2.4. Western blots and quantification

For western blot analysis cell lysates were prepared from monolayer and sandwich cultures, lysates were sonicated for 2 min, rotated for 1 h followed by a high-speed spin. From the supernatant 10/25 μg of total protein were run on SDS/PAGE gels, transferred to nitrocellulose membranes and incubated with various antibodies. Protein bands were detected using the ECL solution reaction and quantified with ImageJ as described in the method outlined at http://lukemiller.org/index.php/2010/11/-analyzing-gels-and-western-blots-with-image-j/.

2.5. Histology

Methanol fixed and PFA-perfused liver sections were obtained as described before [12].

2.6. Hepatocyte isolation and culturing

Primary hepatocytes were isolated from C57BL/6NHsd male mice *via* collagenase perfusion as described previously [40]. Cells were plated onto collagen (0.9 mg/ml) coated 24-well plates at 200,000 cells/well in Williams E medium (PAN Biotech, Aidenbach, Germany), substituted with 10% FBS, 100 nM dexamethasone and penicillin/streptomycin and maintained at 37 °C in an atmosphere with 5% CO_2. After three to four hours of attachment, cultures were washed with phosphate buffer saline (PBS) and either coated with a second layer of collagen (0.6 mg/ml) to obtain a sandwich culture or maintained in medium for the length of the experiment. Medium was changed every day. For insulin stimulation, four days after plating, cells were starved in medium without serum for two hours and the incubated in starvation medium with 170 nM insulin for five to ten minutes. After the cells were harvested for western blot analysis.