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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.

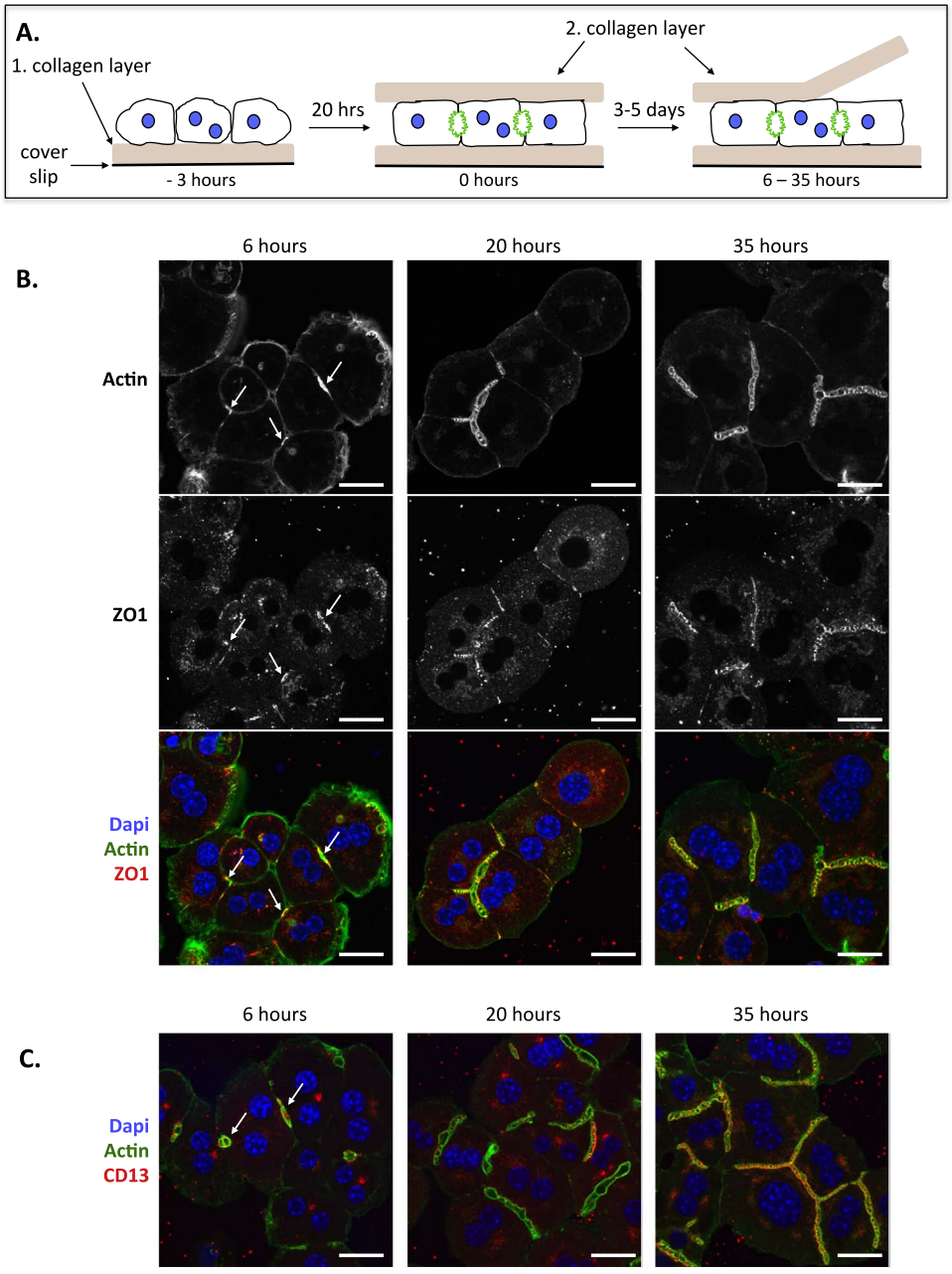

Fig. 1. *Development of cell polarity in collagen sandwich hepatocytes.* (**A**) Schemata of the culturing method for primary mouse hepatocytes in collagen sandwich. Cells are plated onto a manually coated coverslip in 24-wells after isolation. Following an app. Three to four hour attachment the cells are overlaid with a second layer of collagen to allow polarity re-establishment. Application for immunofluorescence is achieved by a soft peeling of the top layer of collagen at indicated times. (**B, C**) Time course of polarity establishment in cultures stained for actin using 488-phalloidin and the tight junction marker ZO1 (**B**) and the transcytotic marker CD13 (**C**). Scale bar=20 µm.

2.7. Immunofluorescence and LDL uptake assay in primary hepatocytes

For immunofluorescence analysis of primary hepatocytes in sandwich culture cells were fixed in 4% paraformaldehyde at room temperature for 30 min, washed twice with PBS, permeabilized for one hour with 0.1% Triton X-100, washed and blocked in 10% horse serum for two hours. Holes were applied to the top layer collagen using fine aspiration to ensure better antibody penetration. Cells were incubated with primary antibodies at room temperature over night, washed for two hours in wash buffer (300 mM NaCl, 0.1% Tween, 10 nM Tris/HCl) with extensive exchanges of buffer. The secondary antibodies were incubated for five hours at 37 °C in a humidified chamber. Thereafter cells were washed extensively and mounted onto glass slides using 0.1 g/ml Mowiol (Calbiochem).

For immunofluorescence of monolayer cultures cells were fixed in 4% PFA for 15 min, washed with PBS, permeabilized for five min with 0.1% Triton X-100, washed and blocked in 10% horse serum for 10 min. Cells were incubated with primary and secondary antibodies at room temperature for 1 h and 30 min, respectively. After, cells were mounted onto cover slips with Mowiol for imaging.

LDL uptake experiments were performed as described before [12]. For EGF uptake kinetics, primary hepatocytes were starved for two hours prior to a555-EGF (200 ng/ml; continuous uptake) addition. Continuous EGF uptake was performed for various time points in full medium followed by a cold wash with PBS and fixation in 4% paraformaldehyde. Nuclei were stained with DAPI and cells were mounted as described above.

Samples were analyzed using a Laser Scanning Confocal Microscope (Olympus Fluoview 1000, LMF facility, MPI-CBG,

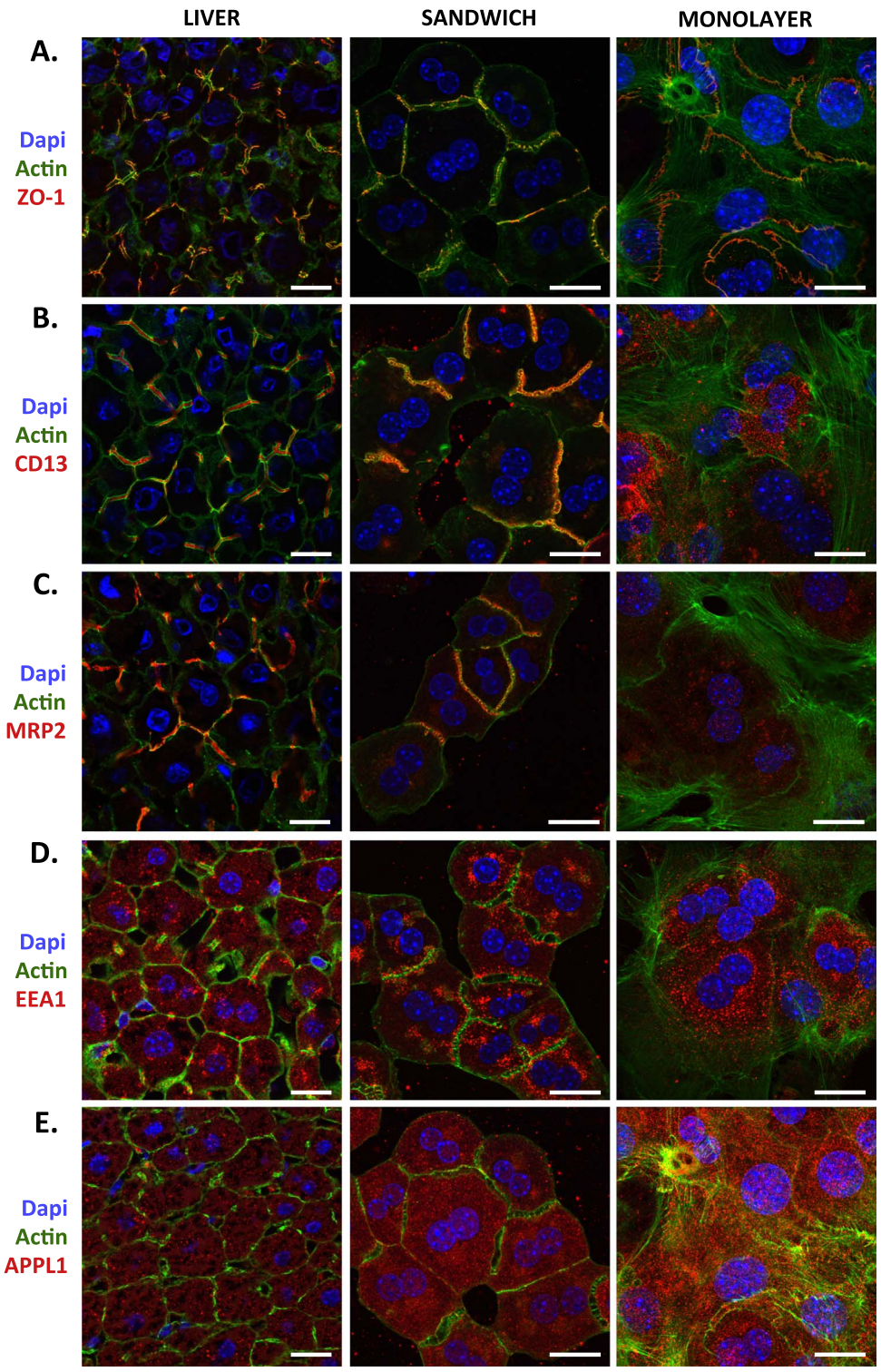

Fig. 2. Polarized distribution of junctional components and endosomal proteins in liver *in vivo* and collagen sandwich hepatocytes *in vitro*. Representative confocal microscopy images of liver sections and primary hepatocytes in monolayer and sandwich configuration five days post-isolation stained with alexa-488 phalloidin (actin), Dapi and ZO1 (**A**), CD13 (**B**), MRP2 (**C**), EEA1 (**D**) and APPL1 (**E**) in red. Scale bar=20 μm.

Dresden) equipped with an Olympus UPlanSApo 60×1.35 Oil immersion objective at a resolution of app. 100 μm/pixel and 500 nm step size. Images were analyzed using Motion Tracking and Fiji software and adjusted for presentation by Adobe Photoshop. All experiments were repeated with similar results.

2.8. Immunofluorescence quantification by motion tracking

Quantitative multi-parametric image analysis was performed as described before [12].

2.9. Immunofluorescence quantification by Fiji

The quantification of LDL receptor surface levels was obtained as

described before [12].

2.10. Glucose secretion assay in primary hepatocytes

The glucose secretion assay from primary hepatocytes was adapted from Sakai et al. [41]. Primary hepatocytes five days post isolation were starved in phenol-free DMEM for over night containing 0.5% FBS, 5 mM glucose, 4 mM glutamine. After the cells were washed twice with PBS at room temperature and stimulated with DMEM containing 4 mM glutamine, 2 mM pyruvate, 20 mM lactate plus/minus 100 nM glucagon for stimulation for 5 h. Collected medium was centrifuged and glucose content measured using an Amplex Red glucose assay kit (Invitrogen). The remaining cells were lysed to measure protein content for well-based normalization.

3. Results

3.1. Hepatocyte sandwich system recapitulates polarized liver morphology

The collagen sandwich culture of primary mouse hepatocytes has proven difficult for the immunohistochemistry analysis of small organelles, such as endosomes and for endocytic uptake assays, because the second layer of collagen acts like a barrier to antibody and cargo penetration. Therefore, to evaluate the subcellular localization of various endosomal markers and internalized ligands, we first had to optimize the culture conditions and protocols for confocal microscopy and quantitative image analysis, using software developed for the morphological analysis of cell and tissue architecture from microscopy images [42]. As previously shown for rat hepatocytes, isolated primary mouse hepatocytes assemble into clusters when plated onto the surface of manually coated collagen coverslips (Fig. 1**A**). After the initial phase of cell attachment (6 h), hepatocytes were overlaid with the second layer of collagen to allow the membrane to re-polarize and form apical plasma membrane (Fig. 1**A, B** and **C**). The apical surface is characterized by the high density of actin filaments, staining for the tight junction marker ZO1 (Fig. 1**B**) and the transcytotic marker CD13 (Fig. 1**C**, 6 h time point, white arrows). These patches grow between hepatocytes and expand laterally with time until they form a small bile canalicular network *in vitro* (Fig. 1**B** and **C**, 20–35 h time point) [36]. To follow the polarization process, cells were fixed at different periods of time after addition of the second layer of collagen and prepared for immunofluorescence staining. For this, we either gently removed the top layer of collagen *via* low-pressure suction or introduced small holes to facilitate the penetration of the antibodies (Fig. 1**A**). In addition, softer top layers of collagen, longer antibody incubation times and extensive washes with detergent-containing buffers were essential to obtain reliable and specific antibody stainings (see Materials and Methods for detailed protocol).

To ensure that the primary culture system recapitulates liver tissue morphology, we compared the hepatocytes in monolayer and collagen sandwich cultures *in vitro* with hepatocytes *in vivo* in liver tissue sections (Fig. 2). We used a series of markers, including the junctional protein ZO-1, the transcytotic marker CD13 and an apical bile acid transporter MRP2 (also called canalicular multi-specific organic anion transporter 1) to label the apical plasma membrane, phalloidin to decorate the actin filaments and two Rab5 effectors, APPL1 and EEA1, to label distinct early endosomal populations [43]. In the liver, the apical bile canalicular membrane is enriched in actin filaments and positive for the tight junction marker ZO-1, which is absent from the basolateral membrane (Fig. 2**A** left panel and [12]). Similarly, CD13 and MRP2 were localized to the apical membrane (Fig. 2**B and C** left panels). These features were recapitulated in the collagen sandwich culture system. ZO-1, CD13 and MRP2 were all localized to the apical surface positioned between adjacent hepatocytes (Fig. 2**A, B and C** middle panel and Fig. 1**B, C**), reflecting the existence of functional polarized sorting machineries in this *in vitro* culture system as reported for rat hepatocytes [36]. In contrast, in monolayer cultures the actin cytoskeleton was spread throughout the cell cortex and stress fibers were frequently observed. ZO-1 was evenly distributed along the membrane (Fig. 2**A** right panel), and CD13 and MRP2 were dispersed throughout the cells (Fig. 2**B, and C** right panels), indicating that the cells were not polarized. Taken together, only the collagen sandwich system reproduces basic morphological features of hepatic cell polarity as in liver *in vivo*.

3.2. Polarized distribution of endosomal components in liver and collagen sandwich hepatocytes

Maintenance of hepatocellular polarity requires distinct basal and apical endocytic and recycling transport routes intersecting specialized endosomal compartments [10]. The canonical marker of early endosomes EEA1 (early endosome antigen 1) is enriched in the somatodendritic domain in hippocampal neurons and basolaterally in epithelial cells [44]. Intriguingly, EEA1 is distributed to both basolateral and sub-apical early endosomes in polarized hepatic WIF-B cells [45]. In order to determine the intracellular distribution of EEA1 we stained liver sections and *in vitro* primary cultures of hepatocytes (Fig. 2**D**). We found EEA1 enriched in the perinuclear region but also underneath the apical membrane in liver *in vivo* and hepatocytes sandwich cultures *in vitro* (Fig. 2**D** left and middle panel), consistent with previous observations [45]. These results suggest a potential function of EEA1-positive early endosomes in the apical early endocytic pathway in hepatocytes. In monolayer cultures, however, EEA1 showed a disperse distribution throughout the cytosol (Fig. 2**D** right panel). In contrast, the APPL1-positive early endosomes were distributed throughout the cytoplasm, showing no clear polarity both in liver sections and in *in vitro* cultures (Fig. 2**E**).

We next quantified the distribution of early endosomes in hepatocytes sandwich cultures as a function of the distance from the apical membrane labeled by ZO-1 (Fig. 3**A, B, D**) using the MotionTracking software [42]. In the sub-apical region of hepatocytes, the density of EEA1-positive endosomes increased by 70%, from 20 to 35 endosome/100 μm^2 (Fig. 3**C**, red line). In contrast, APPL1 endosomes were evenly distributed (Fig. 3**C**, black line). Such a distribution was similar to that observed in peri-portal hepatocytes *in vivo* (Fig. 3**E, F**). Note that although APPL1-positive structures appeared to be enriched in the basal region, these structures most likely belong to the sinusoidal endothelial cells, which are not stained in the image.

Interestingly, the polarized distribution of EEA1 endosomes was subjected to modulation by the actin cytoskeleton and metabolism. First, we found that the sub-apical enrichment of EEA1 endosomes correlated with the apical enrichment of F-actin. Those hepatocytes that displayed an aberrant accumulation of actin stress fibers (mostly in the monolayer but often also in the sandwich culture) had lost the polarized distribution of EEA1 endosomes (Fig. 3**D**, compare cells with and without actin stress fibers). Second, such polarized distribution was not a permanent feature of hepatocytes because starvation of the animals for 12 h caused a dramatic redistribution of EEA1 endosomes throughout the cytoplasm, which resembled that of APPL1 endosomes (Fig. 3**G, H**). This indicates that the metabolic state has profound effects on the intracellular distribution of the EEA1-positive endosomal population.

Taken together these data indicate that the collagen sandwich culture but not the monolayer system recapitulates the intracellular distribution of early endosomal markers in hepatocytes as observed in liver tissue.

3.3. Loss of polarity in monolayer cultures leads to reduced LDL and EGF endocytosis

Hepatocytes in the liver are highly dependent on endocytic trans-

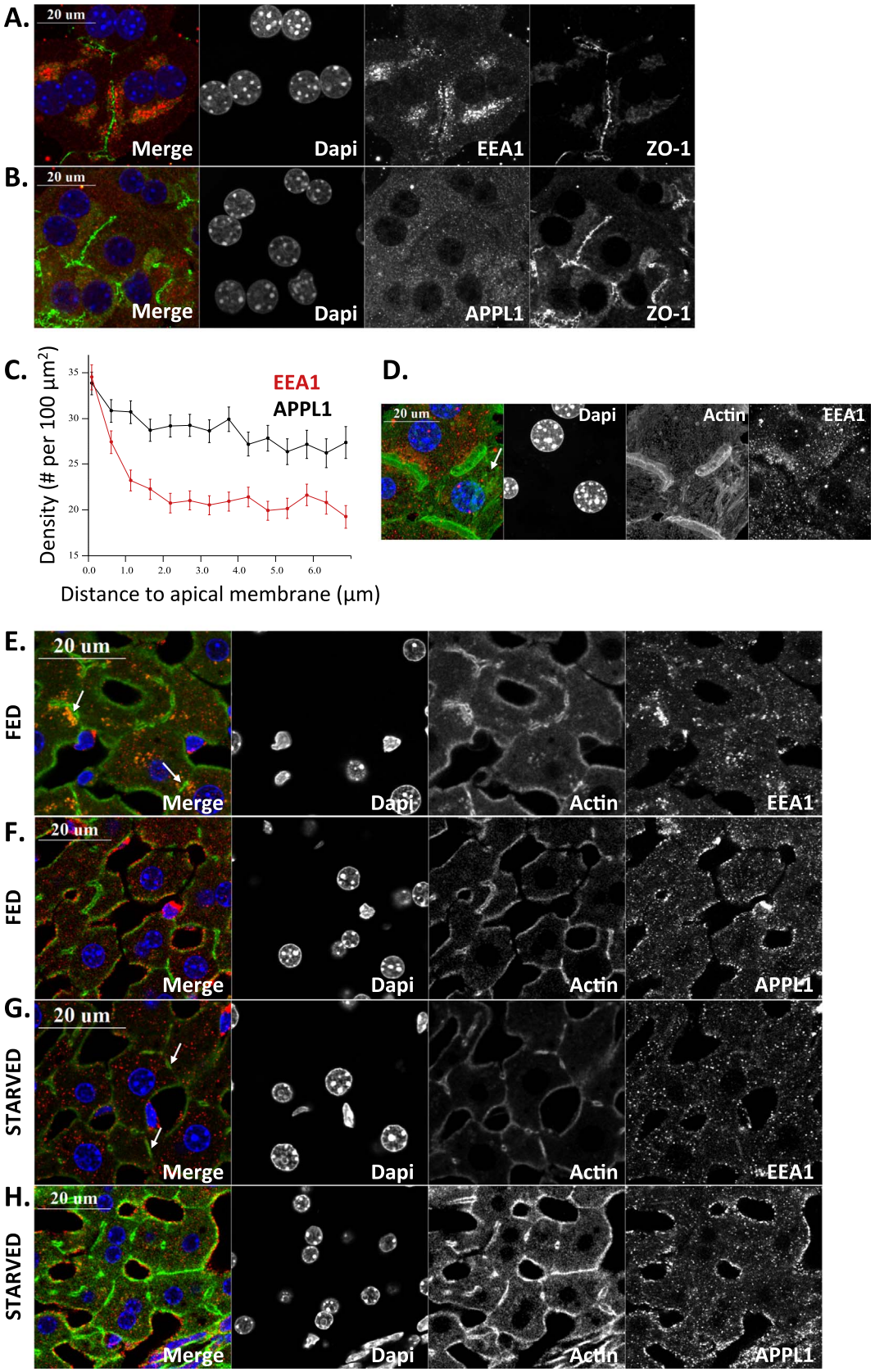

Fig. 3. *Polarized distribution of EEA1 endosomes is sensitive to actin cytoskeleton rearrangements and metabolism.* (**A, B**) Representative confocal microscopy images of EEA1 and APPL1 endosomes in primary hepatocyte sandwich system and quantification of their intracellular distribution with QMPIA (**C**) (mean ± SEM). Apical localization of EEA1 *in vitro* was disturbed in stressed cells evidenced by actin stress fibers (**D**, white arrow). Representative confocal microscopy images of liver sections stained for EEA1 (**E, G**) and APPL1 (**F, H**) under fed (**E, F**) or after 12 h fasting (**G, H**). White arrows point at the apical distribution of EEA1 under fed condition. Scale bar=20 µm.

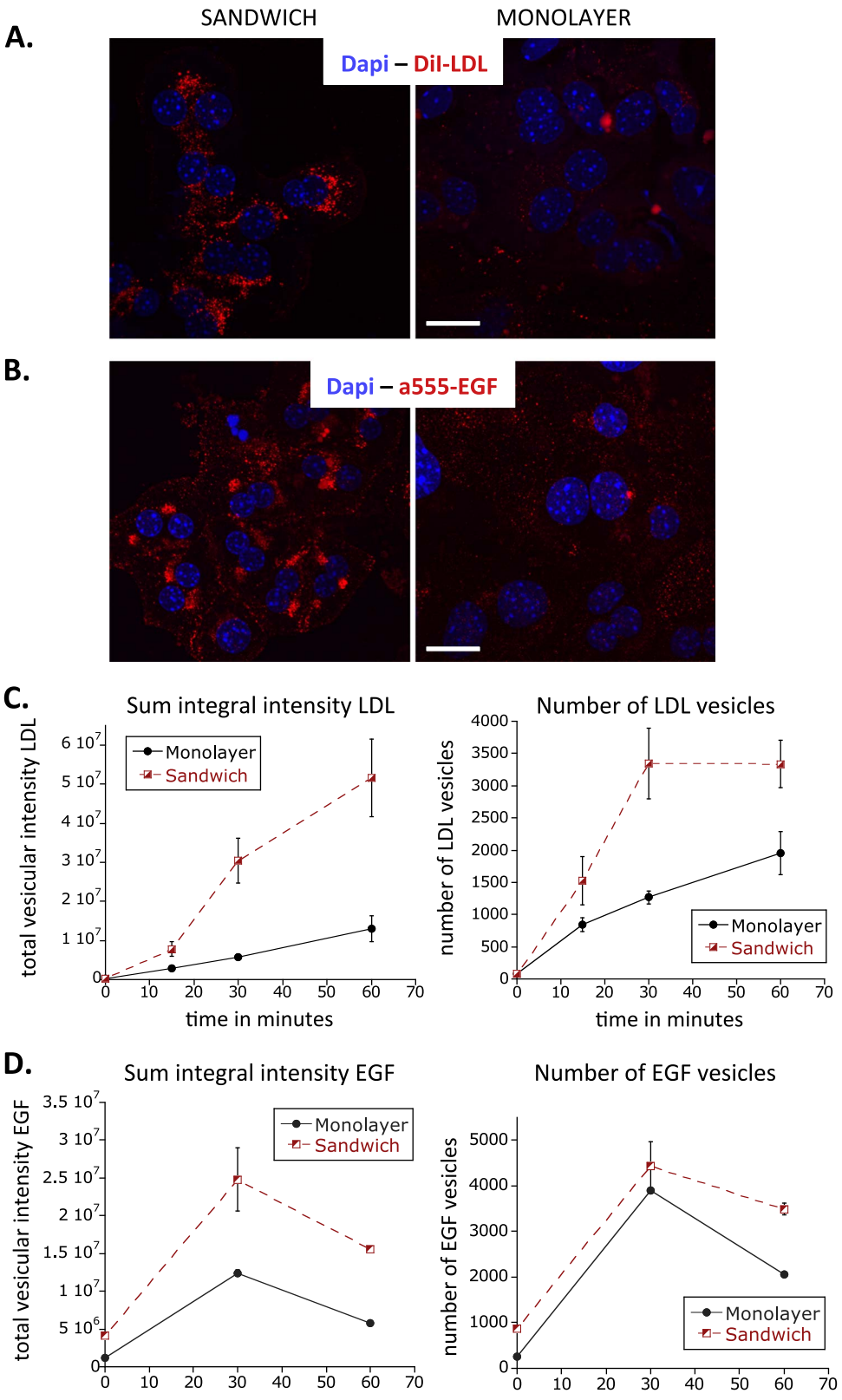

Fig. 4. *Sandwich culture hepatocytes exhibit a fast internalization of LDL.* (**A, B**) Representative confocal microscopy images of monolayer and sandwich cultured hepatocytes after 60 min of DiI-LDL (~2.5 μg/ml; continuous uptake) (**A**) and fluorescently-labeled EGF (~200 ng/ml; continuous uptake) (**B**) internalization and their fluorescence quantification by QMPIA in (**C, D**). Key parameters are presented. (mean ± SEM).

port processes to mediate their metabolic functions [8,12]. Since the spatial distribution of early endosomes correlates with hepatocyte polarity *in vitro* and *in vivo* (see Figs. 2 and 3), we tested whether also endocytosis was altered in non-polarized hepatocytes compared to cells grown in collagen sandwich. As a major task of the liver is the regulation of lipid metabolism and lipoprotein uptake, we investigated the uptake of LDL. Primary hepatocytes grown as monolayer or in the collagen sandwich were compared for their capacity to internalize LDL.

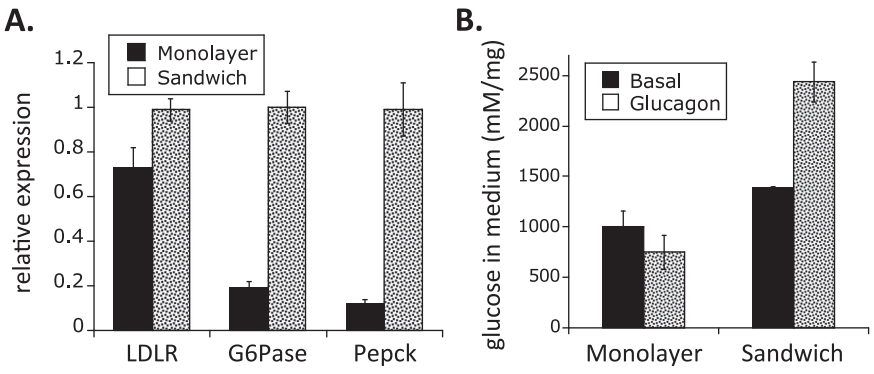

Fig. 5. *Maintenance of glucose production in sandwich cultured hepatocytes.* (**A**) Relative mRNA expression normalized to three housekeeping genes of LDLR, G6Pase, and Pepck1 from monolayer and sandwich grown hepatocytes five days post isolation (representative experiment shown). (**B**) Glucagon (100 nM) -induced glucose production from monolayer and sandwich hepatocytes.

Hepatocytes in collagen sandwich exhibited an efficient uptake of LDL over time (Fig. 4**A**). Using our image analysis software we quantified the number of LDL-positive endosomes and found that they increased over time and stabilized after 30 min, indicating that the number of endosomes containing LDL saturated (Fig. 4**C**), as expected. Strikingly, in monolayer cultures, both the vesicular intensity of LDL and number of LDL-positive endosomes were strongly reduced, indicating a much-reduced endocytosis of LDL (Fig. 4**A, C**). The mRNA levels of LDLR were only slight decreased in monolayer cultures compared to the collagen sandwich system (Fig. 5**A**), which could not account for the reduction in LDL endocytosis. Furthermore, the decrease in LDL internalization was not due to an altered expression of LDLR on the cell surface, as monolayer and sandwich cultures showed similar DiI-LDL binding (Fig. S1).

To test whether the culture conditions determine a general effect on endocytosis, we repeated the same internalization experiments with fluorescently labeled epidermal growth factor (EGF). As for LDL, the internalization of EGF into primary hepatocytes in collagen sandwich saturated after 30 min, as evidenced by their stabilized vesicular intensity and vesicle number (Fig. 4**B, D**). Interestingly, monolayer cultures exhibited a reduction in the vesicular intensity of EGF, but less change in the number of EGF-positive vesicles. This suggests that EGF enters the hepatocytes more slowly in monolayer cultures than in the sandwich system but occupies all endosomes, whereas LDL enters with even slower kinetics and therefore the number of endosomes increases correspondingly more slowly. Altogether, these data strongly suggest that hepatocytes grown in collagen sandwich exhibit a more efficient uptake of LDL and EGF than when grown as monolayers.

3.4. Hepatocytes in collagen sandwich remain glucagon sensitive and are able to perform gluconeogenesis

A recurrent problem with the monolayer culture of primary hepatocytes is the loss of key metabolic activities when grown conventionally [26–28]. Since hepatocytes grown in the collagen sandwich system seem to retain much of their physiological properties as in liver, they may also have improved metabolic functions, as suggested before [37]. A unique function of the liver is to maintain blood glucose homeostasis by inducing gluconeogenesis under fasting conditions. Therefore, we compared the monolayer and sandwich cultures for the expression of the rate-limiting genes involved in gluconeogenesis, glucose-6-phosphatase (G6Pase) and Phosphoenolpyruvate-Carboxykinase (Pepck) (Fig. 5**A**). Interestingly, *G6Pase* and *Pepck* levels were 5-fold higher in collagen sandwich compared to monolayer cultures. Since G6Pase and Pepck are major drivers of gluconeogenesis, we next investigated the response of the cultured hepatocytes to glucagon stimulation by performing gluconeogenesis *in vitro*. Hepatocytes starved overnight were incubated with 20 mM pyruvate with or without 100 nM glucagon for 5 h and the production of glucose into the medium was measured. In line with an increase in *G6Pase* levels, hepatocytes in collagen sandwich cultures showed a 50% increase in glucose production in response to glucagon stimulation (Fig. 5**B**). In contrast, hepatocytes grown in monolayer were glucagon insensitive (Fig. 5**B**). These data show that only hepatocytes grown in the collagen sandwich system retained the ability to perform gluconeogenesis and further support the use of this culture method over the monolayer system for investigating liver-specific metabolic functions *in vitro*.

3.5. Improved insulin sensitivity in sandwich culture hepatocytes

Since metabolism is regulated by signaling pathways, we wondered whether changes in cell polarity can also correlate with signaling outcomes. The maintenance of glucose homeostasis is mediated by an interplay between glucagon and insulin signaling, where insulin is the predominant pathway [46]. Therefore, we measured the ability of primary hepatocytes grown as monolayers or collagen sandwich to respond to insulin stimulation by measuring insulin-induced AKT activation (phosphorylated, p-AKT) as direct readout for insulin receptor signaling. Hepatocytes in monolayer and sandwich culture were starved for 2 h and stimulated with 170 nM insulin for 5 and 10 min (Fig. 6**A, B**). Monolayer hepatocyte cultures exhibited a reduced ability to activate p-AKT (Ser473) in response to insulin stimulation compared to collagen sandwich cultures, demonstrating that proper insulin signaling correlates with cell polarity. Altogether, these data show an improvement of hepatocellular function in hepatocytes grown in collagen sandwich over monolayer culture, emphasizing the necessity of a cell-polarized system for studying liver biology *in vitro*.

4. Discussion

In vitro culture systems amenable to dissecting the mechanisms of hepatocellular organization and function, as well as their dysfunction in disease are in rising demand. In this study, we performed a cell biological characterization and comparison of the most commonly used *in vitro* cultures of primary mouse hepatocytes, the collagen monolayer and sandwich cultures. Our aim was not to demonstrate that such *in vitro* systems can replace the study of liver *in vivo*, but to determine to what extent they are able to recapitulate fundamental properties of hepatocytes in their natural context. Out of the two systems analyzed, we found that the collagen sandwich is the only one that reproduces to a significant degree such a set of cellular properties. The hepatocytes cultured in collagen sandwich exhibit a polarized distribution of junctional components, apical proteins and endosomal markers. Maintenance of cell polarity was accompanied by fast internalization kinetics of two endosomal cargoes (LDL and EGF), and a correct spatial distribution of early endosomes compared to non-polarized monolayer

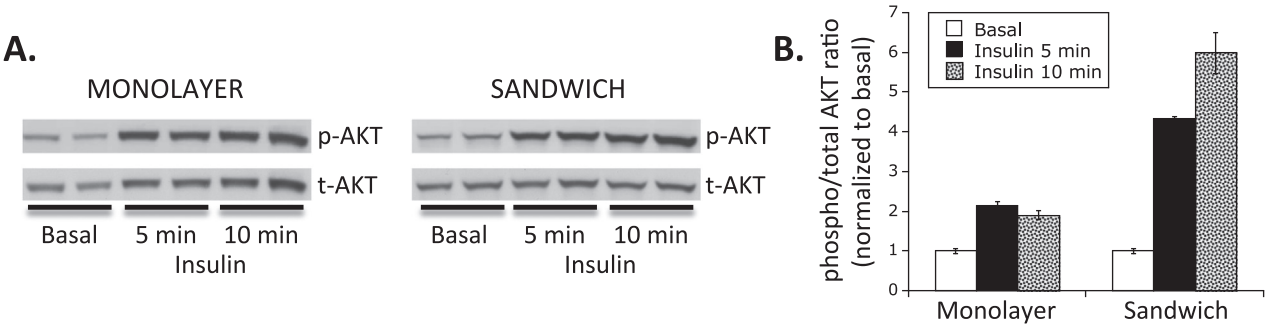

Fig. 6. *Improved insulin sensitivity in sandwich cultured hepatocytes.* (**A**) Western blots of p- and t-AKT following 170 nM insulin stimulation for 5 and 10 min in monolayer and sandwich cultures and quantification thereof (**B**). Scale bar=20 μm, (mean ± SEM).

cultures. Responsiveness to hormonal stimulation with insulin and glucagon was further preserved leading to enhanced signaling and metabolic activities. Altogether, our results encourage the use of the collagen sandwich over the monolayer system to study aspects of liver cell biology *in vitro*.

Hepatocellular polarity is essential for liver structure and function. The apical surfaces of hepatocytes pair with their neighbors to form a three-dimensional bile canalicular network throughout the liver tissue [42,47,48]. It is therefore essential to recapitulate cell polarity in any *in vitro* culture system that aims at studying key cell biological properties of hepatocytes as in the liver. Especially the polarized distribution of liver bile acid transporters should be preserved, since mis-targeting of these transporters leads to perturbation in bile acid metabolism, resulting in Cholestasis [12,13]. Our study shows a striking correlation between apico-basal polarity and other cellular functions such as endocytosis, endosome distribution, glucose metabolism and signaling. Loss of the integrity of the endo-lysosomal pathway has dramatic consequences for the sorting of apical transporters to the bile canaliculi [12]. This and the importance of Rab11a and the endosomal recycling machinery for proper localization of apical transporters [49] underscore the necessity of the early endosomal system for cell polarity [8,12]. A new finding of our study is that two Rab5 effectors and early endosomal markers, APPL1- and EEA1, have a different spatial distribution in hepatocytes, with EEA1 being enriched in the sub-apical region and APPL1 more evenly distributed throughout the cells. These data support the idea that these two markers indeed label two distinct populations of early endosomes [43]. The accumulation of actin stress fibers negatively correlated with the sub-apical enrichment of EEA1 endosomes *in vitro*. This suggests that *in vitro* culture systems, which do not support the proper organization of the actin cytoskeleton as *in vivo*, are not suitable to reproduce the organelle distribution under physiological conditions. This is probably one of the reasons why the monolayer system is less optimal to study endocytosis and endosomal distribution than the collagen sandwich system.

The sub-apical localization of EEA1-positve early endosomes suggests a role of these compartments in polarized endocytic transport. The endosomal system is required for the transport of bile acid transporters to the apical surface [12] and the EEA1 endosomes are the primary candidate compartments for such trafficking. This interpretation is supported by the observation that the subcellular distribution of EEA1 endosomes was also modulated by the metabolic state of the cells. It is know that after a meal, circulating bile acids are reabsorbed by the liver [50] inducing an increase in the translocation but also endocytosis and recycling of apical bile acids transporters to the apical membrane [51]. This process may therefore require an activation of the secretory and endosomal machinery, manifested by the specific redistribution of the EEA1-positive early endosomes, but not APPL1 endosomes, toward the bile canalicular membrane. These results support the tight link between endocytosis and metabolism observed previously [8].

Another remarkable difference between hepatocytes in collagen sandwich *vs.* monolayer was the kinetics of LDL and EGF uptake and the intracellular distribution of early endosomes. Efficient endocytosis reflects the properties of the liver for fast LDL clearance from the blood. Reduced uptake of LDL is known to cause hypercholesterolemia [9]. The reduced uptake of LDL in monolayer cultures therefore reflects a pathological condition and, thus, could be exploited as a disease model. The reduction in EGF internalization suggests a general perturbation of endocytosis, which leads to broader alterations in nutrient availability, turnover of plasma membrane transporters and signaling receptor internalization and recycling. The internalization of signaling receptors and distribution in early endosomes regulates the amplitude and lifetime of the signaling response [52]. Given the severe reduction in endocytic uptake and perturbed endosomal distribution, it is not surprising that hepatocytes in monolayer cultures show impairment in signal transduction (pAKT). Caution should therefore be applied when using hepatocytes in monolayer cultures to study and interpret signaling processes *in vitro*.

Nevertheless, although hepatocytes grown in collagen sandwich show many improvements in recapitulating the functional properties of the hepatocytes in the liver compared to monolayers, there are limitations as with any *in vitro* culture system. Hepatocytes in collagen sandwich become cholestatic over time, since they do preserve the expression and function of some bile acid transporters leading to a constant efflux of bile acid into the canalicular membrane leading to junctional rupture [53]. In addition, BSEP (bile salt export pump) is downregulated in this culture leading to alterations in bile acid metabolism and cholestasis, limiting the usage of this system to 1–2 weeks [32,54]. Despite such pathological signatures, the restoration of cell polarity in the collagen sandwich system makes this *in vitro* culture system suitable to study mechanisms of hepatocyte organization, trafficking, signaling and metabolism.

The occurrence of cholestasis can be overcome by organoid cultures or spheres, where much less extracellular matrix is needed and where the cells are rather supported by a hollow fiber bioreactor [55]. In this bioreactor, cells respond with enhanced albumin and urea secretion, and improved metabolic activity compared to sandwich and monolayer cultures. Nevertheless, sandwich cultured hepatocytes still largely performed better also in this respect compared to monolayers, except for glucose production, which is in contrast to our analysis. One possible explanation is that the sandwich culture method used by Lu et al. employs a higher concentration of collagen I (1.5 mg/ml) compared to our study (0.6 mg/ml), which produces a stiffer matrix known to negatively influence hepatocyte function [56]. In addition, primary hepatocytes require time (3–5 days) to fully polarize and develop metabolic functions in culture. Just 24 h after plating, hepatocytes in sandwich culture fail to properly respond to insulin stimulation and are less suitable to study glucose metabolism [57]. Only after 5–6 days in collagen sandwich can hepatocytes recover insulin and glucagon sensitivity and be usable for glucose metabolism studies.

The sandwich system proved useful for a cell biological characterization of hepatocytes [8,12], something that is difficult in other *in*

vitro systems, *e.g.* spheroid structures. We have defined a number of cellular features that can be used as diagnostic markers to assess the suitability of *in vitro* culture systems to recapitulate properties of the hepatocytes *in vivo*. In addition, the possibility to manipulate these polarized hepatocyte cultures by RNAi provides the necessary tools to ask mechanistic questions in liver cell biology [8,12]. In conclusion, our study supports the use of the sandwich culture of primary hepatocytes as a system that reproduces structural features as well as functional properties of the hepatocyte and thus liver biology.

Conflict of interest statement

The authors declare no conflict of interest.

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Authors' contributions

M.Z. conceived and directed the project. A.Z. designed the experiments, established the primary culture, developed the staining procedures for the tissue sections, the endocytosis assays and staining protocols in primary hepatocytes in collagen sandwich. A.W. performed the endosomal stainings in starved and fed liver tissue sections. S.S. under the supervision of A.Z. performed the Western blot analysis and the hepatocyte isolation and primary culturing. G.M. under the supervision of Y.K. adapted the QMPIA for primary hepatocytes and liver tissue and performed the image analysis. M.Z. and A.Z. wrote the manuscript.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2016.11.027.

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