Original Article

Metabolomics of an *in vitro* liver model containing primary hepatocytes assembling around an endothelial cell network: comparative study on the metabolic stability and the effect of acetaminophen treatment

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ABSTRACT — Recently, a novel culture system consisting of primary hepatocytes structured over a network of endothelial cells on the Engelbreth-Holm-Swarm (EHS) gel has been reported. This *in vitro* liver model on the EHS gel (IVL_{EHS}) has been shown to maintain the expression of hepatic genes and their functional activity. Moreover, the IVL_{EHS} was more sensitive to xenobiotics than hepatocyte monocultures, suggesting the potential utility of this culture system for compound hepatotoxicity screening. However, the effect of this three-dimensional structure formation on the cellular metabolic profile of hepatocytes in the IVL_{EHS} is not well understood. To address this concern, we performed metabolome analysis using capillary electrophoresis-time of flight mass spectrometry. Between the IVL_{EHS} and mono-cultured hepatocytes on the EHS gel, there was no significant difference in the levels of metabolites of the urea cycle and the tricarboxylic acid cycle, essential amino acids, and adenylate energy charge (AEC) which is an important indicator of cellular energy status. On the other hand, acetaminophen-dependent decrease of the AEC in the IVL_{EHS} was greater than that in the monoculture, suggesting the higher sensitivity of IVL_{EHS} to acetaminophen-induced hepatotoxicity which is caused by metabolic activation of this drug. Further analysis showed that the levels of taurocholate, one of the major conjugated bile acids, were higher in the IVL_{EHS} than in the monoculture. Considering that the construction of the IVL_{EHS} did not seem to disturb the major cellular metabolism, our findings would strengthen the concept that IVL_{EHS} would have beneficial effects on the maintenance of hepatic functions.

Key words: Acetaminophen, Adenylate energy charge, CE-TOFMS, EHS gel, Hepatotoxicity,
In vitro liver model (IVL)

INTRODUCTION

The liver is the largest internal organ performing such multiple vital functions as metabolic conversion of xenobiotics, protein synthesis, carbohydrate storage, and bile secretion. Hitherto, *in vitro* liver models faithfully reproducing hepatic functions have been extensively investigated with the aim of developing alternatives to liver transplantation (Sharma *et al.*, 2010; Viollet *et al.*, 2009). Moreover, such *in vitro* liver-mimicking systems

are expected to be used for compound screening to predict potential hepatotoxicity as well as pharmacological effects of drug discovery (Hewitt *et al.*, 2007; Kaplowitz, 2005). One of the major approaches in liver modeling has been the use of primary hepatocytes, which constitute about two thirds of the total cell population in the liver parenchyma (Godoy *et al.*, 2013; Donato *et al.*, 2013; Guguen-Guillouzo and Guillouzo, 2010). However, the use of primary hepatocytes is challenging, because the hepatocytes rapidly lose their function after isolation, and

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their maintenance in simple monoculture condition is difficult.

Considering that the liver is composed of parenchymal hepatocytes as well as of non-parenchymal cells, the structural arrangement of hepatic tissue should be important for supporting the functional activity of hepatocytes (Godoy *et al.*, 2013). It has been established that the survival and functional performance of primary hepatocytes *in vitro* critically depend on the intercellular communication of hepatocytes with the adjacent non-parenchymal cells as well as on the interaction between hepatocytes (Kidambi *et al.*, 2009; Nahmias *et al.*, 2006; Strain, 1999; Bhatia *et al.*, 1999, 1998). In addition to cell-cell contacts, the presence of the extracellular matrix (ECM) is also important, as the reconstitution of the ECM *in vitro* contributes to the maintenance of cell morphology and functional integrity (Gissen and Arias, 2015; Kleinman *et al.*, 2003; DiPersio *et al.*, 1991). When hepatocytes are cultured on ECM-derived gels containing type I collagen or basement membrane proteins, they retain their differentiated morphology for longer time and express liver-specific genes at a higher level compared to monolayer cultures on plastic surface, which have flattened shape and rapidly lose their liver-specific characteristics (Schuetz *et al.*, 1988). In this context, spatially coordinated interaction of non-parenchymal cells and the ECM with hepatocytes would be critical for the *in vitro* maintenance of primary hepatocyte cultures and induction of their liver-specific functions.

We have recently developed an *in vitro* liver model (Toyoda *et al.*, 2012) consisting of primary hepatocytes and endothelial cells cultured on the Engelbreth-Holm-Swarm (EHS) gel, an extract of basement membrane proteins (Kleinman and Martin, 2005). In this model called the IVL_{EHS}, primary hepatocytes gather and assemble in a three-dimensional manner around a capillary-like tubular network of endothelial cells plated on the EHS gel, resulting in a structure that resembles hepatic tissue. In the presence of acetaminophen, an inducer of metabolic conversion-related hepatotoxicity (Coen, 2015; Jaeschke *et al.*, 2003), the IVL_{EHS} tissue model was reportedly more sensitive to acetaminophen-induced toxicity compared to monolayer cultures (Toyoda *et al.*, 2012). Furthermore, the previous study showed that the IVL_{EHS} maintained the drug-metabolizing capacity of primary hepatocytes as evidenced by the expression of liver-specific drug-metabolizing enzymes and biliary efflux transporters, and the higher cytochrome P450 activities as compared with those in the mono-culture conditions.

Whereas the potential utility of the IVL_{EHS} has been pointed out (Ahn *et al.*, 2014; Toyoda *et al.*, 2012), there

is little knowledge about the endogenous metabolism of hepatocytes in the IVL_{EHS} such as ammonia detoxification in the urea cycle and general pathways for energy generation. Since environmental changes can disrupt intracellular homeostasis of cells and cause subsequent alterations in gene expression (de Nadal *et al.*, 2011; Viollet *et al.*, 2009; Dalton *et al.*, 1999), it would be important to clarify whether the metabolic profile of hepatocytes is affected by the construction of IVL_{EHS} characterized by spontaneous formation of three-dimensional structure and hepatocyte migration on the EHS gel.

In this study, we constructed the IVL_{EHS} according to the previous study (Toyoda *et al.*, 2012) to enhance our understanding about the biochemical characteristics of this culture system. The effects of the construction of IVL_{EHS} were examined on the cellular metabolism of hepatocytes using metabolomics approach. There was no significant difference in the levels of metabolites of the urea cycle and the tricarboxylic acid (TCA) cycle as well as in adenylate energy charge (AEC) between the IVL_{EHS} and monocultures of hepatocytes on the EHS gel, suggesting that the IVL_{EHS} could preserve hepatic functions without negative effects on hepatocyte metabolism.

MATERIALS AND METHODS

Materials

The following reagents were used: endothelial cell basal medium (EBM-2) and EGM-2 Single Quots (supplemental factors) (Lonza Japan Ltd., Tokyo, Japan); Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin (100 ×), and trypsin-EDTA (Invitrogen/GIBCO, Grand Island, NY, USA); tissue-culture treated cell culture dishes/plates (Corning Inc., Tokyo, Japan); collagenase, trypsin inhibitor, mannitol, D-camphor-10-sulfonic acid (CSA), 1,3,5-benzenetricarboxylic acid (Trimesate), and gelatin (Wako Pure Chemical Industries Ltd., Osaka, Japan); 2-(N-morpholino) ethanesulfonic acid (MES) (Dojindo, Kumamoto, Japan); growth factor reduced (GFR) Engelbreth-Holm-Swarm (EHS) gel (BD Biosciences, Bedford, MA, USA); and acetaminophen (p-Acetamidophenol) (Nacalai Tesque Inc., Kyoto, Japan). All reagents used in the present study were of analytical grade.

Animals

Six-week-old male BALB/cAJcl mice were purchased from CLEA Japan Inc. (Tokyo, Japan) and treated in accordance with local institutional guidelines for the care and use of laboratory animals. The animal protocol was approved by the Animal Experimentation Committee of

Tokyo Institute of Technology.

Primary cell culture

Human umbilical vein endothelial cells (HUVECs) (Cambrex BioScience Walkersville Inc., Walkersville, MD, USA) were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air in complete EBM-2 containing 2% (v/v) fetal bovine serum, hydrocortisone, hFGF- β , VEGF, R3-IGF-1, ascorbic acid, hEGF, GA-1000, and heparin according to the manufacturer's instructions. Cells were subcultured not more than 10 times. Mouse primary hepatocytes were isolated by the *in situ* 2-step-collagenase perfusion method via portal vein cannulation as described in previously (Toyoda *et al.*, 2012). In brief, after pre-perfusion with Hank's Buffered Salt Solution (HBSS) containing EGTA (0.19 g/L) and glucose (0.98 g/L), the mouse liver was perfused with 0.015% collagenase in HBSS, then minced in ice-cold HBSS. To isolate the hepatocytes, the dispersed cells were filtered, and subjected to 40% Percoll density gradient centrifugation. Isolated hepatocytes were maintained in DMEM supplemented with 100 U/mL penicillin and 100 μ g/mL streptomycin at 37°C in a humidified atmosphere of 5% CO₂ in air.

Construction of the IVL_{EHS}

The IVL_{EHS}, an *in vitro* liver model containing primary hepatocytes assembled around an endothelial cell network on the EHS gel, was constructed according to our previous study (Toyoda *et al.*, 2012). HUVECs (3.5×10^5 cells/well of 6-well plates) were seeded on the EHS gel, and the endothelial cell network was obtained after 3 hr. Four hours after HUVEC seeding, freshly isolated mouse primary hepatocytes (1.0×10^6 cells/well of 6-well plates) were seeded on the HUVEC network structure in 10% FBS-containing medium and allowed to attach; after 4 hr, the medium was replaced with fresh medium. The experiments were started 24 hr after seeding (time 0), when almost all primary hepatocytes completed the migration toward the network and piled up. At that time, medium was changed to fresh one with or without 10 mM acetaminophen, since the previous study showed that this dose of acetaminophen was enough to induce hepatotoxicity in the IVL_{EHS} (Toyoda *et al.*, 2012). Additionally, these common experimental conditions allowed us to interpret the obtained data based on the previous study. Culture medium collected at each time point was centrifuged for 5 min at $300 \times g$ to remove floating cells, and supernatant was stored at -80°C until use.

Albumin detection by ELISA

Mouse albumin in culture medium was detected using the Mouse Microalbuminuria ELISA kit (Albuwell M; Exocell, Philadelphia, PA, USA) according to the manufacturer's introduction. Briefly, 50 μ L of culture supernatant and equal volume of rabbit anti-mouse albumin antibody solution were added to the albumin-coated test plate for 30 min at room temperature. After washing 10 times with rinse solution, an anti-rabbit HRP-conjugated antibody (100 μ L per well) was added for 30 min. After washing 10 times, 100 μ L of Color Development solution per well was added for 5 min; the chromogenic reaction was stopped by an equal volume of Color Stopper solution and evaluated at 450 nm using the iMark™ Microplate Reader. Albumin concentration in each sample was calculated according to a standard curve.

Urea assay

Urea concentration in culture medium was determined by using the QuantiChrom™ Urea Assay Kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instruction. In brief, 5 μ L of culture medium was incubated with 200 μ L of the reaction mixture for 30 min at room temperature, and urea-dependent chromogenic reaction was evaluated at 492 nm using an iMark™ Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Urea concentration in each sample was calculated based on a standard curve constructed using known concentrations of urea.

CE-TOFMS analysis

Intracellular metabolites were analyzed 12 hr after IVL_{EHS} construction the capillary electrophoresis (CE)-time of flight-mass spectrometry (TOFMS) method as described previously (Soga *et al.*, 2006). Briefly, cells on the EHS gel were washed twice, treated with 5% mannitol, and saturated with 1 mL of MeOH containing 25 μ M of each internal standard (MES and CSA) for 10 min; the extraction was performed at 4°C to minimize sample degradation and halt potential enzymatic reactions during sample preparation. The resulting solution was immediately mixed with 400 μ L CHCl₃ and 200 μ L water, and centrifuged at $10,000 \times g$ for 3 min at 4°C. Subsequently, the 400- μ L upper aqueous layer was filtered through a Millipore 5-kDa cutoff filter by centrifugation to remove proteins. The filtrate was lyophilized and dissolved in 50 μ L water containing reference compounds (3-aminopyrrolidine and Trimesate) prior to CE-TOFMS analysis. The extracts were obtained from the IVL_{EHS} and hepatocyte monocultures grown on the EHS gel (control), which were treated or not with acetami-

nophen (10 mM) for 12 hr.

CE-TOFMS was performed using an Agilent CE capillary electrophoresis system, Agilent G3250AA LC/MSD TOF system, Agilent1100 series binary HPLC pump, G1603A Agilent CE-MS adapter, and G1607A Agilent CE-ESI-MS sprayer kit (Agilent Technologies, Waldbronn, Germany) according to previous studies (Soga *et al.*, 2002, 2003, 2006, 2009; Hirayama *et al.*, 2009). For system control and data acquisition, we used the G2201AA Agilent ChemStation software for CE, and the Analyst QS software for TOFMS.

Data normalization

The levels of metabolites in each sample were normalized to that of mouse albumin in culture medium at the time of metabolite extraction for CE-TOFMS analysis according to previous study (Tsutsui *et al.*, 2006), since the amount of cellular protein could not be determined because of the presence of the EHS gel. Our previous study demonstrated that albumin expression levels in primary hepatocytes were hardly affected by the presence of EHS gel and/or HUVECs 48 hr after the seeding (Toyoda *et al.*, 2012). Thus, we focused on the mouse albumin level in the culture medium as a detectable indicator of the number of hepatocytes in each culture system. Furthermore, our previous study showed that LDH levels in HUVECs constituted < 5% of total LDH in the IVL_{EHS}, suggesting that HUVECs did not significantly contribute to the total extracts of the IVL_{EHS} (Toyoda *et al.*, 2012). Hence, we could reliably compare the relative levels of metabolites in IVL_{EHS} hepatocytes with those in hepatocyte monocultures.

Statistical analysis

All statistical analyses were performed by using EXCEL (Microsoft Corp., Redmond, WA, USA) with the Statcel3 add-in software (OMS Publishing Inc., Saitama, Japan). Differences between samples were analyzed by Student's *t* test, and considered statistically significant at the *P* value less than 0.05 and 0.01.

RESULTS AND DISCUSSION

Albumin levels in IVL_{EHS} culture medium

To construct the IVL_{EHS} model, we seeded mouse primary hepatocytes on the EHS gel with or without HUVEC network. After IVL_{EHS} construction, the cells were cultured for further 12 hr in the absence or presence of acetaminophen to induce hepatic injury, then the cellular extracts were collected. At the sampling time point for metabolome analysis, mouse albumin concentration

in culture medium was measured in each acetaminophen condition. In the absence of acetaminophen, there was no difference in medium albumin levels between hepatocytes cultured with or without HUVECs (1.56 ± 0.17 versus 1.55 ± 0.13 $\mu\text{g/mL}$, respectively), which is consistent with comparable albumin expression in hepatocytes cultured on the EHS gel with or without HUVECs for 48 hr (Toyoda *et al.*, 2012). On the other hand, in the presence of acetaminophen, albumin levels in culture medium of the IVL_{EHS} (0.53 ± 0.07 $\mu\text{g/mL}$) were significantly lower than those of mono-cultured hepatocytes on EHS gel (0.66 ± 0.10 $\mu\text{g/mL}$), suggesting higher sensitivity of hepatocytes in the IVL_{EHS} to acetaminophen-induced injuries. Considering that acetaminophen overdose has the potential for causing hepatotoxicity and acute liver failure *in vivo* (Lancaster *et al.*, 2015), together with the previously determined higher cytochrome P450 activities in the IVL_{EHS} (Toyoda *et al.*, 2012), the IVL_{EHS} should be superior to the monoculture on EHS gel in keeping hepatocytes in a state close to the those *in vivo*.

Metabolites in the urea cycle

In a previous study, the urea levels in culture medium of IVL_{EHS}, total amount of cell-secreted urea, was only examined (Toyoda *et al.*, 2012); therefore, in this study, we first focused on the major intracellular metabolites of the urea cycle. The levels of intracellular urea, ornithine, citrulline, aspartate, fumarate, and arginine were determined, whereas the levels of carbamoyl phosphate and arginosuccinate were below the detection limit (Fig. 1a). The urea cycle is regulated by carbamoyl phosphate synthetase 1 (CPS-1), a rate-limiting enzyme allosterically activated by *N*-acetylglutamate (NAG). Therefore, we examined NAG levels in each culture condition. In the absence of acetaminophen, no significant differences were observed in urea cycle metabolites between the IVL_{EHS} and monoculture on the EHS gel (control) (Fig. 1a), which was in agreement with similar concentrations of urea in the medium of the IVL_{EHS} and control cultures (Fig. 1b). In addition, the detected urea concentrations in the culture medium of IVL_{EHS} were comparable with those that were detected in our previous study (Toyoda *et al.*, 2012), suggesting that IVL_{EHS} was well-constructed in the present study. Furthermore, NAG levels were almost the same between the IVL_{EHS} and control. These data suggest that the construction of the IVL_{EHS} did not affect urea metabolism in hepatocytes. Similarly, no significant differences in the urea cycle metabolites were observed between the IVL_{EHS} and control after acetaminophen treatment, with the exception of arginine (Fig. 1a). Regarding this point, we speculate that one

Fig. 1. Urea cycle metabolites in the IVL_{EHS} model and monocultures. **a.** Levels of major urea cycle metabolites in the IVL_{EHS} and control cultures with or without acetaminophen treatment. The y-axis shows relative metabolite levels (nmol/ μg of albumin). White and black columns indicate control and IVL_{EHS}, respectively. Non, no treatment; APAP, acetaminophen; BLOQ, metabolite level below the limitation of quantification. Data are expressed as the mean $\pm$ S.E.M. $n = 4$. **b.** Time-dependent increase of urea levels in hepatocyte culture medium of the IVL_{EHS} (with HUVEC) and monocultures (without HUVEC). Data are expressed as the mean $\pm$ S.D. $n = 3$. Statistical analyses for significant differences were performed according to Student's *t* test (*, $P < 0.05$; **, $P < 0.01$ compared to control). N.S., not significantly different among groups.

plausible explanation of the increase of arginine may be the suppression cytotoxic nitric oxide (NO) production, although we could not determine the NO levels and NO synthase activities in the IVL_{EHS} due to the technical limitations in the present study. However, considering that there was no significant difference in the NAcGlc levels between the IVL_{EHS} and control, it is unlikely that the construction of IVL_{EHS} could affect the overall activity of the urea cycle in IVL_{EHS} hepatocytes. In this context, the ability for ammonia detoxification in the IVL_{EHS} should

be the almost same as in hepatocyte monocultures, supporting that these two culture systems could have similar tolerance to exogenous hepatotoxic stimuli.

Adenylate energy charge and lactate/pyruvate ratio

We next compared the energy status in the two culture systems, IVL_{EHS} and monoculture on the EHS gel. First, we addressed the AEC by CE-TOFMS (Fig. 2a). The AEC is an important indicator of the energy state in the cells

Fig. 2. Energy and redox states in hepatocytes from the IVL_{EHS} and monocultures. Hepatocytes cultures were treated or not with 10 mM acetaminophen (APAP) for 12 hr. **a.** Adenylate energy charge (AEC). **b.** Lactate/pyruvate (L/P) ratio. White and black columns indicate control and IVL_{EHS}, respectively. Data are expressed as the mean $\pm$ S.E.M. $n = 4$. Statistical analyses for significant differences were performed according to Student's *t* test (*, $P < 0.05$; **, $P < 0.01$ compared to control). N.S., not significantly different among groups.

(Atkinson, 1968; Hirayama *et al.*, 2009), defined by the following equation; $([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP])$; accordingly, the AEC values around 1 indicate cell health, while those close to zero suggest cell damage and/or death (Zhang and Vertes, 2015). The results showed that in the absence of acetaminophen, AEC values in the two culture systems were similar (0.815 ± 0.008 in monocultures and 0.822 ± 0.004 in the IVL_{EHS}, respectively) (Fig. 2a). Considering that the AEC value in the liver of rodents is about 0.8 (Chapman *et al.*, 1971), hepatocytes in the two groups were close to the normal physiological state. However, acetaminophen treatment decreased AEC values to 0.699 ± 0.089 in control (monocultures) and to 0.522 ± 0.062 in the IVL_{EHS} (Fig. 2a), indicating the presence of hepatic injury. The decrease of AEC was more significant in the IVL_{EHS}, which should reflect higher sensitivity of the IVL_{EHS} to acetaminophen compared with hepatocyte monocultures.

We then evaluated the ratio of free NAD to NADH (NAD/NADH) indicative of cellular redox status (Williamson *et al.*, 1967), by measuring the lactate/pyruvate (L/P ratio). In the absence of acetaminophen, there was no significant difference in the L/P ratio between the IVL_{EHS} and control cultures (Fig. 2b), suggesting that IVL_{EHS} construction did not negatively affect the redox status in hepatocytes. The balance between free NAD and NADH in the cytosol plays a pivotal role in the regulation of cellular redox homeostasis and, thus, could be used to assess cellular metabolic state (Sun *et al.*, 2012; Debray *et al.*, 2007; Lin and Guarente, 2003). However, there are

technical difficulties in distinguishing between protein-bound and free NAD/NADH; therefore, it has been generally accepted that free NAD/NADH should be estimated based on the L/P ratio according to the principle of chemical equilibrium (pyruvate + NADH $\leftrightarrow$ lactate + NAD) (Debray *et al.*, 2007; Williamson *et al.*, 1967). For example, when cellular respiration is impaired, pyruvate oxidation is decreased and the reduced form of redox coenzyme NADH would be predominant (Debray *et al.*, 2007), i.e., the ratio of L/P would increase and that of NAD/NADH decrease. Consistent with this context, acetaminophen treatment increased the L/P ratio both in the IVL_{EHS} and in control cultures (Fig. 2b); however, the increasing effect was lower in the IVL_{EHS} than in control. While the IVL_{EHS} was more sensitive to acetaminophen than monoculture control in terms of energy status (Fig. 2a), these results imply that the construction of the IVL_{EHS} could enhance molecular mechanism(s) involved in the maintenance of cellular redox potential in response to exogenous damage.

Metabolites of the glycolysis and TCA pathways

We focused on glycolysis and the TCA cycle, which are major pathways regulating cellular energy metabolism. The pathways and relative levels of their major metabolites are presented in Fig. 3. In the absence of acetaminophen, there was no significant difference in the levels of most metabolites between the IVL_{EHS} and control, suggesting comparable ability for energy generation, which is consistent with similar AEC levels (Fig. 2a). Among

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Fig. 3. Effect of acetaminophen on the levels of essential amino acids and major metabolites in the glycolysis and TCA pathways in the IVL_{EHS} and monocultures. The y-axis shows relative metabolite levels (nmol/μg of albumin). White and black columns indicate control and IVL_{EHS}, respectively. Non, no treatment; APAP, acetaminophen; BLOQ, metabolite level below the limitation of quantification. Data are expressed as the mean ± S.E.M. *n* = 4. Statistical analyses for significant differences were performed according to Student's *t* test (*, *P* < 0.05 compared to control).

the TCA intermediates, an exception was 2-oxoglutarate (also known as α -ketoglutarate) whose level was higher in the IVL_{EHS} than in control cultures, and difference was even more pronounced after acetaminophen treatment (Fig. 3). Considering the role of 2-oxoglutarate as an antioxidant involved in reactive oxygen species (ROS) scavenging (Mailloux *et al.*, 2007), the increase in 2-oxoglutarate levels could be attributed to increased tolerance of hepatocytes in the IVL_{EHS} to oxidative stress (Fig. 2b).

Recent studies show that the activation of autophagy protects against acetaminophen-induced hepatotoxicity *in vivo* (Lin *et al.*, 2014; Igusa *et al.*, 2012; Ni *et al.*, 2012); therefore the pool of amino acids should be increased in the presence of acetaminophen as a result of autophagy-dependent proteolysis, which should contribute to cellular energy balance. Consistently, the increase in amino acid levels after acetaminophen treatment tended to be higher in the IVL_{EHS} than in control cultures (Fig. 3), suggesting that the IVL_{EHS} may be superior to hepatocyte monocultures in reproducing the physiological conditions characteristic for the liver.

Bile acids

Bile acid secretion is one of the important functions of the liver. Therefore, we examined the effect of IVL_{EHS} construction on the synthesis of bile acids by ana-

lyzing the primary bile acid cholate and two conjugated bile acids, glycocholate and taurocholate. Although the levels of cholate and glycocholate in cell extracts were below the detection limit, taurocholate was identified and its production in the absence of acetaminophen treatment was higher in the IVL_{EHS} than in control cultures (Fig. 4). This finding shows a possibility that IVL_{EHS} system would contribute to maintain some metabolic functions in hepatocytes that decline *in vitro* even on the EHS gel. Our previous study revealed that bile canalicular membrane transporters involved in biliary excretion of hepatic metabolites showed higher or similar expression in the IVL_{EHS} compared to hepatocyte monocultures on the EHS gel (Toyoda *et al.*, 2012). Hence, the higher levels of intracellular taurocholate in the IVL_{EHS} should be due to relatively increased/maintained taurocholate synthesis rather than decreased excretion. This interpretation is supported by the higher activities of major cytochrome P450 isozymes in IVL_{EHS} as compared with those in monoculture control (Toyoda *et al.*, 2012), since such enzyme-mediated oxidation is involved in the bile acid synthesis in the liver.

Further consideration

In the present study, we compared the metabolic activity of hepatocytes in the IVL_{EHS} and in monocultures on the EHS gel by analyzing the urea cycle, glycolysis, the TCA pathway, and production of essential amino acids. To the best of our knowledge, this is the first metabolomics study on the IVL_{EHS}. Since our study was limited in the metabolomics of cellular extracts that were prepared 36 hr after the hepatocytes seeding, the long-term effects of the IVL_{EHS} construct on the metabolic profile of hepatocytes should be addressed in future investigation. However, our results could support the utility of IVL_{EHS} in the short-term culture experiments. Although all metabolites were not quantified completely because of the detection limit, our results showed that the metabolic profile of hepatocytes was not affected by the IVL_{EHS} construction; moreover, the IVL_{EHS} rather supported hepatocyte metabolism in the presence of exogenous stress. These findings are in agreement with our previous studies on the mouse embryonic stem cell-derived liver tissue model consisting of a hepatocyte layer and surrounding sinusoid-like vascular endothelial cells (Tamai *et al.*, 2013; Tsutsui *et al.*, 2006; Ogawa *et al.*, 2005), and support the pivotal role of the intercellular communication between hepatocytes and endothelial cells in the functional activity of hepatocytes.

Prior work has documented the effectiveness of IVL_{EHS} containing murine hepatocytes and HUVEC network in the maintenance of hepatic functions (Toyoda *et*

Fig. 4. Taurocholate levels in the IVL_{EHS} and monocultures. Hepatocytes cultures were treated or not with 10 mM acetaminophen (APAP) for 12 hr and analyzed for intracellular taurocholate levels. Data are expressed as the mean $\pm$ S.E.M. $n = 4$. Except for the IVL_{EHS} (with HUVECs) in the absence of acetaminophen treatment, each group contained the data which was below the limitation of quantification and thereby treated as zero. Statistical analyses for significant differences were performed according to Student's *t* test (*, $P < 0.05$ compared to control).

al., 2012). In this study we carried out the metabolomics of IVL_{EHS} that was constructed by the same experimental procedure to extend our understanding about the biochemical features of this new culture system. On the other hand, this approach did not allow us to avoid an experimental mismatch in host species of the utilized cells in the IVL_{EHS}. Future study should therefore include the analysis of monospecific IVL_{EHS}.

In conclusion, our data strongly suggest that the IVL_{EHS} construct could contribute to the maintenance of hepatocyte functions without negative effects on their metabolic status, and would be a better model than the existing *in vitro* monoculture systems, both for investigating physiological mechanisms in the liver and for compound hepatotoxicity screening. Further transcriptomic and proteomic studies are required to confirm the utility of the IVL_{EHS} system as a reliable *in vitro* model of the liver.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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