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ORIGINAL ARTICLE

In vitro recapitulation of the urea cycle using murine embryonic stem cell-derived in vitro liver model

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Abstract Ammonia, a toxic metabolite, is converted to urea in hepatocytes via the urea cycle, a process necessary for cell/organismal survival. In liver, hepatocytes, polygonal and multipolar structures, have a few sides which face hepatic sinusoids and adjacent hepatocytes to form intercellular bile canaliculi connecting to the ductules. The critical nature of this three-dimensional environment should be related to the maintenance of hepatocyte function such as urea synthesis. Recently, we established an in vitro liver model derived from murine embryonic stem cells, IVL^{mES}, which included the hepatocyte layer and a surrounding sinusoid vascular-like network. The IVL^{mES} culture, where the hepatocyte is polarized in a similar fashion to its in vivo counterpart, could successfully recapitulate in vivo results. L-Ornithine is an intermediate of the urea cycle, but supplemental L-ornithine does not activate the urea cycle in the apolar primary hepatocyte of monolayer culture. In the IVL^{mES}, supplemental L-ornithine could activate the urea cycle, and also protect against ammonium/alcohol-induced hepatocyte death. While the IVL^{mES} displays architectural and functional properties similar to the liver, primary hepatocyte of monolayer culture fail to model critical functional aspects of liver physiology. We propose that the IVL^{mES} will represent a useful, humane alternative to animal studies for drug toxicity and mechanistic studies of liver injury.

Keywords Embryonic stem cell · Liver · Metabolism · Cell polarity · Urea cycle · Ornithine

Abbreviations

ES	Embryonic stem
IVL^{mES}	Murine embryonic stem cell-derived in vitro liver
OTC	Ornithine transcarbamylase
CPS1	Carbamoyl phosphate synthetase 1
ARG1	Arginase 1
ORNT1	Ornithine/citrulline transporter 1
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
LDH	Lactate dehydrogenase

Introduction

Ammonia, a primary nitrogenous by-product of protein metabolism, must be immediately removed from the circulation to avoid cell/organismal death. In ureotelic organisms, the ammonia in the mitochondria of hepatocytes is converted to urea via the urea cycle. This pathway was discovered by Krebs and Henseleit (1932). L-Ornithine, as well as L-citrulline and L-arginine, is an intermediate of the urea cycle, and it has been reported that the rate of urea formation from ammonia was greatly accelerated by adding any one of these three α-amino acids using thin slices of liver suspended in a buffered aerobic medium.

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Each of them stimulated urea synthesis to a far greater extent than any of the other common nitrogenous compounds tested (Lehninger et al. 1993). Recently, L-arginine has been reported to stimulate protein synthesis, and to show cytoprotective effects using in vitro model, supporting the results shown in animal models (Kong et al. 2012; Zeng et al. 2012). On the other hand, L-ornithine has been reported to decrease blood ammonia concentration in human and rats (Demura et al. 2010; Cutinelli et al. 1970; and Vogels et al. 1997); however, there has been no report that supplemental L-ornithine can activate the urea cycle in vitro.

We hypothesized that culture models might be insufficient to monitor the urea cycle because it has been shown that primary hepatocyte cultures lack some hepatic functions (Toyoda et al. 2012). Recently, we described the recapitulation of hepatic organogenesis from murine embryonic stem (ES) cells (Ogawa et al. 2005). This murine ES cell-derived in vitro liver model, termed IVL^{mES}, includes both the hepatocyte layer and the accompanying sinusoid vascular-like network, and displays cytochrome P450 activities (Tsutsui et al. 2006). Here, we demonstrated the hepatoprotective effect of L-ornithine using the in vitro IVL^{mES}, but failed to show effects of L-ornithine using apolar primary hepatocyte of monolayer cultures. We conclude that L-ornithine does indeed mediate protective effects on the liver, and that the IVL^{mES} culture system is poised to replace animal models of liver function.

Materials and methods

Reagents

L-Ornithine hydrochloride (Kyowa Hakko Bio, Tokyo, Japan) and ammonium chloride (Sigma–Aldrich, Tokyo, Japan) were dissolved in PBS (100 mM and 4 M) and sterilized by filtration.

Animals

Eight-week-old male BALB/cA Jcl mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). All animal studies have been approved by the Animal Experimentation Committee of Tokyo Institute of Technology.

Isolation of primary hepatocytes from murine liver

Hepatocytes were prepared from anesthetized BALB/cA mice by an in situ two-step collagenase perfusion method (Seglen 1976), with slight modifications. Briefly, murine liver was pre-perfused in situ with Hank's Buffered Salt Solution (HBSS) containing 0.5 mM EGTA. Next, the liver was perfused with 0.015 % collagenase in HBSS. Then, the liver was removed, and the cells were dispersed in ice-cold HBSS without EGTA. The cells obtained were filtered through a 100 μm pore mesh nylon cell strainer (BD Biosciences, MA) and centrifuged twice for 2 min at $500\times g$ to remove non-parenchymal cells. The remaining cells were centrifuged for 2 min at $500\times g$, and then subjected to a 40 % Percoll density gradient centrifugation for 10 min at $1{,}200\times g$. At this stage, cell viability as measured by trypan blue was >90 %. The isolated hepatocytes were plated at a density of 3.0×10^5 cells per well in 12-well plates. Cells were grown in Williams' E Medium containing 10 % (v/v) heat-inactivated FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified incubator with 5 % CO_2. The medium was replaced after the first 4 h of incubation, and was replaced daily thereafter.

Establishment of in vitro liver model on EHS gel

Formation of in vitro liver model on EHS gel as previously reported (Toyoda et al. 2012). Briefly, HUVECs (0.4×10^6 cell/well in 6-well plates), a representative type of endothelial cells, were seeded on EHS gel. On EHS gel, HUVECs rapidly elongated and generated a network structure. On this network structure of HUVECs, freshly isolated mouse primary hepatocytes (1.0×10^6 cell/well in 6-well plates) were seeded and cultured. The primary hepatocytes migrated toward the HUVEC network and piled next to one another within 24 h, forming a structure that resembled hepatic tissue. This in vitro liver tissue model on EHS gel, which we term IVL_{EHS}, was used in the following experiments.

Culture and differentiation of murine ES cells

The murine ES cell line, ST1, originally established from the BALB/cA strain, was grown on feeder layers of mitomycin C-treated murine embryonic fibroblasts. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Tokyo, Japan) containing 15 % fetal bovine serum (FBS) (Nichirei Biosciences, Tokyo, Japan), 1 mM sodium pyruvate (Invitrogen), 100 μM nonessential amino acids (Invitrogen), 100 μM 2-mercaptoethanol (Sigma-Aldrich Japan, Tokyo, Japan), and 10^3 U/mL leukemia inhibitory factor (LIF) (Chemicon International, CA).

The IVL^{mES} was differentiated from murine ES cells as previously described (Ogawa et al. 2005). Briefly, murine ST1 ES cells were dissociated with 0.25 % trypsin and re-suspended in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen) containing 15 % FBS, 1 mM sodium pyruvate, 100 μM nonessential amino acids, and 100 μM 2-mercaptoethanol without LIF. The suspension was then

formed into a hanging drop at a concentration of 1,000 cells per 50 μL. Cells were cultured in an atmosphere containing 5 % CO_2 at 37 °C for 5 days. Under these conditions, cells formed embryoid bodies (EBs). Fifty EBs were plated in each well of a gelatin-coated 6-well plate; the day of plating was denoted as day 0 (A0). In this study, IVL^{mES} were used at A18.

RNA extraction and reverse transcription-polymerase chain reaction analysis

Total RNA was prepared by the acid guanidinium isothiocyanate-phenol–chloroform-isoamyl alcohol method. Total RNA samples (5 μg) were reverse transcribed using a Superscript II first-strand synthesis system (Invitrogen) with an oligo (dT) primer (Invitrogen). PCR was performed using Ex Taq DNA polymerase (TAKARA BIO, Tokyo, Japan) as previously reported (Tamai et al. 2011) with primer sets as shown in Table 1.

Quantification of urea and ammonia concentrations

Culture medium collected during each measurement period was centrifuged for 5 min at 300×*g* to remove cell debris, and the supernatant was stored at −80 °C until use. Urea was detected in the culture medium using a QuantiChrom™ Urea Assay Kit (BioAssay Systems, CA). Ammonia was detected in the culture medium using the Ammonia Test Wako (Wako, Osaka, Japan). Absorbance of the reactions (490 nm for urea; 630 nm for ammonia) was measured using an iMark™ Microplate Reader (Bio-Rad Laboratories, CA). Standard curves were used to calculate the urea and ammonia concentrations in each sample. Urea production, expressed per albumin positive cell number (1.2×10^6) and per 24 h. Albumin positive cell number of the IVL^{mES} was calculated by Immunohistochemical analysis.

Quantification of released liver enzyme activities

After the indicated time periods, culture medium was collected and centrifuged for 5 min at 300×*g*. Then, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) activities in the supernatant were assessed using SPOTCHEM™ EZ SP-4430 and SPOTCHEM™ II LDH (ARKRAY, Kyoto, Japan).

Cell viability assay

The viability of cells was evaluated using a Cell Counting Kit (Dojindo Laboratories, Kumamoto, Japan) or with WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H* tetrazolium, monosodium salt (Dojindo, Tokyo, Japan). The reduction of WST-8 was measured photometrically using an iMark™ Microplate Reader at 450 nm.

Immunohistochemical analysis

IVL^{mES} were cultured on gelatin-coated glass coverslips in 6-well plates, and then fixed with 4 % paraformaldehyde/PBS for 10 min, and permeabilized with 0.1 % Triton X for 5 min at room temperature (RT). Then, the sample was incubated in Blocking One (Nacalai Tesque, Kyoto, Japan) for 30 min, followed by incubation with the primary and secondary antibodies for 2 and 1 h, respectively, at RT. The antibodies included: goat anti-pecam1 (1:200; Santa Cruz Biotechnology, CA); Alexa Fluor 488 donkey anti-goat IgG (1:2000; Invitrogen); rabbit anti-albumin (1:200; Santa Cruz Biotechnology); and Alexa Fluor 594 donkey anti-rabbit IgG (1:2000; Invitrogen). The specimens were mounted in Prolong Gold fluorescent mounting medium and observed using a fluorescence microscope (Olympus, Tokyo, Japan).

Statistical evaluation

The results are expressed as the mean ± SEM. The Student's *t* test was used for the comparison of data from the two groups. The difference between groups was considered significant when $P < 0.05$.

Table 1 RT-PCR primer sequences

Gene name	Forward (5′–3′)	Reverse (5′–3′)
Carbamoyl phosphate synthetase 1 (*Cps1*)	TGCCAATGTGACTACGAAGC	AAATTGCAGGGACCTTTTCC
Arginase, liver (*Arg1*)	TCACCTGAGCTTTGATGTCG	TTACCCTCCCGTTGAGTTCC
Ornithine transcarbamylase (*Otc*)	GAAAGGGTCACACTTCTGTGG	GAGCAAAGCCTGTTTCTGTGG
Ornithine transporter 1 (*Ornt1*)	GTGGTCCGTAAAGTGGTTGG	TGAGAGCCCATGGTAGAAGC
Albumin	CAGGATTGCAGACAG	GCTACGGCACAGTGC
Hypoxanthine-guanine phosphoribosyltransferase (*hprt*)	AGCTTTACTAGGCAGATGGC	GTAATGATCAGTCAACGGGG

Results

L-Ornithine enhances urea production in in vitro liver tissue model, but not in cultured primary hepatocytes

Released hepatic enzymes, such as ALT, AST, and LDH, were measured in primary hepatocyte medium 24 h after the addition of NH_4^+ at a variety of doses. The activity levels of the three enzymes increased in a dose-dependent manner in response to NH_4^+ (Fig. 1a). By contrast, cell viability, as determined by WST-8 activity levels, decreased in a NH_4^+ dose-dependent manner (Fig. 1b). Urea production in the cultures increased with the addition of NH_4^+ up to 10 mM, but decreased at NH_4^+ concentrations exceeding 20 mM NH_4^+, i.e., NH_4^+-induced cell death at high concentrations (Fig. 1c). Therefore, to test the protective effects of L-ornithine, we used NH_4^+ concentrations of 20 mM or more in the subsequent experiments.

If NH_4^+ could activate the urea cycle in cultured primary hepatocytes, we would expect to see suppression of ammonia-induced cell death in the cultures. L-Ornithine was added to the medium of the primary hepatocytes or HUVEC co-cultured on type I collagen or EHS gel-coated plastic dishes and IVL_{EHS}. In the case of monolayer culture on type I collagen, no increase in urea production was observed in the culture medium (Fig. 1d). In contrast, the increase in urea levels was detected in the IVL_{EHS} (Fig. 1e), but the signal was not robust enough for the method to be used as a model system for this study.

An in vitro liver model derived from murine ES cells, IVL^{mES}, was prepared from the embryonic stem cell line, ST1, which was originally established from BALB/cA

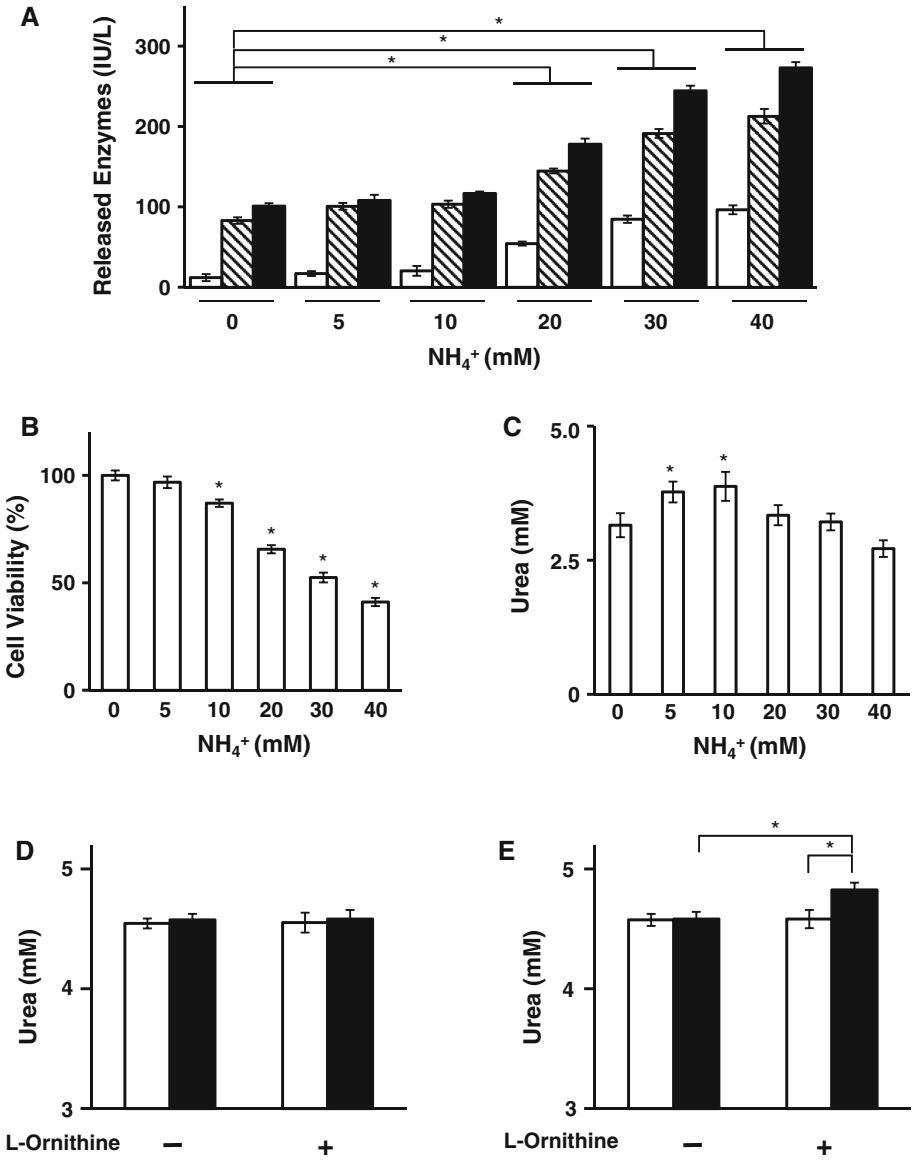

Fig. 1 Ammonia sensitivity of primary hepatocyte cultures. **a** Activity levels of released liver enzymes: alanine aminotransferase (ALT, *white bars*); aspartate aminotransferase (AST, *hatched bars*); and lactate dehydrogenase (LDH, *black bars*). **b** Cell viability, as assessed by the WST-8 assay, of primary hepatocyte cultures 24 h after the addition of 0–40 mM NH_4^+. **c** Urea concentrations in the medium of primary hepatocyte cultures 24 h after the addition of 0–40 mM NH_4^+. **d**, **e** Urea production in the medium of primary hepatocytes, cultured on type I collagen-and EHS gel-coated plastic dishes or IVL_{EHS} and IVL^{mES}, 24 h after the addition of 0–100 μM L-ornithine. All data are expressed as the mean ± SE; $*P < 0.05$

mice. We previously demonstrated that the IVL^{mES} hepatocytes become polarized and express functional transporter proteins (Ogawa et al. 2005; Tsutsui et al. 2006). L-Ornithine was added to the IVL^{mES} culture medium, and the concentrations of urea in the medium were measured after 24 h. Urea production increased with respect to the amount of L-ornithine added to the culture (Fig. 2a).

L-Ornithine protects against ammonium-induced hepatocyte death in the IVL^{mES}

To assess whether L-ornithine was protective against NH_4^+-induced hepatocyte death in the IVL^{mES}, L-ornithine was added to the culture medium in the presence of NH_4^+. The expression of urea cycle-related genes was observed in the IVL^{mES} cells, even after addition of NH_4^+ (Fig. 2b). NH_4^+ decreased cell viability in a dose-dependent fashion in the absence, but not the presence, of L-ornithine (Fig. 2c). In particular, at doses of 30–40 mM NH_4^+, cell viability levels were significantly higher in the presence of L-ornithine as compared to its absence. Because NH_4^+ conversion to urea was enhanced by the addition of L-ornithine in the IVL^{mES} (Fig. 2d), hepatocytes in this culture were protected against NH_4^+-induced cell death.

The protective effect of L-ornithine against ethanol-induced hepatocyte death is observed in the IVL^{mES}, but not in primary hepatocyte cultures

Released hepatic enzymes were measured in the medium of primary hepatocytes 24 h after the addition of ethanol (Fig. 3). The levels of the released hepatic enzymes increased in a dose-dependent manner with increasing levels of ethanol (Fig. 3a), suggesting that ethanol had a dose-dependent effect on cell death. Accordingly, we observed a decrease in cell viability after the same treatments (Fig. 3b). Concentrations of urea and NH_4^+ were measured in the medium of primary hepatocyte cultures 24 h after the addition of ethanol. Treatment with the alcohol decreased urea production (Fig. 3c) and increased the release of NH_4^+ (Fig. 3d) from the hepatocytes. These results indicated that culturing cells in a minimum of 3 % ethanol-induced hepatocyte death; therefore, 3 % ethanol was used in the subsequent experiments.

Next, because L-ornithine could suppress NH_4^+-induced hepatocyte death in the IVL^{mES}, we tested its effects on ethanol-induced hepatocyte death in the same model. Ethanol was added at 3 % to the IVL^{mES} culture medium to induce hepatocyte death. Accordingly, cell viability was decreased, but could be rescued by addition of L-ornithine

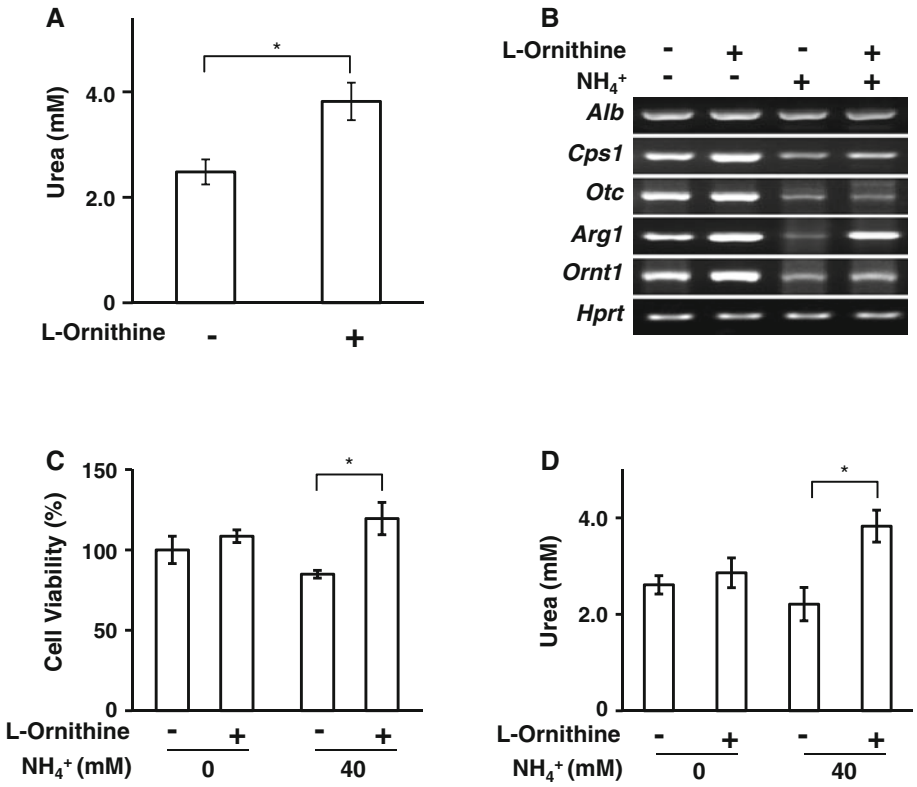

Fig. 2 Protective effects of L-ornithine against NH_4^+-induced cytotoxicity in the murine ES cell-derived liver tissue model, IVL^{mES}. **a** Urea production in IVL^{mES} culture medium 24 h after the addition of 0–100 μM L-ornithine. **b** Expression of albumin and urea cycle-related genes by IVL^{mES} cells 24 h after the addition of 0–100 μM L-ornithine ±40 mM NH_4^+. **c** Cell viability was measured 24 h after the addition of 0–40 mM NH_4^+ ± 100 μM L-ornithine. **d** Urea production in IVL^{mES} culture medium 24 h after the addition of 0 or 100 μM L-ornithine. Data are expressed as the mean ± SE; $^*P < 0.05$

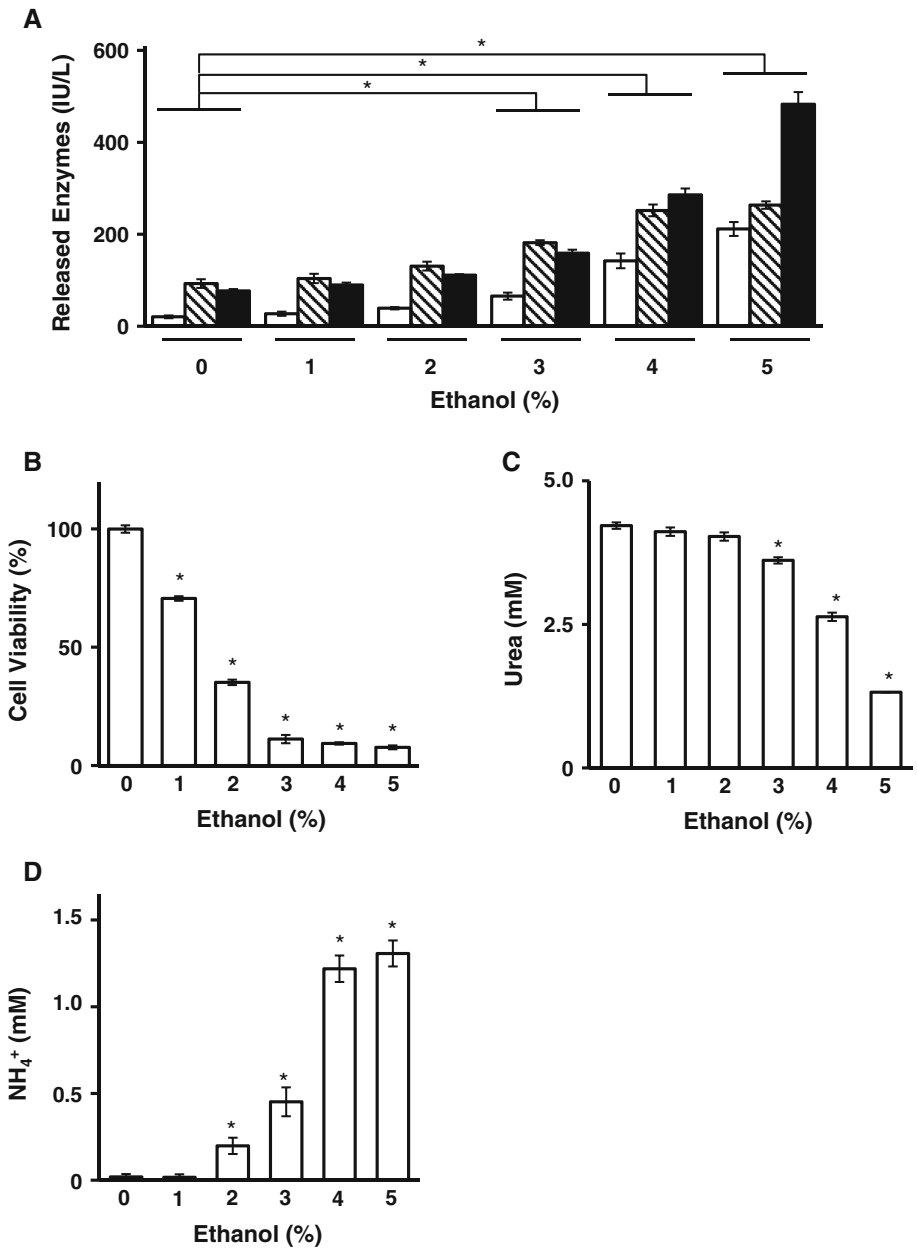

Fig. 3 Ethanol sensitivity of primary hepatocyte cultures. **a** Activity levels of released liver enzymes: alanine aminotransferase (ALT, *white bars*); aspartate aminotransferase (AST, *hatched bars*); and lactate dehydrogenase (LDH, *black bars*). **b** Cell viability, **c** urea production, and **d** ammonia degradation in primary hepatocyte culture medium 24 h after the addition of ethanol. Data are expressed as the mean ± SE; $*P < 0.05$

(Fig. 4a). Concentrations of urea and NH_4^+ in the culture medium were increased and decreased, respectively (Fig. 4b, c), indicating that L-ornithine was protective against ethanol-induced cell death in the IVL^{mES}. Finally, the expression of urea cycle-related genes was also detected in IVL^{mES} cells exposed to ethanol alone or ethanol and L-ornithine (Fig. 4d).

Discussion

It is characteristic that a hepatocyte simultaneously plays a variety of roles in the liver, although other cells generally play a unique role. Cultures of murine primary hepatocytes, obtained by dissociation of the liver into isolated cells, have been used for more than 30 years (Rodriguez-Antona et al. 2000; Tirona et al. 2003), however, it is quite difficult to keep these functions in the in vitro culture. Although many in vitro assays using primary hepatocytes have been reported, when cultured alone, these cells are an imperfect model system to study liver functions in vitro, i.e., experimental results often vary from those obtained by in vivo animal experiments. One reason for this may be that primary hepatocytes cannot proliferate and retain cell type-specific functions when maintained as a monoculture in vitro (Braiterman and Hubbard 2010; Guguen-Guillouzo and Guillouzo 2010; Underhill et al. 2010). In this study, primary hepatocytes were prepared from male BALB/cA

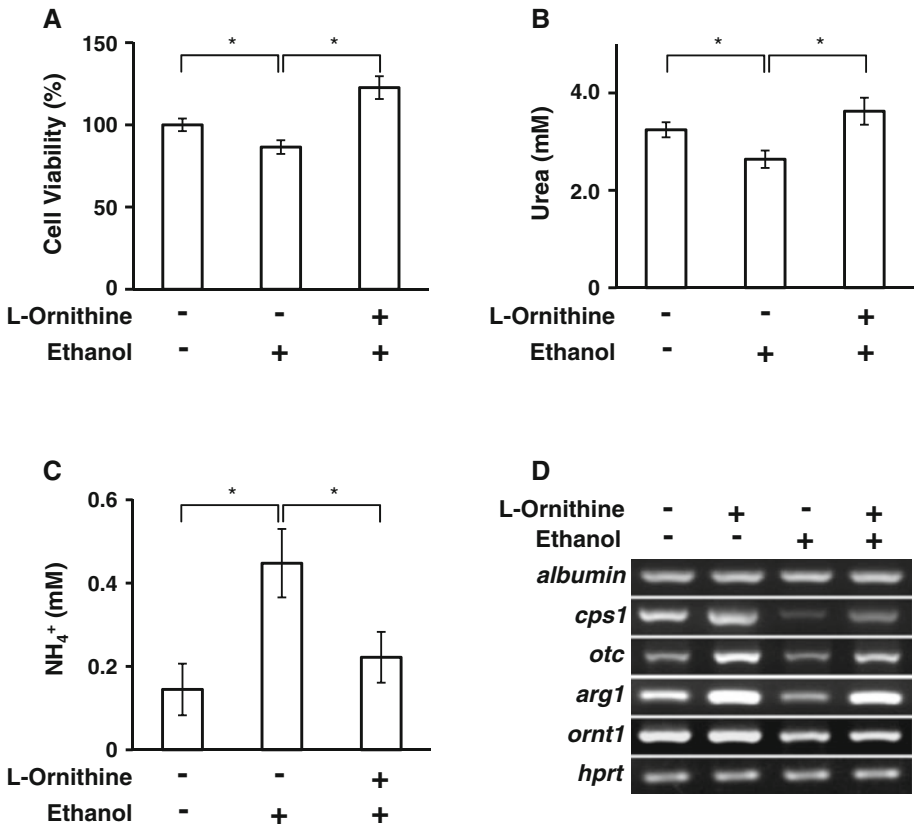

Fig. 4 Protective effects of L-ornithine against ethanol toxicity in the IVL^{mES} system. **a** Cell viability, **b** urea production, and **c** ammonium degradation in IVL^{mES} cells. Data are expressed as the mean ± SE; $*P < 0.05$. **d** Expression of albumin and urea cycle-related genes in IVL^{mES} cells 24 h after the addition of 0–100 μm L-ornithine and 0–3 % ethanol

mice, and cultured on type I collagen-coated plastic dishes. Urea production levels dramatically decreased day-by-day without any stimulation (Supplementary Fig. S1a). To investigate the cause of this reduction, the expression of urea cycle-related genes, such as *carbamoyl phosphate synthetase 1* (*cps1*), *ornithine transcarbamoylase* (*otc*), *arginase 1*(*arg1*), and *ornithine/citrulline transporter 1* (*ornt1*), was assessed by RT-PCR at 0, 1, 4, and 7 days of culture (Supplementary Fig. S1b). The expression of urea cycle-related genes decreased over time, and finally dropped below the limit of detection of the assay, corresponding to the observed decrease in urea production. These results suggested that cultured primary hepatocytes should be used within 1 day of preparation, a directive that we followed for all subsequent experiments in this study.

The liver is composed of parenchymal hepatocytes arranged in cords, and non-parenchymal cells, including sinusoidal endothelial cells forming sinusoidal tubes, and the immune cell population known as Kupffer cells. In particular, sinusoidal endothelial cells are important for the architecture of the liver. Polygonal and multipolar hepatocytes in the liver are surrounded by sinusoids (on their basal side), by the bile canaliculus (at their apical face), and by adjacent hepatocytes (Braiterman and Hubbard 2010). It is only in the context of this tissue architecture that hepatocytes can express their specific functions. Hepatocyte polarity also determines the location of hepatocyte organelles, such as the endoplasmic reticulum and Golgi (Braiterman and Hubbard 2010). For example, the Golgi is positioned between the nucleus and the apical surface. Specific transporters are also preferentially located at specific sides of the hepatocyte. Therefore, hepatocyte polarity exerts a major influence on the cell's physiology. Because primary hepatocytes cultured as a monolayer lose structural polarity, it is difficult to use the culture for assays of hepatic functions, e.g., drug metabolism. Therefore, we aimed to construct a sophisticated culture condition that mimics the situation in the liver, and examine its utility. In our previous reports, we showed that culture of hepatocytes in a model that resembles the structure of hepatic tissue is superior to monolayer culture of primary hepatocytes, both in regard to the maintenance of some hepatic genes and the response to xenobiotics (Toyoda et al. 2012). These findings suggest that this system could be applied to the evaluation of compound.

In the urea cycle, L-ornithine is transported from the cytosol into the mitochondrial matrix via a specific transport system, mitochondrial ORNT1 (Indiveri et al. 1992), and then is carbamylated to citrulline by OTC, which then is exported to the cytosol by ORNT1. Therefore, ORNT1 functions are crucial for hepatocytes to execute the urea cycle. However, in primary hepatocyte cultures, the

expression of the urea cycle-related genes, including *Otc* and *Ornt 1*, dramatically decreased (Supplementary Fig. S1b). As previously discussed, cell polarity affects the distribution of intracellular organelles, including mitochondria (Braiterman and Hubbard 2010), as well as the localization of transporter molecules. Changes in the position of these components likely play a role in the often large experimental gap between assays using primary hepatocyte cultures and animals. We demonstrated this concept by showing that the results we obtained with NH_4^+, addition of L-ornithine to cultures could not suppress the release of ALT, AST, and LDH (Supplementary Fig. S2a), nor maintain cell viability (Supplementary Fig. S1b) of primary hepatocytes monolayer cultured in the presence of 3 % ethanol. Urea and NH_4^+ production by primary hepatocytes was not changed by the addition of L-ornithine in the presence of 3 % ethanol (Supplementary Fig. S1c, d), suggesting that L-ornithine has no effect on primary hepatocytes cultured under these conditions. With regard to these concerns, the liver tissue model includes several improvements that better mimic the in vivo situation. Our system of IVL^{mES} exhibits higher expression of liver-specific genes, improved ammonia degradation (Ogawa et al. 2005), and greater cytochrome P450 activity (Tsutsui et al. 2006) as compared to primary hepatocyte cultures. MRP2 is an efflux transporter expressed on the apical membrane of polarized cells (Harris et al. 2001). We previously showed that the expression of the MRP2 gene was also confirmed in IVL^{mES} by RT-PCR (Tamai et al. 2011). Based on these findings, the IVL^{mES} is expected to be able to mimic in vivo liver function. In support of this proposal, the results of the IVL^{mES} experiment assessing the hepatoprotective abilities of L-ornithine (Fig. 3) were corresponding to those in (Vogels et al. 1997) report in that blood ammonia concentration in L-ornithine-treated rats decreased gradually. These results suggest that L-ornithine in the IVL^{mES} culture medium was incorporated into hepatocyte mitochondria by ORNT1, and then converted to citrulline. The IVL^{mES} represents the possibility for high-throughput drug screening, especially as compared to the liver perfusion system, which is highly labor intensive and expensive. Hepatocytes in the IVL^{mES} are polarized in a similar fashion to their in vivo counterparts, a feature that we hypothesize is critical for their maintenance of hepatocyte functional attributes. We believe that this model system has great promise to supplant animal experiments for drug toxicity studies and experiments dissecting mechanisms of liver injury.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Braiterman LT, Hubbard AL (2010) Hepatocyte surface polarity: its dynamic maintenance and establishment. In: Arias I (ed) The liver: biology and pathobiology, 5th edn. Wiley-Blackwell, New york, pp 73–106

Cutinelli L, Sorrentino L, Tramonti C, Salvatore F, Cedrangolo F (1970) Protection by ornithine-aspartate of the effects of physical exercise. Alzneimittel-Forschung 20:1064–1067

Demura S, Yamada T, Yamaji S, Komatsu M, Morishita K (2010) The effect of L-ornithine hydrochloride ingestion on performance during incremental exhaustive ergometer bicycle exercise and ammonia metabolism during and after exercise. Eur J Clin Nutr 64:1166–1171

Guguen-Guillouzo C, Guillouzo A (2010) General review on in vitro hepatocyte models and their applications. Meth Mol Biol 640:1–40

Harris MJ, Kuwano M, Webb M, Board PG (2001) Identification of the apical membrane-targeting signal of the multidrug resistance-associated protein 2 (MRP2/MOAT). J Biol Chem 276:20876–20881

Indiveri C, Tonazzi A, Palmieri F (1992) Identification and purification of the ornithine/citrulline carrier from rat liver mitochondria. Eur J Biochem 207:449–454

Kong X, Tan B, Yin Y, Gao H, Li X, Jaeger LA, Bazer FW, Wu G (2012) L-Arginine stimulates the mTOR signaling pathway and protein synthesis in porcine trophectoderm cells. J Nutr Biochem 23:1178–1183

Krebs HA, Henseleit K (1932) Untersuchengen über die Harntoffbildung im Tierkörper. Hoppe-Seyler's Z. fur Physiol Chem 210:33–66

Lehninger AL, Nelson DL, Cox MM (1993) Principles of biochemistry, 2nd edn. Worth, New York, p 1013

Ogawa S, Tagawa Y, Kamiyoshi A, Suzuki A, Nakayama J, Hashikura Y, Miyagawa S (2005) Crucial roles of mesodermal cell lineages in a murine embryonic stem cell-derived in vitro liver organogenesis system. Stem Cells 23:903–913

Rodriguez-Antona C, Jover R, Gomez-Lechon MJ, Castell JV (2000) Quantitative RT-PCR measurement of human cytochrome P-450 s: application to drug induction studies. Arch Biochem Biophys 376:109–116

Seglen PO (1976) Preparation of isolated rat liver cells. Meth Cell Biol 13:29–83

Tamai M, Yamashita A, Tagawa Y (2011) Mitochondrial development of the in vitro hepatic organogenesis model with simultaneous cardiac mesoderm differentiation from murine induced pluripotent stem cells. J Biosci Bioeng 112:495–500

Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, Parviz F, Duncan SA, Inoue Y, Gonzalez FJ, Schuetz EG, Kim RB (2003) The orphan nuclear receptor HNF4alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. Nat Med 9:220–224

Toyoda Y, Tamai M, Kashikura K, Kobayashi S, Fujiyama Y, Soga T, Tagawa Y (2012) Acetaminophen-induced hepatotoxicity in a liver tissue model consisting of primary hepatocytes assembling around an endothelial cell network. Drug Metab Dispos 40:169–177

Tsutsui M, Ogawa S, Inada Y, Tomioka E, Kamiyoshi A, Tanaka S, Kishida T, Nishiyama M, Murakami M, Kuroda J, Hashikura Y, Miyagawa S, Satoh F, Shibata N, Tagawa Y (2006) Characterization of cytochrome P450 expression in murine embryonic stem cell-derived hepatic tissue system. Drug Metabol Dispos Biol Fate Chem 34:696–701

Underhill GH, Khetani SR, Chen AA, Bhatia SN (2010) Tissue engineering of the liver. In: Arias IM (ed) The liver: biology and pathobiology, 5th edn. Wiley-Blackwell, New York, pp 935–954

Vogels BA, Karlsen OT, Mass MA, Bovee WM, Chamuleau RA (1997) L-ornithine versus L-ornithine-L-aspartate as a treatment for hyperammonemia-induced encephalopathy in rats. J Hepatol 26:174–182

Zeng L, Tan B, Yin Y, Kong X, Fen Z, Fang J, Lu X (2012) Gene expression profiles in porcine intestinal epithelial cells treated with arginine using a microarray technique. J Food Agri Environ 10:834–839