Supplementary Material (ESI) for Lab on a Chip

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Electronic Supplementary Information

Spheroid array of fetal mouse liver cells constructed on a PEG-gel micropatterned surface: upregulation of hepatic functions by co-culture with nonparenhymal liver cells

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1. Materials and Methods

1.1 Materials Poly(ethylene glycol) diacryloyl (M.W. = 575 Da), 3-(trimethoxysilyl)propyl methacrylate, and 2-hydroxy-4'-hydroxyethoxy-2-methylpropiophenone were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and they were used as received. Dulbecco's modified Eagle's medium (DMEM) containing 4,500 mg/L of glucose, Hanks' balanced salt solution modified (HBSS), insulin, dexamethasone, trypsin-EDTA solution (0.5% trypsin, 5.3mM sodium EDTA), and oncostatin M (OSM) were purchased from Sigma-Aldrich. Fetal bovine serum (FBS) and antibiotic-antimycotic were purchased from Gibco-Invitrogen (Grand Island, NY, USA). Williams' medium E was purchased from MP Biomedicals Inc. (CA, USA). The water used in this study was purified using a Milli-Q system (Nihon Millipore Co., Tokyo, Japan).

1.2 Preparation of medium and buffer Williams' medium E (WE) (10.7 g) for fetal mouse liver cells (FMLCs) culture was supplemented with sodium carbonate decahydrate (4.77 g), dexamethasone solution (1 mL, 10^{-4} mol/L), insulin solution (1 mL, 10^{-4} mol/L), aprotinin (5,000 KIU), hydrochloric acid to adjust pH 7.4, and destilled water (1 L). After filtration, WE was supplemented with fetal bovine serum (FBS) (10%), antibiotic-antimycotic (1%). Commecial DMEM was used for incubation of BAECs and NPCs. HBSS was supplemented with sodium hydrogen carbonate (0.35 g), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (2.38 g), antibiotic-antimycotic (1%), hydrochloric acid to adjust

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pH 7.4 and destilled water (1 L).

1.3 Spheroid culture on a PEG-gel micropatterned surface Fig. S1 shows the process flow for the fabrication of PEG-gel micropatterned surface by photolithography technique (Fig. S1(a)) and spheroid arrays formation of FMLC on the constructed surface (Fig. S1 (b)).

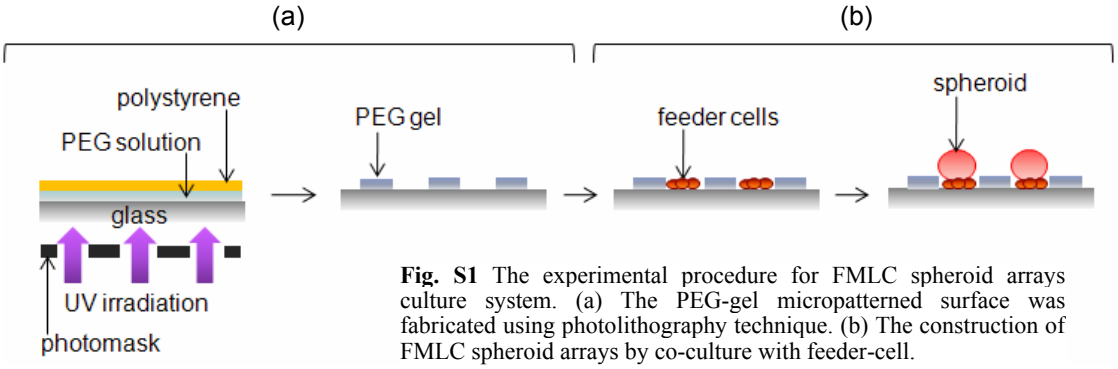

Fig. S1 The experimental procedure for FMLC spheroid arrays culture system. (a) The PEG-gel micropatterned surface was fabricated using photolithography technique. (b) The construction of FMLC spheroid arrays by co-culture with feeder-cell.

Construction of a PEG-gel micropatterned surface: The slide glass purchased from Matsunami Glass Ind. (Tokyo, Japan) was soaked in piranha solution (1:1 volume of H_2SO_4 and 30 w/v% of hydrogen peroxide) for 1h. The surface was modified with an ethanol solution of 3-(trimethoxysilyl)propyl methacrylate (4 vol%) for 3h, followed by rinsing with deinoized water and heating at 120 ºC for 12 h. 15 µL mixture of diacryloyl-PEG (M.W. = 575 Da, 33 vol%) and 2-hydroxy-4'-hydroxyethoxy-2-methylpropiophenone (1 w/v%) as a photoinitiator in 2:1 volume of the methanol/water co-solvent was dropped onto the methacryloyl group-introduced glass surface thus prepared and expanded uniformly by pressing polystyrene chip. As shown in Fig. S1a, the micropattern of PEG-gel was prepared by the irradiation of UV light (254 nm, 3.6 mJ/cm^2) using UV irradiation system (SUPERCURE-352S, SAN-EI ELECTRIC Co., Osaka, Japan) through patterned photomask. The photomask has metalic circle patterns on quartz glass plate which were 100 µm diameter and interval (phase-contrast micrograph of photomask was shown in Fig. 1a), where the metaric patterns area inhibited from exposuring UV for gelation. After irradiation of UV light, the PEG-gel micropatterned surface that had 100 µm diameter cavities was constructed by rinsing distilled water (phase-contrast micrograph of PEG-gel micropatterned surface was shown in Fig. 1b). The exterior of the cavities was modified with cell-incompatible PEG-gel, while the interior of the cavities was cell-compatible glass surface, relatively. The obtained the PEG-gel micropatterned chip was soaked in PBS overnight prior to use.

Cell culture: Bovine aortic endothelial cells (BAECs) were purchased from the Health Science Research Resources Bank (JCRB0099, Osaka, Japan). BAECs were used under 20 passeges in all cell culture experiments. Nonparenhymal cells (NPCs) was isolated from the same mice as described above.

FMLCs were isolated from C57BL/6 fetal mice (14 embryonic days) and this type of mouse was purchased from Japan SLC Inc. (Shizuoka, Japan). Mouse fetuses were extracted from the amnion and placed in HBSS and then the placenta was removed. The livers were separated from mouse fetuses by using two pairs of tweezer and the separated fetal livers were temporarily stored in HBSS containing 5% FBS and dispersed by pipetting of a syringe attached 22 gauge needle. The obtained FMLCs were filtered through a cell-strainer which is a 70 µm nylon mesh.

As shown in Fig. S1b, FMLCs were seeded onto feeder-cell micropatterned surfaces. One hundred µL of culture medium supernatant were carefully substituted with the same volume of fresh medium every 2 days without removing the cells. At 7th days of culture, a fully constructed spheroid array was obtained and the loose FMLCs were removed with all the medium, followd by washing in PBS. The cell morphology was monitored using a phase-contrast microscope (IX71, OLYMPUS Co., Tokyo, Japan) and differential interference contrast (DIC) microscope (Axiovert 200M, Carl Zeiss, Germany).

Cell cultures were demonstrated on 7.8 mm-diameter areas of PDMS wells. The constructed PEG-gel micropatterned chip was placed on the bottom of the well and each well has about 1,100 cavities for

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spheroid formation. According to the 8 μm-diameter for single FMLC cell and the averaged diameter of the constructed spheroid was 70 μm, the number of FMLC cells in single spheroid was calculated as 700 cells. Under the present experimental condition, approximately 800 spheroids can be formed on the bottom of single cluture well.

1.4 LIVE/DEAD assay for the FMLC spheroids The viability of FMLCs constituted spheroids was assessed using fluorescent staining SYTO® 10 and DEAD Red™ (Molecular Probes Inc., CA, USA) that are nucleic acid staining regents for live and dead cells. The micrographs were obtained by DIC microscope at 21th day of culture.

1.5 Quantification of albumin release from the FMLC spheroids The amount of albumin secretion from FMLC spheroids on the constructed surfaces was quantified by sandwich enzyme linked immunnosorbent assay using a Mouse Albumin ELISA Quantitation Kit (BETHYL Laboratories, Inc., TX, USA) and LumiGLO™ Chemiluminescent Substrate Kit (Kirkegaarg & Perry Laboratories, Inc.). The luminescence measurements were carried out by fluorescence plate reader (ARVO™ MX, PerkinElmer Japan Co., Ltd., Yokohama, Japan). The measurments were carried out at 9, 12, 16, 21 days of culture from the 7th days. The number of cells was counted by flowcytometry Guava EasyCyte Mini (Bio-medicine Inc., Hayawrd, CA, USA).

1.6 Determination of enzymatic activity of cytochrome P450 (CYP) 1A2 CYP1A2 is enzyme that appears only in the mature hepatocytes of mice or humans. The CYP1A2 activity was determined using a P450-Glo™ Assays kit (Promega, WI, USA) in accordance with the protcols. This assay is based on the measurements of luminescence from beetle luciferin derivatives, which can be converted to luciferin by the CYP1A2 enzymatic reaction and the luminescence can be observed by reaction with luciferin detection reagent. Before the measurement, the spheroids were retrieved by incubating 200 μL trypsin-EDTA solution under the condition of 37°C in a humidified atmosphere with 5% CO_2 for 10 min. and the spheroids were dispersed as FMLCs by strong pipetting. Twenty μL FMLCs dispersed solution was used to measure the CYP1A2 activity. The activities were monitored at 9, 12, 16, 21 days of culture from the 7th days. The enzymatic activity was normalized with the amount of luminescence of sample solution without cells.

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2. Supporting Data

2.1 Albumin secretion from the FMLC monolayer culture The FMLC monolayer on the flat surface, where BAECs, NPCs, and collagen were cultured as a substrates, was fabricated by using multi-well plate at the same cell concentration of spheroid culture. The cell culture conditions were the same as spheroid culture as described in 1.3 section.

As shown in Fig. S2, the monolayer-cultured FMLCs with BAEC, NPCs, and collagen showed extremely lower amount of albumin secretion than FMLC spheroids (Fig. 3). There were no effect of OSM addition in the amount of albumin secretion from monolayer-cultured FMLCs.

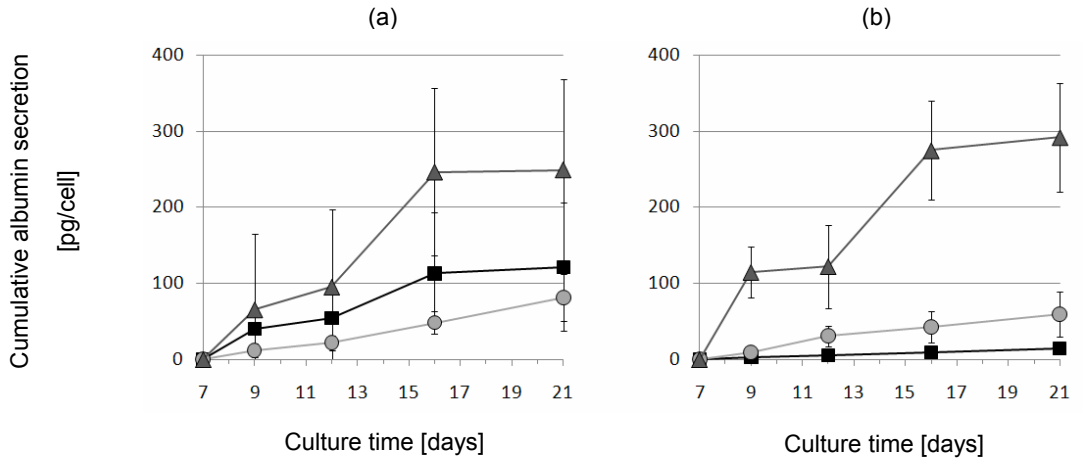

Fig. S2 Cumulative albumin secretion from the FMLC monolayer with different substrates. The squares shows cultured spheroid on BAECs, the circles shows on collagen, the triangles shows on NPCs. (a) Cultured without OSM (OSM(-), n = 3). (b) Cultured with OSM (OSM(+), n = 3).

2.2 Activity of cytochrome P450 (CYP) 1A2 of spheroid with collagen.

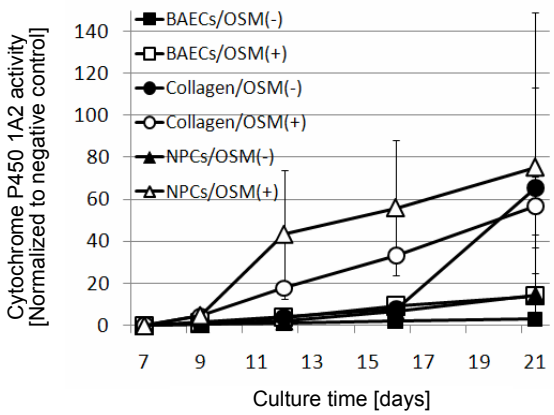

Fig. S3 CYP1A2 activity of FMLCs loosed from a spheroid array with all type of substrate. In this figure, the data of spheroid with collagen/OSM(-) and collagen/OSM(+) were newly added to Figure 4. The vertical axis represents the CYP1A2 activity normalized to negative control values (n = 3). The squares shows using BAECs, the triangles shows using NPCs, and the circles showed using collagen. The closed and open marks show OSM(-) and OSM(+), respectively.

CYP1A2 activity of FMLCs derived from spheroid with collagen was measured and the results were shown in Figure S3. In the case of collagen/OSM(-) (closed circle in Figure S3), the P450 activity was low and nearly equal to that with BAECs(OSM- and OSM+) and NPCs(OSM-) until 15 days of culture. Then the activity significantly increased at 21 days of culture. On the other hand, the P450 activity with collagen/OSM(+)

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showed the liner increase in the P450 activity from 7 days to 21 days of culture and consequently reached to the same activity of FMLCs spheroid with collagen/OSM(-).We are now investigating the effect of feeder layerments in the viability of the FMLCs in spheroids to understand the mechanism of upregulation phenomena.