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Controlled cell morphology and liver-specific function of engineered primary hepatocytes by fibroblast layer cell densities

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Engineered primary hepatocytes, including co-cultured hepatocyte sheets, are an attractive to basic scientific and clinical researchers because they maintain liver-specific functions, have reconstructed cell polarity, and have high transplantation efficiency. However, co-culture conditions regarding engineered primary hepatocytes were suboptimal in promoting these advantages. Here we report that the hepatocyte morphology and liver-specific function levels are controlled by the normal human diploid fibroblast (TIG-118 cell) layer cell density. Primary rat hepatocytes were plated onto TIG-118 cells, previously plated 3 days before at 1.04, 5.21, and 26.1 × 10^3 cells/cm^2. Hepatocytes plated onto lower TIG-118 cell densities expanded better during the early culture period. The hepatocytes gathered as colonies and only exhibited small adhesion areas because of the pushing force from proliferating TIG-118 cells. The smaller areas of each hepatocyte result in the development of bile canaliculi. The highest density of TIG-118 cells downregulated albumin synthesis activity of hepatocytes. The hepatocytes may have undergone apoptosis associated with high TGF-β1 concentration and necrosis due to a lack of oxygen. These occurrences were supported by apoptotic chromatin condensation and high expression of both proteins HIF-1a and HIF-1b. Three types of engineered hepatocyte/fibroblast sheets comprising different TIG-118 cell densities were harvested after 4 days of hepatocyte culture and showed a complete cell sheet format without any holes. Hepatocyte morphology and liver-specific function levels are controlled by TIG-118 cell density, which helps to design better engineered hepatocytes for future applications such as *in vitro* cell-based assays and transplantable hepatocyte tissues.

[**Key words:** Hepatocyte; Fibroblast; Morphology; Albumin; Gene expression; Cell sheet; Tissue engineering]

One of the approaches for liver regenerative medical research was liver tissue engineering using cultured hepatic cell organoids, which were constructed by special technologies, such as the floating culture method, low-attachment surface dishes, and spherical multicellular culture systems (1–5). Hepatic cell organoids with sheet format were also attractive and have already been produced using a temperature-responsive culture dish (TRCD), an electrochemical desorption substrate of alkanethiol, and a magnetic force system (6–9). Cell sheet technology had a great advantage of preventing necrosis by controlling thickness of cultured tissue *in vitro*.

In the field, many researchers challenged the maintenance of cell viability and improvement of liver-specific functions. To maintain liver-specific features in hepatocyte sheets, Kim et al. (10) reported co-cultured hepatocyte sheets with endothelial cells (ECs) were reconstructed bile canaliculi networks *in vitro*. We have previously developed the rapid fabrication technology of multi-layered hepatic cell sheets to combine a feeder layer method and forceful contraction potential of normal human diploid fibroblast TIG-118 cells (11). Engineered hepatocyte/fibroblast sheets (EHFSs), comprised primary human hepatocytes and TIG-118 cells using the same system, were suitable for subcutaneous engraftment without pre-transplant vascularization and/or a mixture of ECs to promote cell survival *in vivo* (12,13). The EHFSs constructed vascularized subcutaneous liver tissue and could advance low-invasive liver regenerative medicine (14).

Controlled two-dimensional (2D) hepatocyte morphology and co-culture system were also important for promoting hepatic functionalities *in vitro*. For example, the hepatocytes with 20-μm lane micropatterns showed spindle shape and increased interactions with 3T3 fibroblasts (15). Micropatterned hepatocytes as a colony surrounded by fibroblasts maintained liver-specific structures and functions (16). A high density of 3T3 fibroblasts accelerated the albumin synthesis activity of hepatocytes (17,18). Although these co-cultured hepatocytes were expected for future clinical applications, these cell lines have risk such as formation of tumor mass after transplantation. In spite of normal diploid fibroblasts with low risk, the cells were scarcely used in such research. Higher functional EHFSs using our concepts have the potential for more effective therapy and are worthy of consideration for liver

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regenerative medicine, but optimal conditions for EHFS production were not discussed.

In this study, we focused on the hepatocyte morphologies and revealed the expanded cell areas, shapes, liver-specific functions, and viabilities of hepatocytes under four culture conditions using a simple method of controlling TIG-118 cell densities. Furthermore, the distributions of hepatocytes and TIG-118 cells, sizes, and thicknesses of hepatocyte sheets were evaluated. The goals of this study were to elucidate optimal density of TIG-118 cells and culture time for EHFS production as functional hepatic tissues.

MATERIALS AND METHODS

Primary rat hepatocyte isolation Ethical approval for primary rat hepatocyte isolation was obtained from the Animal Care and Use Committee of Nagasaki University and performed in accordance with relevant guidelines and regulations. Primary rat hepatocytes were isolated from the whole liver of an adult Wistar rat (male, 7–8 weeks old) by liver perfusion using 130 U/mL collagenase (Wako Pure Chemical, Osaka, Japan) (19). To enrich viable hepatocytes, the cell suspension in 40% Percoll Plus solution (GE Healthcare, Tokyo) was centrifuged at 50 × *g* for 20 min at 4°C. Cell viability was determined by the trypan blue exclusion test, and suspensions with >90% viable cells were used in this study. The medium for isolation was Dulbecco's modified Eagle's medium (Wako Pure Chemical) supplemented with 10% fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Invitrogen, Carlsbad, CA).

Fibroblast culture Normal human diploid fibroblast TIG-118 cells derived from human skin were purchased from Health Science Research Resources (JCRB0535; Osaka, Japan). The TIG-118 cells were cultured as a continuous monolayer in 90-mm tissue culture dishes (Nalgene Nunc International, Rochester, NY), containing 10 mL Minimum Essential Medium (MEM) (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The TIG-118 cells at 90% confluence were treated with 0.25% trypsin-EDTA (Invitrogen), and the cell suspension was obtained.

Co-culture of hepatocytes and fibroblasts Three co-culture conditions were performed in this study. Tissue culture dish was coated with 100 μg/mL collagen type I (Sigma–Aldrich, St Louis, MO) in 10 mM HCl. The coating solution was removed before cell inoculation. Primary rat hepatocytes were plated at a density of 5.21×10^4 cells/cm^2 (5.00×10^5 cells/35-mm dish) onto TIG-118 cells that were plated 3 days previously at 1.04, 5.21, and 26.1×10^3 cells/cm^2 (0.100, 0.500, and 2.50×10^5 cells/35-mm dish) onto collagen-coated dishes (Fib-0.1/RH, Fib-0.5/RH, and Fib-2.5/RH, respectively) (Fig. S1). The TIG-118 cell densities were determined to be confluent at the time of hepatocyte seeding (highest) and cell sheet formation (lowest) (Fig. S2). As control conditions, hepatocytes were inoculated onto a collagen-coated dish after incubation with MEM for 3 days (RH alone). Hepatocytes were cultured with 2 mL of Hepato-STIM Culture Medium (Corning Glass Works, Corning, NY) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. This medium was changed 24 h after hepatocyte inoculation and every two days thereafter. Samples of media were collected and stored at −20°C until assayed.

Hepatocyte morphology analysis At 1, 4, and 7 days of hepatocyte culture, individual adhered hepatocytes were manually traced from the characteristic bright edge of hepatocytes using phase-contrast micrographs. Traced hepatocytes are shown in green (Fig. 1A). The areas and linearities of each hepatocyte were automatically measured using Win ROOF Version 6.3.0 (Mitani Corp, Fukui, Japan). The linearities were calculated as indicated in Fig. S3.

To stain cytokeratin of hepatocytes, cultured cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) (Wako Pure Chemical) for 10 min. Fixed samples were incubated in 0.1% Triton X-100 (Sigma–Aldrich Japan, Tokyo, Japan) in PBS for 5 min and then blocked in PBS containing 1% bovine serum albumin (BSA) for 1 h. The samples were incubated for 1 h in PBS + 1% BSA and mouse anti-cytokeratin 18 (CK18) (Abcam, Cambridge, MA) and then incubated for 1 h in PBS + 1% BSA and tetramethylrhodamine isothiocyanate-conjugated (TRITC) rabbit anti-mouse IgG (Sigma–Aldrich Japan). Nuclei were stained with 4′,6-diamidiono-2-phenylindole (DAPI; DOJINDO, Kumamoto, Japan). The samples were mounted with ProLong gold antifade mounting medium (Invitrogen). Fluorescence images were captured using a confocal laser scanning microscope (TCS SP8; Leica Microsystems, Wetzlar, Germany).

Cell viability and bile canaliculus imaging At day 4 of hepatocyte culture, live and dead cells were stained with calcein-AM and propidium iodide (PI), respectively (Cellstain Double Staining Kit; DOJINDO). To examine the structure of bile canaliculus, cells were treated with 1 μmol/l choly-lysyl-fluorescein (CLF) (Corning Glass Works) in Hank's balanced salt solution (HBSS) for 2 h and washed with HBSS thrice (10,20). These stained cells were visualized using a fluorescence microscope (Eclipse Ti-U; Nikon, Tokyo, Japan).

Albumin and TGF-β1 assays The concentrations of rat albumin in the culture medium were determined by an enzyme-linked immunosorbent assay. Goat anti-rat albumin (40 μg/mL) and horseradish peroxidase-conjugated sheep anti-rat albumin (10 μg/mL) antibodies were used to detect rat albumin (both from MP Biomedicals, LLC-Cappel products, Irvine, CA). The concentration of human transforming growth factor beta 1 (TGF-β1) was measured using DuoSet ELISA Development Systems (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Gene expression analysis (real-time PCR) Hepatocytes that were cultured under four culture conditions for 4 and 10 days were used for mRNA extraction using a spin column (NucleoSpin RNA II; Macherey-Nagel, Düren, Germany). cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Tokyo, Japan). The samples were then stored at −20°C until they were processed for polymerase chain reaction (PCR) analysis.

PCR was performed in an Applied Biosystems StepOnePlus Real-time PCR system using the TaqMan Gene Expression Assay Kit (Applied Biosystems; Table 1). In brief, PCR amplification was performed using a reaction mixture containing 1 μL cDNA sample, 0.5 μL TaqMan Gene Expression Assay probe, 5 μL TaqMan Fast Advanced Master Mix (Applied Biosystems), and 13.5 μL nuclease-free water. Each amplification cycle comprised 1 s at 95°C and 20 s at 60°C. The comparative cycle time method was used to quantify the gene expression levels. The expression levels of rat genes were normalized to that of rat Actb (hepatocyte suspension was set as 1.0). The expression levels of human HIF1A and CASP3 were normalized to that of human ACTB (Fib-0.1/RH at day 1 was set as 1.0).

Construction of hepatocyte sheets To construct hepatocyte sheets, cells were cultured on temperature-responsive culture dishes (TRCDs) (UpCell; CellSeed, Tokyo, Japan) under the same aforementioned conditions. After 4 days of hepatocyte culture, plates were incubated at 20°C and hepatocyte sheets were harvested in accordance with our previous report (11).

Histology of hepatocyte sheets The hepatocyte sheets were fixed with 4% PFA in PBS for 24 h. Fixed samples were embedded in paraffin, cut into 5-μm cross-sections, mounted on MAS-coated slides (Matsunami Glass, Osaka, Japan), and deparaffinized for standard histological staining with hematoxylin and eosin (HE). Slides were mounted with Permount (Fisher Scientific, Atlanta, GA), and bright-field images were captured using an optical microscope (BX53; Olympus, Tokyo, Japan).

For immunofluorescent staining, deparaffinized sections were heated in 10 mM Tris–HCl buffer (pH 9.0) containing 1 mM EDTA using an autoclave for antigen retrieval, incubated in Biotin-Blocking System (Dako Japan, Kyoto, Japan), and then blocked in Tris-buffered saline (TBS) containing 5% BSA for 1 h at room temperature. Blocked sections were incubated overnight at 4°C in TBS + 5% BSA and in the following antibodies: goat anti-rat albumin (rAlb) (MP Biomedicals, LLC-Cappel products), mouse anti-human vimentin (hVIM), rabbit anti-hypoxia inducible factor-1 alpha (HIF-1a), or mouse anti-HIF-1 beta (HIF-1b) (Abcam). Sections were then incubated for 1 h at room temperature in an appropriate secondary antibody: biotin-conjugated rabbit anti-goat IgG, biotin-conjugated goat anti-rabbit IgG, TRITC-conjugated ExtrAvidin, or fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (all from Sigma–Aldrich Japan). Subsequently, the slides were incubated with PBS containing 1 mg/mL DAPI for 5 min to stain the nuclei in the cells and mounted with ProLong gold antifade mounting medium (Invitrogen). Fluorescence images were captured using a fluorescence microscope or a confocal laser scanning microscope.

Cell sheet size and thickness The size (area) and thickness of hepatocyte sheets were measured from the exterior photographs (Fig. S4) and HE stained micrographs of cross sections (11). These sizes and thicknesses were measured using the NIS-Elements software program (Nikon).

Statistical analysis Data are presented as mean ± standard deviation (SD) from at least 6 time points from two independent cell preparations. Means of continuous numerical variables were compared using one-way or two-way analysis of variance (ANOVA) (GraphPad Prism version 6.00 for Windows; GraphPad

TABLE 1. TaqMan gene expression assay numbers for real-time PCR analysis.

Gene symbol	Gene name	TaqMan Assay no.
Casp3 (rat)	Caspase 3, apoptosis-related cysteine peptidase	Rn00563902_m1
Hif1a (rat)	Hypoxia inducible factor 1, alpha	Rn01472831_m1
Tjp1 (rat)	Tight junction protein 1	Rn02116071_s1
Oatp2 (rat)	Solute carrier organic anion transporter family, member 1A2	Rn00756233_m1
Mrp2 (rat)	Multi-drug resistance related protein 2	Rn00563231_m1
Hnf4a (rat)	Hepatocyte nuclear factor 4, alpha	Rn04339144_m1
Otc (rat)	Ornithine carbamoyltransferase	Rn00565169_m1
Arg1 (rat)	Arginase 1	Rn00691090_m1
Actb (rat)	Actin, beta	Rn00667869_m1
CASP3 (human)	Caspase 3, apoptosis-related cysteine peptidase	Hs00234387_m1
HIF1A (human)	Hypoxia inducible factor 1, alpha	Hs00153153_m1
ACTB (human)	Actin, beta	Hs99999903_m1

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