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Generation of mouse hepatobiliary organoids from hepatocyte progenitors and cholangiocytes isolated from healthy adult mouse liver

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Abstract

Number of liver organoids have been reported, though it is not clearly shown whether the functional connection between hepatocytes and cholangiocytes is recapitulated in those organoids. Here, we report generation of a hepatobiliary tubular organoid (HBTO) using mouse hepatocyte progenitors called small hepatocytes (SHs) and cholangiocytes. SHs differentiate and form the bile canalicular network in HBTOs and secret metabolites into the canaliculi, which are then transported into the biliary structure. Hepatocytes in the organoid acquire and maintain metabolic functions including albumin secretion and cytochrome P450 activities, over the long term. We provide the step-by-step protocol for induction of HBTO including isolation of cholangiocytes and SHs and co-culture of these two types of cell to generate functional connections between hepatocytes and cholangiocytes.

Introduction

Epithelial organs consist of multiple types of epithelial tissue, such as alveoli and trachea in the lung, urinary tubules and collecting ducts in the kidney, acini and pancreatic ducts in the pancreas, and bile canaliculi (BC) and bile ducts (BDs) in the liver. The structures connecting two types of tissues are unique to each organ. Various substances, including air, urine, digestive enzymes, and bile are altered in composition as they flow through the connecting structures It is therefore crucial to accurately connect tissue structures composed of different types of epithelial cells, in order to reproduce the function of each organ *ex vivo*.

To generate organoids, or mini-organs, tissue stem cells are cultured in three dimensions (3D), to enable the cells to grow, divide, and self-organize into tissue structures similar to those found *in vivo*. This approach has been used to generate functional components of the gastrointestinal tract, including intestinal (*1*), gastric (*2*), colonic (*3*), and hepatic tissues (*4, 5*). The organoids are likely to contain niches for maintaining tissue stem/progenitor cells, given that these organoids can expand in size. However, each organoid consists of a single type of tissue, and they do not contain structures connecting different types of epithelial cells.

In this study, we present a mouse hepatobiliary tubular organoid (HBTO) with hepatocyte clusters derived from small hepatocytes (SHs), and a biliary network derived from epithelial cell adhesion molecule positive (EpCAM^{+}) cholangiocytes. In HBTO, bilirubin and fluorescein-labeled bile acid were absorbed by the hepatocytes, excreted into BCs, and then accumulated in the biliary system, indicating that a connection between hepatocytes and cholangiocytes had been established. The development of HBTOs enabled us to investigate the transport of hepatocyte metabolites within the liver tissue, and to monitor the metabolism of the hepatocytes in the long-term *ex vivo*.

Reagents

Reagents

- C57BL6 mice
- Isoflurane (Pfizer, NY, Cat. No. 206K0S)
- Collagen I high concentration (Corning Inc., Corning, NY, Cat. No. 354249)
- Collagen Type IC (Koken, Tokyo, Japan, Cat. No. IPC-30)
- Collagenase (Wako Pure Chemical, Osaka, Japan, Cat. No. 032-10534)
- Phosphate buffer saline (PBS)
- Ca-, Mg-free Hanks' balanced salt solution (HBSS) (Sigma-Aldrich, St.Louis, MO, Cat. No. H9269)
- EGTA (Sigma-Aldrich, Cat. No. E0396)
- Butterfly needle, 23 gauge (Terumo, Osaka, Japan, Cat. No. SV-23CLK)
- DNase I (Sigma-Aldrich, Cat. No. DN25)
- Hyaluronidase (Sigma-Aldrich, Cat. No. H3566)
- Autoclaved 250 mm Nylon mesh (Nippon Rikagaku Kikai, Tokyo, Japan�NRS-250)
- Falcon 70 mm Cell strainer (Corning, Cat. No. 352340)
- Laminin mouse (BD Biosciences, Franklin Lakes, NJ, Cat. No. 354232)
- Growth Factor Reduced Matrigel (BD Biosciences, Cat. No. 354230)
- Oncostatin M (R&D systems, Minneapolis, MN, Cat. No. 495-MO)
- EGF (Life Technologies, Carlsbad, CA, Cat. No. PHG0311)
- HGF (R&D systems, Cat. No.294-HGN)
- Gentamicin (Sigma-Aldrich, Cat. No. G1397)
- Penicillin/streptomycin (100×) (ThermoFisher Scientific, Waltham, MA, Cat. No. 15140148)
- Insulin (Sigma-Aldrich, Cat. No. I5500)
- Insulin/Transferrin/Selenium (ITS) (100×) (Life Technologies, Cat. No. 41400-045)
- Dexamethasone (Dex) (Sigma-Aldrich, Cat. No. D-4902)
- Percoll (GE Healthcare Biosciences, Chicago, IL, Cat. No. 17089101)

l 35 mm tissue culture dish (Corning Inc., Cat. No. 430165)

l 100 mm petri dish (Bacterial grade)

l Anti-CD16/32 antibody (Biolegend, San Diago, CA, Cat. No.101-301)

l Biotin anti-EpCAM (Biolegend, Cat. No. 324216)

l Streptavidin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany, Cat. No. 130-048-101)

l L-15 medium (Sigma-Aldrich, Cat. No. L4386)

l DMEM/nutrient mixture Ham F-21 (DMEM/F12) medium

l Decomplemented Hyclone fetal bovine serum (FBS) (Thermo Fisher Scientific, Cat. No. SH30910.03).

l Nicotinamide (Sigma-Aldrich, Cat. No. N0630)

l Dimethyl sulfoxide (Sigma-Aldrich, M81082)

l 30% bovine serum albumin (BSA) (Sigma-Aldrich, Cat. No. A9576)

Reagent preparation

l Pre-perfusion solution: dissolve 0.5 mM EGTA in HBSS (Alternatively, use Liver perfusion medium (Thermo Fisher Scientific, Cat. No. 17701038)

l Perfusion solution: dissolve 1 mg/ml collagenase in pre-warmed 40 ml HBSS in a 50 ml tube by flopping upside down

l Percoll solution: mix 0.6 ml of 10×DMEM/F12 with 5.6 ml of percoll using 10 ml pipette.

l Basic medium: add 10% FBS, 1×Penicillin/Streptomycin, 50 mg/ml Gentamicin, 10 mM Nicotinamide to DMEM/F12 medium

l Growth medium: add 1×ITS, 10^{-7} M Dex, 10 ng/ml EGF, 10 ng/ml HGF to basal medium

l Differentiation Medium-I: add 1×ITS, 10^{-7} M Dex, 10 ng/ml OSM to basal medium

l Differentiation Medium-II: add 1×ITS, 10^{-7} M Dex, 1% DMSO to basal medium

l MACS buffer: add 2 mM EDTA and 0.5 % BSA to PBS

l Wash buffer: add 2 % FBS to PBS

DMEM/NaOH solution: Mix 500 ml of 10 × DMEM with 50 ml 0.5 N NaOH

Equipment

l Centrifuge

l Magnetic cell sorter (MACS): Quadra MACS separator, Multi Stand, and LS column (Miltenyi Biotec).

l CO_2 incubator (Panasonic): The incubator is used to keep culture at 37°C and under 5% CO_2.

l Peristatic pump (Advantec, Tokyo, Japan, Cat. No. PSM070AA)

l Magnetic stirrer: set in an incubator at 37°C

l Tweezers, scissors, clamp, stirring bar

Procedure

Two step collagenase perfusion

1. Anesthetize a mouse with isoflurane
2. Cut the center of the abdominal wall and expose the portal vein
3. Insert a butterfly needle into the portal vein and fix it with a clamp
4. Turn on the peristatic pump and cut the inferior vena cava
5. Perfuse the liver with 25 ml of pre-perfusion solution at 6 ml/min
6. Perfused 40 ml of the perfusion solution at 3 ml/min
7. Transfer the liver to a petri dish
8. Peel off the liver capsule and dissociate hepatocytes using two tweezers
9. Transfer the undigested tissue to a 6 cm dish and keep on ice

Isolation of cholangiocytes

1. Cut the undigested tissue into small pieces with scissors

2. Suspend the tissue pieces in 10 ml of L-15 medium and transfer to a 30 ml beaker containing a stirring bar

3. Add 30 mg collagenase, 25 ml of hyaluronidase solution, 10 ml of 5 mg/ml DNase I to the beaker

4. Stir the solution at 37°C for 40 min

5. Add 10 ml of DMEM/F12 medium containing 10% FBS to the cell suspension

6. Pass the cell suspension through a 70 mm cell strainer

7. Centrifuge at 50 × *g* for 1 min to eliminate hepatocytes and cell clumps

8. Centrifuge the cell supernatant at 350 × *g* for 4 min to collect dissociated cells

9. Resuspend cells in 200 ml of basal medium

10. Add 2 ml of anti-CD16/32 (FcgIII/II receptor) antibody and incubated at 4°C for 30 min

11. Add 2 ml of chilled wash buffer and centrifuge at 350 × *g* for 4 min

12. Resuspend cells in 200 ml basal medium

13. Add 1 ml of biotin-conjugated anti-EpCAM antibody and incubated at 4°C for 30 min

14. Centrifuge the cell supernatant at 350 × *g* for 4 min to collect dissociated cells

15. Resuspend cells in 100 ml of MACS buffer

16. Add 10 ml of streptavidin microbeads and incubated at 4°C for 15 min

17. Centrifuge the cell supernatant at 350 × *g* for 4 min to collect dissociated cells

18. Resuspend cells in 500 ml of MACS buffer

19. Isolate EpCAM^{+} cholangiocytes using MACS

20. Centrifuge the EpCAM^{+} fraction at 350 × *g* for 4 min to collect cholangiocytes

21. Count the number of live cells

22. Resuspend cells in growth medium at the density of $5×10^{4}$ cells/ 300 ml growth medium

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<u>Plate cholangiocytes</u>

1. Mix 100 ml of DMEM/NaOH solution, high concentration type I collagen, and autoclaved water to make 1 ml of 4 mg/ml collagen solution

2. If the solution is yellow, add 5~10 ml 0.5N NaOH, near to neutralize

3. Pour 250 ml collagen solution into each well of a 24 well plate

4. Incubate the plate at $4^{o}C$ for 30 min

5. Add 500 ml basal medium and incubate 1~2 min at room temperature

6. Eliminate the medium by aspiration

7. Feed 5×10^4 cholangiocytes onto each well

8. Culture for 5 days

<u>Isolation of small hepatocytes (SHs)</u>

1. Dissociate hepatocytes from the liver by two-step collagenase perfusion

2. Pass the cell suspension through 250 mm Nylon mesh

3. Pass the cell suspension through 70 mm cell strainer set on a 50 ml tube

4. Centrifuge at 50 × *g* for 1 min and transfer the supernatant to a new 50 ml tube

5. Centrifuge at 50 × *g* for 1 min and transfer the supernatant to a new 50 ml tube

6. Centrifuge at 115 × *g* for 3 min

7. Suspend the pellet in 6 ml of HBSS and add 6 ml of Percoll solution

8. Centrifuged at 180 × *g* for 15 min to eliminate dead cells

9. Resuspend cells in 2 ml basal medium and count cells

10. Resuspend cells in growth medium at the density of 5×10^4 cells/ 300 ml

11. Eliminate medium from cholangiocyte culture

12. Feed 5×10^4 SHs onto each well

13. Place the plate in CO_2 incubator

Induction of organoid formation

1. Replace growth medium with differentiation medium-I at 2 days after SH plating and incubate for 1 day

2. Eliminate differentiation medium and overlay cells with 200 ml of collagen gel containing 20% MG (Col-MG), which was prepared by mixing 2 mg/ml Collagen IPC and MG (v/v = 4:1) on ice

3. Incubated the plate at 37°C for 3–4 hours to form a gel

4. Add 400 ml of differentiation medium-II to each well

5. Replace medium with fresh one every four days.

Troubleshooting

Time Taken

Anticipated Results

l At 1 week after Col-MG overlay, hepatobiliary connections can be recognized by phase contrast microscope (black arrowhead in Fig. 1).

l At 2 weeks after Col-MG overlay, bile canaliculi are well developed in hepatocyte clusters (white arrowheads in Fig. 1). Bile canaliculi are more clearly recognized at 3 weeks.

l With staining with fluorescent dye-conjugated phalloidin after appropriate fixing, the continuous luminal network can be observed under a fluorescent microscope. To visualize luminal spaces both in hepatocyte clusters and biliary tubules, imaging under a confocal microscope is necessary.

l Hepatocyte functions including ALB secretion and CYP activity are gradually increased by two or three weeks after Col-MG overlay. The activities will maintain for more than 2 weeks.

Figures

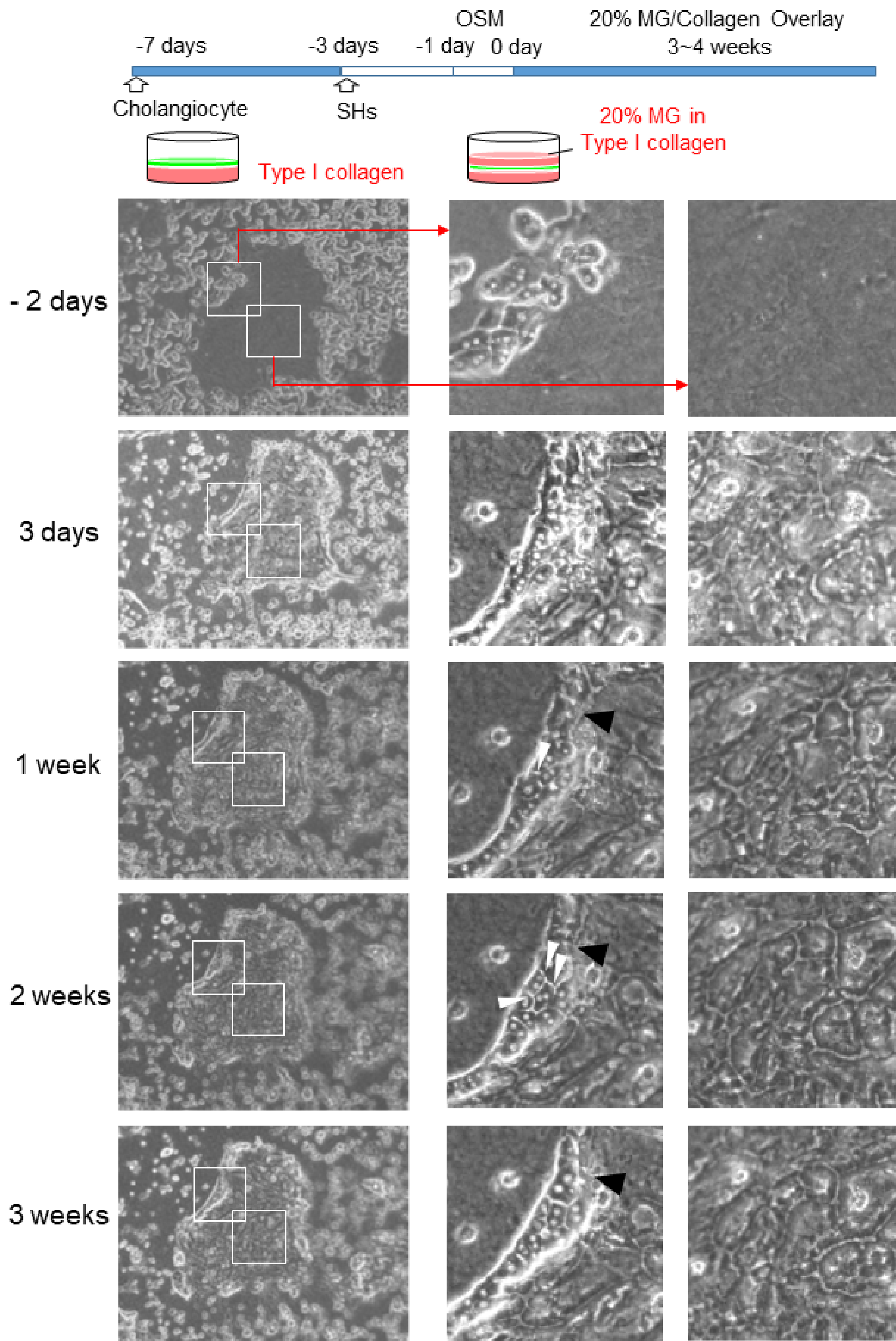

Figure 1

Protocol for induction of heptaobiliary tubular organoid (HBTO) and morphological changes during culture.