Use of biliary organoids in cholestasis research

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Running head: Generation and propagation of biliary organoids.

Summary:

Cholangiocytes play a crucial role in the pathophysiology of cholestasis. However, research on human cholangiocytes has been restricted by challenges in long-term propagation and large-scale expansion of primary biliary epithelium. The advent of organoid technology has overcome this limitation allowing long-term culture of a variety of epithelia from multiple organs. Here, we describe 2 methods for growing human cholangiocytes in organoid format. The first applies to the generation of intrahepatic bile ducts using human induced pluripotent stem cells using a protocol of differentiation that recapitulates physiological bile duct development. The second method allows the propagation of primary biliary epithelium from the extrahepatic ducts or gallbladder. Both protocols result in large numbers of cholangiocyte organoids expressing biliary markers and maintaining key cholangiocyte functions.

Keywords: organoids, cholangiocytes, human pluripotent stem cells, extrahepatic biliary epithelium, cholestasis, endoscopic retrograde cholangiopancreatography, liver biopsy, gallbladder, common bile duct.

1. Introduction

The biliary epithelium plays a crucial role in the pathogenesis of cholestatic disorders. Cholangiocyte proliferation and death, their interaction with bile and their cross-talk with the inflammatory milieu of disease play a key role in the evolution of cholangiopathies ***(1–3)***. However, studies on primary biliary epithelium have been limited by poor access to tissue precluding large scale analyses and functional studies especially in human ***(3, 4)***. Isolation and culture of human biliary epithelial cells can provide unique insights in the pathophysiology and management of cholestatic diseases. It can enable the development of *in vitro* models of cholestasis based on human tissue and advance therapeutics through the generation of high-throughput drug screening platforms ***(4)***.

Organoid technology enables growing primary epithelium in 3-dimensional culture conditions in the presence of growth factors or small molecules modulating Wnt signalling. Organoids were first generated from intestinal epithelium by Hans Clevers and colleagues ***(5)*** and this approach has since been applied to a diversity of organs including the biliary tree. The resulting biliary organoids have the potential to revolutionize the study of human cholangiocytes ***(4, 6–10)***. Indeed, these 3-dimensional structure with an isolated central lumen is ideal for recapitulating key cholangiocyte functions, such as maintaining lumen homeostasis and transfer of water, electrolytes and bile acids ***(11–15)***. Furthermore, organoid culture is compatible with the propagation of both intrahepatic and extrahepatic cholangiocytes from primary tissue or induced pluripotent stem cells (iPSCs) ***(4, 7)***.

Here, we describe 2 methods for generating cholangiocyte organoids (COs) from adult primary extrahepatic biliary epithelium (*i.e.* extrahepatic cholangiocyte organoids (ECOs)) ***(7)*** or iPSC-derived cholangiocyte-like-cells (CLCs) ***(4)***. These 2 systems are complementary, thus ECOs represent mature extrahepatic cholangiocytes but their derivation requires access to primary tissue, while CLCs maintain characteristics of foetal intrahepatic cholangiocytes and can be

easily generated through a skin biopsy, even in cases where access to primary biliary tissue is not possible ***(4, 6, 7)***.

CLCs are generated from iPSCs following a protocol that recapitulates human bile duct development (Fig. 1). Intrahepatic bile ducts develop from bipotent hepatoblasts in the foetal liver, which can give rise to both hepatocytes and cholangiocytes ***(16)***. Periportal hepatoblasts, under the effect of TGF-β start expressing biliary markers, such as SOX9 and form a monolayer of early cholangiocytes surrounding the portal vein, known as ductal plate ***(16, 17)***. The ductal plate subsequently remodels under the effect of Notch signalling ***(16, 18)*** to form tubular structures, which elongate under the effect of non-canonical Wnt signalling and the planar cell polarity pathway ***(19)***. To reproduce this process *in vitro*, iPSCs are plated as a monolayer and differentiated into definitive endoderm (DE), anterior foregut progenitors (FP), bipotent hepatoblasts (HB) and cholangiocyte progenitors (CP) corresponding to the early monolayer of cholangiocytes comprising the ductal plate ***(4, 6)***. Cholangiocyte progenitors are subsequently passaged and re-suspended in Matrigel in 3-dimensional culture. Under these conditions, CPs remodel into organoids, develop a central lumen and exhibit key biliary markers and functions, such as γ-glutamyl transferase (GGT) and alkaline phosphatase (ALP) activity.

The generation of ECOs requires access to primary biliary epithelium in the form of endoscopic retrograde cholangiopancreatography brushings, cholangioscopy biopsies or excised tissue (common bile duct or gallbladder). The biliary epithelium is mechanically dissociated from the rest of the tissue and resuspended in Matrigel in 3-dimensional culture conditions, giving rise to cholangiocyte organoids (ECOs) in 5-14 days. ECOs can subsequently be propagated for multiple passages maintaining their functional properties and expression of key biliary markers ***(7)***. For each passage, the Matrigel needs to be enzymatically digested, the cells are harvested, split typically in a 1:4-1:6 ratio and re-embedded in Matrigel or cryopreserved.

2. Materials

1. Chemically defined medium-PVA (CDM-PVA) for maintenance of iPSCs: mix 250 mL of F12 with GlutaMAX with 250 mL of Iscove's Modified Dulbecco's Medium (IMDM) containing 0.5 g of 87-90% hydrolyzed Poly(vinyl alcohol) (PVA). Add 5 mL of concentrated lipids, 20 µL of 1.25 g/mL thioglycerol, 350 µL of 10 mg/mL insulin, 250 µL of 30 mg/mL transferrin and 5 mL of 10,000 U/mL penicillin/streptomycin. Store at 4°C for up to 1 month.

2. Medium for plating iPSCs: use CDM-PVA complemented with 10 ng/mL Activin and 12 ng/mL bFGF as plating medium.

3. RPMI/B-27 differentiation medium for FPs, HBs and CPs (500 mL): supplement 500 mL of RPMI-1640 with 10 mL of B-27 supplement containing insulin, 5 mL of Eagle□s minimum essential medium (MEM) non-essential amino acids (MEM-NEAA) and 5 mL of 10,000 U/mL pen/strep. Store at 4°C for up to 1 month.

4. Supplemented William's E medium for 3-dimensional culture of CLCs and ECOs (WE+): Prepare 500 mL of William's E (WE) medium containing 10mM nicotinamide, 17 mM sodium bicarbonate, 0.2 mM ascorbic acid trisodium salt, 14 mM glucose, 6.3 mM sodium pyruvate, 20 mM HEPES solution, 5 mL ITS+ premix, 0.1 µM dexamethasone, 2 mM Glutamine and 50,000U pen/strep. Store at 4°C for up to 1 month.

5. 66% (vol/vol) Matrigel solution for 3-dimensional organoid culture: mix thoroughly 1 volume of supplemented WE medium and 2 volumes of Matrigel. Keep at 4°C to avoid Matrigel solidification.

6. Required cytokines for differentiation of iPSCs into CLCs and maintenance of ECOs: the cytokines required are listed below. Reconstitute and store according to the manufacturer's instructions. Prepare appropriate volume aliquots following reconstitution to avoid repeated freeze-thaw cycles.

a. Recombinant human Activin A.

b. Recombinant human bone morphogenetic protein 4 (BMP4).

c. Recombinant human fibroblast growth factor (FGF) basic (bFGF), 146 aa.

d. LY294002.

e. CHIR99021.

f. SB431542.

g. Recombinant human keratinocyte growth factor-2 (also known as fibroblast growth factor 10 or FGF10).

h. Retinoic acid.

i. Rho-associated, coiled-coil containing protein kinase inhibitor Y27632 (ROCK inhibitor).

j. Recombinant human epidermal growth factor (EGF).

k. Recombinant human Dickkopf-related protein 1 (DKK-1).

l. Recombinant human R-pondin-1.

7. Cell dissociation buffer, enzyme-free, for passaging of CPs *prior* to embedding in Matrigel. Store at 4°C according to the manufacturer's instructions.

8. Cell recovery solution for passaging of ECOs and digestion of Matrigel. Store at 4°C according to the manufacturer's instructions.

9. Phosphate-buffered saline (PBS), pH 7.4

10. Centrifuge.

11. Plate heater.

12. Incubator (37°C ± 1ºC, 90% ± 5% humidity, 5% ± 1% CO_2).

13. Laminar air flow cabinet.

14. Thermostated water bath.

3. Methods

3.1. Differentiation of iPSCs to CLCs

3.1.1 Plating of iPSCs for differentiation

1. Plate iPSCs on vitronectin or gelatin-coated plastic tissue culture plates (*see* **Notes 1-4**).

2. Incubate at 37°C overnight.

3.1.2. Differentiation of iPSCs into DE (3 days)

1. First day: replace the medium with CDM-PVA complemented with 100 ng/mL Activin A, 80 ng/mL bFGF, 10 ng/mL BMP-4, 10 μM LY294002 and 3 μM CHIR99021. Incubate the cells at 37°C overnight.

2. Second day: change the medium with CDM-PVA complemented with 100 ng/mL Activin A, 80 ng/mL bFGF, 10 ng/mL BMP-4, 10 μM LY294002. Incubate the cells at 37°C overnight.

3. Third day: change the medium with RPMI/B27 medium complemented with 100 ng/mL Activin A and 80 ng/mL bFGF (*see* **Notes 5-6**). Incubate the cells at 37°C overnight.

3.1.3. Differentiation of DE to FPs (5 days)

Change the medium daily with RPMI/B27 medium complemented with 50 ng/mL Activin A (*see* **Notes 5-6**).

3.1.4. Differentiation of FPs to HBs (4 days)

Change the medium daily with RPMI/B27 medium complemented with 10 μM SB-431542 and 50 ng/mL BMP-4 (*see* **Notes 5-7**).

3.1.5. Differentiation of HBs to CPs (4 days)

Change the medium daily with RPMI/B27 medium complemented with 50 ng/mL FGF10, 50 ng/mL Activin-A and 3 μM Retinoic acid (*see* **Notes 5-7**).

3.1.6 Passaging of CPs and embedding in Matrigel

1. Before starting this step pre-warm a 24-well plate to 37°C on a plate heater placed in the tissue culture laminar air flow cabinet.
2. Wash the cells with PBS once, add an adequate volume of cell recovery solution to cover the surface of the plate and incubate at 37°C for 15 minutes.
3. Transfer the cells to a falcon tube, wash once with WE+ medium and centrifuge at 444xg for 3 minutes (*see* **Note 8**).
4. Resuspend the pellet in 1 mL of WE+ medium.
5. Gently dissociate the cell suspension into small 10-50 cells clumps through gentle pipetting with a p1000 pipette (*see* **Notes 1 and 9**).
6. Centrifuge again at 444xg for 3 minutes.
7. Resuspend the cell pellet in 66% (vol/vol) Matrigel supplemented with 20 ng/mL EGF and Rho kinase inhibitor 10 μM Y-27632 (*see* **Notes 2, 5 and 9-14**).
8. Use a p1000 pipette to plate a 50 μL droplet of the cell suspension in each well of a pre-warmed 24-well plate kept on the plate heater (*see* **Note 15**).
9. Once the droplet starts solidifying (approximately 5 seconds) invert the plate and incubate at 37°C for 30 minutes (*see* **Note 16**).
10. Following incubation, add an adequate volume of WE+ complemented with 20 ng/mL EGF and 10 μM Rho kinase inhibitor Y-27632 to cover the Matrigel domes(*see* **Note 17**).

3.1.7 Differentiation of CPs to CLCs (10 days)

Change the medium on alternate days with WE+ medium complemented with 20 ng/mL EGF. Organoids should emerge after 2-4 days.

3.1.8 Characterization of the resulting CLCs

The resulting CLC organoids can be characterized through quantative real-time PCR (QPCR) or immunofluorescence (IF) for the expression of biliary markers such as CK7, CK19, Sox9, GGT. Functional properties of CLC organoids such as GGT and ALP activity can be assessed at the end of this stage using commercially available kits (*see* **Note 6**).

3.2. Isolation and propagation of ECOs

3.2.1 Isolation of primary cholangiocytes

1. For ERCP brushings wash the ERCP brush with the cells in a 15 mL falcon with WE+ medium and centrifuge the tube at 444xg (*see* **Note 18**).
2. For surgically excised tissue, wash the tissue in a container with WE+ medium 3 times to remove bile and debris (*see* **Notes 18-20**).
3. Transfer the tissue on an empty 10 cm plastic tissue culture plate.
4. Dissect the tissue (bile duct or gallbladder) to expose the lumen.
5. Add an adequate volume of WE+ medium to cover the tissue (*see* **Note 20**).
6. Using a scalpel gently remove the biliary epithelium by scraping the lumen (*see* **Note 21**).
7. Harvest the cell suspension in an appropriate volume falcon tube and collect the cells by centrifuging at 444xg for 3 minutes (*see* **Note 20**).
8. Wash once by resuspending the cell pellet in 1 mL of WE+ media (*see* **Note 20**).
9. Resuspend the cells in a 66% (vol/vol) Matrigel solution and plate them in 24-well plates as described in section 1.6 (*see* **Note 22**).

10. Following incubation add 1 mL of WE+ media per well with 500 ng/mL R-spondin, 50 ng/mL EGF, 100 ng/mL DKK-1 and 10 μM Rho kinase inhibitor Y-27632.

11. Change media on alternate days, using WE+ media with 500 ng/mL R-spondin, 50 ng/mL EGF and 100 ng/mL DKK-1.

12. Organoids should emerge within 2-5 days of culture

3.2.2 Passaging of ECOs

1. Aspirate the medium from the wells to expose the Matrigel domes (*see* **Notes 1, 23-24**).

2. Add 500 μL of cell recovery solution directly on the Matrigel dome.

3. Scrape the well with the tip of a p1000 pipette to disrupt the dome.

4. Transfer the cell recovery solution containing the Matrigel dome fragments in a 15 mL tube using the p1000 pipette.

5. Incubate at 4°C for 30 minutes.

6. Centrifuge at 444xg for 3 minutes.

7. Aspirate the supernatant (*see* **Note 25**).

8. Wash the pellet with a minimum of 1ml of WE+ medium (*see* **Notes 2, 26-27**).

9. Resuspend the cells in a 66% (vol/vol) Matrigel solution and plate them in 24-well plates as described in section 1.6.

4. Notes

1. Cell lines should be regularly karyotyped and tested for mycoplasma contamination. Karyotypic abnormalities or contamination can significantly affect the expression of biliary markers, function, and viability of the cells.

2. Due to physiological variability in parameters such as proliferation capacity, some optimization of parameters such as seeding density and clump size may be required for working with different iPSC or ECO lines.

3. A density of $1x10^6$ cells per 10 cm plate is recommended.

4. All media should be stored at 4°C for a maximum of 1 month. Cytokines should be stored at -20°C or -80°C according to the manufacturer's instructions and aliquoted in appropriate volume aliquots to avoid repeat freeze-thaw cycles.

5. Non-chemically defined components such as FBS, B-27 and Matrigel should be batch tested for their capacity to support proliferation, marker expression and function of the cells compared to primary controls.

6. The efficiency of CLC differentiation depends on the differentiation efficiency of each step, which can be measured by flow cytometry analysis for key markers. The minimum requirements for each differentiation step are outlined below:

DE: >90% SOX17 positive cells.

FP: >95% GATA4 positive cells.

HBs: >95% CK19 and AFP positive cells.

CPs: >75% Sox9 positive cells.

CLCs: > 75% CK7 and CK19 positive cells.

7. The duration of the HB step is critical. The expression of hepatic markers (AFP) is driven by activin/TGF-β blockade (SB431542). Prolongation of this step will commit the cells to a hepatic fate, reducing their ability to reduce the expression of hepatocyte markers in the subsequent CP stage. Conversely, inadequate blockade with SB431542 will impact on the yield of this step for HBs. Therefore, the duration of the HB step can be modified accordingly for lines to fit lines with limited capacity for liver differentiation (prolonged SB431542

treatment and/ or increased concentration) or reduced yield of CPs (reduction in the duration of the HB stage).

8. If the cells have not detached spontaneously following incubation, mechanically dissociate them by scraping the plate with a p1000 pipette.

9. Clump size is crucial for embedding the cells in Matrigel. Single cells and very small clumps demonstrate reduced viability and therefore fail to generate organoids, while very large clumps gravitate to the bottom of the plate and form a monolayer. Clump size should be optimized for each line and smaller sizes are recommended for very proliferative lines.

10. Matrigel should be aliquoted in appropriate volume aliquots to avoid more than 1 freeze thaw cycle.

11. Matrigel should always be kept on ice to avoid solidification. All equipment used with Matrigel such as pipette tips should be pre-cooled to 4°C. Work with Matrigel as quickly as possible, mix thoroughly and keep the suspension on ice to avoid Matrigel solidification.

12. Take care to avoid the introduction of bubbles during the mixing stage which can prevent the optimal formation of Matrigel domes during plating.

13. The total volume of the 66% (vol/vol) Matrigel solution used for resuspension can be calculated by multiplying the number of anticipated wells by 50 µL.

14. We recommend a plating density of 1-2 x 10^5 cells/well. For this density, 1 well of 12 well plate of CPs can yield 8-10 Matrigel domes.

15. As the Matrigel-containing cell suspension droplet comes in contact with the warm surface of the plate, the droplet will start solidifying forming a hemispheric dome.

16. Inverting the plate before the Matrigel solidifies fully is critical. This manoeuvre will prevent large clumps of cells from attaching to the bottom of the plate and expanding as a monolayer.

17. For 1 well of a 24-well plate we recommend using 1 mL of medium.

18. For the successful isolation of primary cholangiocytes it is crucial that the tissue is processed as soon as it is available to avoid reduction in cell viability.

19. Bile is toxic for the cells, therefore adequate washes of the tissue are crucial for preserving cell viability.

20. Wash the tissue during the isolation of primary cholangiocytes with PBS containing 1% (wt/vol) BSA or with WE+ media. Washes with low protein content such as PBS can result in loss of cells.

21. This process will release small clumps of cholangiocytes in the medium.

22. The same considerations as for CLCs apply when embedding primary cholangiocytes in Matrigel. However, primary cells are already broken into very small clumps by the mechanical dissociation and it is crucial that further dissociation of the cells after pelleting is avoided to preserve viability.

23. ECO lines should be passaged promptly when the organoids are approximately 80% confluent. This period can vary between lines but is approximately 5 days. Delays in passaging can affect the quality and proliferation capacity of the organoid culture.

24. Place the aspirator against the wall of the well and aspirate the medium by tilting the plate to avoid disrupting the Matrigel dome.

25. At this stage undigested Matrigel remnants may form a layer above the cells. Even if this layer is not aspirated to avoid loss of cells, it will be cleared in the next wash.

26. Washing with WE+ is crucial. Washes with low protein content such as PBS can result in loss of cells.

27. At this stage the pellet can be split in multiple aliquots, depending on the number of wells required. We recommend a 1:5 split ratio and a plating density of 1-2 x 10^5 cells/well.

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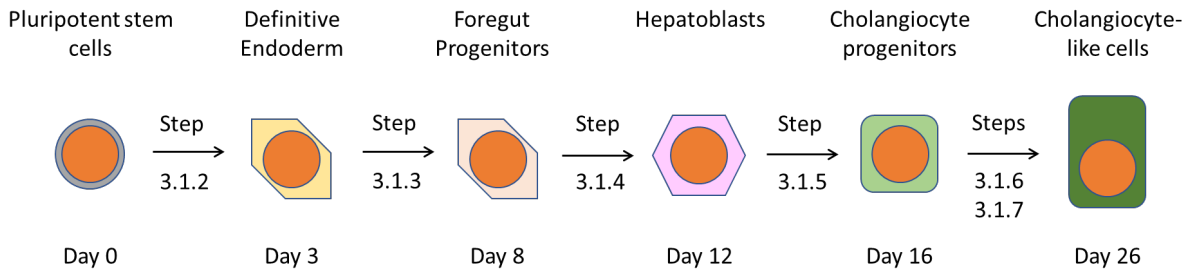
Figure 1



Schematic outline of illustrating the various differentiation stages from induced Pluripotent Stem Cells to Cholangiocyte-like Cells. The corresponding method steps for each stage are indicated (described in the Methods section 3.1)