Generation of hepato-biliary-pancreatic organoid from human pluripotent stem cells

Hiroyuki Koike
Division of Gastroenterology, Hepatology & Nutrition, Division of Developmental Biology, Cincinnati Children's Hospital Medical Center https://orcid.org/0000-0001-9934-6930

Kentaro Iwasawa
Division of Gastroenterology, Hepatology & Nutrition, Division of Developmental Biology, Cincinnati Children's Hospital Medical Center

Takanori Takebe (takanori.takebe@cchmc.org)
Division of Gastroenterology, Hepatology & Nutrition, Division of Developmental Biology, Center for Stem Cell and Organoid Medicine (CuSTOM), Cincinnati Children's Hospital Medical Center, Institute of Research, Tokyo Medical and Dental University (TMDU) https://orcid.org/0000-0002-6989-3041

Method Article

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Abstract

Organogenesis is a complex and inter-connected process, orchestrated by multiple boundary tissue interactions. Here, we established the protocol of the continuous patterning of hepatic, biliary and pancreatic structures from a three-dimensional culture of human pluripotent stem cell (PSC). The boundary interactions between anterior and posterior gut spheroids differentiated from human PSCs enables autonomous emergence of hepato-biliary-pancreatic (HBP) organ domains in the absence of extrinsic factor supply. This anterior-poterior gut interaction protocol can be used to model the early human HBP organogenesis process *in vitro*.

Introduction

The hepato-biliary-pancreatic (HBP) anlage, which is demarcated by HHEX (Hematopoietically-expressed homeobox protein) and PDX1 (Pancreatic and duodenal homeobox 1) expression is specified at the boundary between the foregut-midgut.

Here, we leverage a three-dimensional differentiation approach using human pluripotent stem cells (PSCs) to specify gut spheroids with distinct regional identities comprised of both endoderm and mesoderm. We show that antero-posterior interactions recapitulate the foregut and the midgut boundary *in vitro*, modeling the inter-coordinated specification and invagination in the human hepato-biliary-pancreatic organoid (HBPO).

Reagents

Cells

· Human pluripotent stem cells (for example H1 human embryonic stem cells, NIHhESC-10-0043)

Reagents

· mTeSR1 (STEMCELL technologies, 85850)

· Matrigel, Growth Factor Reduced (Corning Inc., 356231)

· Matrigel, Phenol Red-free (Corning Inc., 356237)

· Accutase (Thermo Fisher Scientific, A1110501)

· Y-27632 (Tocris Bioscience, 1254)

· RPMI 1640 (Thermo Fisher Scientific, 22400089)

· Activin A (R & D Systems, 338-AC-01M)

· FGF-4 (R & D Systems, 235-F4-01M)

· CHIR 99021 (Stemgent, 04-0004-10)

· Defined fetal bovine serum (dFBS) (Hyclone Laboratories Inc, SH3007002)

· Advanced DMEM/F-12 (Thermo Fisher Scientific, 12634)

· B27 Supplement (Thermo Fisher Scientific, 17504044)

· N2 Supplement (Thermo Fisher Scientific, 17502048)

· HEPES (Thermo Fisher Scientific, 15630080)

· L-Glutamine (Thermo Fisher Scientific, 25030164)

· Penicillin-Streptomycin (Thermo Fisher Scientific, 15140122)

· DPBS (for example Thermo Fisher Scientific, 14190250)

· DMEM/F-12 (for example Thermo Fisher Scientific, 11330057)

· BMP-4 (R & D Systems, 314-BP-050)

· Noggin (R & D Systems, 6057-NG-100)

· TrypLE Express (Thermo Fisher Scientific, 12604021)

Equipment

· Cell culture incubator (37°C, 5% CO2)

· Refrigerated centrifuges for 15 ml and 1.5 ml tubes and multiwell plates

· Biosafety cabinet

· Mechanical Pipettes

· Pipette controller

· Sterile filter pipette tips

· Sterile wide bore pipette tips

· Sterile 15 ml centrifuge tube (for example Corning Inc., 352095)

· Sterile 1.5 ml microcentrifuge tubes (for example Eppendorf, 0030120086)

· 96-well Round Bottom Ultra-Low Attachment Microplate (Corning Inc., 7007)

· Transwell (Corning Inc., 3470)

· 6-well Multiwell Cell Culture Plate (for example Corning Inc., 353046)

· 24-well Multiwell Cell Culture Plate (for example Corning Inc., 353047)

· 24-Well Multiwell Cell Culture Plates (VWR international, 10062-896)

· Serological pipettes

· Inverted microscope (for example Olympus, model CKX53)

· Cell counter

· Cell counter slides

Procedure

MEDIUM SETUP

Gut Growth Medium

Advanced DMEM/F12

B27 supplement; 1X

N2 supplement; 1X

HEPES; 15 mM

L-glutamine; 2 mM

Penicillin/streptomycin; 1X

Medium for Primitive streak induction (Day 0 - 1)

RPMI 1640

BMP4; 50 ng/mL

Activin A; 100 ng/mL

Penicillin/streptomycin; 1X

Medium for Definitive endoderm induction #1 (Day 1 - 2)

RPMI 1640

dFBS; 0.2%

Activin A; 100 ng/mL

Penicillin/streptomycin; 1X

Medium for Definitive endoderm induction #2 (Day 2 - 3)

RPMI 1640

dFBS; 2.0%

Activin A; 100 ng/mL

Penicillin/streptomycin; 1X

Medium for Anterior gut cell induction (Day 3 - 7)

Gut Growth Medium

FGF4; 500 ng/mL

CHIR99021; 2 µM

Noggin; 200 ng/mL

Medium for Posterior gut cell induction (Day 3 - 7)

Gut Growth Medium

FGF4; 500 ng/mL

CHIR99021; 3µM

PROCEDURE

Maintenance of PSCs

Maintain the undifferentiated hPSCs on feeder-free conditions in StemFit (Ajinomoto Co., Inc.) or mTeSR1 medium (STEMCELL technologies) on plates coated with iMatrix-511 (Matrixome, Inc.) or Matrigel (Growth Factor Reduced; Corning Inc.) in an incubator with 5% CO_2 at 37°C. Dissolve the Matrigel (Growth Factor Reduced) in ice-cold DMEM/F12 at 1:30 dilution to coat an entire well of culture plate.

Differentiation of PSCs into anterior and posterior gut spheroid

Differentiation of hPSCs into definitive endoderm was induced using previously described methods[1,2] with modifications. Culture the cells in an incubator with 5% CO2 at 37°C. Change the culture medium at the same hour each day without DPBS wash.

Day -2

1) Dissociate colonies of hPSCs by Accutase into single cells and plate the cells with 100,000 - 150,000 cells/cm^2 in mTeSR1 including 10μM Y27632 on Matrigel coated tissue culture plate (Corning). Prepare cells with at least 2 independent wells to generate anterior and posterior gut spheroids at the same time.

Day -1

2) Change medium to mTeSR1 without Y27632.

Day 0

3) Change medium to the Medium for Primitive streak induction (RPMI 1640 containing Penicillin/streptomycin, 100 ng/mL Activin A and 50 ng/mL BMP4). Cell confluency should be around 90 % at this time point.

Day 1

4) Change medium to the Medium for Definitive endoderm induction #1 (RPMI 1640 containing Penicillin/streptomycin, 100 ng/mL Activin A and 0.2 % dFBS).

Day 2

5) Change medium to the Medium for Definitive endoderm induction #2 (RPMI 1640 containing Penicillin/streptomycin, 100 ng/mL Activin A and 2% dFBS).

Day 3-6

6) For anterior gut cell induction, change medium to the Medium for Anterior gut spheroid induction (Gut growth medium including 200 ng/mL noggin, 500 ng/ml FGF4 and 2 μM CHIR99021). Replace the medium every day.

7) For posterior gut cell induction, change medium to the Medium for Posterior gut spheroid induction (Gut growth medium including 500 ng/ml FGF4 and 3 μM CHIR99021). Replace the medium every day.

Day 7

At day 7, confirm that the differentiated cells start to come up from bottom of plate as shown in previous reports[1,2].

8) Dissociate each of anterior or posterior gut cells into single cells by incubation with TrypLE Express at 37°C and collect the each type of cells into 15 mL centrifuge tubes separately .

9) Centrifuge each tubes at 1000 rpm for 3 minutes and, after removing supernatant, re-suspend the pellets in Gut growth medium with 10 μM of Y-27632.

10) Plate the anterior or posterior gut cell suspensions on 96 well round bottom ultra-low attachment plate (Corning Inc.) at density of 10,000 cells/well.

11) Centrifuge the plate at 1000 rpm for 1 minute to gather cells and incubated at 37°C for 24 hours to form spheroid.

Day 8

12) Mix the generated single anterior spheroid and posterior spheroid on 96 well round bottom ultra-low attachment plate in gut growth medium without Y27632.

13) Centrifuge the plate at 1000 rpm for 1 minute to gather spheroids and incubated at for 24 hours to form fused boundary spheroids (anterior-posterior gut spheroid; A-P spheroids).

Day 9 - 13

14) Pickup an A-P spheroid into centrifuge tube by mechanical pipette with wide bore tip. Re-suspend the A-P spheroid in 20 -30 µL/spheroid/well of 100 % Matrigel (Phenol Red-Free) on ice and plate it on 24 well tissue culture plate (VWR international) to make Matrigel drop.

15) Incubate the drop in CO_2 incubator at 37°C for 5 minutes.

16) Flip the plate upside down and incubate for another 5 minutes to prevent the spheroid from attaching bottom.

17) Add Gut growth medium enough to cover entire drop and culture for 4 days to generate Hepato-biliary-pancreatic organoids (HBPOs).

Troubleshooting

Day 7

Problem: Gut cells are not differentiated

Solution:

· Check the pluripotency of the undifferentiated state of PSCs by general undifferentiation maker expression.

· Confirm the cell confluency at day 0 is around 90 %.

· Make sure that each aliquots of small molecule or recombinant protein should be reconstituted and stored appropriately according to manufacture's document.

· Do not store the medium in refrigerator for more than a week after adding small molecules, recombinants, serum or supplements which are not recommended to keep in refrigerator .

· Check the concentration of each component in differentiation medium.

· Make sure that all medium change was performed at the same hour each day.

Day 8

Problem: Gut spheroids are not formed

Solution:

· Use TrypLE Express for dissociation at day 7, not Accutase, EDTA or Trypsin.

· Make sure that 10 μM of Y-27632 was added in medium at day 7.

· Make sure that the seeding cell number was appropriate at day 7.

· Centrifuge the plate at 1000 rpm for 1 minute before starting culture at day 7.

· Check the Solutions described in above for the problem of day 7.

Day 9

Problem: A-P spheroids are not formed

Solution: Centrifuge the plate at 1000 rpm for 1 minute before starting culture at day 8.

Problem: Matrigel drop is not formed appropriately

Solution:

· Remove the supernatant before adding Matrigel.

· Do not dilute the Matrigel.

· The type of tissue culture plate is strongly affect on efficiency of Matrigel drop formation. From our comparison of plates type from different companies, we recommended to use the Multiwell Cell Culture Plates (VWR international, 10062-896) for Matrigel drop formation.

Day 11 - 13

Problem: Hepato-biliary-pancreatic domain is not observed

Solution:

· Check the Solutions described in above for the problem of day 7.

· Confirm that the spheroid was covered by Matrigel drop completely.

· Confirm that the Matrigel drop was covered by medium completely.

· Do not contain Y27632 in the medium at day 8.

Time Taken

· Step 1, Preparation of cell: 1-2 hours

· Step 2, Feeding medium: 30 minutes

· Step 3, Feeding medium: 30 minutes

· Step 4, Feeding medium: 30 minutes

· Step 5, Feeding medium: 30 minutes

· Steps 6-7,Feeding medium: 30 - 60 minutes every day

· Steps 8-11, Preparation of cell: 2 - 3 hours

· Steps 12-13, Preparation of cell: 1 - 2 hours

· Steps 14-17, Preparation of spheroid: 2 - 3 hours

Anticipated Results

The boundary interactions between anterior and posterior gut spheroids differentiated from human pluripotent stem cells enables autonomous emergence of hepato-biliary-pancreatic (HBP) organ domains specified at the foregut-midgut boundary organoids in the absence of extrinsic factor supply.

This enables the subsequent study for early morphogenesis of HBP subdomains, and for generating inter-connected, multi-organ structures within personalized human model systems for organogenesis and disease in vitro.

References

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Figures

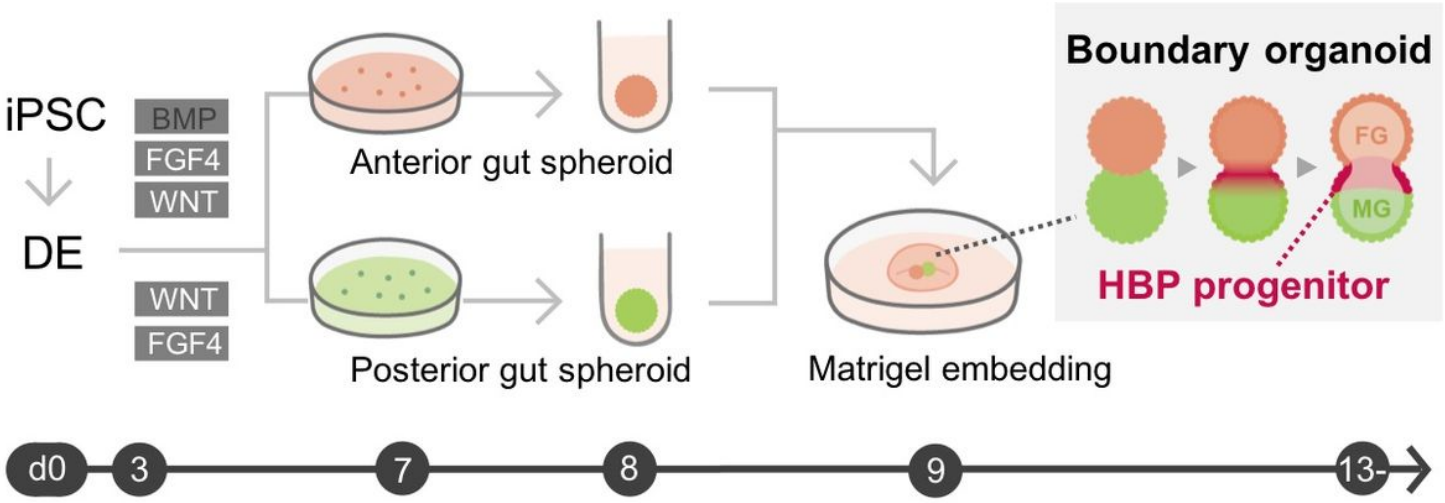

Figure 1

Overview of the HBPO generation protocols.