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Title: Generation of Functional Endodermal Hepatic Organoids

Authors and Affiliations:

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Soheil Akbari[1]*, Nevin Ersoy[1,2,3]*, Alper Bağrıyanık[1,2,4], Nur Arslan[1,5], Tamer Tevfik Önder[6], Esra Erdal[1,7]

[1]Izmir Biomedicine and Genome Center
[2]Department of Histology and Embryology, Health Science Institute, Dokuz Eylul University
[3]Department of Histology and Embryology, Faculty of Medicine, Izmir Tinaztepe University
[4]Department of Histology and Embryology, Faculty of Medicine, Dokuz Eylul University
[5]Department of Pediatric Gastroenterology and Metabolism, Faculty of Medicine, Dokuz Eylul University
[6]Department of Medical Biology, Faculty of Medicine, Koc University
[7]Department of Medical Biology, Faculty of Medicine, Dokuz Eylul University

*These authors contributed equally

Corresponding Authors:

Esra Erdal (esra.erdal@ibg.edu.tr)

Email Addresses for All Authors:

Soheil Akbari (soheil.akbari@ibg.edu.tr)
Nevin Ersoy (nevinersoy@gmail.com)
Alper Bağrıyanık (alper.bagriyanik@deu.edu.tr)
Nur Arslan (nur.arslan@deu.edu.tr)
Tamer Tevfik Önder (tonder@ku.edu.tr)
Esra Erdal (esra.erdal@ibg.edu.tr)

Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

√ Correct

2. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **NO**

3. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **NO**

4. Proposed filming date: To help JoVE process and publish your video in a timely manner, please indicate the proposed date that your group will film here: **MM/DD/YYYY**

When you are ready to submit your video files, please contact our Content Manager, Utkarsh Khare.

Current Protocol Length

Number of Steps: 19
Number of Shots: 55

Introduction

REQUIRED:

1.1. **Esra Erdal:** This research aims to develop human-relevant in vitro liver models for accurate drug efficacy and safety testing, closely mimicking healthy and diseased liver tissues in preclinical studies.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.7.1.*

~~What are the most recent developments in your field of research?~~

~~1.2. **Esra Erdal:** Recent advances include the development of multicellular liver organoids with vascularization and immune components, enhancing their use in disease modeling, drug screening, and regenerative therapy applications.~~

~~1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.~~ NOTE: The authors want to skip this interview statement.

What are the current experimental challenges?

1.3. **Esra Erdal:** Reproducing full liver complexity, achieving long-term functionality, standardizing protocols, and scaling organoid production for high-throughput applications remain major experimental challenges in liver organoid research.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What significant findings have you established in your field?

1.4. **Esra Erdal:** We recently modeled liver fibrosis progression from healthy eHEPOs and established rare disease models, including citrullinemia and pyruvate carboxylase deficiency, using patient-derived cells.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 4.*

What advantage does your protocol offer compared to other techniques?

1.5. **Esra Erdal:** Unlike other methods, our protocol provides functional liver organoids quickly and at scale, supporting long-term culture and multiple assays from one consistent starting population.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 2.*

Ethics Title Card

This research has been approved by the local Clinical Research Ethics Committee of Dokuz Eylul University Medical Faculty

2. Endoderm Differentiation Procedure

Demonstrator: Click here to enter name of demonstrator(s)

2.1. To begin, add 6 milliliters of cold DMEM/F-12 *(D-M-E-M F-twelve)* into a 15-milliliter conical tube **[1]**. Immediately add 80 microliters of thawed basement membrane matrix or BMM *(B-M-M)* to the tube and mix well **[2]**. Coat a tissue culture-treated well plate with the BMM solution and dispense the solution to cover the well plate surface **[3]**.

2.1.1. WIDE: Talent adding DMEM/F-12 into a 15-milliliter conical tube.

2.1.2. Talent adding BMM to the tube and mixing it.

2.1.3. Talent coating a tissue culture-treated well plate with the BMM solution and the solution being dispersed to cover the well plate surface.

2.2. Incubate the plate in a 37 degrees Celsius incubator for at least 1 hour **[1]**. Take an inducible pluripotent stem cell or iPSC *(I-P-S-C)* plate and aspirate the iPSC-specific medium **[2]**.

2.2.1. Talent placing the plate in an incubator.

2.2.2. A shot of the iPSC plate and then talent aspirating the iPSC-specific medium.

2.3. Rinse the plate with DMEM/F-12 or D-PBS *(D-P-B-S)* **[1]** and add 1 milliliter of the specific enzyme to one well of a 6-well plate. After incubating for 1 minute **[2]**, aspirate the enzyme **[3]**, and incubate the plate at 37 degrees Celsius for 5 to 7 minutes **[4]**.

2.3.1. Talent rinsing the plate with DMEM/F-12 or D-PBS.

2.3.2. Talent adding the specific enzyme to one well of a 6-well plate.

2.3.3. Talent aspirating the enzyme.

2.3.4. Talent placing the plate in an incubator.

2.4. Next, add 1 milliliter of pre-warmed iPSC medium to the plate **[1]** and gently tap the side to detach the undifferentiated colonies aggregates from the surface of the plate **[2]**.

2.4.1. Talent adding iPSC medium to the plate.

2.4.2. Talent tapping the side of the plate.

2.5. Gently mix the suspension with a 5-milliliter serological pipette **[1]** and then seed the cell suspension at the desired density onto the BMM-coated well **[2]**. Incubate the cells at 37 degrees Celsius for 3 to 5 days to reach a confluency of 70% **[3]**.

2.5.1. Talent mixing the suspension with a 5-milliliter serological pipette.

2.5.2. Talent seeding the cell suspension onto the BMM-coated well.

2.5.3. Talent placing the plate in an incubator.

2.6. Replace the iPSC medium with the endoderm medium before starting the differentiation **[1]**. The cells gain a spiky shape after the differentiation, indicating the cell-cell interaction **[2-TXT]**.

2.6.1. Talent taking out the iPSC medium and adding the endoderm medium to the plate.

2.6.2. SCOPE: The spiky shape of the cells. **TXT: Control the cell's morphological changes during differentiation**

3. Procedure for Performing eHEPO Establishment

Demonstrator: Click here to enter name of demonstrator(s)

3.1. Rinse the endoderm cells with PBS **[1]**, add 300 microliters of trypsin **[2]**, and incubate the cells for 5 minutes in the incubator **[3]**. Add 3 milliliters of cold DMEM-F12 to the cells **[4]**, and pipet with 1,000-microliter filter tips to generate single cells **[5]**.

3.1.1. Talent rinsing the endoderm cells with PBS.

3.1.2. Talent adding trypsin to the cells.

3.1.3. Talent placing the cells in an incubator.

3.1.4. Talent adding DMEM-F12 to the cells.

3.1.5. Talent pipetting the cells with 1,000-microliter filter tip.

3.2. Collect the cell suspension in a 15-milliliter conical tube **[1]** and centrifuge the cells at 300 *g* for 5 minutes at room temperature **[2]**. After discarding the supernatant, wash the pellet with 10 milliliters of FACS *(Fax)* buffer **[3]**.

3.2.1. Talent collecting the cell suspension in a 15-milliliter conical tube.

3.2.2. Talent placing the tube in a centrifuge.

3.2.3. Talent washing the pellet with FACS buffer.

3.3. To make a single-cell suspension, filter the cells with a 100-micrometer mesh strainer **[1]** and then with a 40-micrometer mesh strainer before centrifuging the cell suspension for 5 minutes **[2]**.

3.3.1. Talent filtering the cells with a 100-micrometer mesh strainer.

3.3.2. Talent filtering the cells with a 40-micrometer mesh strainer.

3.4. Resuspend 15 million cells with 105 microliters of FACS buffer **[1]**, and add 45 microliters of FcR *(F-C-R)* and 15 microliters of EpCAM *(Ep-Cam)* to the suspension **[2]**. Incubate the cells for 10 minutes at 4 degrees Celsius, protecting the tube from light **[3]**. Then, add 500 microliters of FACS buffer to wash the cell suspension **[4]**. NOTE: The VO has been edited.

3.4.1. Talent suspending the cells with FACS buffer.

3.4.2. Talent adding FcR and EpCAM to the suspension.

3.4.3. Talent placing the solution on ice, protecting the tube from light. NOTE: This shot was changed during the shoot.

3.4.4. Talent adding FACS buffer to wash the cell suspension.

3.5. After centrifuging the cells, resuspend the pellet in 500 microliters of FACS buffer **[1]** and add 0.5 micromolar per milliliter DAPI *(Dapi)* to the cell suspension to quantify the dead cells **[2]**.

3.5.1. Talent suspending the pellet in FACS buffer.

3.5.2. Talent adding DAPI to the cell suspension.

3.6. After the cell sorting, centrifuge the cells at 300 *g* for 5 minutes **[1]**, remove the supernatant **[2]**, and resuspend 3,000 to 5,000 cells in 20 microliters of BMM **[3]**.

3.6.1. Talent placing the cells in a centrifuge.

3.6.2. Talent removing the supernatant.

3.6.3. Talent suspending the cells in BMM.

3.7. Seed the cells by adding a droplet of BMM to the center of each 48-well plate **[1]** and incubate the cells for 2 minutes at 37 degrees Celsius **[2]** until the BMM is solidified **[3-TXT]**. Overlay the droplet with the expansion medium **[4-TXT]**.

3.7.1. Talent adding a droplet of BMM to the center of a 48-well plate.

3.7.2. Talent placing the cells in an incubator.

3.7.3. The BMM is solidified. **TXT: Incubate for another 10 min**

3.7.4. Talent overlaying the droplet with the expansion medium. **TXT: 250 µL per well for a 48-well plate**

4. Procedure for Performing eHEPO Mechanical Dissociation and Differentiation

Demonstrator: Click here to enter name of demonstrator(s)

4.1. To split the organoid droplet, discard the organoid medium **[1]** and add 500 microliters of cold Ad-DMEM/F12 *(A-D D-M-E-M F-twelve)* to the well **[2]**. Use 1,000-microliter filter tips to pipette the BMM droplets vigorously **[3]**.

4.1.1. Talent discarding the organoid medium.

4.1.2. Talent adding Ad-DMEM/F12 to the well.

4.1.3. Talent pipetting the BMM droplets vigorously with 1,000-microliter filter tips.

4.2. Collect a maximum of 10 BMM droplets into 15-milliliter conical tubes **[1]** and add 5 milliliters of cold Ad-DMEM/F12 **[2]**. Place the tube on ice for 3 to 5 minutes **[3]**.

4.2.1. Talent collecting BMM droplets into 15-milliliter conical tubes.

4.2.2. Talent adding Ad-DMEM/F12 to the tube.

4.2.3. Talent placing the tube on ice.

4.3. Centrifuge the cells at 200 *g* for 5 minutes at 4 degrees Celsius **[1]** and discard the supernatant until 1 milliliter of the supernatant remains **[2]**. Vigorously pipette the cell pellet 30 to 40 times using 200-microliter filter tips **[3]**.

4.3.1. Talent placing the cells in a centrifuge.

4.3.2. Talent discarding the supernatant until 1 milliliter of the supernatant remains.

4.3.3. Talent pipetting the cell pellet using 200-microliter filter tips.

4.4. After mechanical dissociation, add 5 milliliters of cold Ad-DMEM/F12 before centrifuging the cells for 5 minutes **[1]**. Discard the supernatant **[2]**, and place the suspension on ice **[3]**.

4.4.1. Talent adding Ad-DMEM/F12 to the cells.

4.4.2. Talent discarding the supernatant.

4.4.3. Talent placing the suspension on ice.

4.5. Seed a 50-microliter BMM droplet **[1-TXT]**, and add 25 nanograms per milliliter BMP7 *(B-M-P-seven)* to the expansion medium **[2]**.

4.5.1. Talent seeding a 50-microliter BMM droplet. **TXT: BMM droplet: 35 µL BMM + 15 µL cells and Ad-DMEM/F-12 per well for a 24-well plate**

4.5.2. Talent adding BMP7 to the expansion medium.

4.6. After culturing for 3 days, replace the medium with the differentiation medium **[1]** and culture the cells for at least 14 days **[2-TXT]**.

4.6.1. Talent removing the medium and adding the differentiation medium to the cells.

4.6.2. Talent placing the cells in an incubator to culture the cells. **TXT: Refresh the medium every 3 days**

Results

5. Results

5.1. An immunostaining analysis was performed to validate whether the organoids originating from the EpCAM+ *(Ep-Cam positive)* cells are committed toward the hepatic lineage **[1]**.

5.1.1. LAB MEDIA: Figure 2.

5.2. The presence of CK19+ *(C-K-nineteen-positive)* **[1]** and HNF4α+ *(H-N-F-four-alpha-positive)* cells **[2]** indicates that hepatoblast and bile duct progenitor cells are present in the organoids **[3]**.

5.2.1. LAB MEDIA: Figure 2. *Video Editor: Emphasize A (both the top and bottom image).*

5.2.2. LAB MEDIA: Figure 2. *Video Editor: Emphasize E (top image).*

5.2.3. LAB MEDIA: Figure 2.

5.3. Additionally, organoids expressing epithelial tissue markers, such as **[1]** E-CAD+ *(E C-A-D-positive)* cells **[2]** and cubical or polyhedral epithelial cells expressing ZO-1 *(Z-O-one)* **[3]**, indicate the presence of tight junctions between the cells **[4]**.

5.3.1. LAB MEDIA: Figure 2.

5.3.2. LAB MEDIA: Figure 2. *Video Editor: Emphasize D (both the top and bottom image).*

5.3.3. LAB MEDIA: Figure 2. *Video Editor: Emphasize B (both the top and bottom image).*

5.3.4. LAB MEDIA: Figure 2.

5.4. Immunohistochemistry revealed that the E-CAD+ cells showed a polygonal epithelioid structure, reflecting a hepatocyte-like phenotype **[1]**.

5.4.1. LAB MEDIA: Figure 3. *Video Editor: Highlight B (Both the top and bottom image).*

5.5. The Transmission electron microscopy images of eHEPOs *(E-H-E-P-Os)* are shown here **[1]**. The white circles indicate the intercellular junctional complexes **[2]**, the black arrows show apical surface differentiation **[3]**, and the red arrow shows a transverse section of microvilli **[4]**.

5.5.1. LAB MEDIA: Figure 4.

5.5.2. LAB MEDIA: Figure 4. *Video Editor: Highlight the white circles in A, B, and C.*

5.5.3. LAB MEDIA: Figure 4. *Video Editor: Highlight the black arrows in A, B, and C.*

5.5.4. LAB MEDIA: Figure 4. *Video Editor: Highlight the red arrow in B.*

5.6. qPCR *(Q-P-C-R)* analysis showed that organoids gained mature hepatocyte markers such as ALB *(A-L-B)*, A1AT *(A-one-A-T)*, CYP3A4 *(C-Y-P-three-A-four),* and CYPA7 *(C-Y-P-A-seven)* **[1]**. Pink-stained area in PAS *(P-A-S)* staining indicates the glycogen storage capacity of organoids **[2]**.

5.6.1. LAB MEDIA: Figure 5A, 5B.

5.6.2. LAB MEDIA: Figure 5A, 5B. *Video Editor: Highlight B.*

5.7. Additionally, our analysıs showed the organoids ability to secrete ALB into the culture media, eliminate ammonia from culture media, CYP3A4 enzyme activity and bile acid trasnport capacity **[1]**.

5.7.1. LAB MEDIA: Figure 5C-5G.

Pronunciation Guides:

1. Basement Membrane

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/basement%20membrane
- **IPA**: /ˈbeɪsmənt ˈmɛmˌbreɪn/
- **Phonetic Spelling**: BAYSS-muhnt MEM-brayn

2. Serological

- **Pronunciation link**: https://www.merriam-webster.com/medical/serological
- **IPA**: /ˌsɪrəˈlɑdʒɪkəl/
- **Phonetic Spelling**: SIR-uh-LOJ-ih-kuhl

3. BMP7

- **Pronunciation link**: https://www.howtopronounce.com/bmp7
- **IPA**: /ˌbiːɛmˈpiː ˈsɛvən/
- **Phonetic Spelling**: BEE-EM-PEE SEV-uhn

4. CK19

- **Pronunciation link**: https://www.howtopronounce.com/ck19

- **IPA**: /ˌsiːˈkeɪ naɪnˈtiːn/
- **Phonetic Spelling**: SEE-KAY NINE-teen

5. HNF4α

- **Pronunciation link**: https://www.howtopronounce.com/hnf4-alpha
- **IPA**: /ˌeɪtʃɛnˈɛf fɔːr ˈælfə/
- **Phonetic Spelling**: AYCH-EN-EF FOUR AL-fuh

6. qPCR

- **Pronunciation link**: https://www.howtopronounce.com/qpcr
- **IPA**: /ˌkjuː piː siː ˈɑːr/
- **Phonetic Spelling**: KYOO-PEE-SEE-AR

7. ALB

- **Pronunciation link**: https://www.howtopronounce.com/alb
- **IPA**: /ælb/
- **Phonetic Spelling**: ALB

8. A1AT

- **Pronunciation link**: https://www.howtopronounce.com/a1at
- **IPA**: /ˌeɪ wʌn eɪ ˈtiː/
- **Phonetic Spelling**: AY-one-AY-TEE

9. PAS

- **Pronunciation link**: https://www.howtopronounce.com/pas
- **IPA**: /piː eɪ ɛs/
- **Phonetic Spelling**: PEE-AY-ESS