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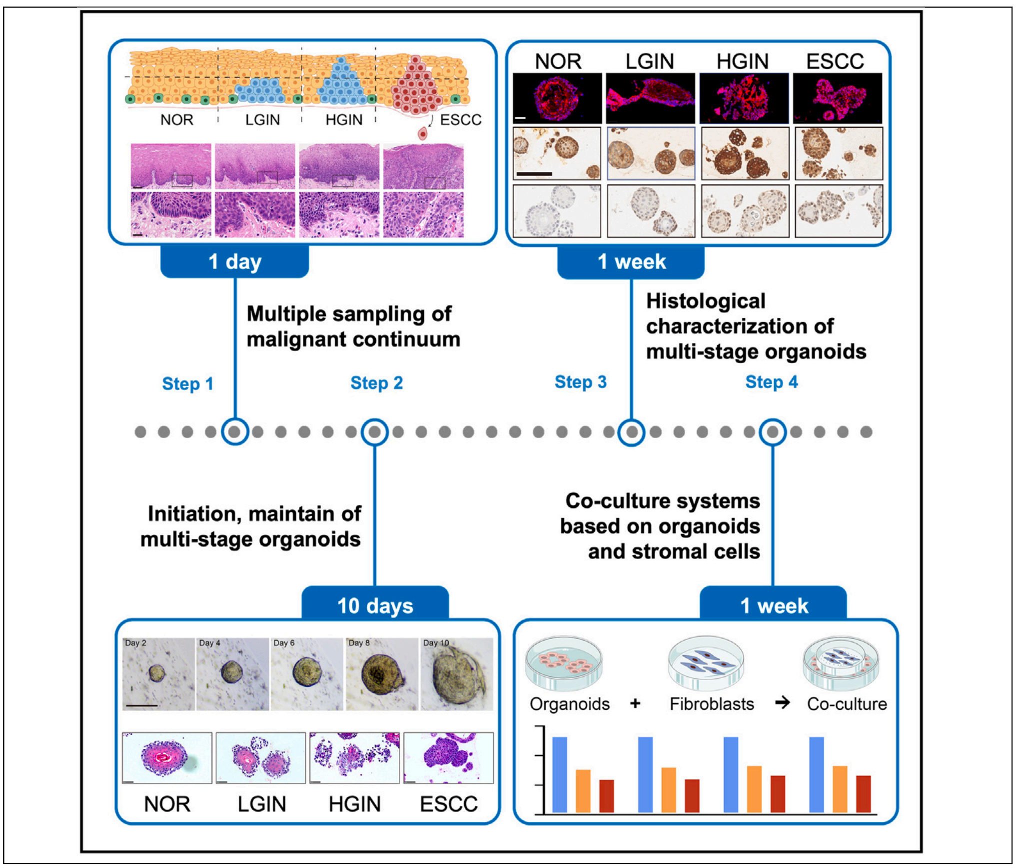
Protocol for the generation, characterization, and functional assays of organoid cultures from normal and cancer-prone human esophageal tissues



Multiple sampling strategies to cover different or dynamic stages of malignant continuum with organoid cultures provide a valuable platform for epithelium homeostasis, transformation, and cancer progression. Here, we present a protocol to initiate, culture, passage, and characterize organoids from normal and cancer-prone human esophageal tissues. We describe steps for multiple sampling of malignant continuum and the initiation and maintenance of multi-stage organoids. We then detail procedures for the histological characterization of organoids and co-culture systems based on organoids and stromal cells.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Multiple sampling strategies to cover dynamic stages of malignant continuum

Multi-stage esophageal organoid cultures

Characterizing multi-stage organoids using multiplex immunofluorescence

Co-culture assays of organoids and fibroblasts

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Protocol for the generation, characterization, and functional assays of organoid cultures from normal and cancer-prone human esophageal tissues

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SUMMARY

Multiple sampling strategies to cover different or dynamic stages of malignant continuum with organoid cultures provide a valuable platform for epithelium homeostasis, transformation, and cancer progression. Here, we present a protocol to initiate, culture, passage, and characterize organoids from normal and cancer-prone human esophageal tissues. We describe steps for multiple sampling of malignant continuum and the initiation and maintenance of multi-stage organoids. We then detail procedures for the histological characterization of organoids and co-culture systems based on organoids and stromal cells.

For complete details on the use and execution of this protocol, please refer to Chen et al.[1]

BEFORE YOU BEGIN

Patient-derived organoids have been widely applied in the research of various types of cancers, such as breast cancer,[2] colorectal cancer,[3] pancreatic cancer,[4] etc. As a novel experimental model, organoids can better mimic the pathological and molecular characteristics of each patient tumor tissue in an individualized manner.[5] Therefore, organoids have been utilized for drug screening and personalized tumor therapy.[5–7] The protocols for constructing organoids from mouse and patient esophageal squamous cell carcinoma (ESCC) have been reported.[8,9] However, their application in the multi-stage progression of tumors is still limited. Previous studies focused solely on culturing organoids of ESCC.[10,11] Here, we constructed organoids derived from four stages of tissues during the development of ESCC, aiming to investigate cellular behaviors and molecular mechanisms involved in the progression of ESCC.

Institutional permissions

This study was approved by the Institutional Review Boards of Cancer Hospital, Chinese Academy of Medical Sciences (20/069–2265). Informed consent was obtained from each patient, and the individual clinical information was collected from medical records.

L-WRN-conditioned medium preparation

Timing: 6 days

This section describes how to prepare the L-WRN-conditioned medium required for configuring organoid culture medium.

Day 1

Timing: 20 min

1. Selection medium preparation.
   a. Add 10% fetal bovine serum (FBS), G-418 (0.5 mg/mL), and hygromycin B (0.5 mg/mL) in the Dulbecco's modified Eagle medium (DMEM) to prepare 500 mL selection medium for the pure selection.
   b. Mix the selection medium fully.
   c. Store the selection medium at 4°C for up to 1 month.
2. Thaw and culture the L-WRN cell line.
   a. Thaw the L-WRN cell line in 37°C water bath for 2 min.

   Note: Ensure that there are at least 1.5×10^6 cells previously frozen.

   b. Centrifuge the cell suspension for 5 min at 200 *g* at 25°C.
   c. Remove the supernatant from the cryovials by gentle aspiration.
   d. Resuspend the cells with 1 mL fresh selection medium.
   e. Seed the cells on a 10 cm culture dish with selection medium and shake them evenly.
   f. Culture the cells in a humidified incubator with 5% CO_2 at 37°C.

Day 3

Timing: 30 min

3. Passage the L-WRN cell line.
   a. Check under the microscope if the cells are 80% confluent.
   b. Remove medium from culture dish by gentle aspiration.
   c. Wash cells with phosphate-buffered saline (PBS).
   d. Add enough 1× trypsin-EDTA to completely cover the cells.
   e. Place them at 37°C for 2 min.
   f. Check under the microscope if the cells have detached from the plate.

   Note: Extend the digestion time until more than 90% of the cells detached from the plate.

   g. Add equal volume of the selection medium to stop the trypsin-EDTA reaction.
   h. Collect all the liquid in a sterile tube.
   i. Centrifuge the cell suspension for 5 min at 200 *g* at 25°C.
   j. Remove the trypsin-EDTA solution by gentle aspiration.
   k. Resuspend the cells with 1 mL fresh selection medium.
   l. Seed the cells on a new culture dish with fresh selection medium with 1:3 subcultivation ratio and shake them evenly.
   m. Culture the cells in a humidified incubator with 5% CO_2 at 37°C.

Day 5

Timing: 15 min

4. Change the culture medium.
   a. Check under the microscope if the cells are 80% confluent.
   b. Change the medium with fresh 10% FBS DMEM.

Day 6

Timing: 45 min

5. Collect the L-WRN-conditioned medium.
   a. Collect the supernatant from the culture dish to a 50 mL centrifuge tube.
   b. Passage the cells for further collection or freeze them for stock (step 6).
   c. Centrifuge the supernatant for 5 min at 200 *g* at 25°C.
   d. Transfer the supernatant to a 20 mL syringe, filter the supernatant with a 0.22 μm filter and collect the filtrated supernatant, which is the L-WRN-conditioned medium, in a new 50 mL centrifuge tube.
   e. Store the L-WRN-conditioned medium at −80°C for further use.
6. Freeze the L-WRN cell line.
   a. Repeat the sub-steps b–k of step 3.
   b. Count the cell number and prepare 1.5×10^6 cells in a new 1.5 mL centrifuge tube.
   c. Centrifuge the cell suspension for 5 min at 200 *g* at 25°C.
   d. Remove the supernatant by gentle aspiration.
   e. Resuspend the cells with 1 mL FBS with 10% dimethyl sulfoxide (DMSO).
   f. Collect the cell suspension in a new cryovial.
   g. Store the cryovials at −80°C for at least 4 h in the BeyoCool Cell Freezing Container.
   h. Transfer the cryovials into liquid nitrogen.

Human esophageal organoid culture medium (H-EOCM) preparation

Timing: 30 min

7. H-EOCM preparation.
   a. Thaw 1.5 mL L-WRN-conditioned medium at 4°C for 4 h and mix the medium evenly.
   b. Add 3% L-WRN-conditioned medium, 1× Anti-Anti, 1× GlutaMAX, 1× N2 supplement, 1× B27 supplement, HEPES (0.15 mM), EGF (40 ng/mL), Y-27632 (10 μM), and A83-01 (50 μM) in the advanced DMEM/F12 medium to prepare 50 mL H-EOCM.

   Note: A83-01 is only required during the initial passage 1–2 to inhibit the growth of fibroblasts.

   c. Store the complete H-EOCM at −20°C for further use.
8. Thaw the H-EOCM at 4°C for 4 h before the experiment.

Human esophageal primary fibroblasts culture medium (H-EFCM) preparation

Timing: 30 min

9. H-EFCM preparation.
   a. Add 1× Anti-Anti, 1× GlutaMAX, 1× N2 supplement, 1× B27 supplement, and HEPES (0.15 mM) in the advanced DMEM/F12 medium to prepare 50 mL H-EFCM.
   b. Store the complete H-EFCM at −20°C for further use.
10. Thaw the H-EFCM at 4°C for 4 h before the experiment.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-KRT6A (dilution 1:800)	Proteintech	Cat# 10590-1-AP; RRID: AB_2134306
Biological samples		
Multistage esophageal tissues	Linzhou Esophageal Cancer Hospital; Cancer Hospital, Chinese Academy of Medical Sciences	N/A
Chemicals, peptides, and recombinant proteins		
B-27 supplement	Gibco	Cat# 17504044
HEPES	Gibco	Cat# 15630080
N-2 supplement	Gibco	Cat# 17502048
GlutaMAX	Gibco	Cat# 35050061
Anti-Anti (Antibiotic-Antimycotic)	Gibco	Cat# 15240062
EGF	Gibco	Cat# PHG0313
Y-27632	Selleck Chemicals	Cat# S1049
A83-01	Tocris Bioscience	Cat# 2939
Fetal bovine serum	Cell Technologies	Cat# 30070
Growth factor-reduced Matrigel	Corning	Cat# 354230
DMEM	Corning	Cat# 10-013-CV
Advanced DMEM/F12	Gibco	Cat# 12634028
Collagenase IV	Gibco	Cat# 17104019
TrypLE Express	Gibco	Cat# 12604021
Trypsin-EDTA	Gibco	Cat# 15400054
G-418	Sigma	Cat# A1720
Hygromycin B	Sigma	Cat# 400050
Cryostor	STEMCELL	Cat# 07930
Agar	Solarbio	Cat# A8190
Gelatin	Solarbio	Cat# G8061
4% Paraformaldehyde	Beyotime	Cat# P0099
Critical commercial assays		
Opal 5-Color Manual IHC Kit	PANOVUE	Cat# 10144100100
Experimental models: Cell lines		
L-WRN	ATCC	CRL-3276 RRID: CVCL_DA06
Other		
Clean bench cabinet	N/A	N/A
CO_2 incubator	N/A	N/A
Refrigerator centrifuge	N/A	N/A
Automated cell counter	Bio-Rad	Cat# 1450102
Inverted microscope	Leica	N/A
Dry baths/block heaters	N/A	N/A
Whole slide image scanner	Hamamatsu	Cat# C13210
10 μL filter tips	N/A	N/A
200 μL filter tips	N/A	N/A
1,000 μL filter tips	N/A	N/A
1.5 mL centrifuge tube	N/A	N/A
15 mL centrifuge tube	NEST	Cat# 601002
50 mL centrifuge tube	NEST	Cat# 602002
6-well plate	Corning	Cat# CLS3335
12-well plate	Corning	Cat# CLS3336
24-well plate	Corning	Cat# CLS3337
10 cm dish	Corning	Cat# CLS3296

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
6 cm dish	Corning	Cat# CLS3295
12 mm cell culture inserts, pore size 0.4 μm	Corning	Cat# CLS3460
6.5 mm cell culture inserts, pore size 8.0 μm	Corning	Cat# CLS3464
70 μm cell strainer	Corning	Cat# CLS431751
24-well low-attachment plate	Corning	Cat# CLS3473
Cell freezing container	Beyotime	Cat# FCFC012

MATERIALS AND EQUIPMENT

Selection medium

Reagent	Final concentration	Amount
DMEM	88%	440 mL
FBS	10%	50 mL
G-418 (50 mg/mL)	0.5 mg/mL	5 mL
hygromycin B (50 mg/mL)	0.5 mg/mL	5 mL
Total	N/A	500 mL

Store at 4°C for up to 1 month.

Human esophageal organoid culture medium (H-EOCM)

Reagent	Final concentration	Amount
Advanced DMEM/F-12	90.7%	45.35 mL
L-WRN-conditioned medium	3%	1.5 mL
Anti-Anti	1×	500 μL
GlutaMAX	1×	500 μL
N2 supplement	1×	500 μL
B27 supplement	1×	1 mL
HEPES	1×	500 μL
EGF (40 μg/mL)	40 ng/mL	50 μL
Y-27632 (10 mM)	10 μM	50 μL
A83-01 (50 mM)	50 μM	50 μL
Total	N/A	50 mL

Store at −20°C for up to 6 months.

Human esophageal primary fibroblasts culture medium (H-EFCM)

Reagent	Final concentration	Amount
Advanced DMEM/F-12	94%	47 mL
Anti-Anti	1×	500 μL
GlutaMAX	1×	500 μL
N2 supplement	1×	500 μL
B27 supplement	1×	1 mL
HEPES	1×	500 μL
Total	N/A	50 mL

Store at −20°C for up to 6 months.

Digestion buffer

Reagent	Final concentration	Amount
DMEM	90%	45 mL
Collagenase IV (20 mg/mL)	2 mg/mL	5 mL
Total	N/A	50 mL

Prepare these reagents fresh each time.

Wash buffer		
Reagent	Final concentration	Amount
PBS	98%	49 mL
Anti-Anti	1×	500 μL
HEPES	1×	500 μL
Total	N/A	50 mL
Prepare these reagents fresh each time.		

Passage buffer		
Reagent	Final concentration	Amount
Advanced DMEM/F-12	98%	49 mL
Anti-Anti	1×	500 μL
HEPES	1×	500 μL
Total	N/A	50 mL
Prepare these reagents fresh each time.		

STEP-BY-STEP METHOD DETAILS

Classical culture of organoids

Timing: 9 days

This section describes how to utilize human esophageal tissue samples, especially small-volume endoscopic biopsy samples, to construct and culture organoids. ESCC tumor, dysplasia lesion (≤ 2 cm to tumor margin) and normal (≥ 5 cm away from tumor margin) tissues were collected from the same individuals with ESCC underwent surgical resection. The histopathological diagnosis of tissue samples was made by two pathologists independently according to the WHO Classification of Tumors of Digestive System 5th Edition.[12] To ensure successful organoid cultivation, biopsy samples should have a minimum size of at least 4 mm^3. For a sample of 100 mm^3, it is recommended to seed cells from the digested sample into 6 wells of a 24-well plate.

Day 1

Timing: 5 h

1. Materials preparation.
   a. Thaw the Matrigel at 4°C for 4 h before the experiment. Use 50 μL Matrigel per well.
   b. Thaw the H-EOCM at 4°C for 4 h before the experiment. Use 500 μL H-EOCM per well.
   c. Add Collagenase IV (2 mg/mL) in the DMEM to prepare the digestion buffer (1 mL for each sample).
   d. Pre-cool the pipette tips at 4°C for further use.
   e. Pre-warm a 24-well plate in the 37°C incubator for further use.
2. Tissue digestion and single cell suspension preparation.
   a. Transfer the sample to an appropriate size of centrifuge tube according to the sample size.

   Note: Prepare 5 mL centrifuge tubes for samples of 100 mm^3 and 1.5 mL centrifuge tubes for samples of 10 mm^3.

   b. Add 1× Anti-Anti and HEPES (0.15 mM) in the PBS to prepare the wash buffer (15 mL for each sample).
   c. Wash the sample with the wash buffer three times by gently flipping the tube upside down.
   d. Cut the sample into 1 mm^3 pieces with surgical scissors.

e. Collect the shredded tissue in a 1.5 mL centrifuge tube.
f. Add 1 mL digestion buffer in the tube and digest the tissue at 37°C for 30 min. Invert the centrifuge tube 10 times to mix thoroughly every 10 min.

Note: The main aim of this step is to digest collagen and loosen the tissue structure. For tissue cut into 1 mm^3 pieces, a digestion time of 30 min is sufficient.

g. Centrifuge the cell suspension for 5 min at 400 *g* at 4°C and remove the supernatant by gentle aspiration.
h. Add 500 μL 0.05% trypsin-EDTA and digest at 37°C for 10 min.
i. Inactivate the digestion with 1 mL 10% FBS DMEM.
j. Filter the cell suspension with a 70 μm sterile strainer and collect the filtrate in a 50 mL centrifuge tube.
k. Transfer the cell suspension to a new 1.5 mL centrifuge tube.
l. Centrifuge the filtrate for 5 min at 400 *g* at 4°C and remove the supernatant by gentle aspiration.
m. Resuspend the cells with 100 μL H-EOCM evenly.

3. Organoid seeding.
   a. Count the cell number and prepare 5,000 cells in a new 1.5 mL centrifuge tube.
   b. Centrifuge the cell suspension for 5 min at 400 *g* at 4°C and remove the supernatant by gentle aspiration.
   c. Resuspend the cells with 50 μL Matrigel evenly to prepare the cell suspension with a density of 2×10^5 cells/mL.

⚠ CRITICAL: Keep the cell suspension and Matrigel on ice to avoid Matrigel solidification.

Note: Pipette gently to resuspend the cells to avoid bubbles. Operate on ice and use pre-cooled pipettes to avoid Matrigel solidification.

   d. Add 50 μL cell suspension and make Matrigel dorms in the center of one well of the 24-well plate.
   e. Incubate the plate in the 37°C cell culture incubator for 30 min and wait for Matrigel solidification. Meanwhile, pre-warm the H-EOCM in the 37°C cell culture incubator.
   f. Add 500 μL H-EOCM in the well to cover the Matrigel.

Note: Add the medium along the wall of the well and be careful not to disperse the Matrigel.

   g. Incubate the plate in a humidified incubator with 5% CO_2 at 37°C.

Note: Multiple samples from different stages of malignant continuum can be treated with the same method for organoid initiation, culture and passage.

Day 3

⏲ Timing: 20 min

4. Change the H-EOCM medium.
   a. Pre-warm the H-EOCM at 37°C.
   b. Remove the old medium by gentle aspiration.

Note: Aspirate the old medium along the wall of the well and be careful not to disturb the Matrigel.

c. Add 500 μL fresh H-EOCM in the well to cover the Matrigel.

Note: Add the medium along the wall of the well and be careful not to disperse the Matrigel.

d. Incubate the plate in a humidified incubator with 5% CO_2 at 37°C.

Note: Change the medium at least every three days until the organoids are passaged.

Semi-floating culture of organoids

Timing: 9 days

The classical organoid culture, while simpler to implement, may compromise some of organ-level characteristics. Compared to classical organoid cultures, semi-floating cultures allow organoids to form spherical structures and maintain size consistency more easily. As these organoids grow in Matrigel diluted with H-ECOM, they are not constrained by the extracellular matrix. In addition, semi-floating culture is more conducive to nanoparticle penetration and is more suitable for evaluating the safety and efficacy of nanomaterials.[13] Therefore, the semi- floating culture system will aid in the development of nanomedicines for tumors, such as chitosan or liposome based nanoparticles drug delivery system.

Day 1

Timing: 5 h

5. Materials preparation.
   a. Repeat sub-steps a–d of step 1.
   b. Pre-warm a 24-well low-attachment plate in the 37°C incubator for further use.
6. Tissue digestion and single cell suspension preparation.
   a. Repeat step 2.
7. Organoid seeding.
   a. Repeat sub-steps a and b of step 3.
   b. Mix 50 μL Matrigel and 450 μL H-EOCM and resuspend the cells with the mixture evenly.

CRITICAL: Keep the cell suspension and Matrigel on ice to avoid Matrigel solidification.

Note: Pipette gently to resuspend the cells to avoid bubbles. Operate on ice and use pre-cooled pipettes to avoid Matrigel solidification.

   c. Add 500 μL cell suspension in one well of the 24-well plate.
   d. Incubate the plate on an orbital shaker at 80 rpm in a humidified incubator with 5% CO_2 at 37°C.

Day 3

Timing: 30 min

8. Change the H-EOCM medium.
   a. Thaw the Matrigel at 4°C for 4 h before the experiment.
   b. Thaw the H-EOCM at 4°C for 4 h before the experiment.
   c. Pre-cool the pipette tips at 4°C for further use.
   d. Collect the cell suspension to a new 1.5 mL centrifuge tube.

e. Centrifuge the cell suspension for 5 min at 400 *g* at 4°C and remove the supernatant by gentle aspiration.

Note: A distinct boundary line between the Matrigel and the supernatant could be seen, do not disturb the Matrigel beneath this boundary line.

f. Mix 50 μL Matrigel and 450 μL H-EOCM and resuspend the cells with the mixture evenly.

Note: Pipette gently to resuspend the cells to avoid bubbles. Operate on ice and use pre-cooled pipettes to avoid Matrigel solidification.

g. Add 500 μL cell suspension in one well of the 24-well plate.
h. Incubate the plate on an orbital shaker at 80 rpm in a humidified incubator with 5% CO_2 at 37°C.

Note: Change the medium at least every three days until the organoids are passaged.

Organoid passaging

Timing: 2 h

This section describes the passage of human esophageal tissue organoids. The growth status of the organoids should be monitored at least every two days after seeding (Figure 1A). Once cornified pearls form in the center of approximately 70%–80% of the organoids, it is necessary to passage them to maintain their optimal growth behavior. The timing for passaging varies based on the growth status of the organoids, typically occurring between 9–14 days based on our previous experience.

9. Materials preparation.
   a. Thaw the Matrigel (50 μL for each well) at 4°C for 4 h before the experiment.
   b. Thaw the H-EOCM (500 μL for each well) at 4°C for 4 h before the experiment.
   c. Pre-cool the pipette tips at 4°C for further use.
   d. Pre-warm a 24-well plate in the 37°C incubator for further use.
   e. Add 1× Anti-Anti and HEPES (0.15 mM) in the advanced DMEM/F12 medium to prepare the passage buffer (1 mL for each well), and pre-cool the passage buffer at 4°C for further use.
10. Organoid digestion.
    a. Organoid collection for classical culture.
       i. Remove the old medium by gentle aspiration.
       ii. Add 500 μL pre-cooled passage buffer in the well to melt Matrigel, blow forcefully to disperse Matrigel, and collect the mixture in a new 1.5 mL centrifuge tube.

Note: If there are still remnants at the bottom of the well, use the pipette tip to scrape off the remaining Matrigel.

    b. Organoid collection for semi-floating culture.
       i. Collect the cell suspension to a new 1.5 mL centrifuge tube directly.
    c. Add another 500 μL pre-cooled passage buffer to rinse the well and collect as many organoids as possible.
    d. Centrifuge the mixture for 5 min at 400 *g* at 4°C and remove the supernatant by gentle aspiration.

Note: A distinct boundary line between the Matrigel and the supernatant could be seen, do not disturb the Matrigel beneath this boundary line.

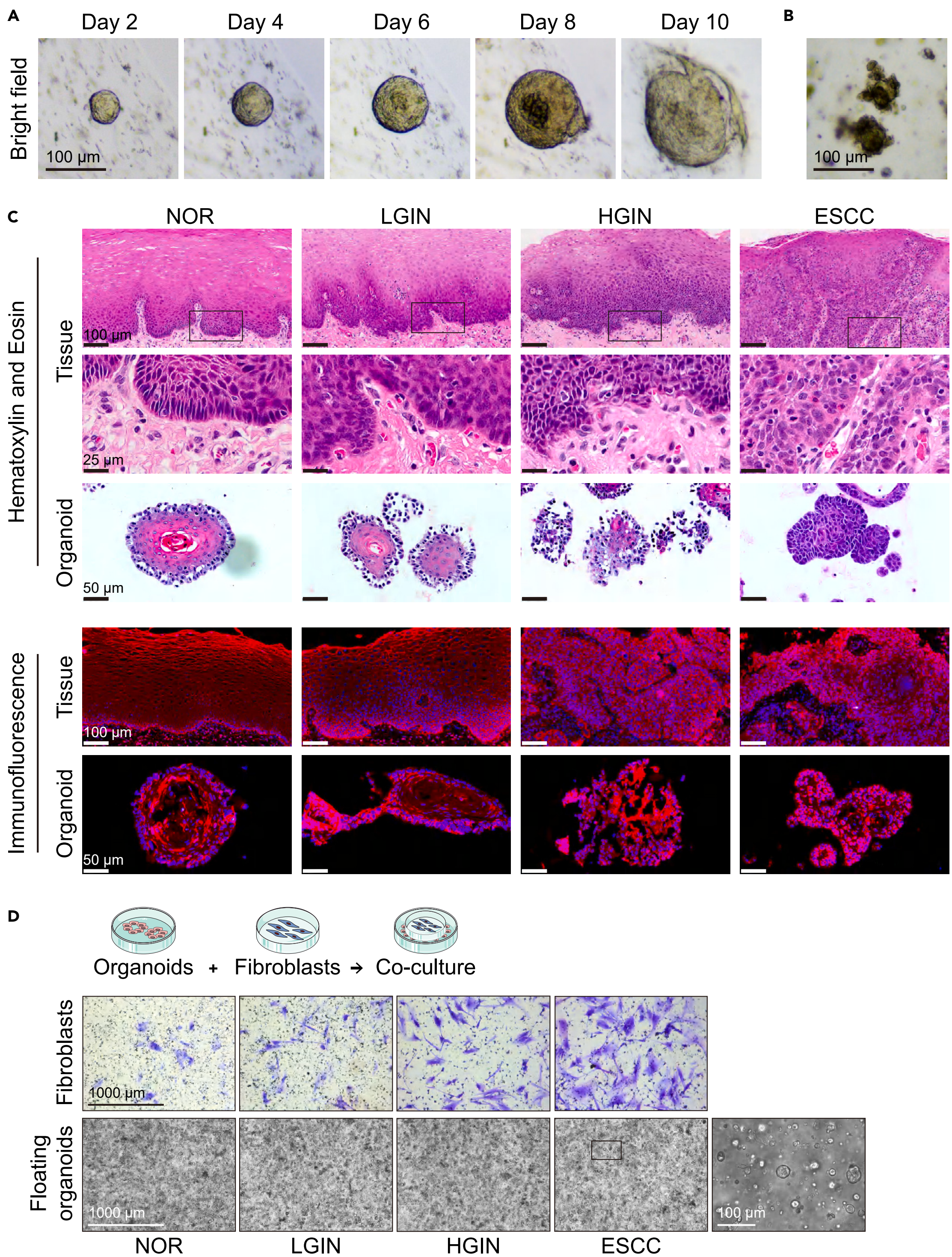

Figure 1. Generation, characterization, and functional assays of multi-stage organoids

(A) Microscopic images of organoids at different time points.

(B) Microscopic images of dying organoids.

(C) The upper panel shows the representative hematoxylin and eosin staining images of primary tissues and organoids. The lower panel shows the representative multiple immunofluorescence images of KRT6A of primary tissues and organoids.

(D) The upper panel shows the schematic of co-culture system and migration assay. The middle panel shows the representative images of fibroblasts migration induced by multi-stage organoids. The lower panel shows the representative bright field images of multi-stage organoids.

e. Add 500 μL TrypLE in the tube and digest the organoids at 37°C for 15 min with full resuspending every 5 min.

Note: The main aim of this step is digesting the Matrigel. At the end of digestion, relative movements between organoids can be observed under the microscope.

f. Centrifuge the mixture for 5 min at 400 *g* at 4°C and remove the supernatant by gentle aspiration.
g. Repeat sub-steps e and f.

Note: The main aim of this step is digesting the organoids. At the end of digestion, the disassembly of organoids can be observed under the microscope.

h. Resuspend the cells with 100 μL H-EOCM evenly.
i. Filter the cell suspension with a 70 μm sterile strainer and collect the filtrate in a 50 mL centrifuge tube.

11. Organoid seeding.
    a. Repeat step 3 for classical culture.
    b. Repeat step 7 for semi-floating culture.
12. Change the H-EOCM medium at least every three days.

Organoid freezing

Timing: 2 h

This section describes how to long-term cryopreserve organoids while ensuring that they maintain their growth vitality upon revival.

13. Materials preparation.
    a. Repeat sub-step e of step 9.
14. Organoid digestion.
    a. Repeat step 10.
15. Organoid freezing.
    a. Count the cell numbers and prepare 10,000 cells in a new 1.5 mL centrifuge tube.
    b. Centrifuge the cell suspension for 5 min at 400 *g* at 4°C and remove the supernatant by gentle aspiration.
    c. Resuspend the cells with 500 μL Cryostor evenly.
    d. Transfer the cell suspension to a new cryovial.
    e. Store the cryovials at −80°C for at least 4 h in the BeyoCool Cell Freezing Container.
    f. Transfer the cryovials into liquid nitrogen.

Histological analysis of organoid

Timing: 4–10 days

This section describes the process of collecting organoids, preparing paraffin sections, and performing multiplex immunofluorescence staining. We recommend initiating histological analysis when approximately 80% of the organoids have formed typical esophageal epithelial tissue structure, usually around 12–16 days of culture. The purpose of histological analysis of organoids is to determine the developmental stage of the derived organoids. Confirmation of multi- stage esophageal organoids is key for organoid establishment as well as co-culture assays based on multistage organoids and stromal cells.

Day 1

⏱ Timing: 2 h

16. Materials preparation.
    a. Add 1× Anti-Anti and HEPES (0.15 mM) in the advanced DMEM/F12 medium to prepare the passage buffer (2 mL for each well), and pre-cool the passage buffer at 4°C for further use.
    b. Add 1 g agar and 1.25 g gelatin to 50 mL water using a 250 mL conical flask, autoclave for 20 min at 121°C to prepare the embedding gel. Dispense the embedding gel into 5 mL aliquots in 15 mL centrifuge tubes and store at 25°C for further use.
17. Organoid fixing.
    a. Repeat sub-steps a–d of step 10.
    b. Add 1 mL passage buffer in a new 1.5 mL centrifuge tube and pipette vigorously to dissociate the precipitation.
    c. Centrifuge the mixture for 5 min at 400 *g* at 4°C and remove the supernatant by gentle aspiration.
    d. Resuspend the organoids with 500 μL 4% paraformaldehyde (PFA).
    e. Incubate the organoids for at least 6 h at 4°C.

Day 2

⏱ Timing: 1 h

18. Organoid embedding.
    a. Centrifuge the organoids suspension for 5 min at 400 *g* at 25°C and remove the supernatant by gentle aspiration.
    b. Add 1 mL passage buffer in the tube and pipette to wash the precipitation.
    c. Centrifuge the organoids suspension for 5 min at 400 *g* at 25°C and remove as much as possible of the supernatant by gentle aspiration.
    d. Place the 15 mL centrifuge tube containing 5 mL embedding gel in a 150 mL beaker containing 100 mL water.

⚠ CRITICAL: Loosen the lid of 15 mL centrifuge tube to ensure safe use of the microwave.

    e. Microwave at the highest power level (700 W) for 1 min until the water starts boiling.

Note: Confirm that the embedding gel has been liquefied.

    f. Resuspend the organoids in a 1.5 mL centrifuge tube with 50 μL embedding gel.
    g. Transfer the tube to 4°C and wait for gel solidification.
    h. Separate the solidified gel containing embedded organoids and transfer into 70% ethanol. Store at 4°C for further use.

Day 3

⏱ Timing: 2 h

19. Paraffin blocks preparation.
    a. Immerse in gradient alcohol from low concentration to high concentration (30%–50% –70%–80% –95%–100%) to dehydrate for 30 min each.
    b. Place in xylene transparent for 30 min.
    c. Soaked in wax and buried using HistoCore Arcadia H - Heated Paraffin Embedding Station.

Note: Paraffin-embedded sections should be stored at room temperature.

d. Continuously cut a 4 μm thick, thin section using the microtome, and carefully attach it to the clean glass slide.
e. Perform hematoxylin-eosin staining and immunohistochemistry analysis.

Day 4–8

Timing: 4 h/day

20. Multiplex immunofluorescence (mIF) staining.
    a. Dewaxing and hydration.
        i. Place the slide into a staining dish containing Histo-Clear II for 40 min at 65°C and transfer the slide into fresh Histo-Clear II for 20 min at 25°C.
        ii. Immerse the slide in anhydrous ethanol for 10 min twice, 95% ethanol for 5 min, 85% ethanol for 5 min, 75% ethanol for 5 min and 4% PFA for 10 min.
        iii. Immerse the slide in sterilized water for 2 min. Repeat this step three times.
    b. Peroxidase blocking.
        i. Remove the remaining sterilized water on the slide.
        ii. Use a histological pen to circle the area of the sample on the slide and add peroxidase blocking solution to cover the sample area.
        iii. Incubate at 25°C with humidity for 30 min.
    c. Antigen retrieval.
        i. Immerse the slide in sterilized water for 2 min. Repeat this step three times.
        ii. Immerse the dewaxing slide in Tris-EDTA antigen retrieval solution (pH 9.0).
        iii. Microwave at the highest power level (700 W) for 3 min until the water starts boiling.
        iv. Maintain at the lowest power level (70 W) for 15 min.

    Note: Pay attention to replenishing the liquid to prevent excessive evaporation and drying of the slide.

        v. Remove the dish and let it cool naturally to 25°C.
        vi. Immerse the slide in 1× TBST buffer for 2 min. Repeat this step three times.
    d. Sheep serum blocking.
        i. Remove the remaining sterilized water on the slide.
        ii. Add sheep serum blocking solution to cover the sample area.
        iii. Incubate at 25°C with humidity for 30 min.
        iv. Immerse the slide in 1× TBST buffer for 2 min. Repeat this step three times.
    e. Primary antibody incubation.
        i. Remove the remaining TBST buffer on the slide.
        ii. Add specific primary antibody solution to cover the sample area.

    Note: Confirm the dilution ratio of the specific antibody.

        iii. Incubate at 4°C with humidity for 8–12 h or at 25°C with humidity for 2 h.
        iv. Immerse the slide in 1× TBST buffer for 2 min. Repeat this step three times.
    f. Secondary antibody incubation.
        i. Remove the remaining TBST buffer on the slide.
        ii. Add specific secondary antibody solution to cover the sample area.
        iii. Incubate at 25°C with humidity for 10–30 min.
        iv. Immerse the slide in 1× TBST buffer for 2 min. Repeat this step three times.
    g. Fluorescent dye staining.
        i. Remove the remaining TBST buffer on the slide.

ii. Add fluorescent dye solution to cover the sample area.

Note: Ensure that each antibody is labeled with a different color fluorescent dye.

iii. Incubate at 25°C with humidity for 10–15 min.
iv. Immerse the slide in 1× TBST buffer for 2 min. Repeat this step three times.

h. Repeat sub-step c for antigen retrieval and noise reduction.
i. Repeat sub-steps d–h to perform additional staining.
j. DAPI staining.
   i. Remove the remaining TBST buffer on the slide.
   ii. Add DAPI staining solution to cover the sample area.
   iii. Incubate at 25°C with humidity for 10–20 min.
   iv. Immerse the slide in 1× TBST buffer for 2 min. Repeat this step twice.
   v. Immerse the slide in sterilized water for 2 min.
k. Slide mounting.
   i. Allow the slide to air-dry slightly, then use a pipette to drop anti-quenching mounting medium onto the slide to cover the sample area.
   ii. Mount the cover slip (#1).

Note: Mount the cover slip slowly to avoid bubble formation.

21. Scan the slide and analyze.

Primary fibroblasts culture

Timing: 3 days

This section describes the process of isolating and culturing fibroblasts from human esophageal tissue samples for subsequent co-culture assays and fibroblast migration assays. For a sample of 100 mm^3, it is recommended to seed cells from the digested sample into a 6 cm dish.

Day 1

Timing: 4 h

22. Materials preparation.
    a. Thaw the H-EFCM at 4°C (5 mL for each dish) for 4 h before the experiment.
    b. Add Collagenase IV (2 mg/mL) in the DMEM to prepare the digestion buffer (1 mL for each sample).
23. Tissue digestion and single cell suspension preparation.
    a. Repeat sub-step a–l of step 2.
24. Primary fibroblasts isolation.
    a. Resuspend the cells with 100 μL H-EFCM evenly.
    b. Seed the cells on a new 6 cm dish with H-EFCM and shake them evenly.
    c. Culture the cells in a humidified incubator with 5% CO_2 at 37°C for 30 min.
    d. Change the medium with fresh H-EFCM.
    e. Culture the cells in a humidified incubator with 5% CO_2 at 37°C.

Day 3

Timing: 30 min

25. Primary fibroblasts passaging.

   a. Check under the microscope if the cells are 80% confluent.
   b. Remove medium from culture dish by gentle aspiration.
   c. Wash cells with phosphate-buffered saline (PBS).
   d. Add enough 1× trypsin-EDTA to completely cover the cells.
   e. Place them at 37°C for 2 min.
   f. Check under the microscope if the cells have detached from the plate.
   g. Add equal volume of the H-EFCM to stop the trypsin-EDTA reaction.
   h. Collect all the liquid in a sterile tube.
   i. Centrifuge the cell suspension for 5 min at 200 *g* at 25°C.
   j. Remove the trypsin-EDTA solution by gentle aspiration.
   k. Resuspend the cells with 1 mL H-EFCM.
   l. Seed the cells on a new culture dish with complete medium with 1:3 subcultivation ratio and shake them evenly.
   m. Culture the cells in a humidified incubator with 5% CO_2 at 37°C.

26. Primary fibroblasts freezing.
   a. Repeat sub-steps a–k of step 25.
   b. Count the cell number and prepare 1.5×10^6 cells in a new 1.5 mL centrifuge tube.
   c. Centrifuge the cell suspension for 5 min at 200 *g* at 25°C.
   d. Remove the supernatant by gentle aspiration.
   e. Resuspend the cells with 1 mL FBS with 10% dimethyl sulfoxide (DMSO).
   f. Collect the cell suspension in a new cryovial.
   g. Store the cryovials at −80°C for at least 4 h in the BeyoCool Cell Freezing Container.
   h. Transfer the cryovials into liquid nitrogen.

Co-culture assays

Timing: 6 days

To investigate the crosstalk between multi-stage organoids (mainly epithelial cells) and fibroblasts or other stromal cells, as well as the phenotypic transition and molecular mechanisms by which they influence each other in cancer initiation and tumor evolution. This section describes the procedure for co-culturing organoids with primary fibroblasts to investigate how epithelial cells influence the phenotypic transition of fibroblasts.

Day 1

Timing: 2 h

27. Materials preparation.
   a. Thaw the Matrigel at 4°C for 4 h before the experiment.
   b. Thaw the H-EOCM at 4°C for 4 h before the experiment.
   c. Pre-cool the pipette tips at 4°C for further use.
   d. Pre-warm a 12-well plate in the 37°C incubator for further use.
   e. Add 1× Anti-Anti and HEPES (0.15 mM) in the advanced DMEM/F12 medium to prepare the passage buffer, and pre-cool the passage buffer at 4°C for further use.
28. Organoid digestion.
   a. Repeat step 10.
29. Organoid seeding.
   a. Count the cell number and prepare 20,000 cells in a new 1.5 mL centrifuge tube.
   b. Centrifuge the cell suspension for 5 min at 400 *g* at 4°C and remove the supernatant by gentle aspiration.
   c. Mix 100 μL Matrigel and 900 μL H-EOCM and resuspend the cells with the mixture evenly.

⚠ CRITICAL: Keep the cell suspension and Matrigel on ice to avoid Matrigel solidification.

Note: Pipette gently to resuspend the cells to avoid bubbles. Operate on ice and use pre-cooled pipettes to avoid Matrigel solidification.

d. Add 1 mL cell suspension to the center of one well of the 12-well plate.
e. Incubate the plate on an orbital shaker at 80 rpm in a humidified incubator with 5% CO_2 at 37°C.

Day 3

Timing: 20 min

30. Change the H-EOCM medium.

Day 5

Timing: 20 min

31. Primary fibroblasts passaging.
    a. Repeat sub-steps a–k of step 25.
32. Primary fibroblasts seeding.
    a. Count the cells.
    b. Install a 12 mm Transwell with 0.4 μm pore polycarbonate membrane insert on a 12-well plate.

Note: The 0.4 μm pore polycarbonate membrane insert can block cell migration and contamination with other type of migrated cells.

c. Seed 100,000 cells on the upper chamber of a Transwell unit with 500 μL H-EFCM and shake them evenly with hand.
d. Add 1 mL H-EFCM in the lower chamber of a Transwell unit.
e. Culture the cells in a humidified incubator with 5% CO_2 at 37°C.

Day 6

Timing: 20 min

33. Change the H-EOCM medium and H-EFCM medium.
34. Install the chamber containing fibroblasts on the well containing organoids.

Note: Slowly install to prevent bubbles forming under the bottom of the chamber.

35. Incubate the plate in a humidified incubator with 5% CO_2 at 37°C for 24 h.
36. Harvest the fibroblasts. Extract proteins of fibroblasts lysate for western blot analysis.

Fibroblast migration assays

Timing: 6 days

This section describes how to co-culture organoids with primary fibroblasts to investigate the recruitment ability of fibroblasts towards epithelial cells.

Day 1

Timing: 2 h

37. Materials preparation.
    a. Thaw the Matrigel at 4°C for 4 h before the experiment.
    b. Thaw the H-EOCM at 4°C for 4 h before the experiment.
    c. Pre-cool the pipette tips at 4°C for further use.
    d. Pre-warm a 24-well plate in the 37°C incubator for further use.
    e. Add 1× Anti-Anti and HEPES (0.15 mM) in the advanced DMEM/F12 medium to prepare the passage buffer, and pre-cool the passage buffer at 4°C for further use.
38. Organoid digestion.
    a. Repeat step 10.
39. Organoid seeding.
    a. Count the cell numbers and prepare 5,000 cells in a new 1.5 mL centrifuge tube.
    b. Centrifuge the cell suspension for 5 min at 400 *g* at 4°C and remove the supernatant by gentle aspiration.
    c. Mix 50 μL Matrigel and 450 μL H-EOCM and resuspend the cells with the mixture evenly.

CRITICAL: Keep the cell suspension and Matrigel on ice to avoid Matrigel solidification.

Note: Pipette gently to resuspend the cells to avoid bubbles. Operate on ice and use pre-cooled pipettes to avoid Matrigel solidification.

    d. Add 500 μL cell suspension to the center of one well of the 24-well plate.
    e. Incubate the plate on an orbital shaker at 80 rpm in a humidified incubator with 5% CO_2 at 37°C.

Day 3

Timing: 20 min

40. Change the H-EOCM medium.

Day 5

Timing: 20 min

41. Primary fibroblasts passaging.
    a. Repeat sub-steps a–k of step 25.
42. Primary fibroblasts seeding.
    a. Count the cells.
    b. Install a Transwell with 8 μm pore polycarbonate membrane insert on a 24-well plate.

Note: The 8 μm pore polycarbonate membrane insert is used to allow cell migration.

    c. Seed 50,000 cells on the upper chamber of a Transwell unit with 200 μL H-EFCM and shake them evenly.
    d. Add 500 μL H-EFCM in the lower chamber of a Transwell unit.
    e. Culture the cells in a humidified incubator with 5% CO_2 at 37°C.

Day 6

Timing: 20 min

43. Change the H-EOCM medium and H-EFCM medium.
44. Install the chamber containing fibroblasts on the well containing organoids.

Note: Slowly install to prevent bubbles forming under the bottom of the chamber.

45. Incubate the plate in a humidified incubator with 5% CO_2 at 37°C for 24 h.
46. Fix the fibroblasts with methanol and stain them with crystal violet. Count the number of migrated fibroblasts.

EXPECTED OUTCOMES

Here, we have established the protocol for constructing organoids from tissue samples at four stages of ESCC development, with a successful rate of approximately 80%. Hematoxylin-eosin (H&E) staining reveals that the organoids exhibit a similar pathological morphology to the primary tissues, and mIF staining demonstrates their faithful recapitulation of the molecular features observed in the primary tissues (Figure 1C).

Multi-stage organoids, as an advanced *in vitro* model for studying carcinogenesis, have been employed in our previous research.[1] The results of immunohistochemical staining on the organoids show that the protein levels of Ki67 gradually increase during the development of ESCC, whereas the protein levels of ANXA1 gradually decrease. The co-culture experiments demonstrate that compared to co-culture with organoids derived from normal esophageal squamous epithelium (NOR), fibroblasts co-cultured with organoids derived from low-grade intraepithelial neoplasia (LGIN) and high-grade intraepithelial neoplasia (HGIN) exhibit a greater tendency to transition towards myofibroblastic cancer-associated fibroblasts (myCAFs), with the highest levels observed during co-culture with organoids derived from ESCC. Transwell assays show that the ability of organoids to recruit fibroblasts gradually increases from NOR, LGIN, HGIN to ESCC (Figure 1D).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis can be performed using R and GraphPad Prism software. The descriptions about statistical details are indicated in the figure legends or text. *p* values were computed with two-sided and unpaired Wilcoxon rank-sum tests.

LIMITATIONS

Shortcomings including these *in vitro* multi-stage organoid cultures contain exclusively epithelial cells, but do not robustly retain the vast heterogeneity within primary tissues. Organoid-based propagation of primary multi-stage epithelial cells but require artificial reconstitution of stromal cells, like fibroblasts and syngeneic tissue infiltrating lymphocytes to recapitulate the microenvironment. These coculture systems will enable the complex full diversity and physical architecture of the microenvironment.

TROUBLESHOOTING

Problem 1

The viability of tissue for culturing organoids is not optimal.

Potential solution

Place the excised tissue samples into 10% FBS DMEM or primary tissue preservation solution as soon as possible and store them at 2°C–8°C for transportation. The tissue viability will decrease at high temperatures. If necessary, the tissue can be divided into several parts to increase the contact area between the tissue and the solution. To assess cell viability, a portion of cell suspension can be extracted for trypan blue staining before seeding the organoids.

Problem 2

The number of single cells digested from the organoids is not enough.

Potential solution

Passaging should be performed before the appearance of keratin pearls in the central region of most organoids. Ensure thorough flushing of the organoids from the bottom of the well plate.

Problem 3

The growth status of the organoids is not good.

Potential solution

Increase the seeding quantity of cells per well.

It is recommended to use a horizontal rotor centrifuge instead of an angled rotor centrifuge, as the latter may subject some cells to uneven force, causing them to excessively adhere to the walls or even become damaged.

Observe and monitor the growth status of the organoids continuously and replace the culture medium in a timely manner. When the organoids show disintegration or a decrease in central opacity, it indicates that the organoids may have died (Figure 1B). If more than 5% of the organoids show signs of cell death, it is essential to passage promptly to maintain their growth vitality.

Problem 4

The Matrigel becomes loose and disintegrates during the cultivation process.

Potential solution

Lysis red blood cell during tissue digestion. During the cultivation of organoids, as the temperature increases, numerous small red blood cells can precipitate out from the Matrigel, attaching to the periphery of the Matrigel and reducing the adhesive force between the Matrigel and the culture plate, leading to Matrigel cracking and disintegration.

Add the culture medium after the Matrigel has fully solidified.

Problem 5

The organoids adhere to the bottom of the culture plate.

Potential solution

For semi-floating organoid culture, it is critical to use 24-well low-attachment plate. The issue would be severe if using the common 24-well plate.

For classical organoid culture, the gelation process can be performed with the culture plate inverted after seeding the organoids.

Problem 6

There are too many cells of other types, but not squamous epithelium.

Potential solution

During tissue processing, efforts should be made to remove non-esophageal squamous epithelium as much as possible. Consider extending the use of A83-01 to suppress the growth of fibroblasts.

Problem 7

It is difficult to determine that the organoid is derived from esophageal squamous epithelium.

Potential solution

The organoids derived from esophageal squamous epithelium exhibit a solid spherical structure with differentiated keratin pearls in the center. Under the microscope, they appear as circular formations with low refractivity in the center and high refractivity at the periphery. Organoids derived from ESCC may exhibit irregular morphology, and those derived from poorly differentiated ESCC may not form keratin pearls.

H&E staining and immunohistochemical staining for esophageal squamous epithelial-specific proteins can also be used to identify the organoids.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be full filled by the lead contact, Shaosen Zhang (zhangss@cicams.ac.cn).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contacts, Shaosen Zhang (zhangss@cicams.ac.cn) and Guoyu Cheng (chenggy@student.pumc.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets or codes.

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AUTHOR CONTRIBUTIONS

S. Zhang, S. Zhu, and G.C. performed the experiments and analyzed the data. S. Zhang and G.C. drafted and D.L. and C.W. reviewed and prepared the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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