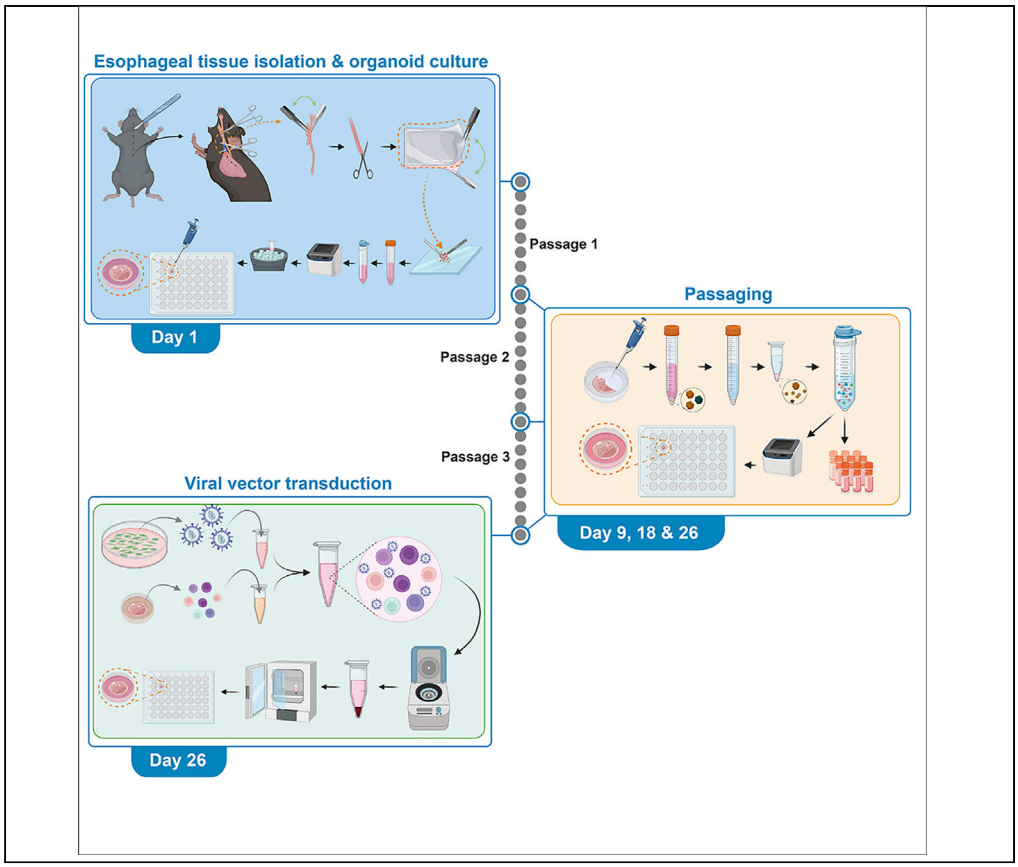
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Establishing transgenic murine esophageal organoids



Organoid systems are commonly used for disease modeling because of their faithful recapitulation of tissue homeostasis, tissue regeneration, and disease processes. However, there is not an optimal approach for the culture of primary mouse esophageal organoids (EOs). Herein, we provide the detailed steps for an efficient and cost-effective protocol for generating and culturing murine EOs. We also describe how to establish transgenic EOs using viral transduction.

Kyung-Pil Ko, Jie Zhang, Jae-Il Park

kko1@mdanderson.org (K.-P.K.)
jaeil@mdanderson.org (J.-I.P.)

Highlights

Generate mouse esophageal organoids with efficient and cost-effective approaches

Provides a streamlined protocol for maintaining mouse esophageal organoids

Optimization of efficient viral transduction techniques of mouse esophageal organoids

Ko et al., STAR Protocols 3, 101317
June 17, 2022
https://doi.org/10.1016/j.xpro.2022.101317

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Establishing transgenic murine esophageal organoids

Kyung-Pil Ko,[1,3,*] Jie Zhang,[1] and Jae-Il Park[1,2,4,*]

[1]Department of Experimental Radiation Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

[2]Genetics and Epigenetics Program, The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences, Houston, TX 77030, USA

[3]Technical contact

[4]Lead contact

*Correspondence: kko1@mdanderson.org (K.-P.K.), jaeil@mdanderson.org (J.-I.P.)
https://doi.org/10.1016/j.xpro.2022.101317

SUMMARY

Organoid systems are commonly used for disease modeling because of their faithful recapitulation of tissue homeostasis, tissue regeneration, and disease processes. However, there is not an optimal approach for the culture of primary mouse esophageal organoids (EOs). Herein, we provide the detailed steps for an efficient and cost-effective protocol for generating and culturing murine EOs. We also describe how to establish transgenic EOs using viral transduction.

For complete details on the use and execution of this protocol, please refer to Zheng et al. (2021).

BEFORE YOU BEGIN

Given that mouse EOs are established from mouse esophageal tissue, animal experiments should be performed with an appropriate protocol approved by the Institutional Animal Care and Use Committee.

Viral transduction-related experiments should be performed with adequate personal protective equipment under institutional guidelines and an approved biosafety protocol.

All animal procedures were performed based on the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care and institutionally approved protocols (IACUC00001141; The University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee). The study was compliant with all relevant ethical regulations regarding animal research.

Institutional permissions

All animal procedures performed here was based on the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care and institutionally approved protocols (IACUC00001451; The University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee). The study was compliant with all relevant ethical regulations regarding animal research.

Matrigel preparation

Timing: 1 day

Thaw the Matrigel at 4°C 1 day before the experiment.

WRN-conditioned medium preparation

Timing: 7 days

This section described how to prepare the Wnt-3A, R-Spondin, Noggin contained conditioned medium for EOs cultivation. Instead of WRN-conditioned medium can be replaced by using each recombinant protein as described by other studies (Kasagi et al., 2018; Giroux et al., 2017).

1. Day 1. Culture the L-WRN cell line.
   a. If the cells were just thawed, culture them in Dulbecco's modified Eagle medium, supplemented with 10% FBS (10% FBS DMEM), G-418 (0.5 mg/mL), and hygromycin B (0.5 mg/mL) for the pure selection.
2. Day 3. Passage the cells when they are 70% confluent.
   a. Remove the medium with an aspirator.
   b. Wash the cells with Dulbecco's phosphate-buffered saline.
   c. Incubate the cells with 0.05% trypsin-EDTA at 37°C for 5 min.
   d. Inactivate the trypsin by adding 10% FBS DMEM.
   e. Spin down the cells (180 g, 3 min), followed by removal of the supernatant.
   f. Seed the cells on a new dish with 10% FBS DMEM with 1:4 subcultivation ratio.
3. Day 5. Change the medium with fresh 10% FBS DMEM.
   a. Change the medium when the cells are 80%–90% confluent.
4. Day 6. Collect the first batch of WRN medium.
   a. Collect the supernatant in a 50-mL conical tube.
   b. Refill the cell culture dish with fresh 10% FBS DMEM.
   c. Spin down the collected medium in a 50-mL conical tube (180 g, 3 min).
   d. Prepare a new 50-mL conical tube, 30-mL syringe, and syringe-installable 0.22-μm filter.
   e. Transfer the supernatant by pipette into a 30-mL syringe with a pre-installed 0.22-μm filter.
   f. Collect the filtrated medium in a new 50-mL conical tube. This is the first batch of WRN-conditioned medium.
   g. Store the first batch at −70°C until it is used.
5. Day 7. Collect the second batch of WRN medium.
   a. Repeat the same procedures for the second batch of WRN medium collection and filtration, as described above.
   b. Store the second batch at −70°C until it is used.
   c. Passage the cells for further collection or freeze them for stock.

Essential minimum organoid culture medium (E-MEOM) preparation

Timing: 1 h

   d. Thaw and mix the first and second batches of WRN-conditioned medium.
   e. Mix the pre-warmed advanced DMEM/F12 medium with WRN-conditioned medium in a 1:1 ratio. To prepare 30 mL of E-MEOM, mix 15 mL of advanced DMEM/F12 medium and 15 mL of WRN-conditioned medium in a 50-mL conical tube.
   f. Add 1 × GlutaMAX, 1 × B27 supplement, 10 mM nicotinamide, 10 μM SB202190, 50 ng/mL EGF, 500 nM A83-01, and 1% penicillin/streptomycin.
   g. Store the prepared E-MEOM at −70°C until it is used.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Ajuba polyclonal antibody	Cell Signaling Technology	Cat#4897, RRID: AB_2280697
Chemicals, peptides, and recombinant proteins		
G-418	Thermo Fisher Scientific	Cat# MT30234CR CAS 108321-42-2
Hygromycin B	Thermo Fisher Scientific	Cat# MT30240CR CAS 31282-04-9
0.05% trypsin-EDTA	Thermo Fisher Scientific	Cat# 25-052-CI
50 × B27 supplement	Thermo Fisher Scientific	Cat# 12587010
Y-27632	Thermo Fisher Scientific	Cat# 12541 CAS 146986-50-7
Penicillin/streptomycin	Life Technologies	Cat# 15140122
100 × GlutaMAX™	Life Technologies	Cat# 35050061
100 × N2 supplement	Life Technologies	Cat# 17502-048
A83-01	Sigma-Aldrich	Cat# SML0788 CAS 909910-43-6
SB202190	Sigma-Aldrich	Cat# S7067 CAS 152121-30-7
Nicotinamide	Sigma-Aldrich	Cat# N0636 CAS 98-92-0
Murine recombinant EGF	PeproTech	Cat# 315-09
Collagenase I	Thermo Fisher Scientific	Cat# 17018029
Matrigel	Corning	Cat# 356231
Polyethylenimine (PEI)	Thermo Fisher Scientific	Cat# AA4389603 CAS 9002-98-6
Polybrene	Sigma-Aldrich	Cat# TR-1003-G
Opti-MEM medium	Invitrogen	Cat# 31985070
Advanced DMEM/F-12	Thermo Fisher Scientific	Cat# 12634010
Experimental models: Cell lines		
L-WRN	ATCC	CRL-3276 RRID: CVCL_DA06
Experimental models: Organisms/strains		
Mouse: 4- to 10- week-old male or female C57BL/6	The Jackson Laboratory	JAX:000664; RRID:IMSR_JAX:000664
Recombinant DNA		
AdCMVCre-eGFP	UI Viral Vector Core	Cat# VVC-I of Iowa-1174
Plasmid: pMD2.G	Addgene	Cat# 12259
Plasmid: psPAX2	Addgene	Cat# 12260
Plasmid: pEGIP	Addgene	Cat# 26777
Plasmid: lentiCRISPR v2	Addgene	Cat#52961
Other		
Clean bech cabinet	n/a	n/a
CO2 Incubator	n/a	n/a
Refrigerator centrifuge	n/a	n/a
Automated Cell Counter	Bio-Rad	Cat# 1450102
Stereomicroscope	KRUSS	n/a
Inverted microscope	ZEISS	n/a
15 mL Conical tube	Fisher scientific	Cat# 14-959-49B
50 mL Conical tube	Fisher scientific	Cat# 05-539-6
1.5 mL tubes	Genesee Scientific	Cat# 24-282
48-well plates	Fisher scientific	Cat# 12-565-322
35 µm Cell Strainer	Corning	Cat# 352235
10 µL filter tips	Genesee Scientific	Cat# 24-401
200 µL filter tips	Genesee Scientific	Cat# 23-412
1,000 µL filter tips	Genesee Scientific	Cat# 23-430

MATERIALS AND EQUIPMENT

Esophageal minimum essential organoid culture medium (E-MEOM)

Reagent	Final concentration	Amount
Advanced DMEM/F-12	50%	15 mL
WRN-conditioned medium	50%	15 mL
GlutaMAX (100×)	1×	300 μL
Penicillin/streptomycin	1%	300 μL
B27 supplement (50×)	1×	600 μL
Nicotinamide (0.5 M)	10 mM	600 μL
SB202190 (10 mM)	10 μM	30 μL
EGF (50 μg/mL)	50 ng/mL	30 μL
A83-01 (1 mM)	500 nM	1.5 μL

Storage condition.
Advanced DMEM/F-12 and GlutaMAX (100×) are stored at 4°C. Penicillin/streptomycin, B27 supplement (50×), Nicotinamide, SB202190, EGF and A83-01 are stored at −20°C.
WRN-conditioned medium is stored at −70°C.
E-MEOM is stored at −70°C up to a month without repeated freeze-thaw cycles.

STEP-BY-STEP METHOD DETAILS

Murine esophageal tissue isolation

Timing: 2 h

Timing: 15 min

Timing: 15 min

Timing: 15 min

Timing: 15 min

This section describes how to extract epithelial cells from the mouse esophagus and seed them with Matrigel for EO culture (Figure 1).

Day 1

1. Euthanize the mice by CO_2 inhalation, followed by cervical dislocation.
2. Excise the esophagus and transfer it to 10-cm Petri dishes containing cold phosphate-buffered saline (PBS) with 1% penicillin/streptomycin.
   a. PBS can be replaced with Hank's balanced salt solution, if available.
   b. The esophagus is attached to the trachea. Cut the esophagus and trachea together and detach the esophagus using 2 surgical tweezers. The esophagus is easily distinguished from the trachea because only the trachea has the rings of cartilage.
3. Wash the isolated esophagus in a new PBS with 1% penicillin/streptomycin by gently moving the dishes back and forth until the supernatant becomes clear.
4. Open the esophagus longitudinally and wash it with cold PBS containing 1% penicillin/streptomycin.
5. Peel off and collect the epithelial cell layer.
   a. The epithelial cell layer exists in the very inner phase of the esophagus.
   b. The epithelial cell layer can be easily peeled off using sterile 2 surgical tweezers.

Note: This step can be made easier with a stereomicroscope.

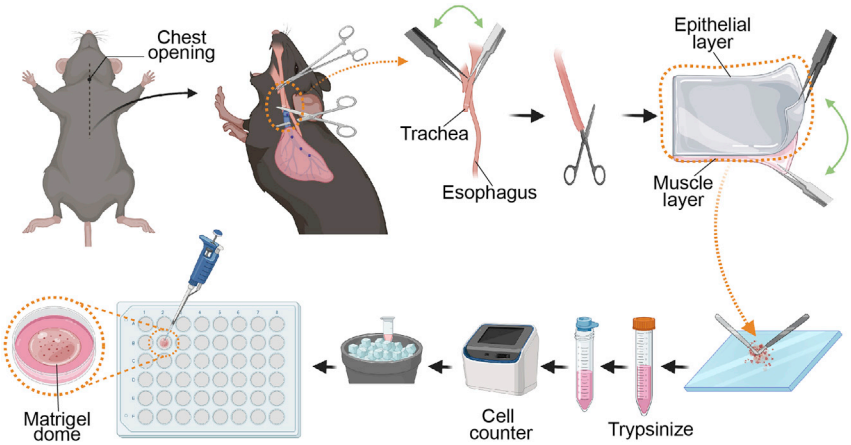


Figure 1. Schematic process of murine esophageal organoid culture

After extracting mouse esophageal tissue, the epithelial cell layer is further isolated physically. The small pieces of epithelial tissue are trypsinized, and the single cells are seeded on the plate with Matrigel.

c. Collect epithelial cell layer and discard the stromal cell and muscle cell layers.

6. Mince the epithelial cell sheet into 0.5-cm^3 pieces with a surgical blade on a glass plate.
7. Collect the minced tissue in a 1.5-mL Eppendorf tube and digest it with 0.05% trypsin-EDTA at 37°C for 45–60 min with vigorous vortexing every 15 min.
   a. Single cells as well as fragments of tissues and aggregates are visible in the microscope at this stage.
   b. A concentrated enzyme such as 0.5% trypsin-EDTA can be used to increase the cell detaching efficiency.
8. Inactivate the trypsin with a 3× volume of 10% FBS + DMEM, followed by vigorous pipetting.
9. Pass the trypsinized cells through a 35-μm sterile strainer to collect the single-cell suspension.
   a. Collect the pass-through cell suspension and discard the strainer containing tissue.
10. Centrifuge the cells (180 g, 4 min) at 4°C and discard the supernatant by aspiration.
11. Resuspend the cells with PBS and centrifuge them (180 g, 4 min) at 4°C.
12. Aspirate the supernatant and resuspend the cells with 100 μL of ice-cold E-MEOM medium.
    a. Prepare E-MEOM with 10 μM Y-27632 before use.
    b. E-MEOM with 10 μM Y-27632 will be used for the initial 3 days from seeding.
13. Count the cell numbers and prepare 5,000 cells in a new 1.5-mL Eppendorf tube.
    a. Add E-MEOM to 10 μM Y-27632 medium, up to 40 μL, and place the tube on ice. If the total volume exceeds 40 μL, centrifuge the cells again and resuspend them with 40 μL of E-MEOM with 10 μM Y-27632.

⚠ CRITICAL: The cell suspension–containing tube should be kept on ice to avoid Matrigel solidification.

14. Add 60 μL of Matrigel to a cell suspension stored on ice.
    a. Matrigel should be pre-thawed at least 3 h at 4°C.
    b. Pipette tips should be ice-cold before use.
    c. Mix and incubate the cell suspension with Matrigel on ice for 5 min.

Note: Apply gentle pipetting to disperse the cells in the Matrigel and to avoid bubbles.

d. Pre-warm a 48-well plate in the 37°C incubator for 5 min.

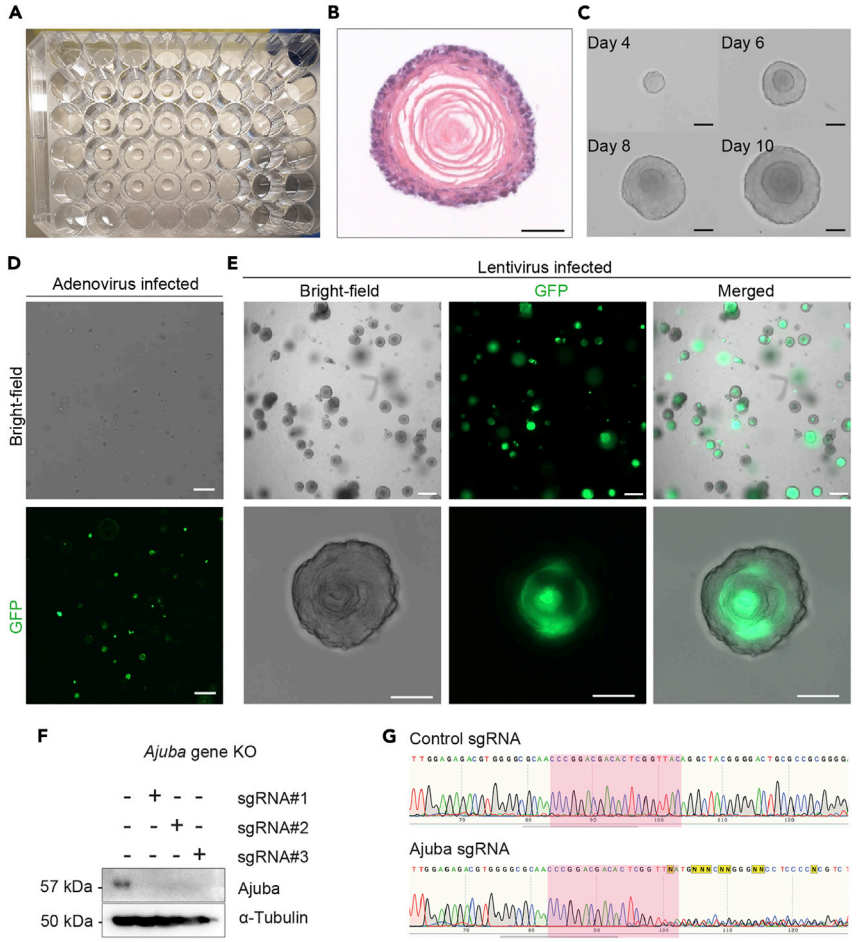


Figure 2. Esophageal organoid culture and viral infection process

(A) Matrigel domes are solidified at the center of each well before the culture medium is added.

(B) Hematoxylin and eosin staining of EO at day 10. Keratin pulp is formed in the central region of the organoid. Scale bar, 50 μm.

(C) Microscopic images of EO at different timepoints. Scale bar, 50 μm.

(D) Adeno-GFP virus was transduced into the murine EOs. Images were obtained 1 day after the viral infection. Scale bars, 100 μm.

(E) Lentivirus (pEGIP)-transduced murine EOs. Images were obtained 4 days after infection. Scale bars, 200 μm (upper) and 50 μm (bottom).

(F) Lentivirus-based gene KO using the CRISPR/Cas9 system in the EOs. 3 different *Ajuba* sgRNA containing lentivirus plasmids were infected and the KO efficiency was evaluated by immunoblot with anti-Ajuba antibody.

(G) Lentivirus-based gene KO in the EOs was further confirmed by genomic DNA sequencing. sgRNA targeting the *Ajuba* gene were highlighted with red boxes.

⚠ CRITICAL: The cell suspension–containing tube should be kept on ice to avoid Matrigel solidification.

15. Aliquot 20 μL of the mixture in each well of the pre-warmed 48-well plate. In total, 4 wells will be used.
    a. Place the drop in the center of the well. The dome will not be created properly if the solution attaches to the wall of the well (Figure 2A).

⚠ CRITICAL: The cell suspension–containing tube should be kept on ice to avoid Matrigel solidification.

16. Incubate the plate in the 37°C cell culture incubator for 15 min to allow the Matrigel to solidify.

17. Add 500 μL of E-MEOM with 10 μM Y-27632 medium to cover the Matrigel and incubate it at 37°C with 5% CO_2.

⚠ CRITICAL: E-MEOM medium should be warmed before use to avoid Matrigel dissociation.

Day 3.

18. Change the medium with fresh E-MEOM medium with 10 μM Y-27632.

Note: Be careful not to aspirate the Matrigel dome during the aspiration of the old medium.

Note: New E-MEOM medium must be pre-warmed and dispensed gently not to disrupt the Matrigel dome structure.

Day 4.

19. Change the medium with a fresh E-MEOM medium.

Note: Be careful not to disturb the Matrigel dome during the aspiration of the old medium.

Note: New E-MEOM medium must be pre-warmed and dispersed gently not to disrupt the Matrigel dome structure.

Day 6.

20. Change the medium with a fresh E-MEOM medium.

Note: Be careful not to disturb the Matrigel dome during aspiration.

Note: New E-MEOM medium must be pre-warmed and dispersed gently not to disrupt the Matrigel dome structure.

Day 8.

21. Change the medium with a fresh E-MEOM medium.

Note: Be careful not to disturb the Matrigel dome during the aspiration of the old medium.

Note: New E-MEOM medium must be pre-warmed and dispersed gently not to disrupt the Matrigel dome structure.

Organoid passaging

Timing: 2 h

Timing: 15 min

Timing: 15 min

Timing: 15 min

Timing: 15 min

This section describes how to passage EOs cultured in Matrigel. We usually perform the passaging 8–10 days after seeding 1,000 cells on the 48-well plate. We have reported that the average size of the EOs is around 25,000 μm^2 at Day 10 in the microscopic images (Zheng et al., 2021). If different scales of culture are used, the morphology of EOs and the culture medium should be carefully monitored. Mature EOs show a thin outward basal cell layer and inward suprabasal and stratified cell layers (Figure 2B). Keratin pulp is formed in the central region of Eos, which is a typical feature and is visible in the light microscope in mature EOs (Figures 2B and 2C). After an optimal passaging time, the dark Keratin pulp area grows larger while maintaining the overall organoid size, indicating that the proportion of the live cells is decreasing. Therefore, EOs should be passaged before this Keratin pulp becomes too large and before the culture medium turns yellow.

Day 1.

Prepare ice, ice-cold 10% FBS DMEM, ice-cold Dulbecco's phosphate-buffered saline, and 0.05% trypsin-EDTA before starting.

22. Discard the culture medium using an aspirator.
23. Dissociate the Matrigel by vigorous pipetting with ice-cold 10% FBS DMEM medium. Scrape the Matrigel from the bottom of the well with a pipette tip.

Note: Ice-cold 1,000 μL pipette tips are used. Before using Matrigel, coat the tip wall with 10% FBS DMEM by pipetting to avoid organoid attaching to the pipette tip.

24. Collect and transfer the sample into a 15-mL tube and place it on ice. Fill the tube with ice-cold 10% FBS DMEM medium, up to 13 mL.
    a. Collect 4 wells together if this process is a continuation from the esophageal tissue isolation section.

Note: Organoid forming efficiency is relatively low in the organoids isolated from the esophagus tissue (0 passage) compared to the established organoids (passaged).

    b. Collecting 2 wells into 1 tube is sufficient if this process is for already established cultured EOs.

25. Centrifuge the sample (180 g, 4 min) at 4°C and discard the supernatant.
26. Wash the sample with 13 mL of cold PBS, centrifuge it (180 g, 4 min) at 4°C, and remove the supernatant.
27. Add 1 mL of trypsin-EDTA (0.05%) to the sample and transfer it to a 1.5-mL Eppendorf tube.
28. Incubate the sample at 37°C (30–45 min) and vortex it every 5 min.
29. Pass the sample through a 35-μm cell strainer.

Note: Cells should be dissociated into single cells after this step. Single cell-grown organoid can be compared with the organoid from the previous passage to assess organoid forming efficiency and growth.

30. Inactivate trypsin with 3 mL of 10% FBS DMEM medium and pipette vigorously.
31. Collect the cells in the 15-mL tube and centrifuge them (180 g, 4 min) at 4°C.
32. Aspirate the supernatant and resuspend it with 50 μL of ice-cold E-MEOM medium.
    a. Prepare E-MEOM with 10 μM Y-27632 before use.
    b. This medium will be used for 3 days after seeding.
33. Count the cells with 10 μL of cell suspension, check the cells' viability, and calculate the cell numbers for seeding.
    a. Generally, 1,000 cells are used in each well of a 48-well plate.

Note: More cells (3,000–5,000 cells) can be seeded in a well according to subsequent experiments. Of note, EOs need to be monitored carefully and passaged earlier than day 10 (e.g., viral transduction can be performed with day 4–5 of organoids. In this case, 3,000–5,000 cells can be seeded to harvest enough cells from a well for the experiment).

34. Transfer the calculated volume of the cell suspension to a new 1.5-mL Eppendorf tube and place it on ice.
    a. Add E-MEOM with 10 μM Y-27632 to the Eppendorf tube, up to 8 μL.
    b. If you plan to seed 3 wells, prepare 3,000 cells in 24 μL of total E-MEOM with 10 μM Y-27632 in a 1.5-mL Eppendorf tube.

35. Add 12 μL of Matrigel to the cell suspension (for a total volume of 20 μL).
    a. The Matrigel should be pre-thawed at least 3 h at 4°C.
    b. Pipette tips should be chilled before use.
    c. Incubate the cell suspension, mixed with Matrigel, on ice for 5 min.

    Note: Apply gentle pipetting to disperse the cells in the Matrigel and to avoid bubbles.

    d. Prepare a 48-well plate in the 37°C incubator for 5 min.
    e. If the cells are subjected to be seeded in 3 wells, mix 36 μL of Matrigel with 24 μL of cell suspension.

36. Aliquot 20 μL of the mixture into each well of a 48-well plate. In total, 4 wells will be used.
    a. Be sure to aliquot the solution in the center of a well. Domes will not be created properly if the solution attaches to the wall of the well.
37. Incubate the plate in the 37°C cell culture incubator for 15 min to allow the Matrigel to solidify.
38. Add 500 μL of pre-warmed E-MEOM with 10 μM Y-27632 medium to cover the Matrigel and incubate it at 37°C with 5% CO_2.

    Note: E-MEOM should be pre-warmed before use. Or it will disrupt the Matrigel dome formation.

Day 3.

39. Change the medium with fresh E-MEOM medium with 10 μM Y-27632.

    Note: Be careful not to aspirate the Matrigel dome during the aspiration of the old medium.

    Note: New E-MEOM medium must be pre-warmed and dispensed gently not to disrupt the Matrigel dome structure.

Day 4.

40. Change the medium with a fresh E-MEOM medium.

Day 6.

41. Change the medium with a fresh E-MEOM medium.

Day 8.

42. Change the medium with a fresh E-MEOM medium.

⚠ CRITICAL: EOs need to be passaged before day 11 on the scale of 1,000 cells seeded in 48 well-plate. Cell viability decreases drastically after day 11. The passaging time point can be shortened if more cells were seeded (e.g., Passaging on days 7–8 is appropriate when 3,000 cells are seeded in a well of 48-well plate).

Organoid freezing

Timing: 1.5 h

This section describes how to freeze and store mouse EOs. Prepare ice, ice-cold 10% FBS DMEM, ice-cold Dulbecco's phosphate-buffered saline, and 0.05% trypsin-EDTA before starting.

Day 1.

43. Discard the culture medium using an aspirator.
44. Dissociate the Matrigel by vigorous pipetting with ice-cold 10% FBS DMEM medium. Scrape the Matrigel from the bottom of the well with a 1,000 μL pipette tip.
45. Collect the sample and transfer it into a 15-mL tube and place the tube on ice. Fill the tube with ice-cold 10% FBS DMEM medium, up to 13 mL. Collect 2–3 wells together.
46. Centrifuge the sample (180 g, 4 min) at 4°C and discard the supernatant.

Note: Matrigel is not visible after the centrifugation. EOs pellet is embedded in a gel after aspiration if the Matrigel is not dissociated enough. In this case, an additional washing step with an ice-cold 10% FBS DMEM medium or PBS is required.

47. Wash the sample with 13 mL of cold PBS, centrifuge it (180 g, 4 min) at 4°C, and remove the supernatant.
48. Add 1 mL of trypsin-EDTA (0.05%) and transfer the sample to a 1.5-mL Eppendorf tube.

Note: Dissociation into a single cell does not have to be perfect. Fragmented EOs can be frozen and restored well after thawing.

49. Incubate the sample at 37°C (30–45 min) with vortexing every 5 min.
50. Pass the sample through the 35-μm cell strainer.
51. Inactivate trypsin with 3 mL of 10% FBS DMEM medium and pipette it vigorously.
52. Collect the cells in the 15-mL tube and centrifuge them (180 g, 4 min).
53. Aspirate the supernatant and resuspend the pellet with an ice-cold E-MEOM medium.
    a. Add 900 μL of E-MEOM per cryovial.
    b. Use 2.7 ml of E-MEOM for 3 vials.
54. Add DMSO to a final volume of 10%.
    a. Keep the vials on ice until moving them to −70°C.
55. Transfer the cells into the cryovials and store them at −70°C for at least 4 h using Corning CoolCell LX Cell Freezing Vial Containers.

Note: Another controlled-rate freezing method is also available such as Mr. Frosty, which uses isopropyl alcohol.

56. Transfer the cryovial to the liquid nitrogen tank.

Adenovirus transduction

Timing: 2 h

Timing: 15 min

Timing: 15 min

Timing: 15 min

This section describes how to transduce adenovirus into mouse EOs. Adenoviral transduction is a widely used method to transiently express the gene of interest into the cells. In this protocol, we described how to introduce adenovirus into the EOs. This method is beneficial for establishing genetically engineered EOs derived from the mouse models carrying the Cre-LoxP system for gene manipulation (knock-out, knock-in, or inducible expression), using adenovirus encoding Cre-GFP.

Day 1.

57. Harvest the cells from EOs and count the single cells as described in the organoid passaging section (steps 1–12).

    Note: Any stage of organoid cells can be used, but we recommend using the cells at day 4- day 6 to increase organoid forming efficiency after viral transduction.

58. Calculate the required volume of adenovirus.
    a. Infected organoids can be visualized if the virus contains fluorescence, such as GFP.
    b. We set the ratio of adenovirus: organoid cell = 1000:1, but the optimized ratio can be different based on the viral vectors and the batch.
    c. For example: If the virus stock is 4×10^7 PFUs/μL, 1 μL of adenovirus is required for 4×10^4 cells.

    Note: We did not perform the adenovirus titration here because it was verified from the provider (See key resources table), but the titration can be done with a titration kit if the viral vector is prepared manually.

59. Mix the required cell suspension, virus-containing medium, and Matrigel and place the drop in the center of the well.
    a. Fill E-MEOM medium up to 8 μL after mixing cell suspension and virus-containing medium.
    b. Add 12 μL of Matrigel into cell suspension and virus mixture on ice.
60. Incubate the plate in the 37°C cell culture incubator for 15 min to allow the Matrigel to solidify.
61. Add 500 μL of E-MEOM with 10 μM Y-27632 medium to cover the Matrigel and incubate it at 37°C with 5% CO_2.

    Note: Since our protocol uses adenovirus encoding GFP, virus-infected EOs can be easily visualized by GFP expression 1 day after infection (Figure 2D).

Day 4.

62. Change the medium with a fresh E-MEOM medium.

    Note: The fluid waste containing Adenovirus should be appropriately discarded. Bleaching (with 10% final bleach volume) for 20 min is recommended.

Day 6.

63. Change the medium with a fresh E-MEOM medium.

Day 8.

64. Change the medium with a fresh E-EMOM medium.

⚠ CRITICAL: The number of GFP-positive cells is decreased 6 days after adeno-GFP virus infection because adenovirus is transiently expressed in the cells. Virus-infected cells should be selected with a marker or fluorescence-activated cell sorting before the signal disappears.

Lentivirus transduction

This section describes how to transduce mouse EOs with lentivirus. This protocol will be useful to introduce genes of interest or perform CRISPR/Cas9-based gene editing using lentivirus. Choosing lentivirus containing fluorescent ORF will be helpful to visualize and isolate the infected organoids. Since EOs have autofluorescence in a Keratin layer, avoiding longer emission wavelengths of fluorescence (e.g., RFP) is recommended.

Lentivirus preparation

Day 1–3. Lentivirus can be produced by using packaging plasmids, Lentivirus plasmid with PEI and Opti-MEM as described elsewhere (Miyoshi and Stappenbeck, 2013; Lo et al., 2021; Kim et al., 2021). We transfected 293T cells with packaging and lentiviral plasmids at Day 1 and changed the medium at Day2. We then collected the media at day 3 using a 0.45 μm filter.

Optional: Concentrate the virus with Lenti-X Concentrator.

a. Produce 500 μL of virus-containing media.
b. We do not perform the virus titration because we establish stably transduced EOs using cell sorting or antibiotics selection. Additionally, we include a positive control, EOs transduced with lentivirus encoding GFP. GFP-positive organoids are traceable in a live cell image and showed more than 80% (46 of 56 organoids) infection rate (Figure 2E).

EO infection

Timing: 7 h

Timing: 10 min

Timing: 10 min

Timing: 10 min

Timing: 10 min

Day 3 (or day 4 if incubated with concentrator).

65. Harvest cells from EOs and count the single cells as described in the organoid passaging section (steps 1–12).

Note: Any stage of organoid cells can be used, but we recommend using the cells at day 4- day 6 to increase organoid forming efficiency after viral transduction.

66. Prepare virus-containing medium with polybrene (7 μg/mL in 500 μL).

67. Add polybrene-containing medium to the cell pellet and transfer the cell suspension into a new 1.5-mL Eppendorf tube.
68. Centrifuge the tube for 1 h with 600 g at ambient temperature (set the temperature at 32°C if possible).

Note: Centrifuge at 20°C–25°C is also applicable. We confirmed that the infection efficiency is comparable to that of the cells infected at 32°C.

69. Incubate the tube in the 37°C incubator for 4 h.
70. Centrifuge the tube (380 g, 5 min) at 20°C–25°C.

Note: Use swinging centrifuge if concentrator is applied.

71. Wash the tube with PBS and centrifuge it again if concentrator is applied.
72. Remove the supernatant, resuspend the cell pellet with an appropriate volume of medium (8 μL in each well of a 48-well plate) and place the tube on ice to cool down.

Note: The fluid waste containing adenovirus should be discarded properly. Bleaching (with 10% final bleach volume) for 20 min is recommended.

73. Add an appropriate volume of Matrigel to the tube (12 μL of pre-thawed Matrigel to each well of a 48-well plate).
74. Seed the cells in the center of the well in a 48-well plate and incubate at 37°C for 15 min.
75. Add E-MEOM medium containing Y-27632 (10 μM) to the well.

Day 5.

76. Replace the E-MEOM medium with the antibiotic based on the selection cassette of the viral vectors (puromycin 0.4 μg/mL, blasticidin 2 μg/mL).

Note: Virus-infected organoids can be visible 48 h after infection if a fluorescent vector such as GFP is used (Figure 2E).

Day 7.

77. Replace the medium with the selected antibiotic (puromycin 0.4 μg/mL, blasticidin 2 μg/mL).

Day 9.

78. Replace the medium with the selected antibiotic (puromycin 0.4 μg/mL or blasticidin 2 μg/mL).

Day 11.

79. Replace the medium with the selected antibiotic (puromycin 0.4 μg/mL or blasticidin 2 μg/mL).
80. Passage the EOs and keep the selected antibiotic if positive cells are not sufficiently selected.

Note: The antibiotic concentration should be optimized to kill only uninfected cells.

EXPECTED OUTCOMES

Similar to our study (Zheng et al., 2021), adenoviral vector transduction into mouse intestinal organoids is feasible (Wang et al., 2014), and this method was successful in our previous study (Zheng et al., 2021). According to our previous report, the viral infection efficiency was more than

80% when we measured the GFP-positive cells in the microscope. Since we knocked out *Trp53* with Cre-LoxP recombination in our previous study (Zheng et al., 2021), we used Nutlin to select *Trp53* KO organoids. The selection of reagents and methods can vary based on the experimental design and the genes of interest. Using FACS (fluorescence activated cell sorting), sorting GFP+ cells at earlier time point such as Day 6 and seeding again in a new well will make the process easier to isolate the transduced cells.

This protocol aims to establish a standard method of establishing and culturing mouse EOs. Although several studies have used mouse EOs (Giroux et al., 2017; DeWard et al., 2014; Natsuizaka et al., 2017; Karakasheva et al., 2020), there is a lack of consensus on optimal culturing methods. In addition, organoid transduction using viral vectors has rarely been performed with mouse EOs (Boj et al., 2015; De Van Lidth Jeude et al., 2015; Wu et al., 2021). Using our lentivirus-based transduction method, we observed the high efficiency of transduction and genetic manipulation of EOs. Moreover, using the method described here, we have succeeded in establishing triple-KO EOs with a lentivirus-based CRISPR gene-editing system.

LIMITATIONS

This protocol is optimized for C57BL/6 mouse-derived EO experiments. All the timelines and scales have been verified in C57BL/6 but not in other species or strains. Therefore, a further test is required to apply this protocol to the different strains or species.

TROUBLESHOOTING

Problem 1

EOs are not forming a typical round shape. (Related to steps 22–42).

Potential solution

Single cells should be isolated through enzyme digestion and cell strainer filtration. Fragmented organoid or multiple cell aggregates make irregular shapes of EOs after passaging.

Problem 2

EOs are not growing well. (Related to steps 22–42).

Potential solution

Use an earlier passage of L-WRN cells. The problem may result from the inefficient production of Wnt-3A, R-Spondin, and Noggin after a continuous culture of L-WRN cells. Although we usually do not QC the WRN protein content with ELISA kit every time for the cost aspect, we confirmed the effect of WRN-conditioned medium on EOs culture was comparable to the impact of recombinant proteins. Our previous study testing each component on EO culture Field (Zheng et al., 2021) makes it likely to predict which reagent or component causes EO growth problems.

Problem 3

Single-cell dissociation from EOs takes too long. (Related to steps 22–30).

Potential solution

Matrigel should be removed with enough cold medium or PBS before being trypsinized. If the medium or PBS is not cold enough, Matrigel dissociation will not be performed properly. Remnant Matrigel with the organoid will inhibit the exposure of trypsin to the organoid. If the organoid pellets are not formed in the bottom of the tube and are dispersed in a transparent gel after centrifugation and supernatant aspiration, this is likely because the Matrigel is not removed completely. This case can be solved by additional washing by vigorous pipetting with ice-cold PBS.

Problem 4

Adenovirus-infected cells are not viable. (Related to steps 57–64).

Potential solution

Decrease the MOI (multiplicity of infection): Optimal infection conditions for each adenoviral vector can be different. The optimized condition can be established after infection at different MOIs, such as 100, 1,000, and 10,000. Non-infected cells should also be used as a negative control for viral infection.

Problem 5

There are too few lentivirus-infected cells. (Related to steps 65–80).

Potential solution

Increase the cell viability.

Minimize the trypsinization time: Cell viability is lower when cells are exposed to trypsin too long. Usually, 15 min of trypsinization is sufficient to obtain a single cell for infection.

Enhance the transduction efficiency.

Concentrate the virus-containing medium: A concentrated virus-containing medium can enhance the viral transduction efficiency. However, an additional washing step is required because the reagent from the concentrating kit can inhibit the assembly of the Matrigel.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jae-Il Park (jaeil@mdanderson.org), or technical contact, Kyung-Pil Ko (kko1@mdanderson.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets or codes.

ACKNOWLEDGMENTS

We are grateful to Ann Sutton (Research Medical Library, MD Anderson) for editing the manuscript. This work was supported by the Cancer Prevention and Research Institute of Texas (RP140563 and RP200315 to J-I.P.) and the National Cancer Institute (2R01 CA193297 and R03 CA256207 to J-I.P.). The core facilities used (DNA Sequencing and Genetically Engineered Mouse Facility) are supported by the Cancer Center Support Grant P30 CA016672 from the National Cancer Institute to The University of Texas MD Anderson Cancer Center.

AUTHOR CONTRIBUTIONS

K.P.K. and J.Z. performed the experiments. K.P.K. and J-I.P. analyzed the data. K.P.K. and J-I.P. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Boj, S.F., Hwang, C.I., Baker, L.A., Chio, Ii, Engle, D.D., Corbo, V., Jager, M., Ponz-Sarvise, M., Tiriac, H., Spector, M.S., et al. (2015). Organoid models of human and mouse ductal pancreatic cancer. Cell *160*, 324–338.

De Van Lidth Jeude, J.F., Vermeulen, J.L.M., Montenegro-Miranda, P.S., Van Den Brink, G.R., and Heijmans, J. (2015). A protocol for lentiviral transduction and downstream analysis of intestinal organoids. J. Vis. Exp. *2015*, e52531.

DeWard, A.D., Cramer, J., and Lagasse, E. (2014). Cellular heterogeneity in the mouse esophagus implicates the presence of a nonquiescent epithelial stem cell population. Cell Rep. *9*, 701–711.

Giroux, V., Lento, A.A., Islam, M., Pitarresi, J.R., Kharbanda, A., Hamilton, K.E., Whelan, K.A., Long, A., Rhoades, B., Tang, Q., et al. (2017). Long-lived keratin 15+ esophageal progenitor cells contribute to homeostasis and regeneration. J. Clin. Invest. *127*, 2378–2391.

Karakasheva, T.A., Kijima, T., Shimonosono, M., Maekawa, H., Sahu, V., Gabre, J.T., Cruz-Acuna, R., Giroux, V., Sangwan, V., Whelan, K.A., et al. (2020). Generation and characterization of patient-derived head and neck, oral, and esophageal cancer organoids. Curr. Protoc. Stem Cell Biol. *53*, e109.

Kasagi, Y., Chandramouleeswaran, P.M., Whelan, K.A., Tanaka, K., Giroux, V., Sharma, M., Wang, J., Benitez, A.J., Demarshall, M., Tobias, J.W., et al. (2018). The esophageal organoid system reveals functional interplay between notch and cytokines in reactive epithelial changes. Cell Mol. Gastroenterol. Hepatol. *5*, 333–352.

Kim, M.J., Cervantes, C., Jung, Y.-S., Zhang, X., Zhang, J., Lee, S.H., Jun, S., Litovchick, L., Wang, W., Chen, J., et al. (2021). PAF remodels the DREAM complex to bypass cell quiescence and promote lung tumorigenesis. Mol. Cell *81*, 1698–1714.e6.

Lo, Y.-H., Kolahi, K.S., Du, Y., Chang, C.-Y., Krokhotin, A., Nair, A., Sobba, W.D., Karlsson, K., Jones, S.J., Longacre, T.A., et al. (2021). A CRISPR/Cas9-Engineered ARID1A -deficient human gastric cancer organoid model reveals essential and nonessential modes of oncogenic transformation. Cancer Discov. *11*, 1562–1581.

Miyoshi, H., and Stappenbeck, T.S. (2013). In vitro expansion and genetic modification of gastrointestinal stem cells in spheroid culture. Nat. Protoc. *8*, 2471–2482.

Natsuizaka, M., Whelan, K.A., Kagawa, S., Tanaka, K., Giroux, V., Chandramouleeswaran, P.M., Long, A., Sahu, V., Darling, D.S., Que, J., et al. (2017). Interplay between Notch1 and Notch3 promotes EMT and tumor initiation in squamous cell carcinoma. Nat. Commun. *8*, 1758.

Wang, N., Zhang, H., Zhang, B.Q., Liu, W., Zhang, Z., Qiao, M., Zhang, H., Deng, F., Wu, N., Chen, X., et al. (2014). Adenovirus-mediated efficient gene transfer into cultured three-dimensional organoids. PLoS ONE *9*, 1–8.

Wu, Z., Zhou, J., Zhang, X., Zhang, Z., Xie, Y., Liu, J.B., Ho, Z.V., Panda, A., Qiu, X., Cejas, P., et al. (2021). Reprogramming of the esophageal squamous carcinoma epigenome by SOX2 promotes ADAR1 dependence. Nat. Genet. *53*, 881–894.

Zheng, B., Ko, K.-P., Fang, X., Wang, X., Zhang, J., Jun, S., Kim, B.-J., Luo, W., Kim, M.J., Jung, Y.-S., et al. (2021). A new murine esophageal organoid culture method and organoid-based model of esophageal squamous cell neoplasia. iScience *24*, 103440.