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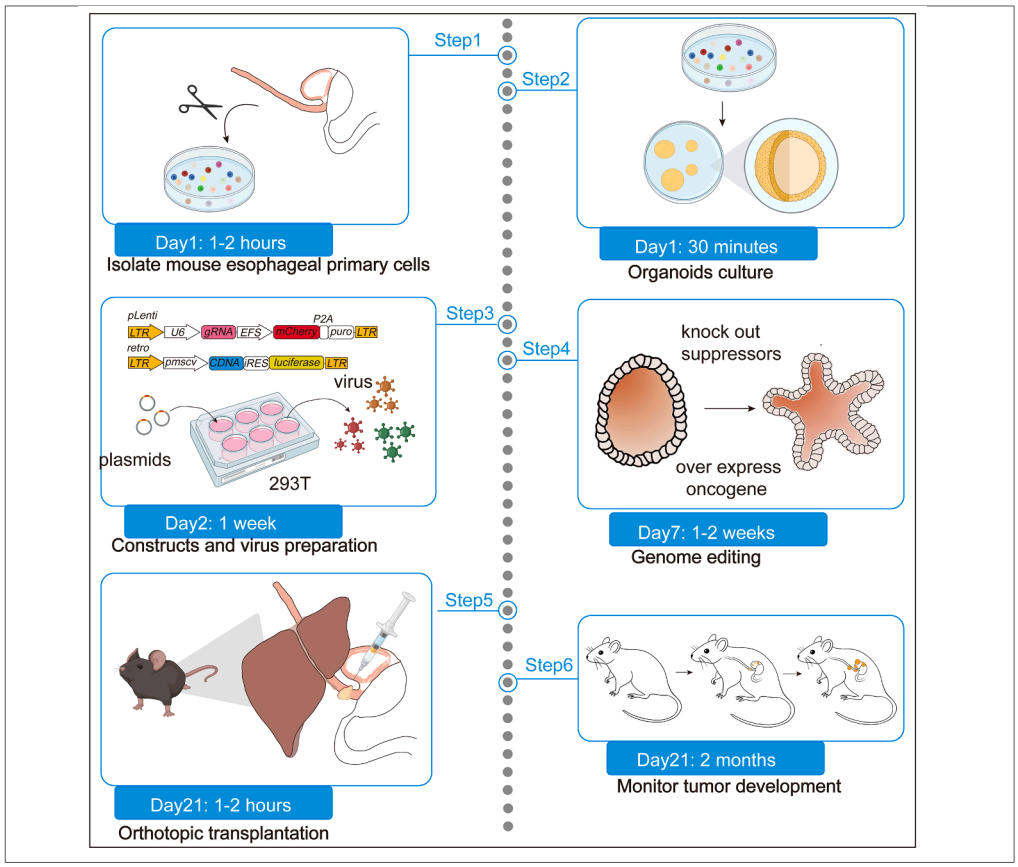
Protocol to generate genetically engineered organoid-initiated mouse models of esophageal cancer



Here, we present a protocol for generating genetically engineered organoid-initiated mouse models of esophageal cancer. We describe steps for isolating mouse esophageal primary cells, organoids culture, construct and virus preparation, and genome editing. We then detail procedures for transplanting engineered organoids into the esophagus, forming squamous cell carcinoma, and tumorigenesis monitoring. The protocol is faster than genetically engineered mouse models.

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

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Highlights

Steps for constructing a primary, orthotopic, esophageal cancer mouse model

Guidance on isolation and organoid development from mouse esophageal epithelial cells

Procedures for organoid passaging, genome editing, and genotyping

Orthotopic transplant of edited organoids into the mouse esophagus by laparotomy

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Protocol to generate genetically engineered organoid-initiated mouse models of esophageal cancer

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SUMMARY

Here, we present a protocol for generating genetically engineered organoid-initiated mouse models of esophageal cancer. We describe steps for isolating mouse esophageal primary cells, organoids culture, construct and virus preparation, and genome editing. We then detail procedures for transplanting engineered organoids into the esophagus, forming squamous cell carcinoma, and tumorigenesis monitoring. The protocol is faster than genetically engineered mouse models.

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

BEFORE YOU BEGIN

The protocol is based on an alternative strategy by in situ transplanting gene-edited normal epithelial organoids to generate primary, orthotopic, and genetically defined tumor models, named the OPCM (Organoid-initiated Precision Cancer Model), including lung cancer,[2] bladder cancer,[3] gastric cancer,[4] and endometrial cancers,[5] generated by our group and others. We isolated esophageal primary cells from *Trp53*$^{-/-}$ *Cas9* mice because *TP53*, a homologous gene in humans, has the highest mutation frequency in esophageal cancer.[6] We also optimized organoid culture conditions for subsequent genome editing.[7] Typically, when isolating 10^6 cells from the esophagus of a mouse, it is possible to generate more than 100 organoids within one week of culture. After one week of gene editing, approximately 500 organoids can be transplanted *in situ*. The mice can develop primary *in situ* esophageal squamous cell carcinoma within about 30 days. All reagents and processes must maintain sterile conditions at the cell-culture-grade level with appropriate attention to personal protection.

Institutional permissions

Animal use protocol in this study was approved by the Sichuan University Animal Ethical and Welfare Committee (IACUC protocol no. 20230303068). Before attempting this protocol, ethical permission should be obtained to use animals in experimental procedures.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
DH5a	Sangon Biotech	Cat# B528413
Experimental models: Organisms/strains		
Mouse: *CAG-Cas9-EGFP* (6–12 weeks, gender not specified, cross with *Trp53*$^{-/-}$ *mice*)	The Jackson Laboratory	Cat# N0026179
Mouse: *Trp53*$^{-/-}$(6–12 weeks, gender not specified, cross with *CAG-Cas9-EGFP mice*)	The Jackson Laboratory	Cat# N0002101
Mouse: BALB/cA-nu (6 weeks, male)	Vital River	Cat# VSM40001
Experimental models: Cell lines		
Human: HEK-293T	ATCC	Cat# CRL-1573, RRID: CVCL_0045
Recombinant DNA		
psPAX2	Addgene	Cat# 12260
pMD2.G	Addgene	Cat# 12260
pCL-Eco	Addgene	Cat# 12371
pCAG-VSVG	Addgene	Cat# 35616
pLenti-U6-gRNA-EFS-mCherry	Na et al.[2]	N/A
pMSCV-*cMyc*-iRES-luci2	Na et al.[2]	N/A
pMSCV-*Kras*^{G12D}-iRES-luci2	Na et al.[2]	N/A
pMSCV-*CDNA*-iRES-luci2	Na et al.[2]	N/A
Reagents for clone		
BsmBI	NEB	Cat# R0739S
EcoRI	NEB	Cat# R0101V
BglII	NEB	Cat# R0144V
T4 DNA ligase	NEB	Cat# M0202V
T4 DNA ligase buffer	NEB	Cat# B0202S
T4 PNK	NEB	Cat# M0236S
Seamless clone enzyme	Gemesand	Cat# SC612
Column recovery kit	Sangon Biotech	Cat# B518131
Phanta Flash super-fidelity DNA polymerase	Vazyme	Cat# P521
RNase-free ddH2O	Sangon Biotech	Cat# B541018
NEB buffer 3.1	NEB	Cat# B6003V
Quick CIP	NEB	Cat# M0525V
rCutSmart	NEB	Cat# B6004V
$CHCl_3$	Merck	Cat# C2432
Ethanol absolute	Sangon Biotech	Cat# A500737
HiScript II Q RT SuperMix for qPCR	Vazyme	Cat# R222-01
Chemicals, peptides, and recombinant proteins		
DPBS	Gibco	Cat# C14190500BT
DMED/F12 (1:1) basic (1×)	Gibco	Cat# C11330500BT
DMEM	Gibco	Cat# C11995500BT
Penicillin/streptomycin	Gibco	Cat# 15140-122
TrypLE	Gibco	Cat# 12604-028
Trypsin	Gibco	Cat# 25200-072
GlutaMAX	Gibco	Cat# 35050-061
B27	Gibco	Cat# A3582801

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
N2	Gibco	Cat# 17502048
N-acetylcysteine	Sigma	Cat# A9165
Nicotinamide	Sigma	Cat# N0636
EGF	PeproTech	Cat# AF-100-15-1000
Noggin	PeproTech	Cat# 120-10C-250
DNase I (1 U/mL)	Thermo Scientific	Cat# EN0521
FGF10	PeproTech	Cat# 100-26-1000
R-spondin-1	PeproTech	Cat# 120-38-1000
A83-01	PeproTech	Cat# 9094360
Protein kinase K	Solarbio	Cat# P9460
D-luciferin potassium salt	Biovision	Cat# 7903-10PK
Matrigel	Corning	Cat# 356237
TRIzol	Applied Biosystems	Cat# 15596026
SYBR	Applied Biosystems	Cat# A25741
FBS	HAKATA	Cat# HB-FBS-500
$CaCl_2$	SELLECK	Cat# S6992
NaCl	Invitrogen	Cat# A57006
Na_2HPO_4	Sangon Biotech	Cat# A610404
HEPES	Sangon Biotech	Cat# E607018
Chloroquine	SELLECK	Cat# S6999
Tris	Sangon Biotech	Cat# A610195
0.5 M EDTA	Sangon Biotech	Cat# B540625
10% SDS solution	Sangon Biotech	Cat# B548118
Antibodies		
Anti-P40 (dilution ratio 1:50)	Abcam	Cat# ab203826; RRID: AB_2934266
Anti-Cytokeratin 5 (dilution ratio 1:1,000)	RTM BIO	Cat# PTM-5040
Anti-Cytokeratin 14 (dilution ratio 1:200)	RTM BIO	Cat# PTM-5391
Anti-Cytokeratin 13 (dilution ratio 1:50)	NOVUS	Cat# NBP1-97797; RRID:AB_3243886
Anti-Ki67 (dilution ratio 1:50)	Abcam	Cat# ab15580; RRID:AB_805388
Anti-Sox2 (dilution ratio 1:200)	HUABIO	Cat# HA721155; RRID:AB_3072277
Anti-CRISPR-Cas9 SP [JM11-55] (dilution ratio 1:100)	HUABIO	Cat# ET1703-85; RRID:AB_3070439
Other		
Medical operation scissors	Sangon Biotech	Cat# F519231
Forceps, sharp end	Sangon Biotech	Cat# F519021
Cell strainers (Falcon)	Sangon Biotech	Cat# A003265
Multi-purpose clinical centrifuge	Sangon Biotech	Cat# Z001346
48-well plate	Sangon Biotech	Cat# D149225
Pipettor (10–100 μL)	Eppendorf	Cat# 3123000047
6-well plate	Sangon Biotech	Cat# F603201
Mini vortex stirrer	Sangon Biotech	Cat# G002035
Agarose gel imaging system	Tanon	Cat# 5200T
For orthotopic transplantation		
Gas anesthesia machine	RWD	Cat# R500
Surgical sutures	MERSILK	Cat# SA845G
Small animal living imaging device	DIGI-UNITED	Cat#V-NIR-II
Insulin syringe 1 mL	Ultra-Fine	Cat# 328421

MATERIALS AND EQUIPMENT

Conditional media for organoids culture

Reagent	Final concentration	Amount
B27	1×	800 μL
N2	1×	400 μL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
EGF	50 ng/mL	40 μL
FGF10	200 ng/mL	40 μL
Y27632	10 mM	20 μL
A83-01	2 mM	8 μL
R-spondin 1	250 ng/mL	200 μL
Noggin	100 ng/mL	40 μL
Wnt-3A	10% Wnt-3A conditioned medium	4 mL
Nicotinamide	1 mM	400 μL
N-acetylcysteine	1 mM	400 μL
Glutamax	2 mM	400 μL
Penicillin/Streptomycin	10000 units/mL	400 μL
DMEM-F12	1×	Add to 40 mL
Total	N/A	40 mL

Note: Media can be stored at 4°C for up to 2 months.

Note: The reason for choosing DMEM/F12 as the organoid culture medium is that DMEM/F12 contains non-essential amino acids and a richer profile of vitamins (such as biotin and vitamin B12), making it more suitable for primary cell culture.

Preparation of dissection wash buffer		
Reagent	Final concentration	Amount
DPBS	1×	500 mL
Penicillin/Streptomycin	10000 units/mL	400 μL
Total	N/A	~500 mL

Note: Buffer can be stored at 4°C for up to 2 months.

Preparation of dissection buffer		
Reagent	Final concentration	Amount
Trypsin	0.25%	10 mL
DNase I (1 U/mL)	0.01 units/mL	100 μL
Total	N/A	~10 mL

Note: The solution should be stored on ice and used within 2 h.

⚠ CRITICAL: Add DNase I immediately before use to avoid degradation.

Note: The solution was freshly prepared just before use.

Preparation of digested end medium		
Reagent	Final concentration	Amount
DMEM	1×	30 mL
FBS	10%	3 mL
Penicillin/Streptomycin	10000 units/mL	300 μL
Total	N/A	~33 mL

Note: This medium should be freshly prepared just before use and stored on ice and used within 1 day.

Preparation of media for HEK-293T		
Reagent	Final concentration	Amount
DMEM	1×	450 mL
FBS	10%	50 mL
Penicillin/Streptomycin	10000 units/mL	5 mL
Total	N/A	~500 mL

Note: The solution should be stored at 4°C and used within 2 months.

Oligos of gRNA and PCR reaction	
Terms	Sequence (5′ to 3′)
sg*Smad4*-F	caccgCCAAGTAATCGCGCATCAA
sg*Smad4*-R	aaacTTGATGCGCGATTACTTGGc
sg*Pten*-F	caccgAACAAAAGGAGATATCAAG
sg*Pten*-R	aaacCTTGATATCTCCTTTTGTTc
sg*Scramble*-F	caccgACATTTCTTTCCCCACTGG
sg*Scramble*-R	aaacCCAGTGGGGAAAGAAATGTc
pcr-*Smad4*-F	CAGTCTCTGCAACCATCCA
pcr-*Smad4*-R	CCACGTTATCAGAGTGACAGAA
pcr-*Pten*-F	GGATCAGGCTCCATACCATAC
pcr-*Pten*-R	CTTTCCAATCCCACTTCATCAC
qpcr-*cMyc*-F	ATGCCCCTCAACGTGAACTTC
qpcr-*cMyc*-R	CGCAACATAGGATGGAGAGCA
qpcr-*Kras*^{G12D}-F	CAAGAGCGCCTTGACGATACA
qpcr-*Kras*^{G12D}-R	CCAAGAGACAGGTTTCTCCATC
qpcr-*Actb*-F	GGCTGTATTCCCCTCCATCG
qpcr-*Actb*-R	CCAGTTGGTAACAATGCCATGT
Sanger seq primer U6	GGACTATCATATGCTTACCG
Sanger seq primer MSCV	CCCTTGAACCTCCTCGTTCGACC

Note: All dissolved oligos should be used within one week and stored at 4°C, while the dry powder can be stored at 25°C for over a year.

Recipe for gRNA phosphorylation and annealing		
Reagent	Final concentration	Amount
gRNA-F	10 μM	1 μL
gRNA-R	10 μM	1 μL
T4PNK	500 units/mL	0.25 μL
T4 DNA ligase buffer	1×	0.5 μL
RNase-free ddH2O	N/A	2.25 μL
Total	N/A	5 μL

Note: The prepared mixture should be used immediately after preparation.

⚠ CRITICAL: Incubate the mix solution at 37°C for 30 min, heat to 95° for 5 min, and then slow down to 4°C (1°C per min). All procedures are performed in the PCR apparatus.

Recipe for pLenti-U6-gRNA-EFS-mCherry linearization		
Reagent	Final concentration	Amount
pLenti-U6-gRNA-EFS-mCherry	200 ng/μL	10 μL
BsmBI	250 units/mL	1 μL
NEB Buffer 3.1	1×	4 μL
RNase-free ddH2O	N/A	25 μL
Total	N/A	40 μL

Note: The prepared mixture should be used immediately after preparation.

⚠ CRITICAL: Incubate the mix solution at 55°C for 2 h, add 1 μL CIP enzyme and incubate at 37°C for 1 h.

Recipe for gRNA ligation		
Reagent	Final concentration	Amount
Annealed gRNA	10 μM	1.5 μL
linearized pLenti-U6-gRNA-EFS-mCherry	20 ng/μL	1 μL
T4 DNA ligase	10000 units/mL	0.25 μL
T4 DNA ligase buffer	1×	0.5 μL
RNase-free ddH2O	N/A	1.75 μL
Total	N/A	5 μL

Note: The prepared mixture should be used immediately after preparation.

⚠ CRITICAL: Incubate the mix solution at 25°C for 30 min. All procedures are performed in the PCR apparatus.

Recipe for pMSCV-*CDNA*-iRES-luci2 linearization		
Reagent	Final concentration	Amount
pMSCV-CDNA-iRES-luci2	200 ng/μL	10 μL
EcoRI	500 units/mL	1 μL
BglII	250 units/mL	1 μL
rCutSmart	1×	4 μL
RNase-free ddH2O	N/A	24 μL
Total	N/A	40 μL

Note: The prepared mixture should be used immediately after preparation.

⚠ CRITICAL: Incubate the mix solution at 37°C for 2 h.

Recipe for CDNA ligation		
Reagent	Final concentration	Amount
CDNA	200 ng/μL	1 μL
linearization pMSCV-*CDNA*-iRES-luci2	20 ng/μL	4 μL
Seamless clone enzyme	1×	5 μL
Total	N/A	10 μL

Note: The prepared mixture should be used immediately after preparation.

⚠ CRITICAL: Incubate the mix solution at 50°C for 30 min. All procedures are performed in the PCR apparatus.

Recipe for 2× HBS		
Reagent	Final concentration	Amount
NaCl	0.28 M	818 mg
Na_2HPO_4	1 mM	7.098 mg
HEPES	50 mM	250 μL
RNase-free ddH2O	N/A	50 mL
Total	N/A	~50 mL

Note: The solution should be stored at 4°C and used within 1 year.

Recipe for TNES buffer		
Reagent	Final concentration	Amount
NaCl	0.4 M	1168.8 mg
Tris	10 mM	60.57 mg
0.5 M EDTA	100 mM	10 mL
10% SDS Solution	0.6%	3 mL
RNase-free ddH2O	N/A	Add to 50 mL
Total	N/A	~50 mL

Note: The solution should be stored at 25°C and used within 1 year.

Recipe for PCR		
Reagent	Final concentration	Amount
primer-F	10 μM	1 μL
primer-R	10 μM	1 μL
Template	100 ng/μL	1 μL
Phanta mix	1×	20 μL
RNase-free ddH2O	N/A	17 μL
Total	N/A	40 μL

Note: The mixture should be immediately subjected to polymerase chain reaction (PCR) under the following conditions: annealing at 58°C, extension for 1 min, for 35 cycles.

Recipe for qPCR		
Reagent	Final concentration	Amount
primer-F	10 μM	1.5 μL
primer-R	10 μM	1.5 μL
Template	100 ng/μL	3 μL
SYBR	1×	7.5 μL
RNase-free ddH2O	N/A	1.5 μL
Total	N/A	15 μL

Note: For qPCR analysis, the mixture should be immediately processed using a two-step amplification protocol.

STEP-BY-STEP METHOD DETAILS

Isolation of mouse esophageal primary cells

Timing: 1–2 h for one mouse

This step outlines the procedure for isolating mouse esophageal primary cells from a *Trp53*$^{-/-}$-*Cas9* mouse (6–8 weeks, male) using enzymatic dissociation.

Dissection of esophagus tissues

1. Thaw 10 mL of Matrigel on ice or in a 4°C refrigerator overnight and aliquot 500 μL Matrigel into sterile 1.5 mL tubes and store at −20°C.
2. Euthanize the mouse using cervical dislocation. Secure the limbs of the mouse with tape and keep the mouse facing upward. Spray the ventral surface of the mouse and the surrounding area with 70% ethanol.
3. Make an incision from the gonads to the neck into the abdominal cavity. Cut the cervical vertebrae, esophagus, and trachea with surgical scissors.
4. Move the liver laterally to reveal the stomach out of the body. Follow the stomach to locate the esophagus and clamp the esophagus with a curved pinch.
5. Aseptically dissect the esophagus (cut at the cardia and excluding the forestomach (1 cm in length); Figure 1A).
6. Dissect the esophagus longitudinally from the inside of the esophageal lumen. Wash the tissues briefly with the pre-cold wash buffer.
7. Place the tissue into fresh wash buffer in a 50 mL tube on ice.

Note: Keep Matrigel matrix on ice all time during handling and avoid freeze-thaw cycles.

⚠ CRITICAL: If the esophagus and trachea are not properly transected at the cervical level, this may result in esophageal rupture, compromising the integrity of the specimen and ultimately leading to insufficient yield of primary cells for subsequent experiments.

Isolation of primary cells

8. Prepare a 10 mL digestion buffer in another 50 mL collection tube on ice.
9. Put the isolated mouse esophagus in a 10 cm dish with sterile tweezers.
10. Cut tissue with scalpels on ice until there are no visible particles.
11. Collect the tissue into the digestive solution with forceps, then use the digestive solution to rinse the tissue from the scissors into the collection tube.
12. Incubate in a 37°C shaking air bath (120 rpm) for 60 min.
13. Add 30 mL digested end media (25°C) to the collection tube to stop digestion.
14. Filter the mixture through a 70 μm cell strainer.
15. Centrifuge at 400 × *g* for 10 min and then discard the supernatant.
16. Resuspend the pellet in 200 μL ice-cold DMEM/F12 and perform a cell count using trypan blue solution.

Note: Non-shaking air baths can also be used with manual agitation every 10 min.

Alternatives: Without a shaking air bath, you can also use a 37°C water bath, turning it upside down once every 10 min.

⚠ CRITICAL: The larger volume of the digested end media helps increase the flow rate to filter more epithelial cells out of the filter. When removing the supernatant, use a 200 μL pipette at the end to avoid sucking out the cells.

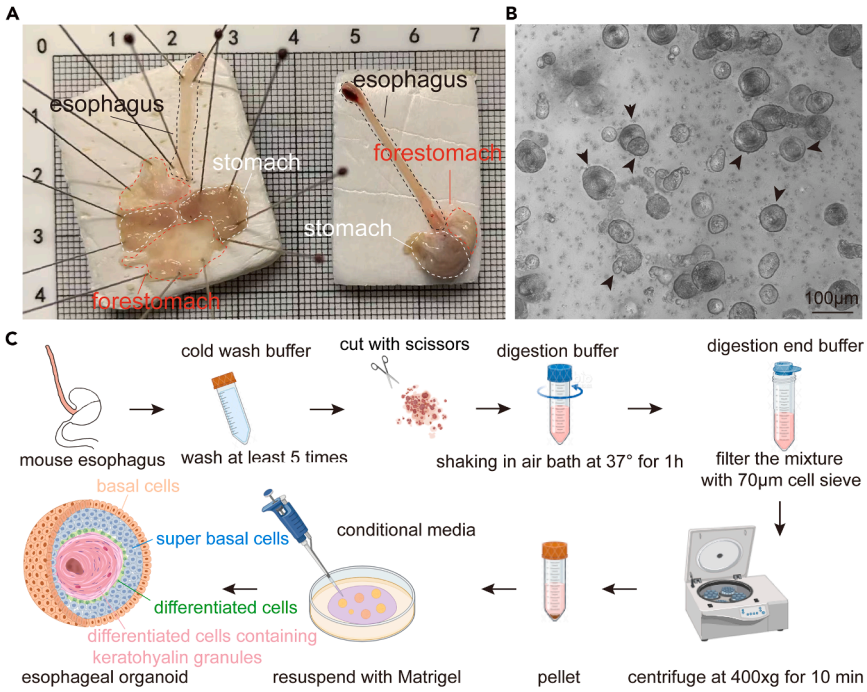

Figure 1. Dissect mouse esophagus and organoids culture

(A) Bright field of murine esophagus (black) stomach (white) and forestomach (red), sectional (left) and whole (right) tissues.

(B) Bright field of murine esophageal organoids, as shown by black arrow. Scale bars, 100 μm.

(C) Strategy for organoids culture.

Establishment of mouse esophageal organoid cultures

Timing: 30 min

This section outlines the steps to seed the esophageal epithelial cells into the Matrigel domes to develop organoid cultures (Figure 1C). Actually, in the isolated products, there are various cellular components, including epithelium, stromal cells, immune cells, etc., but in our culture medium, only epithelial cells can eventually grow into organoids, and other cells cannot proliferate.

Seeding organoid cultures

17. Thaw Matrigel on ice before 1 h. Warm up conditional media at 37°C.
18. Centrifuge at 400 × *g* for 10 min and then discard the supernatant.
19. Resuspend the pellet in Matrigel on ice and mix well.
20. Plate 30 μL of the cell/Matrigel solution onto the center of wells in the prewarmed 48-well plates.
21. Incubate the 48-well plates in a 37°C incubator for 30 min to allow the Matrigel to solidify.
22. Once solidified, add 150 μL of prewarmed conditional media to each well and add 500 μL DPBS to the side holes of the 48-well plate to prevent drying.
23. Incubate the cultures in a humidified 37°C incubator supplied with 5% CO_2.

Note: Resuspend the cells slowly and carefully and ensure that there is always liquid in the tip of the pipettor when blowing to avoid introducing air bubbles, which can affect organoids' growth.

⚠ CRITICAL: Ensure that there are 1000–3000 primary cells in every 30 μL of Matrigel, too few cells will lead to inefficient culture, and too many cells will lead to insufficient space for organoids and stunted development (Figure 1B).

Genome editing

Timing: 1–2 weeks

This section outlines the steps to maintain organoids culture, passage organoid and organoid infection.

Maintaining organoid cultures

24. Replace the conditional media every 3 days.
25. Monitor organoid development under an optical microscope every day. The organoids remain uncontaminated and grow daily until they reach 200 μm or there's insufficient space for further growth, at which point passage is necessary.

Passage organoids

26. Thaw Matrigel on ice before 1 h. Warm up conditional media at 37°C.
27. Discard the conditional media from Matrigel domes in 48-well plate. Resuspend the Matrigel domes in the 1 mL TrypLE by pipetting and scraping the wells using a 1 mL micropipette to collect all the organoid suspension and transfer to a prechilled sterile 15 mL collection tube.
28. Incubate in a 37°C shaking air bath (120 rpm) for 30 min.
29. Centrifuge at 400 × *g* for 10 min and then discard the supernatant.
30. Resuspend the organoid pellet in Matrigel at a ratio of 1:6 on ice and mix well. Plate the mixture into 48-well plates.
31. Incubate the 48-well plates in a 37°C incubator for 30 min to allow the Matrigel to solidify.
32. Once solidified, add 150 μL of prewarmed conditional media to each well and add 500 μL DPBS to the side holes of the 48-well plate to prevent drying.
33. Incubate the cultures in a humidified 37°C incubator supplied with 5% CO_2.

Note: The passage ratio of esophageal organoids is flexible. When we need to expand more organoids, we should keep it from 1:3 to 1:6. When we need to see the morphology of the organoids, we should keep it at about 1:100.

Construct preparation

34. Order *Pten Smad4* and Scramble gRNA oligos from the gene synthesis corporation.
35. Phosphorylated and annealed paired primers.
36. Linearize the pLenti-U6-gRNA-EFS-mCherry vector with BsmBI at 55°C for 2 h, and the terminal phosphate group was removed with CIP enzyme at 37°C for 1 h (Figure 2A).
37. Column recovery linearized vector and clone gRNA to pLenti-U6-gRNA-EFS-mCherry vector with T4DNA ligase enzyme.
38. Transform the ligation product to DH5α competent cells and coat the plate.
    a. Mix 5 μL ligation production and at least 50 μL competent cells on ice for 15 min and then warm at 42°C for 30 s.
    b. Keep them on ice for at least 2 min and coat the plate. Pick a clone to produce plasmids.
    c. Identify the correct clone by sanger sequence with the primer U6.
39. Order the full length of $Kras^{G12D}$ and *cMyc* CDNA from the gene synthesis corporation (Figure 2B).

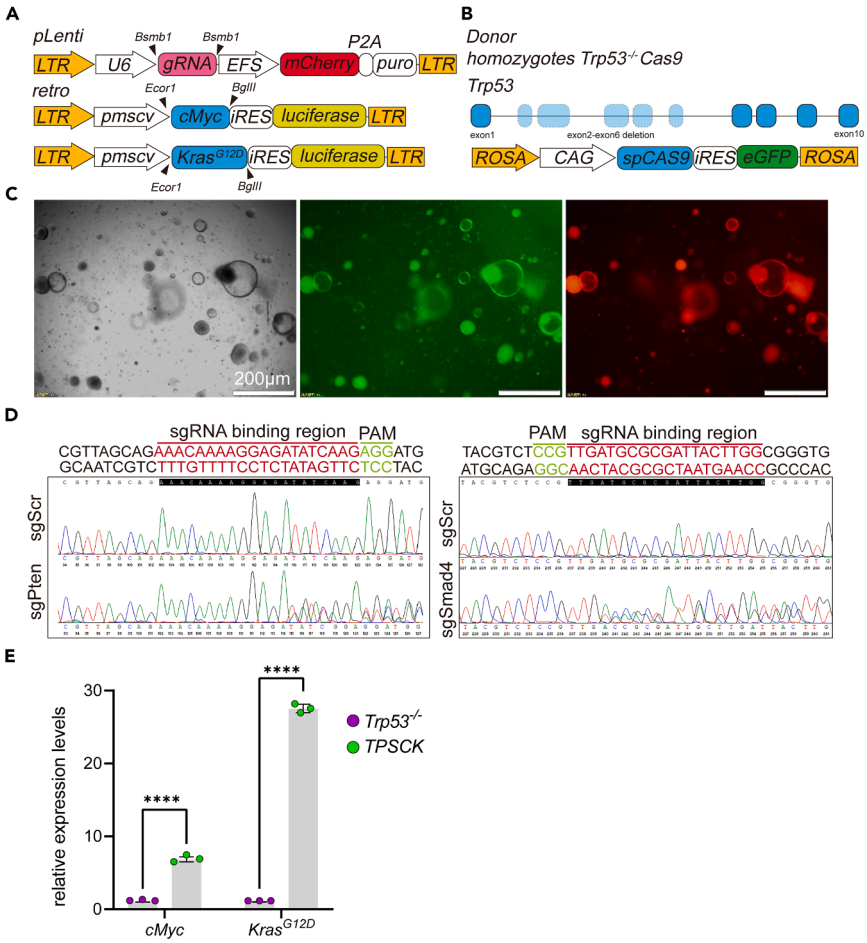

Figure 2. A pipeline with organoid gene editing and genomic analysis for establishing ESCC OPCMs

(A) Schematic of gene editing strategies. CRISPR-mediated knockout of candidate genes was introduced to organoids by lentiviral transfection with red fluorescence, and the proto-oncogene *cMyc* or *Kras*^{G12D} was introduced by retroviruses with luciferase reporter gene.

(B) Organoids from mouse esophageal epithelial cells that were isolated from *Trp53*$^{-/-}$; *Rosa26 CAG-spCas9-IRES-eGFP* mice.

(C) Bright field (left), green fluorescence field (middle) and red fluorescence field (right) of edited organoids. Scale bars, 200 μm.

(D) Sanger sequence show knock out of *Pten* (left) and *Smad4* (right) by Crispr-Cas9 along with gRNAs.

(E) Bar plots show the qPCR assay to quantify the overexpression efficiency of *cMyc* and *Kras*^{G12D} of the TPSCK organoids, normalized by *Trp53*$^{-/-}$ organoids. Data are mean of three technique repeats, and error bars show ±SD. ****p<0.0001, unpaired t-test.

40. PCR amplifies the CDNA with the Phanta Flash Super-Fidelity DNA Polymerase. Prolong the CDNA with homologous arms (primers are not given; you only need to satisfy the 15 bp homologous sequence) and purify them with a column recovery kit.
41. The pMSCV-iRES-luci2 vector was linearized with EcoR1 and BglII at 37°C for 2 h.
42. Column recovery linearized vector and clone CDNA to pMSCV-iRES-luci2 vector with seamless clone enzyme at 50°C for 30 min.
43. Transform the ligation product to DH5α competent cells and coat the plate.
    a. Mix 5 μL ligation production and at least 50 μL competent cells on ice for 15 min and then warm at 42°C for 30 s.
    b. Keep them on ice for at least 2 min and coat the plate.

c. Pick a clone to produce plasmids. Identify the gRNA sequence by sanger sequence with the primer MSCV.

44. Virus preparation.
    a. Spread 293T cells onto 6-well plates 1 day before transfection until the cell density was about 90%.
    b. Fresh medium containing Chloroquine (final concentration 25 μM) was replaced before transfection.
    c. Mix auxiliary plasmids psPAX2 2 μg, pMD2.G 1 μg, and target gRNA plasmids 4 μg with 12.5 μL 2 M $CaCl_2$ solution to a volume of 100 μL by calcium phosphate transfection method.
    d. Add to the 2× HBS solution of equal volume on the vortex meter drop by drop, and finally, add the above 200 μL suspension containing DNA-$Ca_3(PO_4)_2$ precipitation to the medium drop by drop, and gently shake and mix the plate.
    e. Mix auxiliary plasmids pCL-Eco 0.5 μg, pCAG-VSVG 0.2 μg, and target CDNA plasmids 4 μg with 12.5 μL 2 M $CaCl_2$ solution to a volume of 100 μL by calcium phosphate transfection method.
    f. Add the 2× HBS solution of equal volume on the vortex meter drop by drop, and finally, add the above 200 μL suspension containing DNA-$Ca_3(PO_4)_2$ precipitation to the medium drop by drop, and gently shake and mix the plate.
    g. After 8–12 h, refresh the medium, and then collect the supernatant medium of 36 h, 48 h, and 60 h respectively, a total of 6 mL, that is, the virus.

Note: The virus should be stored at 4°C and used up within one week. The virus still poses a risk of contact with infected human tissue and needs to be handled in a biosafety Level II laboratory for pathogenic microorganisms.

Organoid infection

45. Discard the conditional media from Matrigel domes in 48-well plate.
46. Resuspend the Matrigel domes in the 1 mL TryplE by pipetting and scraping the wells using a 1 mL micropipette to collect all the organoid suspension and transfer to a prechilled sterile 15 mL collection tube.
47. Incubate in a 37°C shaking air bath (120 rpm) for 30 min.
48. Centrifuge at 400 × *g* for 10 min and then discard the supernatant.
49. Resuspend the organoid pellet in 1 mL mixed virus (*Pten* 250 μL, *Smad4* 250 μL, *cMyc* 250 μL, $Kras^{G12D}$ 250 μL).
50. Transfer the organoids virus mixture to 12-well plate. Centrifuge at 400 × *g* for 60 min at 31°C.
51. Incubate the plate in a humidified 37°C incubator for 12 h.
52. Collect the cell suspension to 15 mL collection tube. Centrifuge at 400 × *g* for 10 min. Reseed organoids as step passage organoids.

Note: Select positive organoids with puromycin-conditional medium (1 μg/mL) for 1 week (Figure 2C).

Verify efficiency of genome editing

53. For edited organoids, discard the medium, re-suspend with 500 μL TNES buffer and 3 μL Protein kinase K (20 mg/mL) in 1.5 mL EP tube, and heated at 55°C for 2 h.
54. Add 500 μL isopropyl alcohol, mix upside down, white flocculent precipitation can be seen in this step, and centrifuge at 10000 × *g* for 10 min.
55. Discard the supernatant, add 1 mL 75% ethanol, precipitate by suspension, and centrifuge at 4°C, 12000 × *g* for 5 min.
56. Discard the supernatant, dry the remaining liquid at 25°C, and add 20 μL Nuclease-free H_2O to re-suspend the precipitation.

57. Conduct PCR on the fragments targeted by the gRNA, followed by Sanger sequencing with primer-F (Figure 2D).
58. For the edited organoids, discard the medium and resuspend the sample in 1 mL of TRIzol in a 1.5 mL EP tube at 25°C for 5 min.
59. Next, add 200 μL of chloroform ($CHCl_3$), mix gently by turning the tube upside down, and centrifuge at 10000 × *g* for 15 min at 4°C.
60. Carefully transfer the top layer to another EP tube, add 500 μL of isopropyl alcohol, shake the tube repeatedly, and allow it to stand at 25°C for 10 min. Then, centrifuge again at 10000 × *g* for 15 min at 4°C.
61. Discard the supernatant and add 1 mL of 75% ethanol. Mix to precipitate the sample and centrifuge at 4°C for 12000 × *g* for 5 min.
62. After discarding the supernatant, let the remaining liquid dry at 25°C, then add 20 μL of nuclease-free water to resuspend the precipitation.
63. Proceed with the reverse transcription of RNA to cDNA using the HiScript II Q RT-qPCR kit, following the specific instructions provided with the kit.
64. Finally, perform qPCR to assess the amplification efficiency of the cDNA (Figure 2E).

Note: Normal organoids and successfully edited organoids only show morphological differences after long-term culture. Here, we highly recommend detecting fluorescence and gene mutation efficiency instead.

Establishment of an orthotopic esophageal carcinoma mouse model

Timing: 2 months

This step outlines the procedure for orthotopic transplantation surgery and living image monitoring, Recipient mice must be BALB/cA-nu mice aged ≥6 weeks, with no gender restriction.

Orthotopic transplantation

65. Collect the sufficient counts of organoids (2×10^5–5×10^5 cells per mouse, or 2–4 wells organoids) with TrypLE.
66. Resuspend edited organoids with a 20 μL 1:1 ratio of PBS and Matrigel and place on ice. Do not keep organoids on ice for more than 2 h.
67. Anesthetize the recipient mice with isoflurane.
68. Secure the limbs of the mouse with tape and keep the mouse facing upward.
69. Spray the ventral surface of the mouse and the surrounding area with 70% ethanol.
70. Make a 0.5 cm incision lower rib margin on the left side of the body into the abdominal cavity.
    a. Move the liver laterally to reveal the stomach out of the body.
    b. Gently clamp the stomach using tweezers and locate the esophagus. At the gastroesophageal junction, insert the needle from the side of the stomach towards the esophagus.
    c. Position the needle about 3 mm from the point where the esophagus exits the stomach.
    d. Ensure that the needle is inserted parallel to the esophagus, between the muscular layer and the mucosal layer, and insert it approximately 3 mm deep.
    e. Inject the cell suspension about 20 μL into the esophageal mucosa using an insulin needle (Figure 3A).
71. Suture the incision immediately.
72. Monitor the tumor growth using a D-luciferin imaging system 20 days after transplantation (Figure 3B).

Note: The mice selected for orthotopic transplantation should be at least 8 weeks old as possible because the esophagus is too thin when the mice are too young, and the operation is more complicated. Both nude mice and C57BL/6 mice can be used as recipient, but nude

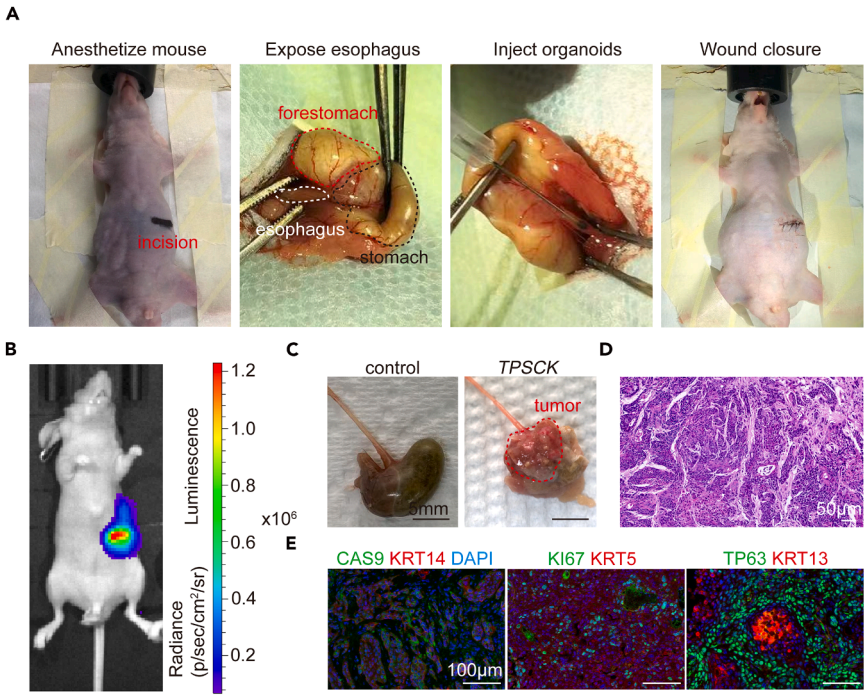

Figure 3. Esophageal OPCMs surgical procedure and pathologic analysis

(A) OPCMs surgical procedure.

(B) D-luciferase image showing the orthotopic tumor growth in TPSMK OPCMs.

(C) Bright field of control esophagus (left) and *TPSMK* esophagus (right) of OPCM mice. Scale bar, 5 mm.

(D) H&E staining of *TPSMK* tumor. Scale bar, 50 μm.

(E) Representative immunofluorescence co-staining images of CAS9 and KRT14 (left), KI67 and KRT5 (middle), and TP63 and KRT13 (right) in TPSMK tumor tissue. Scale bars, 100 μm.

mice form tumors more quickly (within 20 days) compared to C57BL/6 mice, which take about 40 days. The monitoring period for tumor formation in mice is influenced by various factors, including the efficiency of organoid editing, organoid activity, and the success rate of orthotopic transplantation. In our TPSCK model, a fluorescent signal is expected to appear around 20 days after transplantation and can last for approximately 60 days. We defined our endpoints for survival as either tumor-related death in the mice or a significant weight loss (less than 15 g) due to esophageal obstruction.

EXPECTED OUTCOMES

This study established a protocol for generating primary, orthotopic, and genetically defined tumor models. The protocol includes isolating primary cells from the mouse esophagus, developing procedures for culturing esophageal organoids, editing the organoid genome, and orthotopic transplantation. The epithelial cells embedded in Matrigel ultimately developed into an intermediate keratinized organoids with a characteristic 'straw hat' morphology, recapitulating the pathology observed in the mouse esophagus (which undergoes intermediate keratinization and surrounding stem cell proliferation), as well as in human esophageal organoids.[6–8]

In the ESCC patient cohort, loss-of-function alterations (deletions or mutations) in tumor-suppressor genes *TP53*, *PTEN*, and *SMAD4*, along with copy number gains of *MYC* and *KRAS*, represent common genomic events.[1,9] Based on these findings, we hypothesized that combinatorial disruption of these genes would drive tumorigenesis. To test this, we isolated primary esophageal cells from *Trp53*$^{-/-}$ *ROSA-CAG-Cas9-iRES-eGFP* mice and established organoid cultures for genome editing. We then introduced *Pten* and *Smad4* knockout using lentiviral vectors co-expressing *mCherry*

(red fluorescent protein). Achieved oncogene overexpression (*cMyc* and *Kras*^{G12D}) via retroviral vectors containing luciferase reporter genes. Editing efficiency was validated through Sanger sequencing for tumor suppressor gene knockout verification and Quantitative PCR (qPCR) for oncogene overexpression assessment.

Due to the technical challenges posed by the thin mouse esophageal wall and difficult surgical exposure of the thoracic esophagus, we orthotopically transplanted the genetically edited TPSCK organoids (*Trp53*$^{-/-}$*;* sg*Pten;* sg*Smad4; cMyc; KrasG12D*) into the mucosal layer at the gastroesophageal junction. D-luciferase bioluminescence imaging confirmed stable engraftment and progressive growth at the transplantation site. Macroscopic examination revealed complete occupation of the gastroesophageal junction by the graft. Histopathological evaluation demonstrated characteristic ESCC features. Immunohistochemistry confirmed expression of squamous cell carcinoma markers (TP63, KRT5, KRT13, KRT14) (Figures 3C–3E).

LIMITATIONS

The protocol established a method to isolate primary cells from mouse esophageal tissue to produce organoids. However, the esophagus is a non-sterile organ unrestricted to the outside world through the mouth, which will inevitably cause bacterial or fungal contamination during operation. When contamination occurs, it must be treated to prevent further expansion of contamination. In the ESCC patient cohort, esophageal squamous cell carcinoma mainly occurs in the upper and middle esophagus. However, our model makes it difficult to simulate the significant location of esophageal squamous cell carcinoma due to surgical difficulties. The mouse esophagus is very slim; once the tumor is formed, it is easy to cause esophageal obstruction and further causes malnutrition and death of mice.

Due to the high frequency of mutations in ESCC and the complexity of mutations and combinations in patients, our current strategies can only simulate the possible combinations and study specific gene functions, which may have some gaps in the patient's situation. In addition, the cases of deletion and amplification of large chromosome fragments in esophageal cancer are far more than those of gene mutation and function loss. However, the indisposed uniformity of chromosomes between the two species makes it difficult for our mouse model to simulate the effect of chromosomal alterations on esophageal cancer.

TROUBLESHOOTING

Problem 1

Bubbles appear in Matrigel domes during organoid culture, passage, and infection, affecting organoids' development (step 20).

Potential solution

- The bubbles are mainly caused by excessive pipette blowing when the organoids are suspended with Matrigel, which causes air to mix in when the liquid is completely removed from the tips. Retaining part of the liquid when blowing is best to prevent air from mixing.

Problem 2

The domes of Matrigel collapsed during organoid culture or passage, resulting in organoid dysplasia (step 32).

Potential solution

- The main reason for the collapse of the Matrigel domes is that the residual pancreatic Trypsin or TrypLE is not washed away during the resuspension of the organoids, which can be improved by removing them clean with the tip of a 200 μL tip before resuspension.

Problem 3

Organoids grow slowly or apoptosis after infection (step 52).

Potential solution

- The main causes of organoids growing slowly, or apoptosis after infection, are the exorbitant virus titer and overlong incubation time. If the titer is too high, it can be improved by diluting it with the medium or shortening the incubation time.

Problem 4

Organoids cannot be transplanted between the muscular and mucosal layers of the esophagus during surgery (step 72).

Potential solution

- The wound on the mouse's abdomen was misaligned and did not accurately expose the gastric esophagus. To properly access the abdominal cavity, the incision should be made under the left rib margin.
- The insulin needle was injected in an incorrect position, puncturing the esophageal cavity, which caused the organoids to fail to colonize properly. To prevent this, the insulin needle should be placed approximately 3 mm parallel to the esophagus. It is important to avoid puncturing the esophageal envelope and to gently rotate the needle to ensure it does not penetrate the esophageal cavity.

Problem 5

Edited organoids failed to form tumors after orthotopic transplantation (step 74).

Potential solution

- Insufficient organoids at transplantation or inactivity of organoids can lead to tumor formation failure. Transplant 2×10^5 active cells into each mouse at least.
- Enhance the editing efficiency of organoids with puromycin; select edited cells will be improved.
- Leaks organoids into the abdominal cavity when orthotopic transplantation can lead to tumorigenesis failure. Transplantation can be performed with the assistance of stereomicroscopy.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Chong Chen (chongchen@scu.edu.cn).

Technical contact

Questions about the technical specifics of performing the protocol should be directed to the technical contact, Dr. Jian Wang (277894475@qq.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

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AUTHOR CONTRIBUTIONS

Conceptualization, C.C.; methodology, J.W., J.D., L.G., X. Li, L.W., Z.L., X.W., Yuan Li, H. Liu, H. Li, P.T., Y.J., and Yaxin Li; gene selection, X. Luo; writing – original draft, J.W., J.D., and Z.D.; writing – review and editing, C.C.; supervision, Y. Yuan, Y. Yang, L.C., B.H., Y. Liu, F.N., and C.C.

DECLARATION OF INTERESTS

A patent (no. 202010684524.2) for the primary *in situ* mouse model of esophagus based on organoids has been applied for by West China Hospital, Sichuan University.

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