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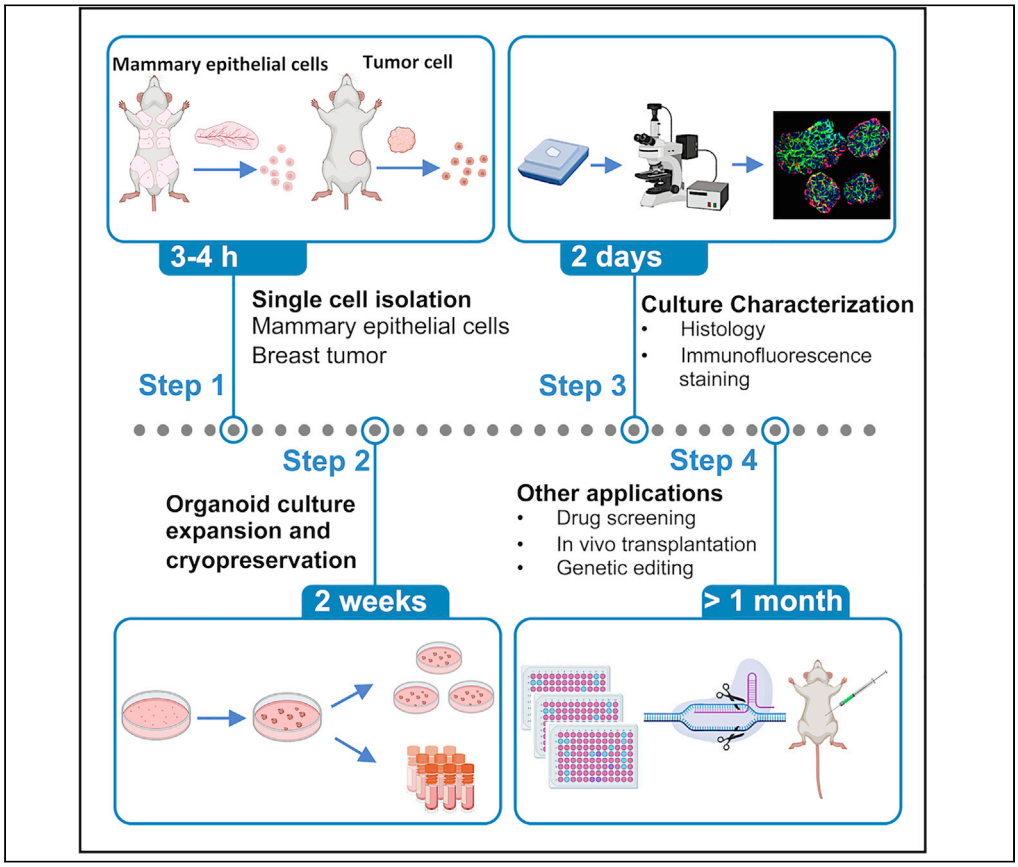
Establishment and long-term culture of mouse mammary stem cell organoids and breast tumor organoids



Mammary stem cells (MaSCs) contribute to mammary epithelium development and homeostasis. They have been proposed as cells of origin for breast cancer. Here, we describe an organoid culture protocol for *ex vivo* expansion of MaSCs from mouse tissues. These organoids maintain the self-renewal of gland-reconstituting MaSCs and can be used to model tumorigenesis by introducing patient-relevant cancer drivers and mutations. Similar organoid culture can be used for long-term expansion of luminal stem/progenitor cells from normal glands and tumor-initiating cells from mammary tumors.

Jihong Cui, Wenjun Guo

jihong.cui@einsteinmed.org (J.C.)
wenjun.guo@einsteinmed.org (W.G.)

Highlights

Protocol to isolate epithelial cells from mammary glands and tumors

Detailed procedure to generate and expand MaSCs and tumor organoids

Organoids can be used for genetic modification and biological analyses

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Establishment and long-term culture of mouse mammary stem cell organoids and breast tumor organoids

Jihong Cui[1,2,*] and Wenjun Guo[1,2,3,4,*]

[1]Ruth L. and David S. Gottesman Institute for Stem Cell and Regenerative Medicine Research, Albert Einstein College of Medicine, Bronx, NY 10461, USA

[2]Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY 10461, USA

[3]Technical contact

[4]Lead contact

*Correspondence: jihong.cui@einsteinmed.org (J.C.), wenjun.guo@einsteinmed.org (W.G.)
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SUMMARY

Mammary stem cells (MaSCs) contribute to mammary epithelium development and homeostasis. They have been proposed as cells of origin for breast cancer. Here, we describe an organoid culture protocol for *ex vivo* expansion of MaSCs from mouse tissues. These organoids maintain the self-renewal of gland-reconstituting MaSCs and can be used to model tumorigenesis by introducing patient-relevant cancer drivers and mutations. Similar organoid culture can be used for long-term expansion of luminal stem/progenitor cells from normal glands and tumor-initiating cells from mammary tumors.

For complete details on the use and execution of this protocol, please refer to Christin et al. (2020) and Zhang et al. (2016).

BEFORE YOU BEGIN

General laboratory preparation

1. All experiments were performed in a BSL2 biosafety hood.
2. All surgery was performed by using aseptic procedures, including sterile gloves, sterile surgical instruments, and aseptic techniques.
3. Set up the incubator for mammary gland digestion at 37°C.
4. Set up the humidified incubator at 37°C with 5% CO_2 for organoid culture.
5. See the key resources table for a complete list of materials.
6. Prepare all solution before sample processing, following the recipes in the Materials and Equipment section.

Preparation of ultra-low attachment plates using poly (2-hydroxyethyl methacrylate)-(poly-HEMA)

Timing: 1 week

7. Poly-HEMA solution preparation
   a. Dissolve 12 g Poly-HEMA in 1000 mL 95% ETOH by stirring. It may take overnight (12–16 h) or longer to completely dissolve (final concentration = 12 mg/mL).
   b. Filter through a 0.22 μm nylon filter top.
8. Plate coating

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a. Coat plates with the poly-HEMA solution as the following:
   i. 5 mL/100 mm dish
   ii. 2 mL/60 mm dish
   iii. 1.5 mL/ well / 6-well plate
   iv. 500 µl/ well / 24-well plate
b. Dry plates in a non-humidified incubator at 37°C–55°C for 2–4 days.
c. Plates can be stored at 22°C–25°C prior to use.

⚠ CRITICAL: Check the coating is even. If observing wrinkles, re-coat with 50% of the original volume to add additional coating. Incubating at 37°C allows more even coating but takes longer to dry.

Matrigel aliquots

Timing: 16 h

9. Thaw a frozen bottle of Matrigel on ice in a 4°C fridge overnight (12–16 h).
10. Resuspend to homogenize the Matrigel solution.
11. Aliquot 1 mL in 1.5 mL micro tubes. Store at −20°C.
12. When purchasing a batch, ensure protein concentration is between 9.5 and 12 mg/mL and test it before use.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-keratin 14 (1:1000)	BioLegend	Cat#905301
Rat anti-keratin 8 (1:60)	Developmental Studies Hybridoma Bank	Cat#TROMA-I
Sox9 (D8G8H) Rabbit mAb (1:200)	Cell Signaling Technology	Cat#82630
Slug (C19G7) Rabbit mAb (1:200)	Cell Signaling Technology	Cat#9585
Chemicals, peptides, and recombinant proteins		
Advanced DMEM/F12	Gibco	Cat#12634028
DMEM	Corning	Cat#10-092-CV
DMEM/Hams F-12 50/50 Mix	Corning	Cat#10-017-CV
Epicult (R) - B Mouse Medium Kit	STEMCELL Technologies	Cat#05610
Fetal bovine serum	VWR	Cat# 97068-085
HyClone Calf Serum, U.S. origin	Cytiva	Cat#SH30073.03
GlutaMAX™ Supplement 100×	Gibco	Cat#35050061
Penicillin-streptomycin 100×	Corning	Cat# 30-002-CI
PBS, 1× without calcium and magnesium	Corning	Cat#21-040-CV
Trypan blue Solution, 0.4%	Corning	Cat#25900CI
Bovine Serum Albumin, Heat Shock Treated (BSA)	Fisher Scientific	Cat# BP1600100
Poly(2-hydroxyethyl methacrylate)	Sigma-Aldrich	Cat#P3932
Ethanol 200 Proof	Beacon Labs	Cat#2701
Collagenase, type III	Worthington Biochemical	Cat# LS004182
Deoxyribonuclease I (DNase I)	Worthington Biochemical; Roche	Cat# LS002139; Cat#104159
Neutral protease (Dispase)	Worthington Biochemical	Cat# LS02109
Trypsin 0.05% with 0.53 mM EDTA	Corning	Cat# 25-052-CI
Epidermal Growth Factor, human (EGF)	Sigma-Aldrich	Cat#E9644
Fibroblast Growth Factor basic Protein, Human recombinant (FGF2)	MillporeSigma	Cat#GF003
Heparin sodium salt, from porcine intestinal mucosa	MillporeSigma	Cat#H3149

(Continued on next page)

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Y-27632 (hydrochloride), ROCK inhibitor	Cayman Chemical Company	Cat#10005583
Hyaluronidase	Worthington Biochemical	Cat# LS002592
Matrigel Basement Membrane Matrix	Corning	Cat#354234
Dimethylsulfoxide (DMSO)	MillporeSigma	Cat#MX-1458
Red blood cell lysing buffer	Sigma-Aldrich	Cat#R7757
Agarose, Low Melting Point	Promega	Cat#V2111
32% Paraformaldehyde (formaldehyde) aqueous	Electron Microscopy Sciences	Cat#15714S
Xylene	Fisher Chemical	Cat# BPX3P1GAL
DAPI Fluoromount-G mounting medium	SouthernBiotech	Cat#0100-20
OCT compound	Fisher Healthcare	Cat#23-730-571
Antigen Unmasking Solution, Citric Acid Based	Vector Laboratories	Cat#H3300
Collagen I, rat tail	Corning	Cat#354236
Non-essential amino acids (100×)	Gibco	Cat#11140050
Sodium Pyruvate (100 mM)	Gibco	Cat#11360070
jetPrime transfection reagent	VWR	Cat#89129-924
Polybrene	MillporeSigma	Cat#TR-1003-G
Experimental models: Organisms/strains		
Mouse: C57BL/6J	The Jackson Laboratory	Cat# JAX:000664
Mouse: FVB-Tg(C3-1-TAg)cJeg/JegJ	The Jackson Laboratory	Cat# JAX:013591
Other		
0.22-μm Pore size sterile syringe filter	Foxx Life Sciences	Cat# 371-2215-OEM
Tissue-Tek® Cryomold® Molds	Sakura	Cat# 4565
10-mL Syringe	BD	Cat#302995
Cell strainer, 40 μm	Fisher Scientific	Cat# 22-363-547
15-mL centrifuge tube	CELLTREAT	Cat#229411
6-well plates	Corning	Cat# 3506
Mini incubator	Denville Scientific	Cat#I4900
Incubator	Quincy Lab	Cat#12-140
100 mm × 15 mm Bacteriological Petri dish	Corning	Cat#351029
GyroMini™ nutating Mixer with dimpled rubber mat	Labnet	Cat#S0500
Hemocytometer	Hausser Scientific	Cat#3200
Eppendorf Centrifuge 5810R	Eppendorf	Cat#022625101
Cryovials	Greiner	Cat#126263
Retriever for antigen unmasking	Electron Microscopy Sciences	Cat#62700-10
500-mL Bottle-top vacuum filter	Corning	Cat#430049

MATERIALS AND EQUIPMENT

Solution and medium used

Advanced D/F	Final concentration	Volume
Advanced DMEM/F12	n/a	490 mL
GlutMAX (100×, 200 mM)	2 mM	5 mL
Pen/Strep (100×, 10,000 I.U. Penicillin 10,000 μg/mL Streptomycin)	Pen: 100 I.U.; Strep: 100 μg/mL	5 mL
Total	**n/a**	**500 mL**

Note: Once prepared, stored at 4°C for 4 weeks

Basic medium	Final concentration	Volume
DMEM	n/a	445 mL
Calf serum	10% (v/v)	50 mL
Pen/Strep (100×, 10,000 I.U. Penicillin 10,000 μg/mL Streptomycin)	Pen: 100 I.U.; Strep: 100 μg/mL	5 mL
Total	**n/a**	**500 mL**

Note: Once prepared, stored at 4°C for 4 weeks

Gland digestion buffer I	Final concentration	Volume
DMEM /F12	n/a	4.94 mL
Collagenase, type III	300 Unit/mL	50 μL (100× stock, 30,000 Unit/mL)
DNase I	10 μg/mL	5 μL (1000× stock, 10 mg/mL)
Y-27632	5 μM	5 μL (1000× stock, 5 mM)
Total	**n/a**	**5 mL**

Note: This solution should be freshly prepared and used immediately.

Tumor digestion buffer I	Final concentration	Volume
DMEM /F12	n/a	4.89 mL
Collagenase, type III	300 Unit/mL	50 μL (100× stock, 30,000 Unit/mL)
DNase I	10 μg/mL	5 μL (1000× stock, 10 mg/mL)
Hyaluronidase	100 Unit/mL	50 μL (100× stock, 10,000 Unit/mL)
Y-27632	5 μM	5 μL (1000× stock, 5 mM)
Total	**n/a**	**5 mL**

Note: This solution should be freshly prepared and used immediately.

Digestion buffer II	Final concentration	Volume
DMEM /F12	n/a	940 μL
Dispase	1 unit/mL	50 μL (20× stock, 20 Unit/mL)
DNase I	100 μg/mL	10 μL (100× stock, 10 mg/mL)
Total	**n/a**	**1 mL**

Note: This solution should be freshly prepared and used immediately.

Suspension buffer	Final concentration	Volume
PBS	n/a	498 mL
BSA	0.1% BSA	0.5 g
EDTA	2 mM	2 mL, (0.5 M stock)
Total	**n/a**	**500 mL**

Note: Filter through a 0.22 μm filter. Once prepared, stored at 4°C for 4 weeks.

MaSC/Tumor organoid culture medium I (without Matrigel)	Final concentration	Volume
Advanced D/F	n/a	47.3 mL
FBS	5%	2.5 mL
EGF	10 ng/mL	50 μL (1000× stock, 10 μg/mL)
FGF2	20 ng/mL	50 μL (1000× stock, 20 μg/mL)
Heparin	4 μg/mL	50 μL (1000× stock, 4 mg/mL)
Y-27632	5 μM	50 μL (1000× stock, 5 mM)
Total	**n/a**	**50 mL**

Note: Once prepared, stored at 4°C for 1 week.

EGF and FGF2 stock reconstituted in 0.1% BSA/PBS and aliquot 100 μL per well.

Before the experiment, add 5% Matrigel into ice-cold medium freshly.

MaSC organoid culture medium II (without Matrigel)	Final concentration	Volume
Epicult-B medium	n/a	47.3 mL
FBS	5%	2.5 mL
EGF	10 ng/mL	50 μL (1000× stock, 10 μg/mL)
FGF2	20 ng/mL	50 μL (1000× stock, 20 μg/mL)
Heparin	4 μg/mL	50 μL (1000× stock, 4 mg/mL)
Y-27632	5 μM	50 μL (1000× stock, 5 mM)
Total	**n/a**	**50 mL**

Note: Once prepared, stored at 4°C for 1 week.

EGF and FGF2 stock reconstituted in 0.1%BSA/PBS and aliquot 100 μL per well.

Before the experiment, add 5% Matrigel to ice-cold medium freshly.

Freezing medium	Final concentration	Volume
Calf serum	92%	46 mL
DMSO	8%	4 mL
Y-27632	5 μM	50 μL (1000× stock, 5 mM)
Total	**n/a**	**50 mL**

Note: Once prepared, stored at 4°C for 4 weeks.

Transfection medium	Final concentration	Volume
DMEM	n/a	44 mL
FBS	10%	5 mL
Sodium Pyruvate (100×, 100 mM)	1%	500 μL
Non-essential amino acids (100×)	1%	500 μL
Total	**n/a**	**50 mL**

Note: Once prepared, stored at 4°C for 1 week.

STEP-BY-STEP METHOD DETAILS

Isolation of mouse mammary epithelial cells

Timing: 3–4 h

1. Euthanize mice according to the protocol approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine.

2. Tissue digestion
   a. For Mammary fat pad digestion
      i. Harvest the #2, 3 & 4 mammary fat pads and wash them in a sterile 15 mL conical tube with 10 mL ice-cold PBS. Multiple mice can be combined for large preparation.
      ii. Transfer glands to a petri dish and mince them into ~1 mm^3 pieces using sterile blade.

iii. Transfer the minced glands to a new 15 mL tube by resuspending them with 5 mL of Gland Digestion Buffer I (see section 'materials and equipment') per mouse.
iv. Place the digestion mixture on a nutator in a 37°C incubator for 1.5 - 2 h.

b. For Tumor digestion
i. Harvest the tumor (~1 cm in diameter) and wash with 10 mL ice-cold PBS in a 15 mL tube.
ii. Mince the tumor into ~ 1 mm^3 pieces using a sterile blade.
iii. Transfer the minced tumor fragments to a new 15 mL tube by resuspending them with 5 mL Tumor Digestion Buffer I (see section 'materials and equipment').
iv. Place the digestion mixture on a nutator in a 37°C incubator for 2 h.

c. After digestion is complete, add 5 mL PBS to each 15 mL tube with digested tissue to reduce the viscosity of the digested tissue mixture.
d. Pipette the tissue mixture up and down for 20 times.
e. Centrifuge at 500 × *g* for 5 min at 4°C–8°C.
f. Carefully aspirate the supernatant (fatty top layer and liquid layer). Do not disturb the cell pellet.
g. Red blood cell (RBC) lysis.
i. For mammary epithelial cell isolation: this step is optional.
If yield is important, perform red blood cell lysis. For each mouse, add 1 mL RBC lysis, resuspend cell pellet, and place tube on ice for 1 min. After 1 min, pipet the cell mixture up and down gently for 5–6 times, add 10 mL PBS to the tube and spin down at 500 × *g* for 5 min.
If yield is not an issue (like in large preps), resuspend the pellet with 10 mL PBS by pipetting, pulse spin at 1,000 × *g* for 1 min. remove the supernatant containing red blood cells.
ii. For tumor organoid cell isolation: perform RBC lysis as above.
h. Resuspend cell pellets in 1 mL 0.05% trypsin-EDTA, place the tube at 37°C for 5 min, mixing occasionally by shaking.
i. After 5 min, pipet the cell mixture to dissociate clumps.
j. Add 10 mL basic medium II (see section 'materials and equipment') to stop the digestion and centrifuge at 700 × *g* for 5 min. Remove the supernatant.
k. Resuspend cell pellets with 1 mL Digestion Buffer II (see section 'materials and equipment') by pipetting and incubate for 5 min at 37°C with occasional mixing by shaking.
l. After 5 min, pipet the cell mixture to dissociate clumps.
m. To stop the digestion, add 10 mL PBS and centrifuge at 700 × *g* for 5 min. Remove the supernatant.
n. Resuspend with 3 mL Suspension Buffer (see section 'materials and equipment').

Note: EDTA helps quench dispase and prevent aggregation.

o. Filter through 40 μm cell strainers to get single cells (Figure 1).

3. Cell counting:
a. Mix 0.1 mL cell suspension with 0.1 mL 0.4% trypan blue. The volume of cell suspension can be reduced to 20 μl if needed.
b. Count the live cell number by Hemocytometer. Live cells will *not* take up trypan blue.

⚠ CRITICAL: All spins should be done in the refrigerated centrifuge at 4°C–8°C.

Establishing MaSC/tumor organoids from freshly isolated cells

Timing: 1 week

4. Before proceeding with the plating, wash cells with organoid culture medium.
5. Pellet down the cell suspension by centrifuging the tube at 700 × *g* for 5 min.
6. Resuspend the pellet

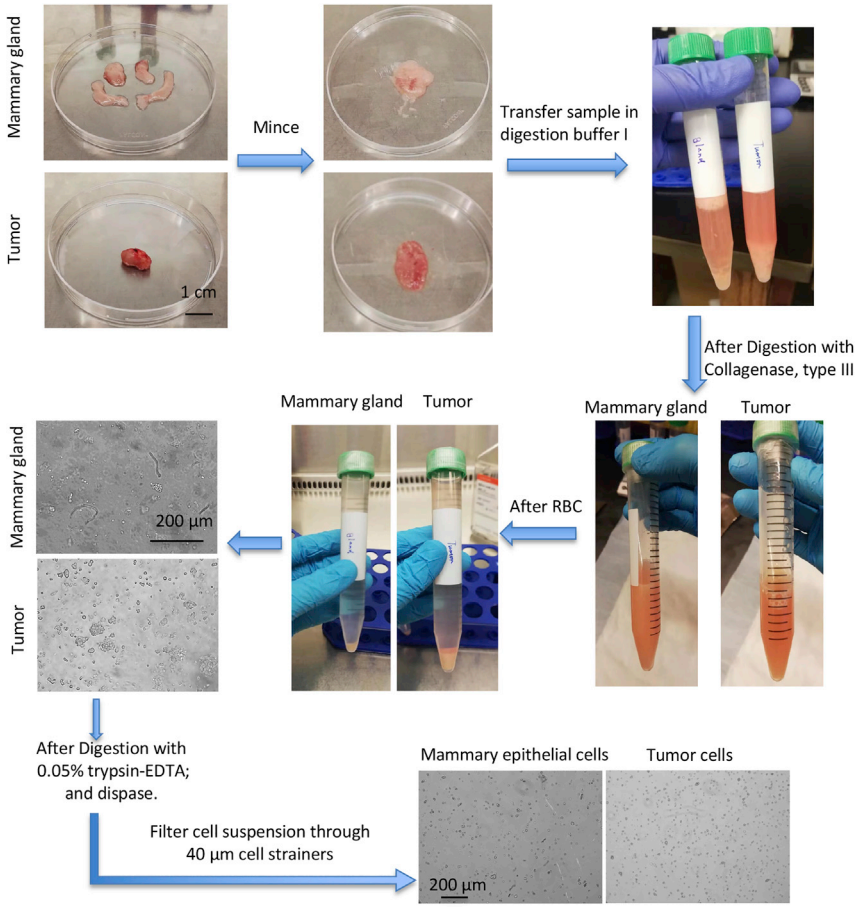

Figure 1. Isolation of mouse mammary epithelial cells from normal mammary glands or tumors

a. Freshly prepare the ice-cold organoid culture medium with 5% Matrigel (see section 'materials and equipment'; For tumor organoid and normal basal stem cell organoid culture, use MaSC/tumor organoid culture medium I; For luminal progenitor organoid culture, use MaSC organoid culture medium II)

b. Resuspend the cell pellet in ice-cold organoid culture medium (containing 5% Matrigel, see section 'Materials and Equipment') at a final concentration of 150,000 cells per 1 mL of organoid culture medium. For one well of poly-HEMA coated 6-well plates (see section 'before you begin'), seed 300,000 cells with 2 mL culture medium.

7. Put the plate in the humidified incubator at 37°C with 5% CO_2 for organoid culture.
8. Replace the medium every 3 days.
9. Check the organoid growth under a light microscope. After 4–6 days, organoids (> 100 μm in diameter) should form.

⚠ CRITICAL: All spins should be done in the refrigerated centrifuge at 4°C–8°C.

Passaging MaSC/tumor organoid culture

Timing: 1 week or experiment dependent

10. Organoids can be passaged once reaching 100–200 μm in diameter (~ 7-day culture from freshly isolated cells). Prepare all the solutions and equipment before the experiment. Thaw Matrigel on ice.

11. Collect organoids into 15 mL tube containing 10 mL ice-cold PBS by pipetting the Matrigel layer containing organoids.
12. Mix and centrifuge at 500 × *g* for 5 min.
13. Digest the organoids with 0.05% Trypsin-EDTA (1 mL per well) for 5–10 min at 37°C. Gently pipette up and down in between.
14. Add 10 mL Basic medium II (see section 'materials and equipment') to stop digestion.
15. Centrifuge at 500 × *g* for 5 min.
16. Remove supernatant without disturbing the pellet and resuspend the cell pellet in 3 mL Basic medium II (see section 'materials and equipment') and do cell counting.
17. Depending on the different aims of the experiment, seed the cells using organoid culture medium (see section 'materials and equipment') at different cell densities. For passage the cells, seed 300,000 cells with 2 mL organoid medium per well for 6-well plates. For quantifying organoid-forming efficiency, seed 200–500 cells per well in 96-well plates.

⚠ CRITICAL: All spins should be done in the refrigerated centrifuge at 4°C–8°C. Refresh the medium every 3–4 days.

Note: Organoid cultures from freshly isolated cells are usually ready for passage after 1 week. They can then be passaged every 4–6 days at a 1:4 ratio for expansion. The exact timing for passaging organoid cultures is determined by the growth rate of the organoids. We usually passage the culture when the majority of organoids are between 100–200 μm in diameter.

Note: Pipetting organoids after Trypsin treatment will result in a single-cell suspension. However, some cell clumps might still remain, which can be filtered out with a 40 μm cell strainer.

Making frozen stocks of MaSC/tumor organoids

Timing: 30 min

18. Prepare Freezing medium (see section 'materials and equipment') and cryovials before the experiment.
19. Dissociate the organoids into single cells based on steps 11–15.
20. Resuspend the cell pellet with 3 mL of Freezing medium (for the cell pellet from one well of 6-well plate).
21. Transfer 1 mL cell suspension into a cryogenic vial, place the vial into a slow-freezing container, and put it at −80°C freezer.
22. The following day, transfer the frozen cryovials to the liquid nitrogen tank for long-term storage.

⚠ CRITICAL: All spins should be done in the refrigerated centrifuge at 4°C–8°C.

Processing MaSC/tumor organoids for histology

Timing: 4–7 days

23. Prepare 2% agarose: (Figure 2)
    a. Mix 20 mg of low-melting agarose with 1 mL of water in a 2 mL tube, put the tube in a heat block set at 70°C to melt the agarose.
    b. Let the agarose solution to cool down to about 45°C–50°C.

Skip step 23 for OCT frozen section preparation.

24. Collet the organoid in a 15 mL tube, add 10 mL ice-cold PBS to wash the organoids.
25. Centrifuge at 500 × *g* for 5 min and discard the supernatant.

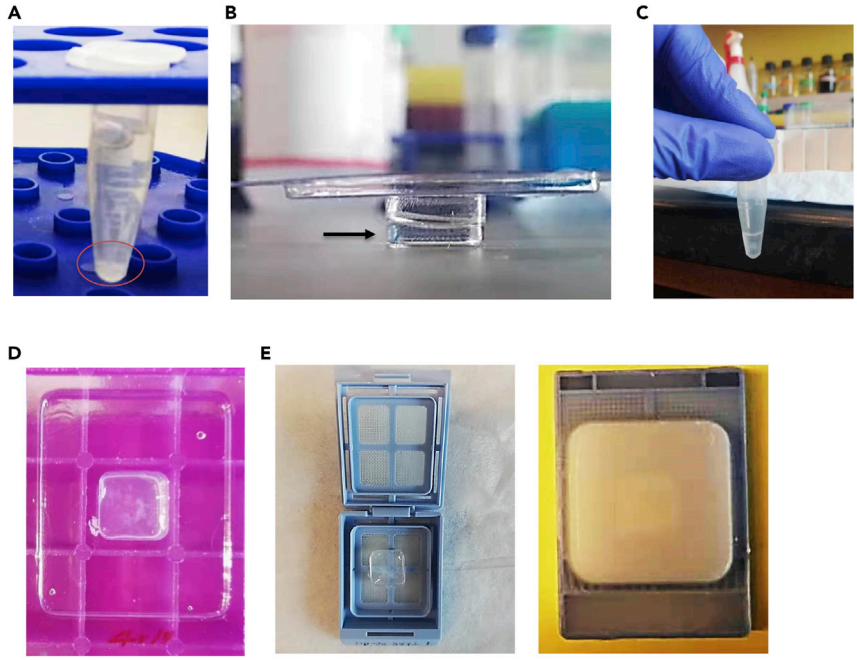

Figure 2. Paraffin block preparations of organoids

(A) Photo of organoids pellet preparation.

(B) Coating the cryomold with 200 μL 2% low-melting agarose.

(C) Resuspend organoids in 100 μL 2% low-melting agarose.

(D) Embedding the organoids in 2% low-melting agarose.

(E) Embedding the organoids/agarose block in the wax.

26. Add 1 mL of 4% PFA or 10% formalin to fix the organoids for 30 min on ice.
27. Add 10 mL PBS to the tube and centrifuge at 500 × *g* for 5 min, discard the supernatant.
28. Repeat step 27 twice.
29. Resuspend the organoids in 1 mL 0.1% BSA/PBS and transfer them to a 1.5 mL tube.
30. Centrifuge at 500 × *g* for 5 min and discard the supernatant.

31. For paraffin-embedding sample preparation
    a. Coat the bottom of a cryomold (10 × 10 × 5 mm) with a layer of 2% agarose by adding 100 μL per cryomold and spreading it with the pipette tip gently. Cool down the agarose at 22°C–25°C for 5 min.
    b. Gently mix the organoids from step 30 with 100 μL of 2% agarose solution.

⚠ CRITICAL: Cut off the end of a P200 pipette tip and use it to pipette the agarose. Bubbles need to be avoided during pipetting.

    c. Gently place the organoids/agarose mixture into the agarose-coated cryomold using a cut P200 tip. Make sure to put the mixture in the middle of the cryomold.
    d. Return the cryomold to the ice for 10–20 min until the gel is completely solidified.
    e. Gently and carefully remove the solidified agarose containing the embedded organoids from the mold and transfer it into a tissue cassette for further processing.
    f. Proceed with the paraffin embedding process following the standard FFPE embedding protocol.
    g. Cut the section at 4–6 μm thickness using a microtome.

32. For OCT-embedding sample preparation

a. After step 30, add 1 mL 30% sucrose solution to the organoid pellet and place at 4°C overnight (12–16 h) to allow organoids to sink into sucrose solution.
b. Remove the sucrose as much as possible.
c. Place a few drops of OCT onto the center of the bottom of cryomold.
d. Add ~300 μl (around 3 drops) of OCT to the organoid pellet. Gently mix and transfer the OCT/organoid mixture into the OCT drop in the cryomold using a cut 200 μL pipette tip.

⚠ CRITICAL: Bubbles need to be avoided. If air bubbles occur in the tissue mold, remove them with a pipette tip.

e. Carefully drop more OCT onto the specimen until it is completely covered. Avoid any bubbles inside the OCT.
f. Snap freeze the block in liquid nitrogen. Frozen blocks could be stored at −80°C.
g. Cut 10 μm thick sections using a cryostat.

33. Immunofluorescence staining of MaSC/tumor organoids
    a. Deparaffinization and rehydration for paraffin section: pass the tissue slides through the following steps (performed in fume hood):
        i. 100% Xylene I for 5 min
        ii. 100% Xylene II for 5 min
        iii. Xylene/ethanol (1:1, v/v) for 5 min
        iv. 100% ethanol for 5 min
        v. 95% ethanol for 5 min
        vi. 75% ethanol for 5 min
        vii. 50% ethanol for 5 min
        viii. Water for 5 min, twice.
    b. Antigen retrieval: incubate slides in antigen unmasking buffer (citrate acid based) and perform antigen retrieval using retriever. The tissues will be processed automatically. In about 2–3 h, the slides will be cool down to 25°C. Wash the slides with water for 5 min twice.
    c. Encircle the section with a hydrophobic barrier pen.
    d. Blocking: incubate the section in blocking buffer (3% BSA, 0.1% TritonX-100 (optional) in PBS) for 1 h at 22°C–25°C.
    e. Primary antibody incubation: discard blocking buffer, incubate the section in diluted primary antibody in 3% BSA of PBS overnight (12–16 h) at 4°C in the dark.
    f. Wash with 0.1% Tween-20/PBS for 5 min, 3 times with shaking.
    g. Secondary antibody incubation: Incubate the sections in fluorescent-dye conjugated secondary antibody diluted in 3% BSA of PBS for 1 h at 22°C–25°C in the dark.
    h. Wash the slides with 0.1% Tween-20/PBS for 5 min, 3 times with shaking
    i. Mount coverslip with the mounting medium (with DAPI).
    j. Capture images under confocal microscopy.

⚠ CRITICAL: Antigen retrieval buffer should be optimal based on different antibodies.

Genetic engineering in organoids

Timing: around 3 weeks

34. Production of Lentivirus:
    a. Day 1: HEK293T cells preparation.
        i. Coat 10 cm plates with 20 μg/mL collagen I in PBS for 1 h at 22°C–25°C.
        ii. Split HEK293T cells 1:4 from 90% confluent culture to the collagen I-coated plates using Transfection medium (see section 'materials and equipment').
    b. Day 2: Transfection.

i. Preparation transfection mixture in a 1.5 mL Eppendorf tube.
Mix DNA in the following ratio:

	VSVG (pMD2.G)	Lenti-pkg (pCMVR8.74)	Lentiviral vector
For each 10-cm plate	1.2 μg	1.8 μg	3 μg

For a 10 cm plate, dilute DNA to 600 μl jetPrime buffer, mix well
Add 12 μl jetPrime reagent, vortex for 10 s, spin briefly.
Incubate the transfection mixture for 10 min at 22°C–25°C.

ii. Add transfection mixture to cells.

iii. Re-fresh transfection medium (see section 'materials and equipment') after 4–5 h.

c. Day 3: Re-fresh transfection medium (10 mL per 10 cm plate).

⚠ CRITICAL: Add new medium dropwise onto cells to avoid dislodging the cells.

d. Day 4: Collect viral supernatant in a 50 mL tube and add fresh transfection medium.

e. Day5: Repeat step d.

f. Day 6: Collect viral supernatant again. Filter through a 0.45 μm filter. Aliquot and freeze at −80°C for long-term storage if the virus is not used within 1 week.

⚠ CRITICAL: Virus can be concentrated with Lenti-X Concentrator (Takara, Cat # 631232) or other methods to achieve higher titer.

35. Lentiviral infection of organoids

a. Day 1: Prepare single cells as section 'Passaging MaSC/tumor organoid culture'. For viral transduction in 6-well, resuspend 300,000 cells with 1 mL virus-containing medium (organoid culture medium containing 5% Matrigel, see section 'materials and equipment'.) with 5 μg/mL polybrene.

⚠ CRITICAL: The virus dilution ratio should be adjusted based on the viral titer.

b. Day 2: Refresh organoid culture medium after overnight (12–16 h) infection.

c. Day 3: Refresh organoid culture medium and supplement with a selection antibiotic (e.g., 2 μg/mL puromycin). Transduced cells can be FACS sorted if using fluorescent marker-containing vectors.

⚠ CRITICAL: The dosage of puromycin may need to be optimized for different cells.

EXPECTED OUTCOMES

This protocol provides an efficient method for establishing long-term organoid cultures from primary mouse mammary epithelial cells and mammary tumor cells. Organoids can be maintained and expanded rapidly in culture (Figure 3). Our previous data indicated that these cells multiplied with an expansion rate of ~100 fold per week even after 4 months in culture. Details of the proliferation rate can be found in our publication (Zhang et al., 2016). But it is possible that the organoid cultures may vary between different tumor samples or mouse strains. To characterize the organoids, histological analysis by immunofluorescence or immunohistochemistry can be performed. For assessing the MaSC phenotype, the expression of transcription factors Slug and Sox9 was examined and found to be expressed in a fraction of organoid cells by immunofluorescence (Guo et al., 2012; Zhang et al., 2016). Organoid cells can be further modified by genetic editing, which enables investigating the function of the defined gene mutations.

LIMITATIONS

Using this protocol, we can enrich the MaSCs and tumor stem cells efficiently, but the purity of stem cells is still not 100%. In our previous publication, we observed some degree of differentiation by co-

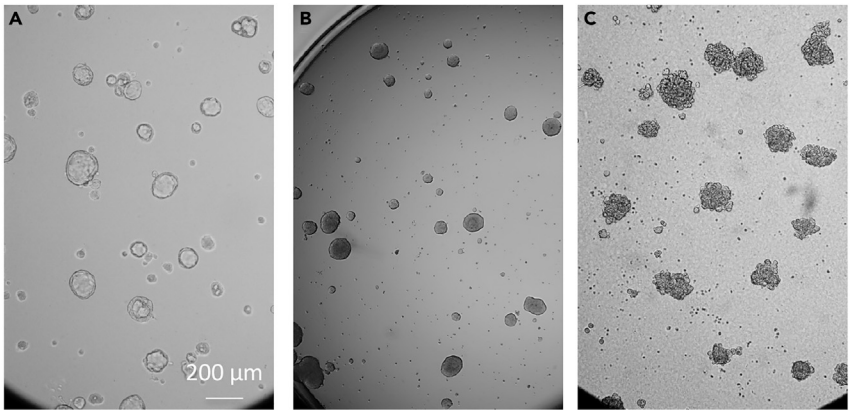

Figure 3. Culture of MaSC/tumor organoids

(A) Representative image of normal acinar structures formed by luminal progenitor cells cultured in Epicult-B medium supplement with growth factors for 6 days (see section materials and equipment, use MaSC organoid culture medium II recipe).

(B) Representative image of normal organoid structures cultured in Advanced DMEM/F12 medium supplement with growth factors for 6 days (see section materials and equipment, use MaSC organoid culture medium I recipe).

(C) Representative image of tumor organoid structures cultured in Advanced DMEM/F12 medium supplement with growth factors for 6 days (see section materials and equipment, use MaSC organoid culture medium I recipe).

staining KRT14 (basal cell marker) and KRT8 (luminal cell marker) in cultured organoids. Hence, only a subset of organoid cells is MaSCs.

For tumor organoids, it may fail to fully recapitulate the *in vivo* tumor heterogeneity. The clonal selection may occur during long-term culture. The effect of such selection could not be fully predicted in advance.

TROUBLESHOOTING

Problem 1

Low cell yield after digestion

Potential solution

1. Over mincing of the tissue will lead to excessive cell death. Decrease the mince time properly. Generally, 40–50 minces are sufficient.
2. Insufficient digestion: if large tissue clusters remain after digestion, increase the incubation time for a period. Pipette up and down to promote the tissue disruption process.

Problem 2

Organoid growth rate is slow. It is possible that cell density is too high and the cell medium may not be sufficient to support the growth of the organoids (Figure 4).

Potential solution

Re-fresh the medium every 3–4 days.

Split the organoids every 4–6 days.

Problem 3

Organoids in culture medium distribute unevenly (Figure 5).

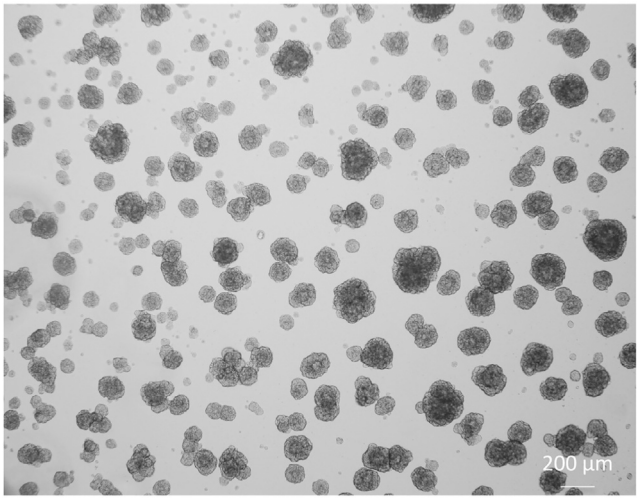

Figure 4. Representative image of MaSc organoids ready for passaging

Potential solution

Mix the single cell in the organoid medium up and down several times.

After seeding the cells in the plate, leave it in the hood for 15 min to let the cells settle down to the bottom layer.

Problem 4

Tumor organoids fail to recapitulate the properties of *in vivo* tumors.

Potential solution

Although this is unavoidable due to the clonal selection under culture, we could reduce the risk by doing quality control before applying the established organoids as a model for the tumor from which it is derived. Analyze the expression levels of cell markers (like keratin 14, keratin 8, CD49f, Epcam, CD61, Sox9, et al.) by immunofluorescent/immunohistology staining, qRT-PCR, or flow cytometry.

For big tumors, take tumor fragments from different locations for digestion.

Problem 5

The lentiviral infection efficiency in organoids is too low.

Potential solution

Increase the amount of lentivirus.

Concentrate the lentiviral supernatants by a commercial kit (eg. Lenti-X™ Concentrator from Takara).

Polybrene can greatly enhance the efficiency of the retroviral or lentiviral infection to mammalian cells. Usually, the range of polybrene concentration is around 4–8 µg/mL depending on the cell lines. But the dosage of polybrene may need to be optimized for different cells.

Change to other transduction enhancers such as protamine sulfate or DEAE-dextran.

Avoid freeze and thaw of virus stock.

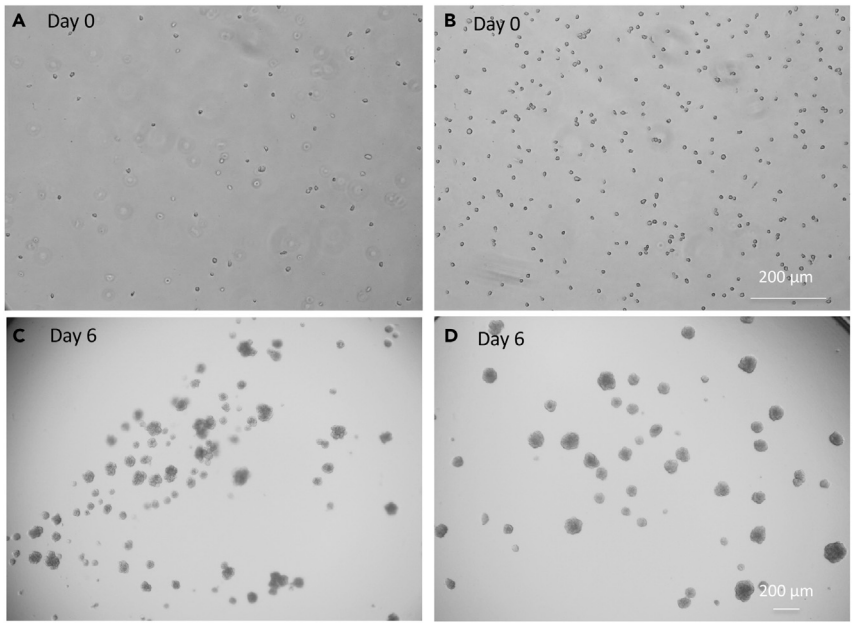

Figure 5. .Improving even distribution of organoids in culture

(A) Representative bright-field images of single cell suspension right after seeding (Day 0).

(B) Representative bright-field images of single cell suspension after seeding and sitting in the hood after 15 min (Day 0).

(C) Representative bright-field images of organoids after 6 days culture from (A).

(D) Representative bright-field images of organoids after 6 days culture from (B).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wenjun Guo (wenjun.guo@einsteinmed.org).

Materials availability

This study did not generate new unique reagents or mouse strain.

Data and code availability

This study did not generate any new data or codes that are not presented in the published article.

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AUTHOR CONTRIBUTIONS

J.C. and W.G. conceived the study and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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