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A Robust Mammary Organoid System to Model Lactation and Involution-like Processes

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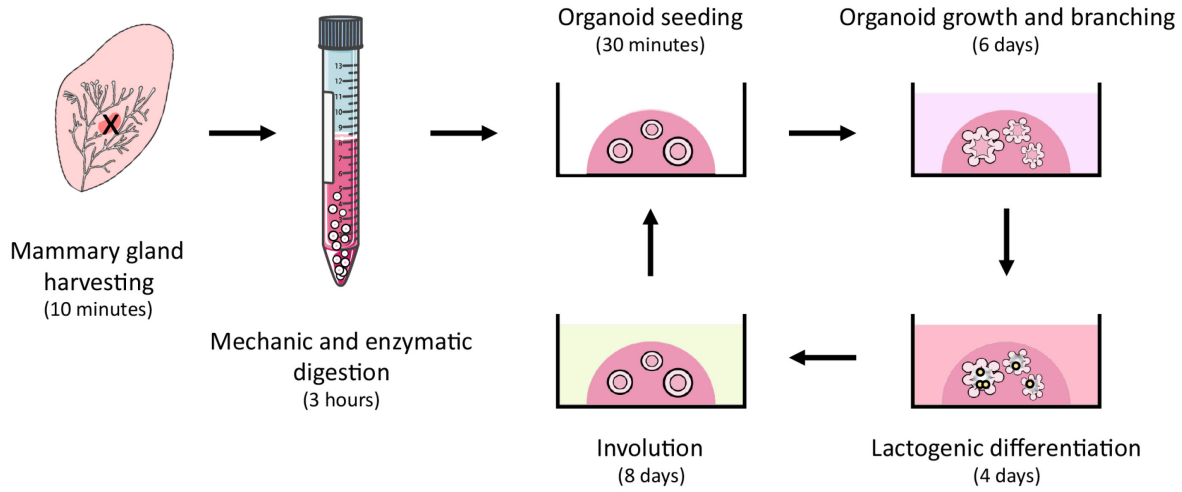
[Abstract] The mammary gland is a highly dynamic tissue that changes throughout reproductive life, including growth during puberty and repetitive cycles of pregnancy and involution. Mammary gland tumors represent the most common cancer diagnosed in women worldwide. Studying the regulatory mechanisms of mammary gland development is essential for understanding how dysregulation can lead to breast cancer initiation and progression. Three-dimensional (3D) mammary organoids offer many exciting possibilities for the study of tissue development and breast cancer. In the present protocol derived from Sumbal et al., we describe a straightforward 3D organoid system for the study of lactation and involution *ex vivo*. We use primary and passaged mouse mammary organoids stimulated with fibroblast growth factor 2 (FGF2) and prolactin to model ~~the~~ three cycles of mouse mammary gland lactation and involution processes. This 3D organoid model represents a valuable tool to study late postnatal mammary gland development and breast cancer, in particular postpartum-associated breast cancer.

Graphic abstract:



Mammary gland organoid isolation and culture procedures

Keywords: Mouse, Mammary gland, 3D organoid, *Ex vivo*, Lactation, Involution

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[Background] The primary function of the mammary gland is to provide nutrition to newborns via milk production. The development of the mammary gland is a highly dynamic process that occurs mainly after birth and is regulated by several factors including hormones and growth factors (Brisken and Rajaram, 2006; Sternlicht, 2006). During puberty, hormones and growth factors regulate ductal morphogenesis from a rudimentary embryonic ductal tree (Brisken and O'Malley, 2010). During each pregnancy, the mammary gland begins a new morphogenetic step initiated by hormonal stimulation, which is characterized by massive proliferation for epithelial expansion and alveolar development accompanied by adipocyte regression (Brisken and O'Malley, 2010). Importantly, prolactin signaling plays a crucial role in the terminal differentiation of luminal cells to enable milk production (Ormandy *et al.*, 1997). At the end of lactation after weaning of the progeny, the mammary gland enters the involution stage characterized by programmed cell death, tissue remodeling, and redifferentiation of adipocytes (Hughes and Watson, 2012; Macias and Hinck, 2012; Zwick *et al.*, 2018; Jena *et al.*, 2019).

Histologically, the mammary gland is composed of a bilayered epithelium consisting of an inner layer of luminal cells (keratin 8+) and an outer layer of contractile basal cells (keratin 5+). Luminal cells are responsible for milk production during lactation, while basal cells aid milk ejection. The epithelium is surrounded by a stromal fat pad that comprises fibroblasts, nerves, vasculature, lymphatics, immune cells, adipocytes, and extracellular matrix (ECM) (Richert *et al.*, 2000).

Over the past decade, organoids of various tissues, such as stomach, colon, lung, and pancreas, have been developed (Huch and Koo, 2015), offering many exciting possibilities for the study of tissue development and disease. The organoid system is a powerful tool that combines the advantages of a 2D culture (easy manipulation, precise control of cell composition and microenvironment, live imaging) with the opportunity to study complex cell–cell and cell–ECM interactions in a more controlled *ex vivo* manner (Huch and Koo, 2015; Shamir and Ewald, 2015; Koledova, 2017; Artegiani and Clevers, 2018).

Several models have been developed to study the mechanisms of mammary branching morphogenesis in primary mammary epithelium using different protocols (Ewald *et al.*, 2008; Huebner *et al.*, 2016; Neumann *et al.*, 2018), cell lines (Xian *et al.*, 2005), sorted cells *(*Jamieson *et al.,* 2017*;* Linnemann *et al.*, *2015*), or induced pluripotent stem cells (Qu *et al.*, 2017). However, an organoid system modeling key aspects of the late postnatal developmental stages of the mammary gland has remained challenging to establish.

Previously, there have been several attempts to model lactation in 3D culture: spheroids of a breast adenoma cell line were used to study copper secretion into milk (Freestone *et al.*, 2014); organoids of primary epithelium were shown to produce milk following the administration of a lactogenic stimulus (Mroue *et al.*, 2015; Jamieson *et al.*, 2017); and co-culture of breast epithelium and pre-adipocyte cell lines was shown to initiate an involution-like process (Campbell *et al.*, 2014). However, in-depth characterization of milk production and involution or the proper bilayered architecture of mammary epithelium remained to be carried out.

Recently, we developed a model of lactation and involution of mammary epithelium based on organoids of primary mammary gland tissue cultured in 3D Matrigel® (Sumbal *et al.*, 2020b). Under lactogenic stimuli, primary organoids maintain long-term milk production, retain the contractile

myoepithelial layer, and enter involution following hormone withdrawal. Moreover, after involution, the organoids remain hormonally sensitive and are able to enter another round of lactation (Sumbal *et al.*, 2020b). Here, we present a methodological guideline to establish the primary mammary organoid-based *ex vivo* model of lactation and involution, with detailed procedures for obtaining tissue, isolating organoids, establishing and maintaining 3D culture, and preparing organoid samples for subsequent RNA or protein expression analysis or histological examination. This model can be used for studies on lactation biology, mammary stem cell plasticity, regulatory mechanisms of mammary epithelial cell differentiation and death, or other interesting biological phenomena. We believe that this model will initiate the further development of organoid technology, including creative applications in biotechnology and regenerative medicine (Sumbal *et al.*, 2020a).

Materials and Reagents

1. 100-mm tissue culture Petri dish (*e.g.*, Corning, catalog number: 353003)
2. 0.2-µm filters and 50 ml syringes (*e.g.*, GVS, catalog number: FJ25ASCCA002DL01)
3. No. 22 disposable scalpel blades (*e.g.*, Swann-Morton, catalog number: 0508)
4. 50-ml tubes (*e.g.*, Corning, catalog number: 352070)
5. 15-ml tubes (*e.g.*, Corning, catalog number: 352096)
6. 10-ml disposable plastic pipettes (*e.g.*, Corning, catalog number: 357551)
7. 25-ml disposable plastic pipettes (*e.g.*, Corning, catalog number: 357535)
8. 24-well tissue culture plates (*e.g.*, Corning, catalog number: 353047)
9. 30 G insulin syringes (*e.g.*, BD Microfine, catalog number: 324826)
10. Plastic histology molds (*e.g.*, Thermo Scientific, catalog number: 1830)
11. Plastic embedding cassettes (*e.g.*, Simport, catalog number: M492-2)
12. Histology tissue molds (*e.g.*, Simport, catalog number: M474-3)
13. Microscope slides for histology (*e.g.*, Thermo Scientific, catalog number: J1800AMNZ)
14. Mice: virgin females, 7–10 weeks old, inbred strain C57BL/6J (*e.g.*, The Jackson Laboratory, catalog number: 000664)
15. Ethanol (EtOH), 70%, 95%, and 100% (*e.g.*, VWR, catalog number: 83813)
16. Phosphate-buffered saline (PBS) (*e.g.*, Sigma-Aldrich, catalog number: D1408)
17. Dulbecco's modified Eagle medium (DMEM)/F12 (*e.g.,* Gibco, catalog number: 21331-020)
18. Bovine serum albumin (BSA) (*e.g.*, Sigma-Aldrich, catalog number: A3608)
19. Fetal bovine serum (FBS) (*e.g.*, Sigma-Aldrich, catalog number: F0804)
20. Collagenase A (*e.g.*, Roche, catalog number:11088793001)
21. Trypsin (*e.g.*, Dutcher Dominique, catalog number: P10-022100)
22. Insulin (*e.g.*, Sigma-Aldrich, catalog number: I6634-100MG)
23. Gentamicin (*e.g.*, Sigma-Aldrich, catalog number: G1397)
24. Glutamine (*e.g.*, Gibco, catalog number: 35050-061)
25. DNase I (*e.g.*, Sigma-Aldrich, catalog number: D4527-40KU)

26. Dispase II (*e.g.*, Roche, catalog number: 13 75 2000)
27. Growth factor-reduced Matrigel® (*e.g.*, Corning, catalog number: 354230)
28. Insulin-transferrin-selenium (ITS) (*e.g.*, Gibco, catalog number: 41400-045)
29. Penicillin/Streptomycin (*e.g.*, Gibco, catalog number: 15140-122)
30. FGF2 (*e.g.*, Gibco, catalog number: PM60034)
31. Prolactin (*e.g.*, Sigma-Aldrich, catalog number: SRP4688)
32. Hydrocortisone (*e.g.*, Sigma-Aldrich, catalog number: S H6909)
33. Oxytocin (*e.g.*, Sigma-Aldrich, catalog number: O3251)
34. RNeasy Micro Kit (*e.g.*, Qiagen, catalog number: 74004)
35. β-Mercaptoethanol (*e.g.*, Sigma-Aldrich, catalog number: M6250)
36. Phosphatase inhibitor cocktail II (*e.g.*, Millipore, catalog number: 524625)
37. RIPA buffer (*e.g.*, Sigma-Aldrich, catalog number: R0278)
38. Protease inhibitor cocktail I (*e.g.*, Sigma-Aldrich, catalog number: 539131)
39. Pierce Coomassie (Bradford) Protein Assay Kit (*e.g.*, Thermo Scientific, catalog number: 23200)
40. Paraformaldehyde (PFA), 32% (*e.g.*, Electron Microscopy Sciences, catalog number: 15714)
41. Low gelling temperature agarose (*e.g.*, Sigma-Aldrich, catalog number: A9414)
42. Xylene (*e.g.*, Sigma-Aldrich, catalog number: 534056)
43. Paraffin (*e.g.*, Sigma-Aldrich, catalog number: 1071642504)
44. Dissociation solution (see Recipes)
45. BSA solution (see Recipes)
46. Basal organoid medium (BOM) (see Recipes)
47. Morphogenesis medium (see Recipes)
48. Lactation medium (see Recipes)
49. 4% PFA (see Recipes)
50. RNA lysis buffer (see Recipes)

Equipment

1. Surgical tools
   Forceps (*e.g.*, Phymep, catalog numbers: 11050-10 and 11051-10)
   Scissors (*e.g.*, Phymep, catalog number: 14088-10)
2. Dissection board (*e.g.*, Thermo Scientific, catalog number: 36-119)
3. P1000 pipette
4. Laminar flow hood
5. Fridge 4°C (*e.g.*, Liebherr, catalog number: 7083 001-01)
6. Freezer –80 °C *(e.g.*, Thermo Scientific, catalog number: 88400V)
7. Liquid nitrogen tank *(e.g.*, Air Liquide Espace 151, catalog number: 2433867)
8. Shaking incubator at 37°C (*e.g.*, Infors HT Multitron)
9. Centrifuge (*e.g.*, Thermo Scientific, model: Sorvall ST40)

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10. Incubator for cell culture, 37°C, 5% CO_2 (*e.g.*, Thermo Scientific, model: HERAcell 150i)
11. Heating plate at 37°C (*e.g.*, Techne DRI-Block DB-2A)
12. Microscope and camera *(e.g.*, Olympus model: CKX41)
13. NanoDrop™ (*e.g.*, Implen Nanophotomoter NP80)
14. Sonicator (*e.g.*, Diagenode Bioruptor Pico)
15. Incubator at 65°C (*e.g.*, Memmert Incubator I)
16. Embedding workstation (*e.g.*, Leica EG1150C)

Procedure

A. Isolation of mammary primary organoids

1. Dissection of a virgin mouse to harvest mammary glands (see Video 1).

Video 1. Mammary gland harvesting. This video was made at Pasteur Institute. according to guidelines from the regulations of Institut Pasteur Animal Care Committees (CETEA). on Animal Care and approved by the French legislation in compliance with European Communities Council Directives (A 75-15-01-3).

a. Euthanize the donor mouse using an ethically approved method (*e.g.*, cervical dislocation) and immediately proceed to mammary gland collection.
Notes:
   i. *Cervical dislocation is a common method for animal euthanasia and provides a fast and painless death. With this method, cell/tissue survival in culture is not altered if collected immediately.*
   ii. *In the case of processing multiple mice, euthanize one animal and collect the glands immediately, then proceed to the next animal.*

b. Sanitize the ventral side of the animal by spraying 70% EtOH on the skin.
Note: After disinfection, work inside a laminar flow hood to maintain aseptic conditions. Application of aseptic work procedures, together with the presence of antimycotic and

antibiotic supplements (gentamicin in digestion solution; penicillin and streptomycin in culture medium) will prevent the occurrence of contamination.

c. Pin the mouse by its four paws to a dissection board, with the abdomen facing upward (see Figure 1A, pins 1–4).
d. Using forceps, tightly grasp the skin of the lower part of the abdomen at half the width (see Figure 1A, point A).
e. Using surgical scissors, make the first incision in the skin at point A.
Note: Be careful to incise only the skin and not rupture the underlying peritoneum.
f. Continue to incise the skin cranially to the throat of the animal (see Figure 1A, from point A to point B).
g. From this median line, use forceps to grasp the skin and cut toward each of the four paws (see Figure 1A, incise to join the middle line to points C, D, E or F, respectively).
h. Using forceps and a cotton swab, gently separate the skin from the peritoneum on one side of the animal. Attach the skin to the dissection board with three pins (see Figure 1B, pins 5–7).
i. Repeat step 8 on the other side of the animal (see Figure 1B, pins 8–10). The mammary glands are now exposed.
j. Identify the lymph node of the mammary gland #4 (a small dense structure, round in shape; see Figure 1B, surrounded). Remove the lymph node from both glands using forceps and scissors and discard.
k. Proceed to the harvest of the mammary glands #3 and #4. Using curved forceps, grasp the mammary glands and gently separate them from the skin and other tissues with scissors.
Note: Carefully separate the mammary glands #3 (whitish and shiny) from the muscles (light brown ribbed structure) since this protocol does not prevent muscle contamination.
l. Place all the collected glands in the same sterile Petri dish containing cold PBS (approximately 3 ml, previously stored at 4°C) for washing prior to tissue processing.
m. Properly dispose of the animal corpse and continue with mechanical and enzymatic dissociation of the mammary glands.

2. Mechanical and enzymatic dissociation
Reminder: Work inside a laminar flow hood to maintain aseptic conditions.
a. Freshly prepare 10 ml dissociation solution for the four glands collected from one mouse, pass through a 0.2-µm filter, and pre-heat at 37°C.
Note: Do not exceed the maximum 30 ml dissociation solution in a 50-ml tube to ensure correct dissociation.
b. Transfer the freshly collected mammary glands to a new sterile Petri dish.
c. Use three scalpels to finely chop the mammary glands and obtain a homogeneous mince of 1-mm^3 mammary fragments (see Figure 1C).
d. Transfer the mince to a 50-ml tube containing the pre-warmed dissociation solution.
e. Place the tube in a shaking incubator for 30 min at 37°C, 100 rpm.

Notes:

i. All the following steps are performed at room temperature except incubation with dispase.

ii. From here on, pre-coat all the pipettes, tips, and tubes with 2.5% BSA solution. Prepare the BSA solution in a 50-ml tube and aspirate/remove from every consumable following coating; this will prevent stickiness and loss of organoids. The BSA solution can then be filtered, stored at 4°C, and re-used.

f. After incubation, resuspend the dissociated mammary glands by performing ten up-and-down motions with a 10-ml pipette. Centrifuge for 10 min at 400 × *g*.

g. After centrifugation, handle the 50-ml tube carefully to prevent disturbance of the three separated layers (see Figure 1C). Keep the epithelial pellet intact and transfer the middle aqueous phase and the top fatty layer into a clean 15-ml tube.

h. Resuspend the epithelial pellet in 5 ml DMEM/F12 and set it aside.

i. Focus on the fatty and aqueous solutions in the 15-ml tube: resuspend by performing ten up-and-down motions with a 10-ml pipette. Centrifuge for 10 min at 400 × *g*.
Note: This step allows recovery of epithelial fragments trapped in the fatty layer.

j. Again, handle the 15-ml tube carefully to avoid disturbing the three separated layers. Discard the fatty and aqueous layers.

k. Take the 5 ml resuspended pellet from the 50-ml tube to resuspend the pellet in the 15-ml tube.

l. Wash the 50-ml tube with 5 ml DMEM/F12, pool with the suspension in the 15-ml tube, and mix.

m. Centrifuge for 10 min at 400 × *g*.

n. Discard the supernatant. Use 4 ml DMEM/F12 to resuspend the pellet. Subsequently, add 80 µl DNAse I at 100 µg/ml and agitate for 5 min by hand or on an orbital shaker at 100 rpm.

o. Add 6 ml DMEM/F12 and resuspend the solution by performing 5 up-and-down motions with a 10-ml pipette.

p. Centrifuge for 10 min at 400 × *g*.

q. Discard the supernatant. Use 4 ml DMEM/F12 to resuspend the pellet. Subsequently, add 150 µl dispase II at 0.5 mg/ml and incubate for 5 min at 37°C.

r. Add 6 ml DMEM/F12 and resuspend the solution by performing 5 up-and-down motions with a 10-ml pipette.

s. Centrifuge for 10 min at 400 × *g*.

t. Discard the supernatant. Resuspend the pellet in 9 ml DMEM/F12.

u. Perform differential centrifugation to separate the mammary epithelium from the stromal fraction: centrifuge the suspension for 15 s at room temperature, 400 × *g*. Discard the supernatant containing the stromal fraction and resuspend the epithelial pellet in 9 ml DMEM/F12.

Note: Set the time on the centrifuge to 1 min. Once a speed of 400 × g is reached, time 15 s precisely and stop the centrifuge manually.

v. Repeat the previous step (t) 4 times, for a total of 5 differential centrifugations, to efficiently remove stromal contamination.

w. Resuspend the final pellet in 1 ml basal organoid medium (BOM) and place on ice. The organoids are now ready to be counted and cultured.
Note: Adjust the volume of resuspension according to pellet size. From a pool of 2–3 mice, the final pellet was resuspended in 1 ml basal organoid medium, for an expected range of 3,000–6,000 organoids. Adjust the volume of BOM for resuspension of the pellet according to the number of mice pooled.

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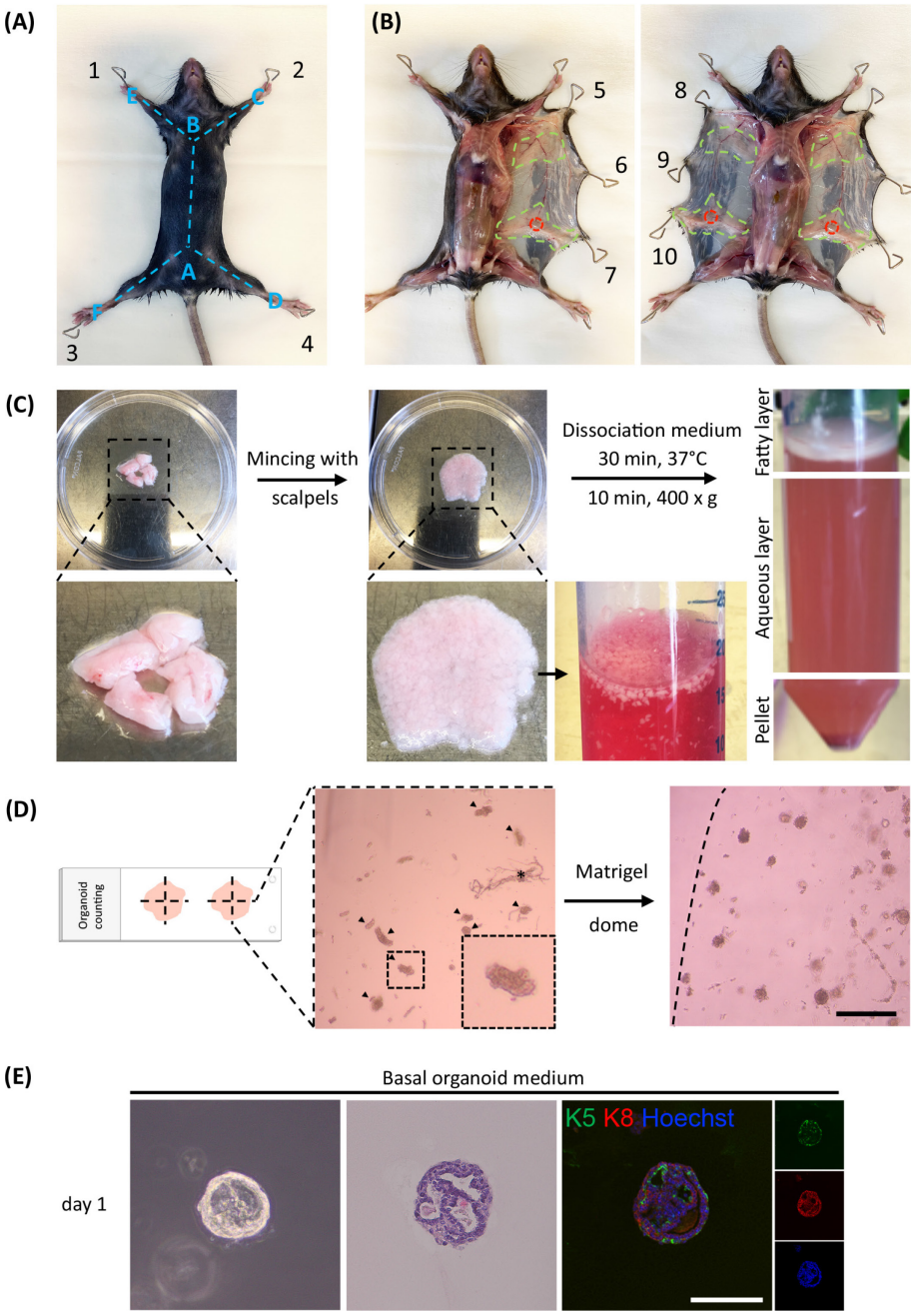

Figure 1. Key steps of mammary gland collection for organoid isolation and 3D culture. A,B. Images of mouse dissection to access the mammary gland. A. Needles 1–4 represent the points at which to pin the mouse. Needles 5–7 and 8–10 represent the points at which to pin the skin of the mouse. Letters A–F with the blue dotted lines indicate the cuts. B. Green dotted lines denote the mammary gland. The lymph node is denoted in red and must be removed. C. Mammary gland before (left panel) and after (middle panel) mincing with a scalpel. Mammary organoids after transfer to dissociation medium (right panel). D. Example of mammary organoid counting. Left panel: organoids are surrounded by dotted lines. Star represents nerves. Right panel: organoids after embedding in Matrigel®. Arrow represents the edge of the Matrigel® dome. Scale bar = 500 µm. E. Freshly isolated primary organoid. Left panel: image of a mammary

organoid on day 1 post-isolation. Middle panel: Hematoxylin & eosin staining of an organoid on day 1 post-isolation. Right panel: immunofluorescence staining showing the distribution of myoepithelial (keratin 5+, green) and luminal cells (keratin 8+, red) in organoids on day 1 post-isolation. Hoechst, blue (nuclei). Scale bar = 100 µm.

3. Organoid counting

   Reminder: Work inside a laminar flow hood to maintain aseptic conditions.

   a. Draw two large crosses with a marker on a microscope slide.

   b. Take the organoid suspension and homogenize by performing five up-and-down motions with a P1000 pipette.

   c. On the reverse side of the slide, spread 10 µl solution around the center of each cross.

      Note: Use a 20-µl tip or cut the extremity of a 10-µl tip to avoid large organoids becoming trapped.

   d. Count the organoids under the microscope at 4× magnification (see Figure 1D).

      Notes:

      i. Take each quarter of the cross as a landmark to avoid double-counting of the same organoid.

      ii. Organoids appear as rounded structures with a smooth perimeter. Occasionally and unavoidably, nerves and endothelium are also present. The nerves appear as rope-like structures and can be organized in bundles (see Figure 1D). The endothelium has a somewhat ragged look in comparison with the smooth-looking organoids. The minor presence of primary nerves and endothelium does not interfere with organoid lactation or involution.

      iii. Count only the organoids with a diameter greater than 30–50 µm since the smaller ones may not develop properly.

   e. Calculate the average of the two counts in 10 µl solution and multiply according to the volume of BOM used to resuspend the pellet to obtain the total number of organoids.

      Note: Freshly isolated organoids can be viably frozen in a solution of FBS containing 10% DMSO for long-term storage in liquid nitrogen and later use.

B. 3D culture of mammary organoids

1. Embedding in Matrigel®

   Reminder: Work inside a laminar flow hood to maintain aseptic conditions. Wash the ice bucket and heating plate thoroughly with 70% EtOH prior to placement in the laminar flow hood.

   a. Thaw the Matrigel® on ice or at 4°C.

      Notes:

      i. Matrigel® solidifies really fast at room temperature. Always keep it on ice before use and during the plating procedure.

ii. Keep in mind that Matrigel® thawing takes time; therefore, begin thawing prior to the procedure (2 h for a 1-ml aliquot, 6 h for a 10-ml bottle).

b. Place a 24-well plate on ice. Calculate the number of wells needed and spread 20-µl Matrigel® in a round patch on the bottom of each well.
Note: Start by placing the tip containing Matrigel® at the center of a well and expand circularly towards the edges of the well, without touching them.

c. Incubate the 24-well plate in a cell incubator (5% CO_2) for 15 min at 37°C.

d. In the meantime, pre-heat a heating plate to 37°C.

e. Prepare the organoid suspension in the Matrigel®: calculate the volume of organoid suspension required to obtain the desired number of organoids. Pipette this volume of suspension into a fresh 1.5-ml tube and centrifuge for 3 min at 400 × *g*.
Note: Adjust the number of organoids per well depending on the type of experiment: 200 organoids per well for morphology and histology, 400 for gene expression, and 1000 for western blotting analysis.

f. Carefully remove the supernatant and place the tube on ice. Subsequently, carefully resuspend the pellet in the required volume of cold Matrigel® (50 µl per well), avoiding bubble formation. Keep on ice.

g. Remove the 24-well plate from the cell incubator and place on the 37°C heating plate.

h. In each Matrigel®-precoated well, cautiously seed the suspension of organoids (in Matrigel®) as a dome on top of the solidified Matrigel® patch.

i. Place the 24-well plate back in the cell incubator (5% CO_2) for 30 min at 37°C to solidify the Matrigel® (see Figure 1D).

j. In the meantime, pre-warm BOM at 37°C.

k. Following incubation, carefully add 1 ml pre-heated BOM to each well and culture in the cell incubator at 37°C, 5% CO_2.
Notes:
i. Add medium against the edges of the well to avoid disruption of the dome.
ii. Characterization of the organoids can be performed using regular histological stains (e.g., hematoxylin & eosin) or immunostaining on day 1 post-recovery in BOM (see Figure 1E and Step B2 of the procedure).

2. Morphogenesis with FGF2

Reminder: Work inside a laminar flow hood to maintain aseptic conditions.
Note: Overnight recovery is optimal for organoid culture; however, FGF2 treatment can be administered immediately after plating the organoids.

a. Pre-heat the BOM at 37°C.

b. Add fresh FGF2 at a final concentration of 2.5 nM to pre-heated BOM to obtain the morphogenesis medium.

c. Aspirate the medium from the wells without touching the Matrigel® dome and replace with 800 µl fresh morphogenesis medium.

d. Renew all medium with fresh morphogenesis medium every 3 days, for a total of 6 days of treatment.

3. Lactogenic differentiation with prolactin
   a. Pre-heat the BOM at 37°C.
   b. Add 1 µg/ml prolactin and 1 µg/ml hydrocortisone to the pre-heated BOM to obtain the lactation medium.
   c. Aspirate the medium from the wells without touching the Matrigel® dome and replace with 800 µl fresh lactation medium.
   d. Renew all medium with fresh lactation medium every two days, for a total of 4 days of treatment.
4. Myoepithelial cell contraction with oxytocin
   a. Prepare fresh lactation medium, filter, and pre-heat at 37°C.
   b. Add 40 µg/ml recombinant oxytocin to the lactation medium.
   c. Aspirate the medium from the wells without touching the Matrigel® dome and replace with 800 µl fresh lactation medium supplemented with oxytocin.
   d. Using live cell imaging, record contraction images every second for 120 s.
5. Mimicking involution by hormonal withdrawal
   a. Pre-heat the BOM at 37°C.
   b. Aspirate the medium from the wells without touching the Matrigel® dome and replace with 800 µl fresh BOM.
   c. Renew all medium with BOM every two days, for a total of 8 days of treatment.
6. Replating

   Note: Use tips pre-coated with 2.5% BSA.
   a. Aspirate the supernatant and wash the wells twice with 800 µl cold PBS.
   b. Add 1 ml cold PBS and disrupt the Matrigel® dome using an up-and-down motion with a P1000 pipette.
   c. Check for successful disintegration of the Matrigel® under a microscope.
   d. Transfer the suspension to a 15-ml tube and add cold PBS to a total volume of 10 ml.
   e. Centrifuge for 3 min at 400 × *g*.
   f. Carefully remove the supernatant, resuspend the organoid pellet in fresh Matrigel® and plate as described in B1.

C. Organoid processing for further analysis

Note: We suggest carefully following organoid development under the microscope before renewing the media. Morphogenesis with FGF2 should induce branching after 3–4 days of treatment, while organoids in culture with BOM only, as the negative control, should remain round. Lactogenic differentiation can be confirmed by analysis of Csn2 mRNA using qPCR, comparing organoids before and after prolactin treatment (d6 versus d10). The involution process can also be confirmed using qPCR by detecting decreased expression of Csn2 mRNA following prolactin withdrawal (d10

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versus d18), or at the morphological level by the progressive disappearance of branching (see Figure 2B and Figure 3B).

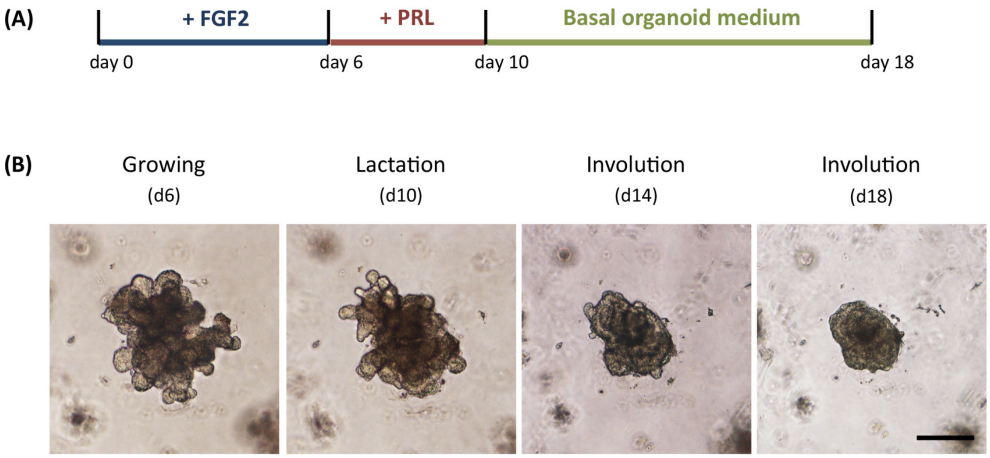

Figure 2. Modeling lactation and involution-like processes in primary mammary organoids. A. Scheme depicting the experimental design. B. Morphology of primary mammary organoids during lactation and involution-like processes. Bright-field images of organoid morphology following morphogenic and lactogenic stimulation and on days 4 or 8 after hormonal withdrawal. Scale bar = 100 µm.

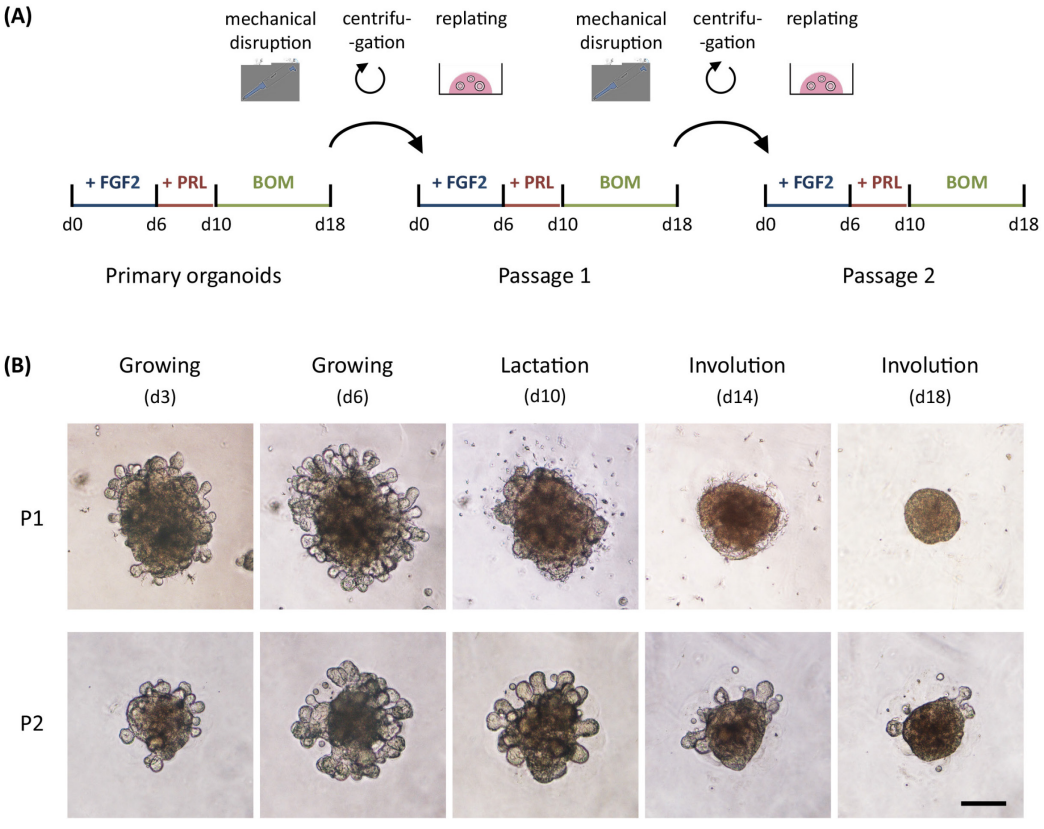

Figure 3. Passage of involution-like organoids. A. Scheme depicting the experimental design. PRL: Prolactin; BOM: basal organoid medium. B. Morphology of passaged organoids during the lactation and involution-like processes. Brightfield images of passage 1 (upper panel) and passage 2 (lower panel) organoids following morphogenic and lactogenic stimulation and on days 4 or 8 after hormonal withdrawal. Scale bar = 100 µm.

1. RNA isolation

   Note: Embedding in Matrigel® does not interfere with the quality of extracted RNA.

   a. Aspirate the culture medium.
   b. Add 350 µl RLT buffer (RNeasy Micro Kit) contiaining 3.5 µl β-mercaptoethanol to each well.
   c. Disintegrate the organoid culture in lysis buffer by performing ten up-and-down motions with a P1000 pipette.
   d. Transfer the solution to a fresh 1.5-ml tube and vortex well.

      Note: Samples can be stored at –80°C until RNA extraction. To perform RNA extraction, thaw samples on ice and proceed according to the following instructions.
   e. Homogenize RNA lysates by performing ten up-and-down motions with a single-use 30 G insulin syringe.
   f. Process samples as described in the RNeasy Micro Kit booklet, starting from Step C1b.
   g. Measure the RNA concentration using a NanoDrop™.

2. Protein extraction

   Note: Embedding in Matrigel® interferes with western blotting analysis. Follow these steps to remove the Matrigel® prior to protein extraction.

   a. Aspirate the culture medium and dissociate the 3D culture with 800 µl cold PBS supplemented with phosphatase inhibitor cocktail II.
   b. Transfer the suspension to a clean 1.5-ml tube and centrifuge for 3 min at 400 × *g*, 4°C.
   c. Rinse twice with PBS supplemented with phosphatase inhibitor cocktail II.
   d. Discard the supernatant and resuspend the pellet in 100 µl ice-cold ready-to-use RIPA buffer supplemented with protease inhibitor cocktail I and phosphatase inhibitor cocktail II.

      Note: Samples can be stored at –80°C until protein extraction. To perform protein extraction, thaw samples on ice and proceed according to the following instructions.
   e. Sonicate the samples twice at 4°C using a 60-kHz ultrasonic wave frequency program (30 s ON/30 s OFF).
   f. Vortex the samples, cool on ice, and repeat the sonication according to Step C2e.
   g. Centrifuge for 20 min at >10,000 × *g*, 4°C.
   h. Transfer the supernatant to a clean 1.5-ml tube.
   i. Measure the protein concentration using a Coomassie Protein Assay Kit.

3. Fixation and embedding for histology

   a. Aspirate the culture medium and rinse the culture twice with 800 µl cold PBS.

b. Incubate with 800 µl 4% PFA for 30 min. Following removal of the 4% PFA, wash twice with PBS.

Notes:

i. Domes should be entirely covered with the solution. Add a greater volume if required.

ii. The fixed cultures can be stored in PBS at 4°C until embedding.

c. Prepare 3% low gelling temperature agarose in PBS and melt slowly in a microwave for 1.5–2 min at 1000 W (homogenize every 30 s by hand rotation).

d. Detach the fixed culture using the flat side of a spatula and transfer to a plastic histology mold containing melted agarose. Overlay with more agarose.

e. After solidification of the agarose, unmold the block. Use a scalpel to remove the excess agarose surrounding the Matrigel® dome and place in a plastic embedding cassette for histology.

f. Proceed to sample dehydration: incubate the embedding cassettes in successive 1-h baths of 70% EtOH, 95% EtOH, 100% EtOH (twice), xylene (twice), 50% xylene-50% melted paraffin, and 100% melted paraffin.

g. Incubate overnight at 65°C in a second bath of 100% melted paraffin.

h. Embed in a histology tissue mold using an embedding workstation.

i. Unmold the paraffin blocks after 24 h of solidification.

j. Cut 5-µm sections and spread on microscope slides. Keep the slides at room temperature until further analysis.

k. Remove the paraffin prior to any staining by successive 5-min baths of xylene (twice), 100% EtOH (twice), 95% EtOH, 70% EtOH, and H_2O.

Recipes

1. Dissociation solution

   Note: This solution is prepared inside a laminar flow hood under aseptic conditions and does not need to be filter-sterilized.

   2 mg/ml collagenase

   2 mg/ml trypsin

   5 µg/ml insulin

   50 µg/ml gentamicin

   5% FBS

   2 mM glutamine

   in DMEM/F12

2. BSA solution

   Note: This solution can be filter-sterilized and reused several times when stored at 4°C.

   2.5% BSA in PBS

3. Basal organoid medium (BOM)

Note: This solution is prepared inside a laminar flow hood under aseptic conditions and does not need to be filter-sterilized.

1× insulin-transferrin-selenium (ITS)

100 U/ml penicillin

100 µg/ml streptomycin

2 mM glutamine

in DMEM/F12

4. Morphogenesis medium

Note: This solution is prepared inside a laminar flow hood under aseptic conditions and does not need to be filter-sterilized.

2.5 nM FGF2 in BOM

5. Lactation medium

Note: This solution is prepared inside a laminar flow hood under aseptic conditions and does not need to be filter-sterilized.

1 µg/ml prolactin

1 µg/ml hydrocortisone

in BOM

6. 4% PFA

Note: This solution is prepared inside a chemical hood and does not need to be filter-sterilized.

4% paraformaldehyde in PBS

7. RNA lysis buffer

Note: This solution is prepared inside a chemical hood and does not need to be filter-sterilized.

10 µl β-mercaptoethanol per 1 ml RLT lysis buffer (from the RNeasy Micro Kit; this solution can be stored for up to one month at room temperature).

8. Phosphatase inhibitor cocktail II

Note: This solution is prepared inside a chemical hood or on a bench and does not need to be filter-sterilized.

2 mM imidazole

1 mM sodium fluoride

1.15 mM sodium molybdate

1 mM sodium orthovanadate

4 mM sodium tartrate dihydrate

in RIPA buffer

9. Protease inhibitor cocktail I

Note: This solution is prepared inside a chemical hood or on a bench and does not need to be filter-sterilized.

500 µM AEBSF hydrochloride

150 nM aprotinin

1 µM protease inhibitor E-64

0.5 mM EDTA
1 µM leupeptin hemisulfate
in RIPA buffer

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Competing interests

The authors declare that they have no competing interests.

Ethics

The animal study was reviewed and approved by French legislation in compliance with European Communities Council Directives (A 75-15-01-3) and the regulations of the Institut Pasteur Animal Care Committees (CETEA).

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