Mammosphere cultures

(prepared by P. Sun, amended by K. Watanabe)

Materials:

- HyClone Classic Liquid Media RPMI 1640 w/L-Glutamine, Phenol Red (HyClone, 500ml, Cat# SH3002701)
- Collagenase from Clostridium histolyticum (Sigma Chemical, 100 mg, Cat# C9891-100MG)
- Hyaluronidase from bovine testes Type I-S, lyophilized powder (Sigma Chemical, 100 mg, Cat# H3506-100MG)
- SPINDRIVE* Orbital Shaker Platforms, SCIENCEWARE*, orbit size 22 (7/8), Bel-Art No. F37041-0000 (VWR Scientific, 20903-879)
- Fetal Bovine Serum (HyClone, 500 ml, Ca# SH30071.03)
- Red Blood Cell Lysing Buffer (Sigma, 100 ml, Cat# R7757)
- 0.25% trypsin-EDTA (Invitrogen Corp., Cat# 25200-056)
- Deoxyribonuclease I from bovine pancreas (Sigma Chemical, 100 mg, Cat# DN25-100mg)
- Dispase Solution (5mg/mL) in HBSS (StemCell Technologies Inc, 100 ml, Cat# 07913)
- 40 μm cell strainer (BD Falcon, Cat# 352340)
- LB Agar, Miller, 500g (Fisher Scientific, BP1425-500)
- mammary epithelial basal medium (MEBM) (Lonza Walkersville Inc. Cat#CC-3151)
- B27 Supplement (50X) (Invitrogen, Cat#17504-044)
- Insulin from bovine pancreas (Sigma Chemical, Cat#I6634-250MG)
- EGF, human recombinant (100mg) (Millipore Corp. Cat#01-107)
- Recombinant Human FGF-basic (Peprotech, Cat#100-18B)
- heparin (STEMCELL, Cat#07980)

1. Preparation of agar-coated plate

(1) Dissolve 5% LB agar in PBS by autoclave.
(2) Keep agar/PBS solution in 95°C at water bath
(3) Warm MEBM at 42°C
(4) Mix MEBM and 5% agar at 4:1, then pour into plate immediately (1.5 ml/6mm dish). Let plates set until cool.
(5) Add 1 ml MEBE to prevent dry.
(6) Agar-coated dishes are sealed with Parafilm and can be stored at 4C.

2. Isolation of mammary cells

(1) Kill female mice (9 to 10 weeks old) and dissect out 5 pairs of mammary glands.
(2) Transfer mammary tissue to a 10 mm dish and wash them once time with 10 ml of

2%FBS/PBS.

(3) Mince mammary tissue with scissors into pieces smaller than 1 mm, and transfer the tissue to a new 10 mm dish.
(4) Prepare 10 ml medium 1640 mixture with collagenase (300U/ml), hyaluronidase (100U/ml) and 5% FBS. (collagenase is prepared just before using. hyahuronidase is dissolved in DMEM/F12 without serum in advance, aliquot and freeze down at -20°C until needed)
(5) Add this mixture solution to the minced tissue in the dish such that the tissue is well suspended in the solution and dissociate at 37°C for 1.5 hr with a gently shaking (speed is 7.5). (check all tissue fragments are digested)
(6) Centrifuge at 1000 g for 5 min at RT and discard the supernatant.
(7) Add 2 ml of Red Blood Cell Lysing Buffer and pipette 5 times with moderate force and avoid bubbling.
(8) Centrifuge at 1000 g for 5 min at RT and discard the supernatant.
(9) Add 3 ml of pre-warm 0.25% trypsinEDTA and pipette 5 times with moderate force and avoid bubbling.
(10) Incubate at 37°C for 5 min, then pipette 20 times with moderate force and avoid bubbling.
(11) Add 12 ml of 2% FBS/PBS and centrifuge at 1000 g for 5 min at RT. Discard supernatant.
(12) Add 2 ml of Dispase (5mg/mL) and 20 μl of DNase (10 mg/ml) and pipette for 1-3 min with moderate force and avoid bubbling. And then add 10 ml of 2% FBS/PBS.
(13) Filter the cell suspension through a 40 μm cell strainer into a new 50 ml centrifuge tube.
(14) Transfer filtrated from above to 15mL tube and spin 1000g for 5 min. Discard the supernatant.
(15) Add 0.5 ml of 2% FBS/PBS, resuspend. Count cell number.

3. Primary mammospheres

(1) Mammary cells are plated onto agar-coated plate at a density of 100,000 viable cell/ml in a serum-free mammary epithelial basal medium (MEBM), supplemented with 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, B27 (1:50), 20 ng/ml EGF and bFGF, and 4 μg/ml heparin.

(2) Incubate cultures in a 5% CO_2, at 37°C for 7 days.

(3) Harvest mammospheres after 7 days in culture. Collect the entire culture into a 15 mL conical tube and centrifuge at 1000g for 5 minutes at RT
(4) Aspirate as much supernatant as possible without disturbing the pellet
(5) Add 0.5 ml of 0.05% pre-warmed Trypsin-EDTA, suspend and incubated for 3-5 minutes at 37°C
(6) After checking for single cells (Using a hemacytometer), Add 2 mL of 2%FBS/PBS and centrifuge the cell suspension at 1000g for 5 minutes at RT.
(7) Aspirate the supernatant and resuspend the pellet in 0.5 mL of complete MEBM.

(8) Count cell using Trypan Blue.
(9) 10,000 cells from disaggregated primary-mammospheres were plated and culture as described in step(1).