Fat pad transplantation of mammary cells
(prepared by P.Sun, consulted the method of Mike Lewis lab/Ricardo)

Materials:

1. Isolation of cells

- 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)
- 40 μm cell strainer (BD Falcon, Cat# 352340)

2. Collect Lin-negative cells

- FACS tube: 5 ml Polystyrene Round-bottom tube (BD Falcon, Cat# REF352052)
- Biotinylated-CD31: (BD Pharmingen), Cat No: 558737, rat x mouse
- Biotinylated-CD45: (BD Pharmingen), Cat No: 553077, rat x mouse
- Biotinylated-TER119: (BDPharmingen) Cat No: 553672, rat x mouse
- Anti-Biotin MicroBeads (MASC, Cat# 130-090-485)
- LD Column (MASC, 25 column, Cat# 130-042-901)
- MidiMACS™ Separation Unit (MASC, Cat# 130-042-302) (borrow from Anshu)
- MACS MultiStand (MASC, Cat# 130-042-303) (borrow from Anshu)
- 1x PBS (Cellgro, 500 ml, sterile, Cat# 21-040-CV)
- UltraPure 0.5 M EDTA, pH 8.0 (Invtrogen, 100 ml, Cat# 15575-038)

3. Transplantation

- 3 weeks old SCID/Beige mice (Charles River Laboratories, Cat#250)
- Sterile water (for injection): VWR, cat# 68099-180
- Ketamine: Western Medical Supply, cat# 4165, 100 mg/ml
- Xylazine: Western Medical Supply, cat# 5530, 20 mg/ml
- Buprenorphine: Western Medical Supply, cat# 7292
- Basement Membrane Matrix, 10 ml *LDEV-Free (BD Bioscience, Cat# 354234)
- 25G needle 1 inch (Becton Dickinson, Cat# 305125)
- Hamilton Syringe 1705LT Gt 50UL (Hamilton,Cat# 80901)
- 9mm Autoclip* Wound Clip Applier (Becton Dickinson)
- Wound clips 9mm (Becton Dickinson, Cat# 427631)
- Change-A-Tip™ (Bovie AARON MEDICAL)
- 1-ml insulin syringe U-100 (BD, Cat#329652)

1. Isolation of mammary cells

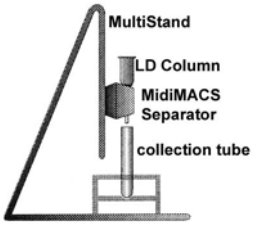
(1) Kill female mice (9 to 10 weeks old) and dissect out 5 pairs of mammary glands.
(2) Transfer mammary tissue to a 10 cm dish and wash them once with 10 ml of 2%FBS/PBS.
(3) Mince mammary tissue with scissors into pieces smaller than 1 mm, and transfer the pieces to a new 10 mm dish.
(4) Prepare 10 ml RPMI 1640 media containing collagenase (300 U/ml), hyaluronidase (100U/ml) and 5% FBS. (collagenase is prepared just before using, hyahuronidase is dissolved in DMEM/F12 (1:1) 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. Dissociate at 37°C for 1.5 hr with gentle shaking (speed is 7.5). (check if all tissue fragments are digested)
(6) Transfer the solution mix to 15 ml tube with pipetting (5 ml) up and down to dissociate cell from fat clums.
(7) Centrifuge at 1000 g for 5 min at RT and discard the supernatant.
(8) Add 2 ml of Red Blood Cell Lysing Buffer and pipette 5 times with moderate force and avoid bubbling.
(9) Centrifuge at 1000 g for 5 min at RT and discard the supernatant.
(10) Add 3 ml of pre-warmed 0.25% trypsinEDTA and pipette 5 times with moderate force and avoid bubbling.
(11) Incubate at 37°C for 5 min, then pipette 20 times with moderate force and avoid bubbling.
(12) Add 12 ml of 2% FBS/PBS and centrifuge at 1000 g for 5 min at RT. Discard supernatant.
(13) Add 2 ml of 2% FBS/PBS 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.
(14) Filter the cell suspension through a 40 μm cell strainer into a new 50 ml centrifuge tube.
(15) Transfer filtrate from above to 15mL tube and spin 1000g for 5 min. Discard the supernatant.
(16) Add 0.5 ml of 2% FBS/PBS, resuspend. Count cell number.

2. Remove Lin-positive cells with anti-biotin magnetic micro-beads

(1) Suspend 2-5 x 10^6 cells in 200-500 μl of PBS containing 2% FBS in FACS tube.
(2) Add linage antibodies (CD31, CD45, TER119; 1:50) and incubate at RT for 25 min.
(3) Wash cells with cold microbeads buffer (0.5% FBS, 2 mM EDTA, 1 x PBS) 2 times.
(4) Suspend cells in 80 μl cold microbeads buffer, then add 20 μl anti-biotin mcrobeads, mix well and incubate on ice for 15 min.
(5) Wash cells by adding 2 ml cold microbeads buffer and centrifuge at 1000g for 5 min at 4C. Aspirate supernatant completely.

(6) Resuspend cells in 5 ml cold microbeads buffer and keep on ice.
(7) Attach MidiMACS Separator to the MultiStand and place LD Column in the separator. Place a collection tube under the LD Column (Fig1).

Fig. 1



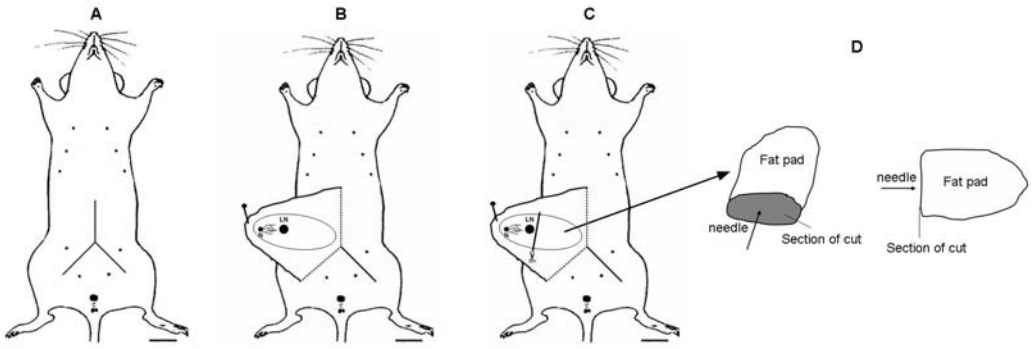
(8) Prepare LD Column by rinsing with buffer: apply 2 mL of rinsing buffer (2 mM EDTA, 1 x PBS) on top of the column and let the buffer run through. LD Columns are "flow stop" and do not run dry. Then discard effluent and change collection tube.
(9) Apply cells suspension onto the rinsed column.
(10) Collect unlabeled cells that pass through column. Wash column with 2 x 1 ml of cold microbeads buffer. Collect total effluent. This is the unlabeled cell fraction.
(11) Spin 1000g for 5 min at 4 C. Discard the supernatant.
(12) Add 0.5 ml of 2% FBS/PBS, resuspend. Count cell number.

3. Transplantation

(1) Thaw matrigel (Basement Membrane Matrix) on ice in advance.
(2) Prepare anesthesia solution (katamine 10 mg/ml and xylazine 1 mg/ml, IP). Example: add 1 ml katamine and 0.5 ml xylazine into 8.5 ml sterile water
(3) Resuspend Cells in a 1:1 solution of PBS/Matrigel at limiting dilutions and keep cells on ice.
- Although only 10 ul cell suspension is injected into each mammary fat pad, you need to prepare at least 300 ul volume in 1.5 ml tube to make it easy to draw with Hamilton Syringe
- As soon as you suck up the cell suspension into the syringe, keep the syringe on ice until you are ready to inject (step7) ---- otherwise matrigel will set.

(4) Measure recipient mouse (3-week old) weight and inject anesthesia solution (90 µl per 10g) using 1-ml syringe with needle.
(5) Open ventral skin and pin it on a styrofoam plate (Fig. 2-A, B). Cut off blood vessel connecting mammary fat pad No.4 to No.3 and No.5 using Change-A-Tip™.

Fig.2



(6) No.4 mammary fat pads are cleared by removing the distal end of the fat pad, including lymph node and budding mammary epithelium with Change-A-Tip™ (Fig. 2-C).
(7) Pierce needle into the fat pad at its cross-section of the cut at the indicated position and inject cells (Fig. 2-D).
(8) Sew up skin with Autoclip* Wound Clip Applier.
(9) Immediately inject Buprenorphine (0.05-0.1 mg/kg IP) post-operation for pain relief. Be sure to record buprenorphine use in the controlled substance inventory.
(10) Analyze by whole-mount at 8 weeks after transplantation.