Microinjection of KOMP JM8, JM8.N4, JM8.F6 and VGB6 ES cell clones into blastocysts
August 26, 2009

Materials and Reagents:

Item	Vendor	Catalog Number
PMSG	National Hormone & Pituitary Program	1190
HCG	Sigma	CG-10
M2 medium	Speciality Media	MR-015
KSOM+AA	Specialty Media	MR-106-D
hepes buffered DMEM	Gibco	12430-054
FBS (ES cell tested)	Hyclone	SH300 70.03
Mineral Oil	Sigma	M-8410
BALB/c Donor Mice	Harlan	4701F

Selection of Donor strain:

JM8, JM8.N4, JM8.F6 and VGB6 parental cell lines were derived from C57BL/6N strain. Inject these cells into blastocysts from BALB/c donor will get white, agouti and black chimeras.

Microtool Preparation:

Holding pipettes are prepared from glass capillaries (custom glass tubing #9-000-3000), injection pipettes from glass capillaries (custom glass tubing #9-000-2155) using a horizontal micropipette puller (Model: 97, Sutter Instrument co.). The holding pipette has an external diameter of 60-80□m and an opening of 10-□□□m. The injection pipette has an external diameter of 12-17□m and an internal diameter of 10-15□m.

Superovulation of Donor Females:

1. Day 0: Give PMSG (5 I.U.) to 3-4 weeks old 15 BALB/c females by I. P. injection at 2 pm.,
2. Day 2: Give HCG (5 I.U.) to the females at 1 pm by I. P. injection. Mate the females to stud males.
3. Day 3: Check plugs.

Preparation of Blastocyst:

1. Day 6 - Sacrifice plugged female mice by CO2 asphyxiation or cervical dislocation at 3.5dpc at 8:00 am.
2. Dissect open the abdomen, locate the complete uterine horn and remove. Taking as much fat from the uteri off as possible.

3. Flush the uterine horn into a petri dish by inserting a 26g needle (attached to a 3ml syringe) into the oviduct/uterine junction and flush approximately 0.5ml of M2 through. Fluid should be visible flushing through the uterus and out of the cervical opening.
4. Collect all the eggs using a mouth transfer pipette and sort blastocysts, morulae and undeveloped embryos into separate 40μl drops of KSOM+AA covered with oil (pre-equilibrated) into the incubator (5% CO_2 at 37^{o}C) Record the number of embryos.

Expansion of JM8, JM8.N4 and JM8.F6 derived ES cell clones for microinjection:

Follow "Thawing and Expansion of JM8-derived KOMP Clones for Microinjection and Future Use" section under Culturing JM8-derived KOMP Clones for JM8, JM8.N4 and JM8.F6 derived ES cell clones

Expansion of VGB6 derived ES cell clones for microinjection:

1. 1day prior, prepare one 10cm MEF Treated Feeder dish, using the plating density guide below.
2. The next day (day of passaging); 3-4 hours prior to passaging, reefed the 70-80% confluent 6-well dish with 4ml pre-warmed VGB6 medium (VERY IMPORTANT).
3. After the 3-4hours; aspirate off the media, and rinse once with 4ml PBS.
4. Cover the cells with 0.5ml of 0.25% trypsin solution and incubate at 37°C for 10 minutes.
5. Add 5ml of VGB6 medium to inactivate the trypsin, and pipette very gently to make single cell suspension (we recommend 3-4 times only).
6. Transfer the cell suspension from each well into 15ml centrifuge tubes (labeled 'Expansion' or 'Microinjection').
7. Spin tubes for 4-7 minutes at 1,200rpm.
8. For the 'Expansion' cells; aspirate off the supernatant and resuspend the pellet in10ml VGB6 medium. Transfer the cell suspension onto the 10cm MEF Treated Feeder dish prepared the day before.
9. Culture in a 37°Chumidified 5% CO2incubator. Change medium daily until 70-80% confluent (should take 2-3days). You may continue to passage the cells as described - we would recommend a splitting ratio of1:1-1:10 depending on dish size (not anymore dilute).
10. For the 'Microinjection' cells from the 6-well (1daypriorto microinjection); aspirate off the supernatant and resuspend the pellet in1.1ml VGB6medium. Into each well ofa12-well feeder plate, plate the following dilutions of cell suspension:

    - 100μl, 200μl, 300μl, 500μl

On the day of microinjection, examine all wells, and determine which dilution is optimum (confluency should be 30-60%, and morphology should be bright, small, smooth and round aggregates).

For the optimum cell dilution, trypsinize as standard, and resuspend the cell pellet in Microinjection Medium (Hepes Buffered D-MEM with5% FBS; filtered through0.2 µM filter unit, can be aliquoted and stored at $-20^{o}C$ for up to 12 months).

Immediately place the tube of cells on ice, and microinject within1-2.5 hours.

Blastocytst Microinjection:

1. Prepare a 60mm petri dish to place injected blastocysts into. Pipette four 40µl drops of KSOM+AA, cover with oil and place in a 5% CO_2 incubator to equilibrate.
2. Prepare a cavity slide by making a large (~40µl) drop of ES injection buffer into the center of the well and cover with mineral oil.
3. Place an appropriate number of blastocysts onto the slide, then add the prepared embryonic stem cells. Lower the holding pipette into the small drop of medium and fill to the shoulder, by capillary action. Place into the main drop.
4. Fill the tip of the injection needle with medium from the other small drop, by capillary action; place the needle into the main drop. Collect about 100 round and small ES cells with the injection pipette.
5. Hold a blastocyst using the holding pipette, with the inner cell mass closest to the holding pipette. Gently push the injection pipette into the blastocyst. Once inside release 10 to 15 ES cells and gently withdraw the needle. Continue until all blastocysts on the slide have been injected. Try not to exceed 30 min for each set of blastocysts.
6. Place injected blastocysts into the pre-equilibrated KOSM-AA drops in a 5% CO_2 incubator.

Embryo transfer:

Transfer 6 to 7 injected blastocysts into each uterine horn per 2.5 dpc pseudopregnant CD1 female.