Microinjection of KOMP-Regeneron VGB6JM8ES clones into m morulae (modified —"Vellocimouse"ᶜtechnique)

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Materials And Reagents:

Item	Vendor	Catalog Number
PMSG	National Hormone & Pituitary Program	1190
HCG	Sigma	CG-10
M2 medium	Specialty Media	MR-015
KSOM+AA	Specialty Media	MR-106-D
Mineral Oil	Sigma	M-8410
Swiss Webster	Harlan	
PVP	Irvine Scientific	99219

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 60-80µm and an opening of 10-15µm. The injection pipette has an external diameter of 12-17µm and an internal diameter of 10-15µm.

Microinjection set up: Place about 0.5ul mercury in the proximal end of the injection pipette, attach the pipette to the manipulator and push the mercury to the tip. Using a cavity slide, place a small drop (~40µl) of ES cell injection media +5% FBS into the center of the well and cover with mineral oil. Place a small drop (~5µl) of 10% PVP in M2 on the right at the edge of the oil. Add a small drop (~5µl) of M2 on the left hand side of the slide. Place the appropriate number of pre-compacted morulae onto the slide, and then add the prepared ES cells.

Selection of Donor Strain: Parental cell line for KOMP Regeneron clones is VGB6, which is derived from C57BL/6N strain. Inject these cells into blastocysts from Balb/c will get black, and agouti chimeras.

Superovulation:

1Day 0: Give PMSG (5 I.U.) to 3 weeks old 15 Swiss Webster females by I. P. injection at 2 pm.,
2Day 2: Give hCG (5 I.U.) to the females at 1 pm by I. P. injection. Mate the females to stud males.
3Day 3: Check plugs.

Harvesting Morulae:

1Day 5: Sacrifice plugged female mice by CO_2 asphyxiation or cervical dislocation at 2.5dpc at 8:00 am.

2Dissect open the abdomen, locate the complete uterine horn and remove. Taking as much fat off from the uteri as possible. Trim the uterine horn to ~1cm long, leaving the oviduct intact, the ovary can be removed.
3Flush each oviduct through infundibulum with 0.2 ml M2 with a 30g needle attached to a 3ml syringe. Collect all the eggs using a mouth transfer pipette and sort morulae into pre compacted, compacted and fragmenting embryos, place into 40µl drops of KSOM+AA covered with oil (pre-equilibrated) into the incubator (5% CO_2 at 37°C) Record the number of embryos.

Expansion of VGB6-derived KOMP (REGEN.) Clones for Microinjection and Future Use**:**

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 ofVGB6medium; 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°C for up to 12 months).

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

Morulae Injection:

1Wash the injection pipette in the PVP drop, leave a small amount of PVP in the pipette, lift out of the PVP drop and then lower into the mineral oil so that the pipette is at the edge of the injection medium. Blow out some PVP and then suck a small amount of mineral oil into the pipette, then slide the pipette into the injection medium and suck a small amount of injection medium into the pipette, the pipette is ready to use.
2Lower the holding pipette into the small drop of injection medium on the left of the slide and fill to the shoulder, by capillary action. Place into the main drop.
3Hold one morulae using the holding pipette, try to ensure there is a space between the blastomere and the zona pellucida on the opposite side to the holding pipette. Gently suck the zona at the 3 o'clock position using the injection pipette and apply several piezo –pulses (intensity 2-4, speed 2) to penetrate the zona.
4Remove the pipette and expel the zona from the tip, collect 8 ES cells and gently insert the pipette into the hole and inject the ES cells with as little media as possible.
5Remove the injection pipette.
6Continue until all the pre-compacted morulae have been injected. Aim to inject all the embryos in 30 min. Placed injected morulae into the pre equilibrated drops KSOM+AA in a 5% CO_2 incubator.

Embryo transfer:

Transfer 12-16 injected morulae per 0.5dpc pseudopregnant CD1 female.