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Isolation and handling of primary mouse embryonic fibroblasts (MEFs)

accompanying protocol to "Mouse embryonic stem (ES) cell culture - basic procedures"

Isolation of embryonic fibroblasts

Embryonic stem cells are usually grown on a layer of mitotically inactivated primary mouse embryonic fibroblasts to promote growth and prevent differentiation. These cells stop dividing after a couple of passages, so embryonic fibroblasts need to be isolated freshly from time to time. This requires pregnant female mice at day 13 post coitum (assuming day one is the first day the plug is observed). F1 hybrid or outbred mice are favored because of increased litter size

- preparation: sterilize (autoclave) dissecting instruments (fine scissors, tweezers, razor blades)
- sacrifice the pregnant female at day 13p.c. by cervical dislocation. Dissect out the uterine horns, briefly rinse them in 70% (v/v) ethanol and place into a petri dish containing PBS (Gibco 14190-169, without bivalent cations)
- separate each embryo from its placenta and surrounding membranes. Cut away brain and dark red organs, wash with fresh PBS ,remove as much blood as possible
- using a minimal amount of PBS and razor blades, finely mince the embryos until they become "pipettable". Suspend cells/tissue in several ml of trypsin-EDTA (about 1-2ml per embryo, Gibco 25300-096) and incubate with gentle shaking at 37°C for 15min together with a few 5mm glass beads. Ideally, the resulting cell suspension should be essentially free of any larger pieces of tissue and should not be too viscous (genomic DNA - lysed cells). High viscosity due to cell lysis may prevent efficient pelleting of cells in the subsequent centrifugation step. Addition of some DNAse during trypsin treatment may solve this problem (we use 100 Kunitz units per ml)
- transfer suspension to a 50ml falcon tube and add about 2 volumes of fresh...

...MEF medium:

DMEM (high glucose, Gibco 41966-052, store in fridge)
10% (v/v) FCS (store aliquots at -20°C)
1/100 (v/v) L-glutamine (200mM: Gibco 25030-024, store aliquots at -20°C), stable in solution for 10d only
1/100 (v/v) pen/strep (Gibco 15140-122, store aliquots at -20°C)

- to remove remaining pieces of tissue, let these settle down to the bottom of the tube within a few minutes and carefully take off the supernatant to be subjected to low-speed centrifugation (5min)
- resuspend the resulting cell pellet in warm MEF medium and plate out at 1 embryo equivalent per 10cm dish (this is "passage No. 0"). Change medium on the following day. The fibroblasts will be the only cells that attach to the dishes. Plates will be confluent within 1 to a few days

This is the time to do something to the cells - freeze them or split them...

...Splitting of MEFs (max. ratio: 1:5)

- wash MEFs with PBS once, then trypsinize (1-2ml per 10cm dish). In contrast to treatment of ES cell colonies, trypsinization of MEFs proceeds quite quickly: rock plates at room temperature after a few min. Inactivate trypsin by adding some MEF medium

- pipette up and down several times to dissociate into single cells, then pellet at low speed for 5min
- soak off supernatant, resuspend cells in fresh medium and plate out at the desired splitting ratio - about 10ml total volume per 9cm dish (Nunc), about 12ml per 10cm dish (TPP). Again, grow until confluence

...Freezing of MEFs (usually 1 embryo equivalent per vial)

- wash confluent (passage No. 0 - "P0") dishes once with PBS, then trypsinize (1-2ml per 10cm dish). Rock plates at room temperature after a few min. Inactivate the trypsin by adding some MEF medium.
- pipette up and down several times to dissociate into single cells, then pellet at low speed for 5min
- soak off supernatant, resuspend cells in cold freezing medium (10% DMSO, Sigma D2650, in MEF medium). Transfer cell suspension into cryo-vials (1ml per vial) and cool on ice before transferring the vials to a -80°C freezer in a pre-cooled styrofoam box. By this procedure, it is assured that cells freeze slowly.
- the next day or later, transfer the cells to liquid nitrogen.

Thawing and replating of MEFs

- quickly thaw frozen MEFs in a warm waterbath, clean the outside of your vial(s) with 70% (v/v) ethanol, then transfer the fibroblasts to a few ml of warm MEF medium and pellet by centrifugation (5min, low speed). This is to remove the DMSO contained in the freezing medium.
- soak off supernatant, resuspend cells in MEF medium, and plate out (=P1 if thawing P0 cells). The time required until cells grow to confluency depends on the splitting ratio (1:1 to 1:5).

*Before generating feeder layers from the MEFs, split cells several times (otherwise you would waste too many embryos) letting the cells grow to confluence, respectively. Making feeders means to mitotically inactivate and replate the MEFs. Cell division can be interrupted either by **g**-irradiation or mitomycin C treatment. As we do not have any device for to producing **g**-irradiation in our institute, we treat MEFs with mitomycin C (Roche 107409 - dissolve in PBS at 0.5 mg/ml and filter-sterilize; store in fridge):*

Making feeders

- add mitomycin C diluted in MEF medium (10µg/ml final conc.; diluted by 1/50 from stock solution) to an almost confluent MEF dish and incubate for 2h at 37°C
- soak off the mitomycin-C-containing medium and wash twice with PBS, then trypsinize, centrifuge, resupend, and replate cells as above. Use a splitting ratio of a bit less than 1:1 in this step (remember that the cells won´t divide any more). The reason to go for "a bit less than 1:1" here is to achieve a feeder cell density that almost completely covers the bottom of the dish but at the same time leaves enough space for each cell to "spread" a bit. In general, feeder quality is very important since it directly affects the growth of the ES cells growing on top. Only experience tells you how a good feeder looks like...

The feeders can be used after one day until up to about 1 week (change medium every 3 days or so). There are some advantages in using feeders that are a few days old: You can be quite sure about contamination, and the cells will have flattened nicely, which improves the growth of your stem cells in some respect. Switch to ES medium immediately before plating out the ES cells (see accompanying protocol "Mouse embryonic stem (ES) cell culture - basic procedures")