**Culture of Trophoblast Stem cells**

Most details from Methods in Molecular Medicine Quinn, Kunath, and Rossant

**Split TS cells:**

1. Wash cells w/PBS
2. Add 1.5/4 ml 0.125% trypsin to 6-well-well/10cm plate
3. Incubate 3 minutes at RT, or until cells within colonies are all rounded and individually visible
4. With plugged Pasteur pipette, squirt trypsin over plate surface, paying special attention to dislodging cells on the outer the rim of the plate, which usually has a higher density of colonies. The goal is to dislodge from the bottom of the plate without breaking colonies into a single-cell suspension. TSCs do not tolerate single cell suspensions well. I typically spray trypsin over the plate surface 3 – 5 times to ensure I have released all colonies into solution.
5. Add cell suspension to 10 – 20 ml of DME with 10% serum
6. Spin cells at 1000rpm (or setting 5) for 5 minutes
7. While cells are spinning, prepare appropriate amount of TSM with FGF4 and Heparin added
	1. Both are 1:1000 dilution
	2. Add 20% more growth factors than there is media present
8. Aspirate all but 100-200ul of media
9. Flick bottom of tube with your index finger 3-5x to help re-suspend cell pellet
10. Add 2 ml of TSM (with growth factors)
11. Using a plugged Pasteur pipette, pipette up and down 5-10x to re-suspend cells. Both this step and the flicking of the tube are essential to bring the trypsinized colonies down to an appropriate size.

When growing TSCs on MEFs, it is almost impossible to break colonies up too much using this method, so err on the side of rigor, rather than being overly gentle. When growing TSCs off MEFs, err on the side of being gentle, because the colonies might not recover from being broken up too much.

1. Add media to appropriate volume and plate
2. Split the cells again just after the colonies start to harbor a visible outer ring of cells and an inner flat center. Confluency of the plate is irrelevant when culturing TSCs for passage purposes (rather than an endpoint assay).

**MEF removal Culture Notes:**

1. When removing TS cells from MEFs:
	1. be gentle during trypsinization so as not to disrupt small colonies
	2. plate for 40 minutes then transfer to a clean, non-gelatinized plate for culture
	3. Prior to split off MEFs, ideally want a large number of medium sized-colonies as these contain the highest concentration of undifferentiated cells
	4. During passage, split colonies off MEFs before they start to touch, as once this happens they start to differentiate and don’t propagate well
	5. For final passage off MEFs, let cells become confluent to increase numbers of undifferentiated cells

**Plating MEFs for TSC culture:**

1. One vial is 6e6 cells, plate 1e6 per 10cm plate – first gelatinize all plates
2. Spin out DMSO
3. Plate @ 1e6 per 10 cm

**Making MEF Conditioned media:**

1. 1 vial of irradiated feeders at 6e6 cells per vial is enough for 1 15m plate.
2. spin out DMSO, R/S irradiated MEFs in 5-10ml TSM per 6e6 vial thawed.
3. [optional] Count to make sure 6e6 are plated per 15cm plate
4. Add 6e6 of irradiated MEFs, 28ml TSM per 15cm plate
5. Take media every 3 days for 9 days
	1. Pool media from each plate, spin down at 1000g for 5 mins, sterile filter in Millipore Filters (SCGPT01RE), and freeze in 40ml aliquots at -80C.
6. Each 15cm plate will yield about 75 ml media over the course of 9 days
7. Conditioned media is good for a long time at -80C

**Making primary MEFs for TS feeders:**

1. Dissect 1 d12.5 embryo, removing head and internal organs (anything red)
2. Place embryo into 2ml media in 6 well well (10-15% FCS DMEM)
3. Take 1ml syringe and 19.5 gauge needle and plunge embryo 5-10 times until it is dissociated into little pieces
4. Add additional 2-3 ml media
5. ~48 hours later (or whenever the dish is super 100% confluent, split into equivalent of 2 60mm dishes. Use 0.1% trypsin.
6. Freeze 2 embryo’s worth in 1 vial for later expansion

**To expand primary MEFs for irradiation**

1. P2 DR4’s can be purchased from ATCC or Applied Stem cell
	1. ATCC DR4’s are thawed from ATCC onto 3 10 cm plates, then frozen one vial per plate after confluency has been reached
2. Thaw 1 vial into 2 15cm plates
	1. Grow MEFs in 10 or 15% FBS in standard DMEM – use 15% serum for faster growth like when growing DR4’s.
	2. It is essential to count cells after every pass, and relate the count # to the visual density on the plate.
	3. ATCC recommends seeding MEFs at 1e6 per 15cm plate
	4. Cells are ready to split at 100% confluency, best case is when swirls begin to appear on plate
3. Split to 6 15cm plates, then 20 15cm plates
	1. Again, count cells after every pass and relate the count # to the visual density on the plate. A confluent 15cm plate of DR4 MEFs will have ~5 million cells.
	2. ATCC recommends seeding MEFs at 1e6 per 15cm plate
4. Trypsinize, quench and spin out trypsin, R/S @ 6.5e6 per ml or in 30 ml total per 10 dishes and irradiate (in 2 50ml conicals).
	1. **Prior to trypsinization**, ensure you have enough 0.125% trypsin (usually you will need to make a bunch in a 250ml bottle)
	2. **Prior to trypsinization**, aliquot 15ml of media per plate to be trypsinized in a sterile 500ml bottle, to be used later for quenching of trypsin
	3. **Prior to trypsinization**, examine each plate under the microscope to ensure none are contaminated, and to gauge the confluency of the population
	4. Aspirate media on each plate
	5. Wash each plate with 15ml of PBS
	6. Add 10ml of fresh 0.125% trypsin to each plate
	7. Wait ~4 to 5 minutes at room temp until nearly all MEFs have balled up on plate
	8. Spray off MEFs on each plate and pipette against the bottom of the plate once to break up clumps, then unite suspensions in the 500ml bottle with 15ml of media per plate trypsinized
	9. Swirl 500ml bottle with cell suspension well and aliquot into 50ml conical tubes. To avoid getting uneven cell counts from the different conicals, between the making of each aliquot, swirl cell suspension and mix by pipetting
	10. Count cells from each or most aliquots and average counts to arrive at the final number of MEFs that will be irradiated
	11. Make certain that the counts match what your eye estimated for confluency prior to trypsinization (step c)
5. Go to blood bank in hospital to irradiate -- 3100 rads (phone 6-4011). Let them know you are irradiating cells for research.
6. Spin out media and freeze in 10% DMSO 20% serum.
7. Plate at 6e6 cells per 15cm dish in 29 ml TS cell media to make conditioned media, 1e6 per 10cm plate for TSC passage.

**TS Media:**

1. Stock reagents:
	1. Heparin: Sigma H3149 10,000 Units: R/S in sterile PBS to a final concentration of 1.0mg/ml (1000x)
	2. Recombinant Human FGF4: Sigma F2278 (or Gibco PHG0154) 25ug: R/S in 1ml of sterile PBS/0.1% BSA Fraction V
	3. ES qualified FBS from Gibco
	4. 100x Sodium Pyruvate, l-glutamine, pen/strep from Gibco
	5. 100mM beta-mercaptoethanol 1000x stock (add 70ul of stock 14.3M to 10ml of sterile PBS)
2. Final Media concentrations:
	1. RPMI
	2. 20% serum
	3. 1x Sodium Pyruvate, l-glutamine, pen/strep from Gibco
	4. 1x FGF4 and 1x Heparin (add fresh or within a week of use)
	5. 1x BME
3. Freezing media:
	1. 25% FBS
	2. 10% DMSO
	3. 65% TSM