Protocol: EBs culture with graded Activin A concentrations (250c/EB)

Day 1: Thawing of mES cells (E14Tg2a)

Pre-considerations:

- Pre-heat medium at 37°C (previously prepared according to Lab's protocol)
- Coat a 6 cm culture plate with 0.1% gelatine (4°C)– adding 1.5mL/6cm dish (add to half of the plate and spread it to all of it) and leave it for 1-5 min then aspirate all gelatine.
- Take a vial containing $0.8*10^5$ - $1*10^6$ cells from -80°C and dip it in the bottom in the water bath (move a bit) and remove it when some ice is still present - clean the vial with sprayed ethanol and a tissue.
- Put all vial content in a 15mL falcon using a wide tip and add pre-warmed SCM dropwise (4mL).
- Centrifuge for 5 min at 1500 rpm at RT.
- Aspirate supernatant (carefully - do not touch cells) and re-suspend the pellet in 5mL SCM+LIF adding slowly medium to the pellet and pipet until cells are well suspended and plate it considering to uniform distribution of cells.
- Keep the plate in the incubator (37°C + 5% CO_2).

Day 2: Check cells growth:

- If there are many dead cells (floating cells) the medium must be changed –aspirating gently without touching the cells and replace it adding 5 mL SCM + LIF with slow pipetting and target the wall of the dish. In case of no cells attached discard the plate or in case of 60-80% confluence (round colonies as little differentiation possible) passaging must be done.

Day 3: Passaging mES cells

Pre-considerations:

- Pre-heat trypsin, DPBS, 2i + LIF + 1.5 % FBS medium (previously prepared according to Lab's protocol) at 37°C.
- Coat the TC dishes with 0.1% gelatine (4°C) for 1-5 min.
- Aspirate the spent medium from the dish containing mESCs (carefully - do not touch cells), and rinse the dish once with dPBS (removal of trypsin inhibitors: ions, serum…): ≅5 mL – slow pipetting and target the wall of the dish.
- Add 0.5 mL of trypsin-EDTA (combined method for detaching cells) and incubate for 5' at 37°C
- Add 0.5 mL trypsin inhibitor (previously prepared in a small quantity to stock) and pipet several times using a 1mL narrow tip to get a single cell suspension (check under the microscope to confirm the absence of clumps from the suspension).
- Count the cells (dilution of mESCs suspension using a 0.4% Trypan Blue dye solution -1:1-) and plate the appropriate amount of cells (add 1 ml of medium and suspend the cells in it: 6.10^5 - 8.10^5 cells) in the 6 cm TC dish that already contain 4 ml of 2i+LIF + 1.5% FBS medium.

Day 4: Check cells growth

- Same considerations as mentioned before just contemplate the difference in the conditions (growth in 2i+ LIF is slower).

- Considering that mEBs culture will be done afterwards, is important to do the following one day in advance:
  *Prepare 1.2% POLYHEMA: For 20 ml (not in a big quantity) is required ≅ 19,2 ml ETOH 99,98% + 0,8 ml (H_2Od) + 0,24g polyhema in a 50 ml FALCON Rock over night to reach the optimum dissolution and next day centrifuge 20' max rpm at room temperature (checking centrifuge max speed and max weight) and transfer supernatant to a new FALCON and discard the pellet.
  NOTE: Polyhema recipient is open in the flow, put the approximate amount of powder into a falcon tube, close it and then measure it. Everytime the falcon tube enters the flow should be sterelize (as is mention before).
  * Prepare the 96 well plates U-SHAPE: Coat with 1.2% POLYHEMA adding 100 µL to the first column, pipetting up and down several times and passing to the second column repeating the same procedure (twice per plate) and taking care that all the content is remove from the wells every time. After reproducing this procedure in all plates, put the lid on and leave it in the flow for at least 1 hour. If the plates won't be used immediately can be seal with parafilm and kept in the fridge (4 ^{o}C).

Coat 96 well plates U-SHAPE with 1.2% POLYHEMA adding 100 µL to the first column, pipetting up and down several times and passing to the second column repeating the same procedure (twice per plate) and taking care that all the content is remove from the wells every time. After reproducing this procedure in all plates, I put the lid on and leave it in the flow for at least 1 hour. If the plates won't be used immediately can be seal with parafilm and kept in the fridge (4 ^{o}C).

Is also possible coating the plates, following the subsequent protocol:

Coat 96 well plates U-SHAPE with 1.2% POLYHEMA adding 10 µL to each well, and taking care that all the content is remove from the wells every time after aprox. 10 min. and after I put the lid on and leave it in the flow ON and the next day I sealed them with parafilm and kept in the fridge (4 ^{o}C).

Day 5/6: Passaging mES cells and EBs differentiation

- Pre-heat trypsin, dPBS, SFM (previously prepared according to Lab's protocol) at 37°C.
- Wash each well of the coated plates with 100 µL dPBS and aspirate all the content after.
- Aspirate the spent medium from the dish containing mESCs (carefully - do not touch cells), and rinse the dish once with DPBS (removal of trypsin inhibitors: ions, serum…): ≅5 mL – slow pipetting and target the wall of the dish.
- Add 0.5 mL of trypsin-EDTA (combined method for detaching cells) and incubate for 5' at 37^{o}C
- Add 0.5 mL trypsin inhibitor (previously prepared in a small quantity to stock) and pipet several times using an 1mL narrow tip to get a single cell suspension (check under the microscope to confirm the absence of clumps from the suspension).
- Count the cells (dilution of mESCs suspension using a 0.4% Trypan Blue dye solution -1:1-) and add the appropriate amount of cells (considering 2,5*10^{2} cells/well) to the SFM, mix well and add the appropriate volume per well (considering 50 µL SFM/well, 2.5*10^{4} cells were added to 5 ml SFM)
- Centrifuge the plates 3' 100-120g (optimum speed: 110g) at ≅ 37^{o}C (average temperature used 20 ^{o}C - 25 ^{o}C) and take pictures (6 wells should be chosen from each plate)

- Collect the rest of the cells in an eppendorf , centrifuge it 5 min 1500 rpm (37^{o}C), discard supernatant, add 300 µL Tryzol and keep it at -80 ^{o}C as **day 0**.

MEDIA

* **Serum Containing Medium: 100 ml**
100 ml KO-DMEM (for ES cells)
18 ml Fetal Calf Serum (Hyclone or Milipore #ES-009-B – ES cell certified, Heat Inactivated)
1 ml NEAMN
1 ml P/S
1 ml L-glut
200 µl β-mercaptoethanol (50mM)
+ LIF (stock L5283-10UG): 10µL

* **2i+LIF + 1,5 % Fetal Bovine Serum**
N2B27 base: 10 ml
4,8 mL neurobasal
4,8 mL DMEM:F12
100 µl L-Glutamine
100 µl P/S
50 µL N2
100 µl B27 **without vit. A #12587-010**
20 µl 2-mercaptoethanol
100 µl 5% BSA (0.05%)
2i+LIF:
1 µL PD0325901 10mM (1 µM)
3 µL CHIR99021 10mM (3 µM)
+ LIF (stock L5283-10UG): 1 µL
+ 1,5 % FBS: 150 µl

*** Serum Free Medium: 5 ml**
75% IMDM:25% Ham's F12 : 3.75 mL IMDM : 1,25 mL Ham's F12
0.5X N2 (25 µl -100x)
0.5X B27 without vit. A #12587-010 (50µl - 50x)
1% P/S (50 µl - 100x)
2mM L-glutamine (50 µl - stock 200 mM 100x)
0.05% BSA (50 µl - 5% BSA)
0.5mM ascorbic acid (5 µl - 500mM stock)
$4.5x10^{-4}$ M (0.2 µL 1-Thioglycerol 11.5M)