SOP SCB D 001	Title: Embryoid Bodie's (EB's) formation and three germ layer differentiation.

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OBJECTIVE

This protocol aims for the formation of EB's from IPSC or ESC cell lines and their subsequent differentiation to the three germ layers: Ectoderm, Endoderm and Mesoderm.

MATERIALS:

- 96 well plate. [Thermo scientific REF.]
- 60mm ultra-low attachment (ULA) plate x 2. [Corning REF.3261]
- 100mm plate with IPSC/ESC with 90% of confluency [Corning 430167]
- Slide flask ([Thermo Scientific. REF. 170920]) previously coated with Matrigel 1:2 x 3
- PBS [Biowest REF. L0615-500]
- EDTA 0.5mM [Invitrogen REF. 15575-038]
- EB's differentiation media (For the preparation Media instructions see below):
  - Ectoderm (N2/B27).
  - Endoderm (EBm).
  - Mesoderm (EBm + AA).
- mTSER-1 [Stem Cell Technologies REF. 85851]
- Multichannel pipette reagent reservoir. [Corning REF. 4870]
- Multichannel pipette.
- 50 ml centrifuge tube. [Falcon. REF 352070]
- Centrifuge.
- P1000 micropipette.
- Matrigel [Corning REF. 356234] 1:2 (Diluted in KO-DMEM [GIBCO REF. 1082958])
- L-ascorbic acid powder [SIGMA REF. A4544-25G]

PROCEDURE

Day 1:

1. Once colonies are compact and reach approximately 90% of confluency of a 100mm plate, aspirate the medium and wash the plate with 5 mL of PBS.
2. Eliminate PBS and add 3 mL of EDTA solution.
3. Incubate for 2 min at 37 °C and 5% CO_2.

4. Remove EDTA.
5. Lift colonies flashing with fresh mTSER-1 medium and collect them in a 50 mL falcon tube.
6. Take 2/3 of the final volume (The other 1/3 is for other characterization procedures) and transfer it to a 50 mL falcon tube.
7. Complete with mTSER-1 until getting a final volume of 15 mL.
8. Transfer the 15 mL to a multichannel pipette reagent reservoir and using a multichannel pipette seed 150 µl per well in a 96-well plate.
9. Centrifuge the 96-well plate at 800 g for 10 minutes.
10. Incubate for 24 hours at 37 °C and 5% CO_2.

Day 2:

1. Prepare two 60mm ultra-low attachment (ULA) plates, one of them with 5 mL of mTSER and the other empty.
2. Using a microscope and a p1000 micropipette pick up the EB's formed in the 96 well plate one by one and place them at the empty ULA plate.
   Note: If some of the EB´s break when they are being collected, let them incubate for another 24 hours.
3. After assuring that every EB's were collected from de 96 well plate transfer all of them to the ULA plate with the 5mL of fresh medium, trying not to transfer a large quantity of the previous media.
4. Incubate the EB's at 37 °C and 5% CO_2 for 24-48 hours.

Day 3:

1. Label 3 matrigel-coated Slide flasks with each one of the three-germ layer names (ECTO, ENDO, MESO).
2. Add 2 mL of the respective differentiation media and 40ul of Matrigel 1:2 to each one of the slide flasks.
3. Pick up approximately between 30 to 33 EB's and seed them in each slide flask.
4. Incubate at 37 °C and 5% CO_2 .
5. Proceed to change the medium every 2 days. (Now without Matrigel)
   Note: For the Ectoderm differentiation media (N2/B27) changing, only eliminate 1 mL and add 1 mL of fresh medium.
6. Cultured conditions in the differentiation media would be held for 21 days for Endoderm and Mesoderm, and for 28 days in the case of ectoderm.
7. After the indicated days have passed, the slide flasks are fixed and analyzed by immunocytochemistry, taking confocal pictures of the immune-marked cells. (see SOP XXX)

EB'S DIFFERENTIATION MEDIA PREPARATION:

- **Ectoderm - N2/B27 Media:**
  50% Neurobasal medium [Gibco REF.21103-049]
  50% DMEM/F12 [GIBCO REF. 21331020]

0,5% N2
1% B27
1% Glutamax [GIBCO REF. 35050-038]

1% Penicillin-Streptomycin [Gibco REF. 15140-122]

- **Endoderm – EBm:**
  87% Knockout-DMEM [GIBCO REF. 1082958]
  10% Hyclone FBS [Cytiva REF. SV3016003]
  1% NEAA [GIBCO 11140035]
  0.1% β-mercaptoethanol [31350-010]
  1% Glutamax[GIBCO REF. 35050-038]
  1% Penicillin-Streptomycin [Gibco REF. 15140-122]

- **Mesoderm – EBm + AA:**
  100% Endoderm medium
  0.5mM ascorbic acid solution*
  Note: Ascorbic acid must be added just before the media change.

  ***AA solution preparation:**
  1. Turn off the air conditioner before weighing the L-ascorbic acid powder.
  2. Using a scale, weigh 88 mg of L-ascorbic acid powder in an Eppendorf tube.
  3. Dissolve it in 1mL of KO DMEM media.
  4. Distribute the final volume in 20 µl aliquots.
  5. Freeze them at -20°C until their use.
  NOTE: The aliquots ONLY must be kept for 1 month maximum.