**Haematopoietic progenitor and lymphoid differentiation from human pluripotent stem cells**

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2) Lentiviral Infection of Human Embryoid Body-Derived Haematopoietic Progenitor Cells (hEB-HPCs)

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**Differentiation of haematopoietic progenitors from human embryoid bodies (hEBs)**

Adapted from Chadwick et al. *Blood*, 20031

hEB Media

400 mL KnockOut (KO) DMEM (Invitrogen 10829-018)

100 mL Fetal bovine serum FBS (non-heat inactivated)

2.5 mL L-Glutamine (Invitrogen 11140-050)

5 mL MEM-NEAA (Invitrogen 11140-050)

5 mL Pen/Strep (Corning CellGro 30-002-CI)

3.3 uL β –ME (Sigma M7522-100 mL)

250 uL Ascorbic Acid (100 mg/mL stock in H2O, stored at -80°C)

1 mL Holotransferrin (Sigma T0665-1G; 100 mg/mL stock in IMDM, stored at -80°C)

Section A: Human Embryoid Body Production

1. Generally, one week after splitting human pluripotent stem cells (hPSCs), cells are ready to make embryoid bodies (EBs). Colonies should appear thick, dense, white and fairly free of differentiation.
2. Aspirate media from each 10 cm dish. Replace with 5 mL of 1X collagenase IV diluted in 0.22 uM filtered KO-DMEM (Gibco Cat#10829-018)

 Collagenase IV (Gibco Cat#17104-019)

 Measure out 0.5g collagenase IV and dilute with 50 mL DMEM/F12. Filter with 0.22 uM steriflip (500mg/50mL = 10X). Before use, dilute 10X collagenase with KO-DMEM to 1X (1mg/mL).

1. Incubate at room temperature in the hood for 5 minutes.
2. Aspirate collagenase IV and replace with 5 mL of filtered KO-DMEM.
3. Using a cell lifter, scrape colonies only once, taking care to preserve the entire colony. Dip the cell lifter in media after each strip to wash colonies stuck to the edge of the cell lifter
4. With the pipettor set on the slow setting, gently and slowly transfer the cells to a Falcon-15 tube using a 5 mL pipet. Pool 2 starting plates of hESCs to one Falcon-15. If there are residual colonies, wash plates with additional 5 mL and add to same Falcon-15.
5. Allow cells to settle in the Falcon-15 by gravity (~3-5 minutes). Aspirate the supernatant to get rid of most of the MEFs that remain in suspension. Gently add ~10 mL of KO-DMEM to wash cells, and spin down 1000 rpm for 1 min.
6. While cells are spinning, add 11 mL of hEB media to low-adherent 10 cm dishes.
7. Aspirate the media and resuspend cells gently with 1 mL of hEB media.
8. Using the p1000 wide bore tips, gently transfer the 1 mL of cells to each low-adherent 10 cm containing hEB media. Using the same p1000 wide bore tip, pipet up 1 mL from an area on the plate without any cells and wash the Falcon-15. Add back the 1 mL to the Falcon-15; final volume of each plate now containing 2 starting plates of hPSCs is 12 mL.
9. Transfer to incubator and place hEB plates on rotator at 37°C. 4-5 plates can be stacked on top of one another, with one plate filled with PBS at the bottom of the stack to prevent evaporation. This is day 0 of hEB culture.
10. Day 1- change media of hEB plates. With pipettor on slow settings, use a 10 mL pipette and transfer hEBs to a Falcon-15. Wash plate with a few mLs of filtered KO-DMEM to collect leftover hEBs and pool to same Falcon-15. Allow hEBs to setting by gravity (~3-5 mins) and aspirate the supernatant. Gently wash the hEBs with 10 mLs of filtered KO-DMEM and allow to settle by gravity (~3-5 mins). Prepare 12 mLs hEB media supplemented with the following cytokines per plate:

SCF (Peprotech 300-07) 200 ng/mL

FLT3 Ligand (Peprotech 300-19) 200 ng/mL

IL3 (Peprotech 200-03) 10ng/mL

IL6 (Peprotech 200-06) 20 ng/mL

GCSF (R&D 214-CS-025) 50 ng/mL

BMP4 (R&D 314-BP-010) 50 ng/mL

Resuspend the hEBs with 1 mL of hEB media + cytokines. Add 11 mL of hEB media to a low-adherent 10 cm dish. Using the wide bore tips, gently transfer the hEBs to the 10 cm dish. Using the same p1000 wide bore tip, pipet up 1 mL from an area on the plate without any cells and wash the Falcon-15. Return to rotator at 37°C.

1. Change media + cytokines on days 5 and 9. Dissociate hEBs on day 14.

Section B: Human Embryoid Body Dissociation

1. On day 14, collect and transfer hEBs using a 10 mL pipet to a Falcon-15. Wash plate with 5 mL of 0.22 uM filtered IMDM and add to same Falcon-15. Spin down cells at 1300rpm 5 mins.

Aspirate and resuspend in 5 mL of 1X collagenase B diluted in IMDM.
Stock is 10X (10 mg/mL). Dilute 1 mL of stock in 9 mL of IMDM (1mg/mL). Use 5 mL 1X collagenase B per plate.

1. Use p1000 and pipet vigorously. Place Falcon-15 laying horizontally on the rotator and allow to shake for 2-2.5 hours at 37**°**C. Pipet vigorously with a p1000 every 20 minutes to help break up EBs. EBs should mostly dissociate into small particles.
2. Refill Falcon-15 with IMDM to the top of the tube. Spin down at 1300rpm 5 mins.
3. Aspirate media and resuspend in 2 mL of 0.22 uM filtered enzyme-free dissociation buffer (Millipore Cat#S-014-C). Pipet using a p1000 thoroughly.
4. Place in 37**°**C water bath for 15 mins. EBs should be fully dissociated at this point.
5. Fill Falcon-15 all the way up with 2% FBS/PBS(no calcium, no magnesium). Pass through a 70 uM strainer and collect in a Falcon-50. Transfer the filtered cells back to a Falcon-15 to visualize the pellet more easily and spin at 1300 rpm for 5 mins.
6. Aspirate and resuspend cells in 200 uL (or an appropriate, convenient volume for counting) of 0.22 uM filtered 2% FBS/PBS. Count cells.
7. Add up to 300 uL of filtered 2% FBS/PBS (total volume of 500 uL) and add 500 uL of 0.22 uM filtered 2X freezing media (FBS + 20% DMSO) to freeze down cells in a final volume of 1 mL. Mix by pipetting with p1000 1-2X and label number of cells. Place in a room temperature Mr. Frosty and freeze at -80**°**C. Transfer to -160**°**C liquid nitrogen tank.

**Lentiviral Infection of Human Embryoid Body-Derived Haematopoietic Progenitor Cells (hEB-HPCs)**

hEB-HPC Infection Media
StemSpan Serum Free Expansion Medium (SFEM, StemCell Technologies # 09650)

supplement with:

IL3 10ng/mL (Peprotech)

IL6 50 ng/mL (Peprotech)

TPO 50 ng/mL (R&D)

SCF 50 ngmL (R&D)

FLT3 ligand 50 ng/mL (R&D)

1% Penicillin/Streptomycin

Section A: Thawing Haematopoietics

1. Spray down vortexer with 70% EtOH and bring into hood.
2. Thaw vial of hEB-HPCS at 37°C until a small pellet of ice remains.
3. Using a p1000, transfer cells to a Falcon-50 conical tube.
4. Wash vial with 1 mL of warm IMDM. With the vortexer set on the lowest rotational speed that allows mixing cells evenly, add the 1 mL of media to CB in a dropwise manner. The slower the drops are added, the better the recovery.
5. Using a 10 mL pipette, slowly add 9 mL of warm IMDM in a dropwise manner to CB on the vortexer.
6. Spin down cells at 1100 rpm for 10 minutes.
7. Aspirate and resuspend in 300 uL of 0.22 uM filtered 2% FBS/PBS.

Section B: MACS Enrichment of CD34+ Cells (Miltenyi Biotec 130-046-702, as per manufacturer’s protocol)

1. Add 100 uL of FcR Blocking Reaging for up to 10^8 cells.
2. Add 100 uL of CD34 MicroBeads for up to 10^8 cells.
3. Mix well and incubate for 30 mins on ice (2-8°C).
4. Wash cells by adding 5-10 mL of 2% FBS/PBS for up to 10^8 cells and centrifuge 1300 rpm x 5 mins. Aspirate supernatant completely.
5. Resuspend up to 10^8 cells in 500 uL 2% FBS/PBS.
6. Prepare LS column by rinsing with 3 mL of 2% FBS/PBS.
7. Apply the cell suspension onto the column. Collect flow-through containing unlabeled cells.
8. Wash the LS column 3 times with 3 mL 2% FBS/PBS each wash.
9. Remove the column from magnetic board and place on a Falcon-15 conical.
10. Pipet 5 mL of 2% FBS/PBS into the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
11. Spin down enriched CD34+ cells at 1300 rpm x 8 mins.
12. Resuspend in an appropriate volume of 2% FBS/PBS. Count cells and record recovery.

Section C: Lentiviral Infection of CD34+ hEB-HPCs

1. Prepare fresh infection media + cytokines.
2. Spin down sorted cells at 1700 rpm for 8 minutes. Aspirate and resuspend in 200 uL of hEB-HPC infection media.
3. Take a 2 uL aliquot and dilute with trypan blue accordingly. Count cells and record post-sort counts.
4. In a 96-well non-tissue culture treated, retronectin-coated (CH296, Takara Cat# T100B) (10ug/cm2) plate, combine:
 30,000-50,000 cells/well
 high-titer lentivirus at: HOXA9 MOI 5, ERG MOI 5, RORA MOI 3, SOX4 MOI 3,

MYB MOI 3, shRNA MOI 2
 Fresh hEB-HPC Media + cytokines + protamine sulfate up to total volume of 150 uL per well
\*Depending on the total volume of virus added, may need to add hEB-HPC media with higher (e.g. 2X) cytokine concentrations to adjust the final concentration of cytokines per infection to the same as in 1X hEB-HPC media.
Fill surrounding wells with sterile PBS to prevent evaporation.

1. Seal plate with parafilm and spin down 2300 rpm at 25°C for 30 mins. Remove parafilm and infect 24-36 hours at 37°C.
2. Wash off virus and culture in hEB-HPC media + cytokines.

**Culturing Human Embryoid Body-Derived Haematopoietic Progenitor Cells (5F cells)**

5F cell media
StemSpan Serum Free Expansion Medium (SFEM; StemCell Technologies # 09650)

1% Penicillin/Streptomycin

Filter through a 0.22 uM filter.

Keep at 4°C.

Then, supplement with cytokines (final concentration):

IL3 10ng/mL (Peprotech)

IL6 50 ng/mL (Peprotech)

TPO 50 ng/mL (R&D)

SCF 50 ngmL (R&D)

Flt3 50 ng/mL (R&D)

Doxycycline (Dox) 2 ug/mL (Sigma)

Puromycin: 0.3 ug/mL (add if cells have puromycin-selectable shRNA stably integrated) (ThermoFisher)

Section A: Culturing 5F cells

1. Replate cells in 5F cell media plus cytokines, Dox and puromycin in a 6 well plate, at a density of <2 million cells/well. Count and replate cells in freshly prepared media every ~4 days.

**Differentiation of Human T Cells on OP9-DL1 Stroma**

Adapted from Holmes and Zúñiga-Pflücker, *Cold Spring Harb Protoc*, 20092

OP9-DL1 media

Powder α-MEM (Gibco REF: 12000-022)

20% FBS (non-heat inactivated)

1% Penicillin/Streptomycin/L-Glutamine

1. Seed OP9-DL1 cells onto 6-well NUNC plates.
2. To passage: Prepare a 40 um filter and a single Falcon-50 conical tube for each well.
3. Using P1000, gently pipet up and down all around the well to dislodge cells from stroma; do not disrupt the stroma at this point.
4. Pipet the suspension through the filter
5. Add 2 ml of fresh media into the well, and pipet up and down as before with more force; small pieces of stroma should come off. Pipet through the filter.
6. Add 2 ml of fresh media into the well, and pipet forcefully lifting off stroma; pipet enough times to break up the stroma into visible pieces but not into single cells. Pipet through the filter.
7. Wash the filter with 2 ml of fresh media. The tube should contain about 10 ml of media. Repeat with new tube for each of the wells.
8. Can transfer cells to a Falcon-15 conical tube to visualize the pellet more easily. Spin down 1500 rpm for 5 min.
9. Resuspend in 3 ml media media with a pre-screened batch of serum + 30 ng/mL SCF (R&D), 5 ng/ml FLT3 (R&D), 5 ng/nl IL-7 (R&D) and Dox 2 ug/mL (see point 12).
10. Plate suspension onto a pre-seeded OP9-DL1 well (>90% confluent).
11. Replate onto fresh stroma every 5-7 days.
12. 5F cells are maintained on +Dox for the first 20 days of differentiation, then Dox is no longer added during the last 15 days of differentiation.
13. At the end of 35 days differentiation, harvest cells for flow cytometric analysis by pipetting and filtering supernatant and stroma through a 40 uM strainer.

**Differentiation of Human B Cells on MS-5 Stroma**

Adapted from Nishihara et al*. Eur J Immunol,* 19983

MS-5 Base Media

Myelocult H5100 (Stemcell Technologies Cat# 05150)

1% Penicillin/Streptomycin

+Cytokines

50 ng/mL SCF (R&D)

10 ng/mL FLT3 (R&D)

25 ng/mL IL7 (R&D)

25 ng/mL TPO (R&D)

Doxycycline (Dox) 2 ug/mL (Sigma)

Section A: MS5 Expansion/Freezing aliquots

1. Thaw Myelocult H5100 in 37°C water bath. Make 50mL media aliquots and freeze at -20°C. Prior to use, thaw one aliquot of media in water bath and ensure that the majority of salts are dissolved but be sure to not overheat the media. Add 1% pen/strep and filter through a 0.22 uM steriflip filter.
2. Thaw 1 vial of MS-5 into four 6-well NUNC plates that have been gelatinized for at least 20 mins.
3. Allow 4-5 days for MS5 to expand. Then freeze down 2 aliquots per plate. Try to keep the MS5 stromal cells as low passage as possible, as higher passage cells have a propensity to differentiate into adipocytes.

Section B: B cell differentiation

1. MS5 stroma can be directly thawed into any size gelatinized NUNC plates (96w, 6w, etc) depending on assay.
2. For population-based B cell differentiation, plate a confluent plate of MS5 onto a gelatinized 6w NUNC plate. For clonal analysis, plate into 96w NUNC plates and perform single-cell sorting directly onto confluent stroma. \*Once the stroma is plated, it will remain for the entirety of the 5-6 week differentiation – do not repassage/disturb the stroma\*
3. Plate 5x104 hPSC-derived CD34+ cells or ~1K cord blood CD34+ cells per well of a confluent 6well plate using 3 mL of MS5 media + cytokines.
4. Once plated, perform ½ media changes (pipet up 1-1.5mL of media and discard) every 4-5 days. \*note: cells will proliferate a lot as they differentiate. Try to keep the density of cells low because differentiated macrophages will chew up stromal cells, hence, discard half of the media supernatant containing myeloid cells every 4-5 days\*
5. Replace media+cytokines by adding back 1-1.5mL slowly on the side of the well, so to not disturb the stroma.
6. For differentiation of 5F cells, +Dox is only added to the media for the first 10 days of differentiation.
7. Differentiate for 5-6 weeks. B cells will appear around week 4-5. B cells will migrate under the stroma, and will appear muted. Differentiated myeloid cells will remain in the supernatant/loosely interacting with stromal layer. Lineages that are present should include: CD19+ B cells, CD56+ NK cells, CD33+ myeloid cells.
8. After 5-6 weeks of differentiation, harvest cells for flow cytometric analysis by pipetting and filtering supernatant and stroma through a 40 uM strainer.

**Flow Cytometry**

The following antibodies were used for human cells: For hEB-HPC panel: CD34–PE–Cy7 (8G12, BD Biosciences), CD45–PE–Cy5 (IM2652U, Beckman Coulter Immunotech), DAPI.

For T cell panel: CD45 APC–Cy7 (557833, BD Biosciences), CD4 PE–Cy5 (IM2636U, Beckman Coulter Immunotech), CD8–BV421 (RPA-T8, BD Horizon), CD5–BV510 (UCHT2, BD Biosciences), TCR–APC (555718, BD Biosciences), TCR–BV510 (T10B9.1A-31, BD Biosciences), CD3–PE–Cy7 (UCHT1, BD Pharmigen), CD7–PE (555361, BD Pharmigen), CD1a–APC (559775, BD Pharmigen).

For B cell panel: CD45–PE–Cy5 (IM2652U, Beckman Coulter Immunotech), CD19–PE (4G7, BD Biosciences), CD56–V450 (B159, BD Biosciences), CD11b–APC–Cy7 (557754, BD Biosciences).

All stains were performed with <1 × 106 cells per 100 µl staining buffer (PBS plus 2% FBS), with a 1:100 dilution of each antibody, for 30 min at room temperature in the dark. Compensation was performed by automated compensation with anti-mouse Igk and negative beads (BD Biosciences). All acquisitions were performed on BD Fortessa or BD Aria cytometer.

**References**

1 Chadwick, K. *et al.* Cytokines and BMP-4 promote haematopoietic differentiation of human embryonic stem cells. *Blood* **102**, 906-915 (2003).

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3 Nishihara, M. *et al.* A combination of stem cell factor and granulocyte colony-stimulating factor enhances the growth of human progenitor B cells supported by murine stromal cell line MS-5. *Eur J Immunol* **28**, 855-864 (1998).