Severe congenital neutropenia (CN) is a pre-leukemic bone marrow failure syndrome that can progress to acute myeloid leukemia (CN/AML). Patient material to study leukemogenesis, especially hematopoietic progenitor cells (HPCs) is limited and hard to access. We have established a protocol for generation of HPCs from iPSCs followed by HPC expansion on Sl/Sl feeder cells expressing FLT3L. We performed drug treatment of iPSC-derived HPCs on feeder cells or under feeder-free conditions. Our protocol is also suitable for primary leukemia blasts.

Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol
Generation, expansion, and drug treatment of hematopoietic progenitor cells derived from human iPSCs

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SUMMARY
Severe congenital neutropenia (CN) is a pre-leukemic bone marrow failure syndrome that can progress to acute myeloid leukemia (CN/AML). Patient material to study leukemogenesis, especially hematopoietic progenitor cells (HPCs) is limited and hard to access. We have established a protocol for generation of HPCs from iPSCs followed by HPC expansion on SI/Sl feeder cells expressing FLT3L. We performed drug treatment of iPSC-derived HPCs on feeder cells or under feeder-free conditions. Our protocol is also suitable for primary leukemia blasts.

For complete details on the use and execution of this protocol, please refer to Dannenmann et al. (2021), (2020), and (2019).

BEFORE YOU BEGIN
General information on SI/Sl (FLT3L) cell line
SI/Sl is a stromal cell line derived from SI/Sl mouse strain (Hogge et al., 1996). We were using SI/Sl (FLT3L) cell line (kindly provided by C. Eaves, Vancouver, Canada) for expansion of CN and CN/AML iPSC-derived CD34⁺CD45⁺ cells and primary AML or CN/AML blasts. SI/Sl (FLT3L) cell line has been genetically engineered to produce human FLT3L to support expansion of HSCs, HPCs, LSCs and leukemic blast. To further support the expansion of these cells we add additional cytokines (as described below).

Maintenance of SI/Sl (FLT3L) cell line
Maintenance (culturing and passaging) of SI/Sl cells is also described in the manual for SI/Sl (IL-3, SCF) cell line (STEMCELL Technologies, https://cdn.stemcell.com/media/files/pis/29301-PIS_1_0_3.pdf). Every second week, G418 (Geneticin) should be added to SI/Sl (FLT3L) cell line to positively select for the retroviral infected cells producing FLT3L. SI/Sl cells require G418 at a final concentration of 0.8 mg/mL. SI/Sl cells should be passaged for maximum 20 passages or 4 months. The level of growth factors produced by SI/Sl feeder cells must be tested regularly. For this, freeze supernatants from SI/Sl cells of several culture time points and determine growth factor levels (4 ng/mL) by enzyme-linked immunosorbent assay (ELISA), i.e., human FLT3L solid-phase sandwich ELISA (Thermo Fisher Scientific).

Passaging protocol for SI/Sl (FLT3L) feeder cells
© Timing: 30 min
Sl/Sl cells should be almost confluent, 5–7 days after passage.

1. Aspirate medium.
2. Wash with warm PBS twice (4 mL/dish).
3. Add 2 mL of Trypsin (0.05%) /EDTA (0.02%).
4. Incubate 5–10 min at 37°C until cells start to detach. Control this step under microscope.
5. Add 2 mL of Sl/Sl medium (DMEM + 15% FBS) to stop trypsin and collect cell suspension into a 15 mL Falcon tube.
6. Add again 4 mL Sl/Sl medium to rinse the plates and add to collected cell suspension.
7. Centrifuge cell suspension with 300 g for 5 min at 20°C.
8. Aspirate supernatant and add 1 mL of Sl/Sl medium.
9. Count cells.
10. Seed cells in Sl/Sl medium at a density of 5–10 × 10^5 cells / 10 cm dish.
11. Incubate at 37°C, 5% CO₂.

**Preparation of mitomycin C (MMC) solution to inhibit Sl/Sl feeder cells proliferation**

© Timing: 30 min

12. Add 2 mL of dH₂O to MMC vial using syringe and needle.
13. Aliquot the solution in Eppendorf tubes (500 μL/tube with 1 mg/mL MMC).
14. Keep one aliquot at 4°C as running solution. Keep other aliquots at 20°C for further use for up to 6 months.

**Mitomycin C (MMC) treatment and freezing of Sl/Sl (FLTL3) feeder cells**

© Timing: 3.5 h

Use 70% confluent Sl/Sl (FLTL3) cells.

**Note:** Steps 18–22 should be done as quickly as possible.

15. Add 1:50 MMC 1 mg/mL to each dish (e.g., 160 μL MMC to 8 mL medium) to get a final concentration of MMC at 20 μg/mL.
16. Incubate 3 h at 37°C.
17. During this time, prepare one collagen coated 3.5 cm-dish to control successful MMC treatment (1 mL collagen, 1 h incubation at 20°C) and appropriate amount of freezing medium (DMEM + 15% FBS + 10% DMSO).
18. Collect cells using Trypsin/EDTA (0.05%) to detach cells.
19. Count cells.
20. Seed 3 × 10^5 cell on a collagen coated 3.5 cm dish and incubate at 37°C for 24 h.
21. Centrifuge again and add appropriate freezing medium to the cell pellet (1.5 × 10^6 / 500 μL freezing medium).
22. Aliquot cells in cryovials and put them in a Mr. Frosty freezing container (Thermo Fisher Scientific) that ensures a freezing rate of 1°C / minute. Put Mr. Frosty for at least 24 h to a – 80°C freezer. For long-term storage transfer aliquots to a liquid nitrogen tank.
23. Check control dish after 2–3 days. Cells should stop to proliferate.

**Aliquoting of Geltrex (an extracellular matrix) stock solution**

© Timing: 30 min

24. Thaw 5 mL Geltrex vial for 24 h at 4°C.
25. Prepare 20 mL DMEM-F12 with 1% Penicillin-Streptomycin and keep it on ice.
26. Take defreezed 5 mL Geltrex vial and transfer immediately to cold 20 mL DMEM-F12.
27. Mix gently several times (avoid air bubbles) with 10 mL pipette on ice.
28. Distribute 300 μL/vial to 1.5 mL Eppendorf tubes.
29. Freeze immediately at −20°C.
30. This will be your 1:5 prediluted solution for 1 × 6 well plate (6 mL).

Geltrex coating of 6-well plates

© Timing: 1.5 h

31. Take out one vial of 1:5 prediluted Geltrex solution.
32. Put on ice for defreezing for 1 h.
33. Pipette 6 mL DMEM F12 + 1% Penicillin-Streptomycin in a 15 mL Falcon tube.
34. Transfer with a 1,000 μL pipette Geltrex stock solution into your DMEM vial.
35. Mix Geltrex solution gently with a 5 mL pipette. Geltrex solution is now diluted 1:100 and ready to use.
36. Pipette Geltrex solution immediately to a 6 well plate, 1 mL/well.
37. Keep at 37°C, 5% CO2 for 1 h or put it in +4°C for 24 h (parafilm sealed!).

Aliquoting of MethoCult medium for CFU assay

© Timing: 1 h

38. Thaw MethoCult medium for 24 h at 2°C–8°C.
39. Shake vigorously for 1–2 min and wait until bubbles are disappeared.
40. Use a 5 mL Luer-Lock syringe attached to a 16-gauge Blunt-End needle to dispense MethoCult medium into 5 mL sterile FACS tubes.
41. If you plan to use MethoCult H4435, dispense 3 mL per tube for 1.1 mL duplicate cultures, or 4 mL per tube for 1.1 mL triplicate cultures.

Alternatives: If you plan to use MethoCult H4230, dispense 2.4 mL per tube for 1.1 mL duplicate cultures, or 3.2 mL per tube for 1.1 mL triplicate cultures.

Note: Do not expel the medium to the “0” mark on the syringe when aliquoting. For example, measure from 4 mL to 1 mL rather than 3.0 mL–0 mL.

42. Tubes of complete medium can be used immediately, stored at 2°C–8°C for up to 1 month, or stored at −20°C for at least until expiration date. After thawing aliquoted tubes of MethoCult, mix well and use immediately. Do not re-freeze.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | | |
| D-MEM F12 | Sigma | Cat# D6421 |
| KnockOut SR | Gibco | Cat# 10828-028 |
| NEAA | Gibco | Cat# 11140-035 |
| GlutaMAX | Gibco | Cat# 35050061 |
| 2-Mercapto Ethanol | Gibco | Cat# 31350-010 |

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### MATERIALS AND EQUIPMENT

#### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Penicillin/Streptomycin | Sigma | Cat# P0781 |
| PBS | Gibco | Cat# 14190-094 |
| EDTA 0.5 M | Sigma | Cat# E7889 |
| bFGF (FGF2) | PeproTech | Cat# 100-18B |
| BMP4 | R&D | Cat# 314-BP |
| VEGF | R&D | Cat# 293-VE |
| SCF | PeproTech | Cat# 300-07 |
| TPO | PeproTech | Cat# 300-18 |
| FLT3L | BioLegend | Cat# 550-606 |
| IL-3 | PeproTech | Cat# 200-03 |
| IL-6 | PeproTech | Cat# 200-06 |
| Rock-inhibitor Y-27632 | Tocris | Cat# 1254 |
| Geltrix | Thermo Fisher Scientific | Cat# A1413202 |
| STEMdiff APEL2 Medium | STEMCELL Technologies | Cat# 05270 |
| MethoCult H4435 | STEMCELL Technologies | Cat# 04435 |
| MethoCult H4230 | STEMCELL Technologies | Cat# 04230 |
| IMDM with 2% FBS | STEMCELL Technologies | Cat# 07700 |
| Antibiotic-Antimycotic | Thermo Fisher Scientific | Cat# 15240-096 |
| May-Grünwald-Solution | Merck | Cat# 101424 |
| Giemsa-Solution | Merck | Cat# 109204 |
| 0.05% Trypsin-EDTA | Gibco | Cat# 25300-054 |
| FBS | Sigma | Cat# 7524 |
| G418, Geneticin | Thermo Fisher Scientific | Cat# 10131035 |
| DMEM (1X) | Gibco | Cat# 41966-052 |
| Bovine Collagen Solution | Sigma | Cat# 804592 |
| Mitomycin C | Sigma | Cat# M0503 |
| DMSO | Sigma | Cat# D8418 |
| MyeloCult H5100 | STEMCELL Technologies | Cat# 05150 |
| Hydrocortisone 100 mg | STEMCELL Technologies | Cat# 74142 |
| CTS PSC Cryomedium | STEMCELL Technologies | Cat# A4238801 |
| Stem Span SFEM II | STEMCELL Technologies | Cat# 09655 |
| 7AAD | BD Biosciences | Cat# 559925 |

#### Antibodies

| Antibodies | Source | IDENTIFIER |
|-----------|--------|------------|
| CD34 - PE-Cy7 | BD Biosciences | Cat# 348811 |
| CD45 - BV510 | BioLegend | Cat# 304036 |

#### Experimental models: Cell lines

| Experimental models: Cell lines | Source | IDENTIFIER |
|---------------------------------|--------|------------|
| SNL-feeder cells | Public Health England | Cat# 07032801 |
| Si/Si (FLT3L) Cell Line | Connie Eaves, Terry Fox Laboratory, BC, Canada or STEMCELL Technologies | N/A contact techsupport@stemcell.com |
| Si/Si (IL-3, SCF) Cell Line | STEMCELL Technologies | Cat# 00302 |

#### Other

| Other | Source | IDENTIFIER |
|-------|--------|------------|
| Blunt-End Needles, 16 Gauge | STEMCELL Technologies | Cat# 28110 |
| Microscope slides | R. Langenbrink | Cat# 300030 |
| Mr. Frosty Freezing Container | Thermo Fisher Scientific | Cat# 5100-0001 |

#### iPSC maintenance medium

| Reagent | Source | Final concentration | Amount [mL] |
|---------|--------|---------------------|-------------|
| D-MEM F12 | Sigma | n/a | 384 |
| KnockOut SR | Gibco | 20% | 100 |
| NEAA | Gibco | 1% | 5 |

(Continued on next page)
### Continued

| Reagent                        | Source   | Final concentration | Amount [mL] |
|-------------------------------|----------|--------------------|-------------|
| L-Glutamine (200 mM)          | Gibco    | 2 mM               | 5           |
| 2-Mercapto Ethanol (50 mM)    | Gibco    | 0.1 mM             | 1           |
| Penicillin-Streptomycin       | Sigma    | 1%                 | 5           |
| **Total**                     |          |                    | **500**     |

### List of cytokines for EB-based serum-free iPSC to HPC differentiation

| Cytokine | Final concentration [ng/mL] | Source     |
|----------|----------------------------|------------|
| bFGF     | 10                         | PeproTech  |
| BMP4     | 40                         | R&D        |
| VEGF     | 40                         | R&D        |
| rh SCF   | 100                        | PeproTech  |
| rh TPO   | 50                         | PeproTech  |
| rh FLT3L | 50                         | BioLegend  |
| rh IL-3  | 50                         | PeproTech  |
| rh IL-6  | 20                         | PeproTech  |

### List of antibodies for flow cytometry analysis of iPSC-derived HPCs

| Antibody | Conjugation | Company | Volume [µL] |
|----------|-------------|---------|-------------|
| CD34     | PE-Cy7      | BD      | 5           |
| CD45     | BV510       | BioLegend | 2          |
| 7AAD     | 7AAD        | BD      | 2           |

### Types of MethoCult media, complete (H4435, enriched) or incomplete (H4230), and their usage

| Product       | Contains  | Application                          | Recommended cells                                                                 |
|---------------|-----------|--------------------------------------|-----------------------------------------------------------------------------------|
| H4435 enriched| rh SCF    | Detection of:                        | CD34* enriched and cells isolated by other purification methods from BM, CB, PB,  |
|               | rh GM-CSF | BFU-E                                | Mobilized PB (MPB)                                                                |
|               | rh IL3    | CFU-GM                               |                                                                                   |
|               | rh IL6    | CFU-GEMM                             |                                                                                   |
|               | rh G-CSF  |                                      |                                                                                   |
|               | rh EPO    |                                      |                                                                                   |
| H4230         | No additional cytokines | Allows to add cytokines of your choice | Depends on application                                                           |

### Preparation of cells in IMDM

| Cell source                  | 10× concentration to be prepared | Plating concentration (cells per 35 mm dish) |
|------------------------------|----------------------------------|----------------------------------------------|
| CD34* cells (BM, CB, MPB)    | $1 \times 10^5$ cells/mL         | 1,000                                        |
| CD34*CD45* cells (iPSC-derived) | $1 \times 10^5$ cells/mL         | 10,000                                       |

### Preparation of MethoCult H4435/cell mixture

| Reagent                     | Volume for duplicates | Volume for triplicates |
|-----------------------------|-----------------------|------------------------|
| Methocult (H4435)           | 3 mL                  | 4 mL                   |
| Cells in IMDM*              | 300 µL                | 400 µL                 |
| Antibiotic-Antimycotic      | 30 µL                 | 40 µL                  |
| **Total**                   | 3.33 mL               | 4.44 mL                |
**PREPARATION OF METHOCULT H4230/CELL MIXTURE**

| Reagent                  | Volume for duplicates | Volume for triplicates |
|--------------------------|-----------------------|------------------------|
| MethoCult (H4230)        | 2.4 mL                | 3.2 mL                 |
| Cells in IMDM*           | 300 µL                | 400 µL                 |
| Cytokines in IMDM**      | 600 µL                | 800 µL                 |
| Antibiotic-Antimycotic   | 30 µL                 | 40 µL                  |
| **Total**                | **3.33 mL**           | **4.44 mL**            |

* Cell concentration should be 10 times higher than a final concentration. 1,000 cells/dish in a final concentration corresponds to 10,000 cells/mL in IMDM.

**OVERVIEW OF CYTOKINES FOR iPSC-DERIVED CD34+CD45+ CELL EXPANSION**

| Cytokine  | Final concentration [ng/mL] | Source   |
|-----------|-----------------------------|----------|
| rh SCF    | 100                         | PeproTech|
| rh TPO    | 100                         | PeproTech|
| rh FLT3L  | 100                         | BioLegend|
| rh IL-3   | 20                          | PeproTech|
| rh IL-6   | 20                          | PeproTech|

* Cell concentration should be 5 times higher than final concentration.

**STEP-BY-STEP METHOD DETAILS**

**Generation of CN and CN/AML iPSC-derived CD34+CD45+ cells (HPCs) using embryoid body (EB)-based serum-free iPSC differentiation protocol with subsequent analysis of HPCs.**

**Note:** Our differentiation protocol is mainly designed for iPSCs maintained under feeder-free conditions on Geltrex or Matrigel using StemFlex or mTESR medium. Before EB formation, iPSCs are transferred for only one day to SNL feeder cells to synchronize cell cycle and facilitate induction of differentiation. However, it also works for iPSCs maintained on feeder cells. In this case, the transfer to feeder cells before EB formation should be skipped.

**EB-based iPSC differentiation**

**© Timing:** 16 days

1. Two days before EB formation, seed $4 \times 10^5$ mitotically inactivated MMC-treated SNL feeder cells on a 3.5 cm² cell culture dish. SNL is a ready-made immortal mouse embryonic fibroblasts (MEFs) cell line.

**Δ CRITICAL:** SNL feeder cells are helpful for efficient differentiation of many iPSC lines. However, it can be tried out to differentiate any iPSC clone first without the use of SNL feeder cells.

**Note:** Mitotically inactivated SNL feeder cells should be prepared before starting iPSC differentiation experiment as described for SI/SI feeder cells in the ‘before you begin’ section. Procedure is the same for SNL feeder cells.

2. One day before EB formation, seed $7 \times 10^5$ feeder-free iPSCs dissociated into single cell suspension from one well of a 6-well plate to a 3.5 cm² dish with SNL feeder cells in iPSC maintenance medium supplemented freshly with bFGF (30 ng/mL) and ROCK inhibitor (Y-27632 dihydrochloride) (10 nM).

**Note:** iPSC maintenance medium without bFGF or ROCK inhibitor can be stored up to 4 months at 4°C.
CRITICAL: Seeding density of iPSCs on SNL feeder cells is critical. iPSC cannot be counted again at the day of EB formation since PBS/EDTA solution for detachment of cells results in cell clumps instead of single cells. For some iPSC lines, iPSCs show no efficient attachment to feeder cells. In this case, seeding density should be increased. If seeding density varies too much, EB size will be incorrect, which results in inefficient iPSC differentiation.

3. On the day of EB formation (day 1), pre-cool centrifuge to 4°C.
4. Prepare APEL medium for EB formation. From one 3.5 cm² dish, you can produce up to 30 EBs. Prepare 3 mL of APEL medium supplemented with ROCK inhibitor (10 nM), bFGF (10 ng/mL) and BMP4 (40 ng/mL) for 30 EBs.
5. Remove iPSC maintenance medium from iPSCs and wash once with 1 mL warm PBS. Detach SNL feeder cells by pipetting gently up and down several times during washing step. SNL feeder will detach quite easily, while iPSCs stay attached. An example of iPSCs pre and post SNL feeder removal is shown in Figure 1.

CRITICAL: It is important to get rid of the majority of SNL feeder cells to obtain compact EBs. If too many feeder cells remain, EBs may be very fragile. The presence of feeder cells may also prevent proper outgrowth of endothelial cells after plating of EBs to Geltrex coated plates on day four.

6. Add 1 mL PBS/0.02% EDTA for 7 min, remove PBS/EDTA solution and detach all iPSCs in prepared APEL medium.

CRITICAL: Do not use Accutase or any other cell detachment solution in this step since it will prevent proper formation of compact EBs. Further, do not incubate cells longer than maximum 7 min in PBS/0.02% EDTA. This would also lead to fragile EBs.

7. Collect iPSCs in a Fugealcon tube and distribute 100 μL per well of iPSC suspension to a 96-well plate with conical bottom. One EB consists of approximately 20,000 cells.
8. Centrifuge plate for 5 min at 435 g and 4°C.

CRITICAL: It is necessary to centrifuge iPSCs for EBs at 4°C since it will increase efficiency of compact EB formation.

9. Incubate plate at 37°C, 5% CO₂.
10. On day three, prepare Geltrex coated 6 well plates (see described above).
11. On day four, plate EBs on Geltrex coated wells (10 EBs/well) in 2 mL per well of APEL medium supplemented with VEGF (40 ng/mL), SCF (100 ng/mL) and IL-3 (50 ng/mL).

Figure 1. Morphological examination of iPSC maintenance on- and without feeder cells
(A and B) iPSCs pre (A) and post (B) SNL-feeder removal.
CRITICAL: Do not touch EBs for at least 24 h, since it might interrupt EB attachment and outgrowth.

12. On day eight, change medium to 2 mL APEL medium supplemented with SCF (100 ng/mL), TPO (50 ng/mL), FLT3L (50 ng/mL), IL-3 (50 ng/mL) and IL-6 (20 ng/mL). Hematopoietic cells start to appear from day 8.

13. On day eleven, add 2 mL of APEL with SCF (100 ng/mL), TPO (50 ng/mL), FLT3L (50 ng/mL), IL-3 (50 ng/mL) and IL-6 (20 ng/mL).

14. On day 14, harvest floating hematopoietic cells which are mainly CD34^+CD45^+ hematopoietic progenitor cells (HPCs). This should be confirmed by the flow cytometry analysis panel of HPCs and morphological evaluation of cytospin preparations (see below).

**Note:** HPC numbers can be highly variable between various iPSC clones.

15. These HPCs can be used for various analyses like colony-forming unit (CFU) assay. Further, they can be expanded for at least 14 days on feeder cells or used for drug treatment studies on feeder cells or under feeder free conditions.

The scheme of EB-based iPSC to HPC differentiation protocol is shown in Figure 2.

**Analysis of iPSC-derived CD34^+CD45^+ cells (HPCs) by flow cytometry**

- **Timing:** 4 h

16. Harvest 6 x 10^4 floating cells from EB-differentiation system at day 14 and centrifuge for 5 min at 300 g. 3 x 10^4 cells are usually used for staining with specific antibody and 3 x 10^4 cells for unstained control.

17. Resuspend cells in 180 μL ice-cold FACS Buffer.

18. Add 90 μL of cell suspension into each FACS tube on ice.

19. Add antibodies as described in the 'List of antibodies for flow cytometry analysis of iPSC-derived HPCs'.

20. Incubate for 20 min on ice and protected from light.

△ CRITICAL: It is important to keep antibodies protected from light. Especially tandem dyes like PE-Cy7 are extremely sensitive and degrade easily.
21. Add 1 mL ice-cold FACS Buffer to wash stained cells.
22. Centrifuge 5 min at 300 g, 4°C and discard supernatant.
23. Resuspend cell pellet in 150 µL ice-cold FACS Buffer.
24. Evaluate stained cells on BD FACS Canto II analyze data with FlowJo software (BD Biosciences).
   Representative images of flow cytometry are shown in Figure 3. CD34^+ expression should be > 80%.

*Note:* Comprehensive multicolor flow cytometry analysis of iPSC-derived cells at early stages of hematopoietic differentiation was already described by us in Methods of Molecular Biology (Dannenmann et al., 2020).

**Cytospin preparations and Pappenheim Staining (May-Grünwald & Giemsa staining) of iPSC-derived HPCs**

© Timing: 2 h

25. Harvest and spin down 20,000 cells/cytospin slide from EB-differentiation system at day 14 at 300g for 5 min.
26. Prepare cytospin slides either by centrifugation using Thermo Scientific Cytospin 4 Centrifuge (4 min at 250 rpm/10 g) or add a drop of your cell suspension and let it dry, if your cells may be damaged by centrifugation.
27. Stain your cells for 5 min in May-Grünwald-Solution.
28. Rinse slides with dH2O.
29. Stain your cells for 20 min in 1:20 diluted Giemsa-Solution.
30. Rinse slides with dH2O.
31. Let slides dry for at least 1 h.
32. Take pictures of slides with any bright-field microscope using 100X objective with oil. Examples are shown in Figure 4.

*Note:* If you plan to expand iPSC-derived HPCs after analysis, CFU-Assay could be skipped (33–39). Freezing of iPSC-derived HPCs is not recommend, since survival rate after defreezing is only approximately 50%. It is recommended to perform the complete protocol without pause points.
Analysis of HPC differentiation potential: Colony forming unit assay (CFU-Assay) of iPSC-derived HPCs

© Timing: 14 days

Note: CFU-Assay (steps 33–39) can be performed in parallel with expansion of iPSC-derived HPCs (steps 40–53).

CFU-Assay is used for measurement of proliferation and differentiation ability of individual cells within a sample, in this case mainly CD34+CD45+ cells (HPCs). The potential of these cells is measured by the observation of the colonies (consisting of more differentiated cells) produced by each input progenitor cell. 14 days of culture is sufficient to allow colonies to grow to a size which allows accurate counting and identification, though shorter periods may be used in certain situations.

Colony assay protocol

33. Add cells in IMDM and Anti-Anti to the MethoCult H4435 tube as described in ‘Preparation of MethoCult H4435/cell mixture’.

Alternatives: If MethoCult H4230 is used: Add cells in IMDM, cytokines in IMDM, and Anti-Anti to the MethoCult H4230 tube as described in ‘Preparation of MethoCult H4230/cell mixture’.

34. Vortex for 30s.
35. Wait for 10 min till bubbles go up.
36. Seed 1.1 mL each into 3.5 cm dishes.
37. Put two dishes with cells and one dish filled with PBS without lid (to prevent MethoCult medium and cells from drying) in 10 cm dish.
38. Incubate at 37°C.
39. Count colonies after 14 days. Counting criteria and examples for CFU colony types are described in the MethoCult Manual from STEMCELL Technologies (https://www.stemcell.com/methocult-h4435-enriched.html). iPSC-derived HPCs form primarily three CFU colony types: CFU-granulocyte (CFU-G), CFU-granulocyte-macrophage (CFU-GM) and CFU-macrophage (CFU-M) (Figure 5).
Expansion of CN and CN/AML iPSC-derived HPCs (CD34⁺CD45⁺ cells) and primary AML or CN/AML blasts on Sl/Sl (FLT3L) feeder cells

Note: Expansion of iPSC-derived HPCs can be performed in parallel with CFU-Assay.

Thawing MMC treated Sl/Sl cells

© Timing: 20 min (thawing) + 24–48 h (incubation)

Note: MMC treated Sl/Sl cell should be thawed 1–2 days before adding iPSC-derived CD34⁺CD45⁺ cells for expansion.

40. Prepare four 3.5cm dishes coated with collagen (as described above).
41. Thaw one vial 1.5 × 10⁶ MMC treated cells in warm medium (DMEM + 15% FBS).
42. Centrifuge 5 min at 300 g.
43. Resuspend in 4 × 2.5 mL HLTM (MyeloCult H5100) medium with 10⁻⁶ M Hydrocortisone and 1% Penicillin-Streptomycin.
44. Pipet 2.5 mL cell suspension / dish.
45. Leave for 24 h in the incubator before seeding cells for expansion.

Note: Since iPSC-derived CD34⁺CD45⁺ cells proliferate more slowly than primary CD34⁺ cells, it is recommended to culture these cells on 24-well plates. For this purpose, thaw 1.5 × 10⁶ Sl/Sl cells on the inner 8 wells of a 24-well plate and fill the outer wells with PBS.

Protocol for expansion of iPSC-derived CD34⁺CD45⁺ cells on Sl/Sl (FLT3L) feeder cells

© Timing: 1 h (seeding, medium preparation) + up to 14 days for expansion

46. Prepare HLTM medium supplemented with Hydrocortisone (10⁻⁶ M) and cytokines freshly before adding cells for expansion as described in ‘Overview of cytokines for iPSC-derived CD34⁺CD45⁺ cell expansion’.
47. Seed 1–2 × 10⁵ iPSC-derived CD34⁺CD45⁺ cells/well of a 24 well-plate with Sl/Sl (FLT3L) feeder cells in 0.5 mL HLTM medium supplemented with cytokines (see materials and equipment section). Depending on the cell number needed for later experiments, you can scale up well-size to 12 well or 6 well plate. Cells double in average every 2–3 days.
48. Add 500 µL HLTM supplemented with Hydrocortisone (10⁻⁶ M) and cytokines (see materials and equipment section) twice per week or change medium completely.
49. Expand cells to a maximum density of 2 × 10⁶ cells/mL. This density is typically reached after 10–14 days depending on starting cell number and iPSC-clone.

Figure 5. Examples for CFU-G, CFU-GM, and CFU-M of iPSC-derived HPCs after 14 days

Expansion of CN and CN/AML iPSC-derived HPCs (CD34⁺CD45⁺ cells) and primary AML or CN/AML blasts on Sl/Sl (FLT3L) feeder cells

Note: Expansion of iPSC-derived HPCs can be performed in parallel with CFU-Assay.
Note: CN-iPSC derived CD34⁺CD45⁺ cells expand well for at least 2 weeks, CN/AML-iPSC derived CD34⁺ cells for more than 4 weeks.

50. CD34⁺ cells on Sl/Sl feeder cells are partially attached at the bottom in a quite loose fashion or in suspension. CD34⁺ cells can be harvested by gently pipetting up and down several times without detaching Sl/Sl feeder cells.

51. Harvested CD34⁺ cells can be directly used for further experiments (e.g., terminal differentiation to mature hematopoietic cells, RNA-seq, CFU-assay) when desired cell number is reached, or can be frozen using CTS PSC freezing medium, which is a single cell & stem cell freezing medium that ensures high cell survival rate after thawing. Depending on the desired downstream analysis and the percentage of CD34 expression after expansion, enrichment of CD34⁺ cells by fluorescence-activated cell sorting (FACS) could be performed.

Note: The expansion protocol described above can be also applied for primary AML blasts or CN/AML blast. Whereas expansion of iPSC-derived HPCs is possible for most iPSC lines for only 14 days, primary AML or CN/AML blasts can be expanded with our Sl/Sl (FLT3L) feeder system for several months.

Quality control of expanded CD34⁺CD45⁺ cells by flow cytometry analysis

⊙ Timing: 4 h

52. Analyze your iPSC-derived CD34⁺CD45⁺ cells before expansion on Sl/Sl feeder when you harvest them from your iPSC differentiation system by flow cytometry.

53. Perform flow cytometry analysis every 2–3 days to monitor your proliferation experiment and quality of HPCs.

Note: If your cells in expansion still show a high proliferation rate, they should express CD34. If proliferation rate slows down, you will notice a decrease in CD34 expression. To monitor proliferation rate, count cell number every 3–4 days.

Drug treatment of primary AML or CN/AML blasts and CN/AML iPSC-derived CD34⁺CD45⁺ cells on Sl/Sl (FLT3L) feeder cells

⊙ Timing: up to 7 days

Treatment of primary AML or CN/AML blasts and CN/AML iPSC-derived CD34⁺CD45⁺ HPCs on Sl/Sl feeder (FLT3L) cells with small molecules or other drugs is recommended if primary AML and CN/AML blasts do not expand under feeder-free conditions or if the treatment of blasts is planned as a long-term experiment (> 2 weeks).

54. Before you start to apply any drug to your co-culture system, test its toxicity for Sl/Sl feeder (FLT3L) cells first. If feeder cells are undergoing apoptosis, try feeder free conditions.

55. Expansion medium preparation and seeding of cells for expansion long-term culture. Add the drug you want to test to your expansion medium.

56. Small molecule/drug should be refreshed depending on its half-life. For most small molecules it is recommended to refresh half of the expansion medium with freshly added drugs every 3 days.

57. If your cells expand slowly, it is also sufficient to add fresh medium with your drug by changing a half of the medium once a week.

58. Check viability of your feeder cells regularly.

59. For counting of cell number, pipet gently up and down culture medium to mix cells evenly without detaching feeder cells. Use the cell counting device of your choice.
Drug treatment of CN/AML iPSC-derived CD34+CD45+ cells under feeder-free conditions

@ Timing: up to 7 days

If drug treatment of CN/AML iPSC-derived CD34+CD45+ cells on Sl/Sl (FLT3L) feeder cells is leading to apoptosis of feeder cells, it is recommended to perform drug treatment under feeder free conditions. However, not many samples of CD34+CD45+ cells may expand under feeder-free conditions.

60. Prepare serum free CD34+ expansion medium (Stem Span SFEM II medium supplemented with cytokines, see materials and equipment section) freshly before adding your cells for expansion.
61. Add your drug in the concentration needed.
62. Seed 1–2 x 10^5 iPSC-derived CD34+CD45+ cells/well of a 24 well-plate in 1 mL CD34+ expansion medium.
63. Small molecule /drug should be refreshed depending on its half-life. For most small molecules it is recommended to refresh half of the expansion medium with freshly added drugs every 3 days.
64. If your cells expand slowly it is also fine to add medium fresh medium with your drug and change half of the medium once per week.
65. For counting of cell number, pipet gently up and down cells suspension to mix cells evenly. Using the counting device of your choice.

Alternatives: You can timely monitor your experiment using Live Cell Imaging System IncuCyte (Sartorius).

EXPECTED OUTCOMES
The successful differentiation of hiPSCs to HPCs is monitored by CD34 and CD45 surface marker expression measured by flow cytometry (Figure 3) and morphological analysis (Figure 4). The proliferation potential of iPSC-derived HPCs is tested by expansion on Sl/Sl (FLT3L) feeder cells for up to 14 days, whereas the differentiation potential is measured by CFU assay. Using our approach of iPSC to HPC differentiation and subsequent expansion (up to 10-fold/week) of HPCs, we solve the problem of limited numbers of HPCs for translational research. iPSC-derived HPCs can be used for several further applications like RNA-sequencing, ATAC-sequencing, drug screening and many more. iPSC-derived HPCs can also be terminally differentiated to neutrophils or any other blood lineages which was described by our group in Methods of Molecular Biology 2115 (Dannenmann B, Nasri M et al. Methods Mol Bio. 2020. Chapter 27) (Dannenmann et al., 2020).

LIMITATIONS
Our protocol described above allows only short-term expansion (< 14 days) of iPSC-derived HPCs. Maximum expansion time highly dependent on iPSC clone and underlying disease. We were able to expand CN-iPSC-derived HPCs for approximately 2 weeks, whereas CN/AML-derived HPCs were expandable for at least 4 weeks. Besides, we noticed that CFU potential drops over time during expansion phase. Further optimization of protocols for iPSC-differentiation or HPCs expansion may be further optimized to enable an extended long-term culture of HPCs derived from different types of iPSC-lines independent of genetic background and disease.

Our Sl/Sl (FLT3L) feeder cell co-culture system can only be used for drug treatment experiments if drugs are not affecting feeder cell survival. If drugs are killing Sl/Sl (FLT3L) feeder cells, experiment should be performed under feeder-free conditions. Main advantages of feeder-free conditions are firstly that this system is cleaner, because feeder cells might uptake most of the drugs, and secondly that proliferation can be monitored using an automated cell counter, for instance IncuCyte (Sartorius). Nonetheless, feeder free cell culture is not recommended for long-term cell culture (> 2 weeks).
TROUBLESHOOTING

Problem 1
EBs are not compact and degrade during plating step (step 11).

Potential solution
Too many remaining SNL feeder cells could interrupt formation of compact EBs. Try to get rid of most feeder cells. If EBs are too big (> 25,000 cells) they also tend to degrade. Try to decrease EB size. Therefore, seed less iPSC on SNL feeder cells before EB generation.

Problem 2
EBs do not outgrow (step 11).

Potential solution
Too many remaining SNL feeder cells in EBs also prevent EBs from outgrowing since feeder cells outgrow faster than iPSCs. Try to seed iPSCs on less SNL feeder cells (e.g., 2–3 \times 10^5).

Problem 3
HPCs counts are low for a specific iPSC clone (step 14).

Potential solution
Differentiate additional iPSC clones from the same patient.

Problem 4
Staining of cells on cytospins is very weak (step 32).

Potential solution
Repeat staining for these cells and increase staining time or prepare fresh staining solutions. If you store cytospins for months-years you might also repeat staining procedure.

Problem 5
CFU counts are low (below 50 colonies) (step 39).

Potential solution
Increase seeding density of HPCs for CFU assay to 15,000 cells /dish or harvest iPSC-derived HPCs earlier from EB-system (e.g., day 10–12). Kinetics of EB-based iPSC differentiation can vary between iPSC clones and patients.

Problem 6
HPCs tend to become adherent during expansion (step 49).

Potential solution
Transfer HPCs to new SI/SI (FLT3L) feeder cells.

Problem 7
Proliferation rate of HPCs is very low or decreases over time rapidly (step 49).

Potential solution
Do not expand HPCs with too high or too low density. If iPSC-derived HPCs are seeded at very low density they might not proliferate. To solve this problem, increase starting cell number before expansion. Do not overgrow expansion cultures, since this will decrease proliferation potential and induce differentiation.
RESOURCES AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact: Julia Skokowa, Julia.Skokowa@med.uni-tuebingen.de.

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate or analyze datasets or code.

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AUTHOR CONTRIBUTIONS
B.D. wrote the protocol, made the figures, and graphical abstract. J.S. reviewed, edited, and corrected the protocol. B.D. and J.S. designed the study. J.S. supervised the study and secured funding.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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