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# Reconstruction of Human AML Using Functionally and Immunophenotypically Defined Human Haematopoietic Stem and Progenitor Cells as Targeted Populations

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**[Abstract]** Acute myeloid leukaemia (AML) is a highly heterogenous blood cancer, in which the expansion of aberrant myeloid blood cells interferes with the generation and function of normal blood cells. Although key driver mutations and their associated inhibitors have been identified in the last decade, they have not been fully translated into better survival rates for AML patients, which remain dismal. In addition to DNA mutation, studies in mouse models strongly suggest that the cell of origin, where the driver mutation (such as MLL fusions) occurs, emerges as an additional factor that determines the treatment outcome in AML. To investigate its functional relevance in human disease, we have recently reported that AML driven by MLL fusions can transform immunophenotypically and functionally distinctive human hematopoietic stem cells (HSCs) or myeloid progenitors resulting in immunophenotypically indistinguishable human AML. Intriguingly, these cells display differential treatment sensitivities to current treatments, attesting the cell of origin as an important determinant governing treatment outcome for AML. To further facilitate this line of investigation, here we describe a comprehensive disease modelling protocol using human primary haematopoietic cells, which covers all the key steps, from the isolation of immunophenotypically defined human primary haematopoietic stem and progenitor populations, to oncogene transfer via viral transduction, the *in vitro* liquid culture assay, and finally the xenotransplantation into immunocompromised mice.

**Keywords:** AML, MLL fusion, HSPC, CD34, Human disease modelling

**[Background]** Acute myeloid leukaemia (AML) is a highly heterogenous blood cancer driven by diverse mutations and distinctive cell populations, which is generally associated with poor prognosis in particular for older patients aged over 60. Recent research efforts have not only brought to light key AML driver mutations but also greatly enhanced our mechanistic understanding of how these mutations transform normal blood cells into leukaemia cells (Zeisig *et al.*, 2012). Despite this progress, the survival rates of most AML patients have only marginally improved and remain dismal (Shallis *et al.*, 2019).

Disease modelling is a powerful tool to study cancer biology, as it allows the researchers to readily access bona fide diseased cells for subsequent cellular and molecular studies. Mouse models have been particularly instrumental given the easy accessibility and well-established protocols for genetic manipulation, *in vitro,* and *in vivo* propagation of the cells. They have provided important insights into the biology of AML cells transformed by key driver mutations including MLL fusions and revealed

promising novel therapeutic targets (Almosailleakh and Schwaller, 2019; Zeisig and So, 2021). Murine disease models also led to the identification of candidate leukaemia cell of origins and their potential roles in influencing treatment responses (Krivtsov *et al.*, 2013; Stavropoulou *et al.*, 2016; Siriboonpiputtana *et al.*, 2017). While human and mouse cells share significant similarities, there are also clear differences including their contrasting transformation requirements, telomere lengths, and the non-coding regulatory genomes. As compared with mouse cell models, reconstruction of human AML using human primary cells has significantly laged behind (Barabé *et al.*, 2007; Horton *et al.*, 2013; Wunderlich *et al.*, 2013). Until recently, it was not clear if human AML can also originate from multiple cells of origin, which may have different biology and treatment responses. Using immunophenotypically and functionally defined hematopoietic populations isolated from umbilical cord blood as the targeted cells to reconstruct MLL-AML, we have shown that human HSCs and common myeloid progenitors (CMPs) can be the cellular origins for MLL-AML, in which HSC-derived MLL-AML with a different transcriptional programme is more resistant to current chemo treatment than their myeloid progenitors-derived counterparts (Zeisig *et al.*, 2021). Here we describe in detail the experimental protocols for reconstruction of human MLL-AML using functionally and immunophenotypically defined hematopoietic cell populations as leukemia cells-of-origin.

## Materials and Reagents

1. Antibodies (HSPC):
   CD34 (clone: 581; APC-Cy7)
   CD38 (clone: HB-7; FITC)
   CD90 (clone: 5E10, PE)
   CD123 (clone: 6H6;PE-Cy7)
   CD45RA (clone: HI100,Pacific Blue)
2. Antibodies (Lineage) (all conjugated to the same fluochrome, *e.g.*, PE-Cy5):
   CD2 (clone: RPA-2.10)
   CD3 (clone: S4.1)
   CD4 (clone: S3.5)
   CD7 (clone: CD7-6B7)
   CD8 (clone: 3B5)
   CD10 (clone: MEM-78)
   CD11b (clone: ICRF44)
   CD14 (clone: HCD14)
   CD19 (clone: HIB19)
   CD20 (clone: 2H7)
   CD56 (clone: B159)
   CD235a (clone: GA-R2)
3. CD34 MicroBead Kit UltraPure (Miltenyi Biotec, catalog number: 130-100-453)

4. Cell Strainer 40 µm (Greiner Bio-One, catalog number: 542040)
5. Dulbecco's Modified Eagle Medium (DMEM) (Gibco, catalog number: 41966-029)
6. Fetal Bovine Serum (FBS) (Sigma, catalog number: F7524)
7. Ficoll-Paque Plus (GE Healthcare, catalog number: 17-1440-03)
8. Fresh cord blood or adult BM
9. Gag/pol plasmid (Addgene, plasmid number: 14887)
10. HEK293T cells (ATCC, catalog number: CRL-11268573)
11. Human cytokines:
    Human IL-6, premium grade (Miltenyi Biotec, catalog number: 130-093-932);
    Human IL-3 premium grade (Miltenyi Biotec, catalog number: 130-095-069);
    Human SCF, premium grade (Miltenyi Biotec, catalog number: 130-096-695);
    Human Flt3-Ligand, premium grade (Miltenyi Biotec, catalog number: 130-096-479);
    Human TPO, premium grade (Miltenyi Biotec, catalog number: 130-108-339);
    Prepare 10 µg/ml stock solutions in sterile filtered PBS + 0.1% FBS.
12. Isocove's Modifed Dulbecco's Medium (IMDM) (Gibco, catalog number: 31980-022)
13. LS Columns (Miltenyi Biotec, catalog number: 130-042-401)
14. Minisart Filter unt 0.45 µm (Sartorius, catalog number: 16555-K)
15. MSCV plasmid (TaKaRa, catalog number: 634401)
16. Needles: 27G (BD, catalog number: 300635), 29G (BD, catalog number: 324824)
17. NSG mice (The Jackson Laboratory, catalog number: 05557)
18. OneComp eBeads Compensation beads (Thermo Scientific, catalog number: 01-1111-41)
19. Penicillin/Streptomycin (P/S) (Sigma, catalog number: P4333)
20. pMSCV-MLL-AF6, pMSCV-MLL-ENL (request to: eric.so@kcl.ac.uk)
21. Polybrene infection/transfection Reagent (10 mg/ml) (Merck, catalog number: TR-1003-G)
22. Polyethylenimine (PEI) 25kD linear ; Polysciences, calalog number: 23966-2)
23. Propidium iodide
24. Rely+On™ Virkon® tablets (VWR, catalog number: 115-0020)
25. Syringes: 1 ml (Terumo, catalog number: SS+01T1); 10 ml (Terumo, catalog number: SS+10ES1)
26. Tissue culture plastic:
    10 cm dish (Thermo Scientific, catalog number: 150350)
    96-well U-bottom plate (Falcon, catalog number: 353077)
    48-well plate (Sarstedt, catalog number: 83.3923.500)
    24-well plate (Greiner, catalog number: 662160)
    12-well plate (Greiner, catalog number: 665180)
27. Tubes:
    50 ml tubes (Greiner Bio-One, catalog number: 227261)
    15 ml tubes (Greiner Bio-One, catalog number: 188261)
    1.5 ml Eppendorf tubes (Starlab, catalog number: S1615-5550)

28. Ultracentrifuge tube (Thermo Scientific, catalog number: 3117-0380)
29. VSVG plasmid (Addgene, plasmid number: 14888)
30. Culture media (see Recipes)
31. D10 (see Recipes)
32. Expansion media (see Recipes)
33. FACS buffer (see Recipes)
34. MACS buffer (see Recipes)
35. Polyethyleneimine (PEI) (see Recipes)
36. Red cell lysis buffer (see Recipes)

## Equipment

1. Pipettes
2. Aspirator
3. Cell counter (Hemocytometer)
4. Cell sorter (*e.g.*, BD FACS Aria)
5. Centrifuge for 96-well plates
6. Freezer (-80°C)
7. Gamma-irradiation irradiator
8. Incubator (5% $CO_2$ 37°C)
9. Inverted microscope
10. Laminar flow cabinet
11. MACS MultiStand (Miltenyi Biotec, catalog number: 130-042-303)
12. MidiMACS Seperator (Miltenyi Biotec, catalog number: 130-042-302)
13. Pipette aid
14. Swinging bucket rotor (Beckman Coulter, model: SW32 Ti)
15. Ultracentrifuge (Beckman Coulter, model: Optima L-100 XP)
16. Water bath

## Software

1. Microsoft Excel

## Procedure

Important information before you start: Carry out the procedures in a category II lab in line with local rules. Obtain all the necessary local Health and Safety and ethic approvals for viral work, primary human sample handling and manipulation as well as xenotransplantation studies. A schematic overview of the procedures described in this protocol is provided in Figure 1.

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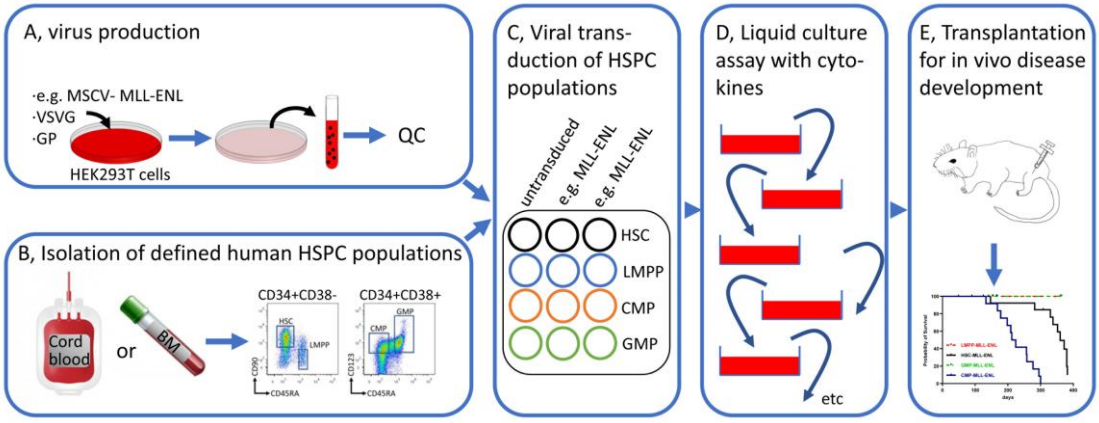


**Figure 1. Schematic overview of the main steps to perform *in vitro* and *in vivo* transformation assay using highly purified human haematopoietic stem and progenitor cell populations as targeted populations.**

A. Virus preparation

1. Generation of concentrated retrovirus carrying the oncogene of interest
   a. Plate 4 million HEK293T cells in 9 ml of D10 into each 10 cm plate. Prepare 3 plates for each viral construct to be tested in this protocol.
   b. Put plates into a 37°C, 5% $CO_2$ incubator overnight.
   c. The next day, bring all reagents to room temperature (RT), including IMDM, PEI, plasmids.
   d. Change media. Aspirate media and replace with 8 ml of warm D10 media. Place the plates into a 37°C, 5% $CO_2$ incubator. For retrovirus production, pipette 3 ml of IMDM into a 15 ml Falcon tube. Add 7.5 µg Gag/Pol plasmid, 7.5 µg VSVG plasmid and 15 µg retroviral plasmid containing the gene of interest, *e.g.*, MSCV-MLL-ENL-IRES-GFP. Mix by vortexing.
   e. Add 90 µl (1 mg/ml) PEI to a 15 ml Falcon tube and vortex for 20 s. Incubate at RT for 15 min.
   f. After incubation, briefly vortex the 15 ml Falcon tube. Label your plates accordingly and transfer 1 ml of this mixture into each of the three HEK293T plates. This is best done slowly in a drop-wise fashion covering most areas of the plates. Gently swirl the plates and return them to the 37°C, 5% $CO_2$ incubator overnight.
   g. The next day, change media. Aspirate media and replace with 12 ml of warm D10 media.
   h. Place the plates into 37°C, 5% $CO_2$ incubator overnight.
   i. The following day, and at least 48 h after transduction, collect the supernatant of the transfected HEK293T plates using a 10 ml syringe. Attach a 0.45 µm filter to the syringe and pass the supernatant into a labelled ultracentrifuge tube. Repeat for other plates transfected with the same construct until all supernatant is collected in the same ultracentrifugation tube. Discard syringe and filter into freshly prepared 1% virkon solution.
   j. Place the filled ultracentrifuge tube into the bucket and close lid. Prepare a balance if needed. Place all buckets onto the rotor and insert rotor following the manufacturer's

 

instructions.

k. Spin at 25,000 rpm (>80,000 × *g*) for 3 h at 4°C.

l. After the spin, retrieve the labelled ultracentrifuge tube. Aspirate media, leaving ~3 ml in the tube. Vortex the remaining media (= concentrated retrovirus) gently and aliquot 220 µl each into pre-labelled Eppendorf tubes. Store virus at -80°C. Cells can be transformed by virus stored over 1 year at -80°C for this protocol.

2. Viral titration by FACS

a. Plate 50K HEK293T cells in 500 µl of D10 into each well of a 24-well plate. Prepare 7 wells.

b. Place plates into a 37°C, 5% $CO_2$ incubator overnight.

c. The next day, thaw one Eppendorf tube containing the concentrated virus and prepare several serial dilutions using D10, *e.g.*, four 1:5 serial dilutions by mixing 30 µl of virus with 120 µl of D10.

d. For viral transduction of the HEK293T cells, add 0.25 µl of polybrene to each well of HEK293T cells. Label the wells 'untransduced', 'stock', and accordingly to your serial dilution, *e.g.*, '1:5', '1:25', '1:125' and '1:725'. Add 100 µl of stock or the corresponding serially diluted virus to the labelled wells. Add 100 µl of D10 to the well labelled 'untransduced'.

e. Put the 24-well plate in a pre-warmed centrifuge and spin at 800 × *g* for 1 h at 33°C.

f. Put the 24-well plate into 37°C, 5% $CO_2$ incubator overnight.

g. The following day, and at least 24 h after adding the virus, aspirate the media. Resuspend the HEK293T cells in 400 µl of PBS, and transfer to a FACS tube. Analyse the GFP expression by FACS, using the untransduced cells as control to set the gate.

h. The virus titer in 100 µl of concentrated virus can be calculated by multiplying (number of plated cells) * (fraction of $GFP^+$ cells) * (dilution factor).

B. Isolation of defined HSPC populations from human cord blood or adult bone marrow/mobilized peripheral blood

*Note: In our experience, fresh cord blood or adult BM/PBMC gives much more robust transformation results compared to frozen ones.*

1. Bring Ficoll-Paque Plus to RT.

2. Dilute fresh cord blood or adult BM/mobilized peripheral blood 1:1 in PBS, by transferring 15 ml of cord blood or adult BM into a 50 ml tube containing 15 ml PBS. Use multiple tubes to dilute all the original cord blood or adult BM/mobilized peripheral blood product if applicable.

~~3.~~ Add 20 ml of Ficoll-plaque Plus to a separate 50 ml tube. Slowly overlay Ficoll with 30 ml of the diluted cord blood or adult BM/PB. Repeat for other tubes if applicable. Centrifuge RT for 30 min at 400 × *g* with acceleration and deceleration set to 0.

4. Carefully aspirate around 2/3 of the top fraction containing the serum. Then collect the interphase containing the low-density mononuclear cells and transfer to a new 50 ml tube. This is best done by using a 10 ml pipette attached to a pipette aid. Repeat for the other tubes if

 

applicable. Add PBS to make up 50 ml, to wash the mononuclear cells. Mix by inverting tubes several times and centrifuge at 700 × *g* for 7 min with acceleration and deceleration set to maximum.

5. Aspirate most of the supernatant. If multiple tubes are used, leave around 3-5 ml in each tube. Resuspend cells in the remaining media and combine into a single 50 ml tube. Add PBS to make up 50 ml and centrifuge again at 700 × *g* for 7 min.
6. Aspirate most of the supernatant. Resuspend the cell pellet (which should be red/ pink in color, due to the presence of residual red blood cells) in 5 ml of red cell lysis buffer, mix by pipetting and leave at RT for 10 min. After the incubation, add 45 ml of PBS and mix the tube by inverting. Filter cells through a 40 µm cell strainer into a new 50 ml tube. Count cells using a hemocytometer. Transfer 100K cells into one 1.5 ml Eppendorf tube labelled 'MNC unstained' and place at 4°C. Centrifuge the 50 ml tube with the remaining cells at 700 × *g* for 7 min.
7. The cell pellet should appear white now (If still red, repeat Step B6 again). Aspirate supernatant and resuspend pellet in an appropriate volume of MACS buffer according to the manufacturer recommendation, *i.e.*, 300 µl for up to $10^8$ cells. Add 100 µl of FcR blocking reagent for up to $10^8$ cells and mix. Add 100 µl of CD34 microbeads for up to $10^8$ cells, mix, and incubate for 30 min at 4°C protected from light. After the incubation, wash the cells by adding 45 ml of MACS buffer. Mix and centrifuge at 700 × *g* for 7 min.
8. Put the MidiMACS separator onto the stand and insert a LS column. Place a 50 ml Falcon under the column and load 3 ml of MACS buffer to equilibrate the column. Buffer will flow through the column by gravity.
9. Aspirate supernatant from cells in Step B7 and resuspend in an appropriate volume of MACS buffer (500 µl MACS buffer per $10^8$ cells) and load onto column. Wait until all volume passed through the column. Wash three times by loading 3 ml of MACS buffer each time onto column.
10. After the final wash, remove the column from the MidiMACS separator and place onto 15 ml Falcon tube. Add 5 ml of MACS buffer to column, insert, and slowly push the plunger. Count the eluted cells using a hemocytometer. Centrifuge Falcon tube at 700 × *g* for 7 min. Aspirate supernatant.

*Note: From a cord blood unit of 90 g, typically 100 × $10^6$-200 × $10^6$ MNC can be isolated. Around 0.3-1% of those are $CD34^+$ cells, depending on the product. In our experience, very large cord blood units over 130 g yield higher numbers of MNC and $CD34^+$ cells exceeding those obtained from pooling two smaller units of up to 90 g together.*

11. Resuspend cells in 1 ml of MACS buffer and transfer into a 1.5 ml Eppendorf tube. Label this Eppendorf tube 'CD34 stain'. Centrifuge at 2000 × *g* for 2 min. Aspirate supernatant and resuspend pellet in 156 µl of MACS buffer. Add 4 µl (200 ng) of each HSPC antibody (working concentration 1 ng/µl). Add 2 µl (100 ng) of each lineage antibody (working concentration 0.5 ng/µl). Mix by pipetting and incubate at 4°C for 30 min.
12. After incubation, add 1 ml of MACS buffer to both Eppendorf tubes, labeled 'MNC unstained' and 'CD34 stain'. Centrifuge at 2,000 × *g* for 2 min, aspirate supernatant, and resuspend in

 

300 µl and 1 ml FACS buffer, respectively. Filter through 40 µm cell strainer into sterile FACS tubes.

13. Using a FACS sorter, *e.g.*, BD Aria or equivalent, sort hematopoietic progenitors such as HSC (lin$^-$CD34$^+$CD38$^-$CD90$^+$CD45RA$^-$), LMPP (lin$^-$CD34$^+$CD38$^-$CD90$^-$CD45RA$^+$), CMP (lin$^-$CD34$^+$CD38$^+$CD123$^+$CD45RA$^-$), GMP (lin$^-$CD34$^+$CD38$^+$CD123$^+$CD45RA$^+$). Compensation of different fluorochromes must be performed before running the samples, according to the instruction of the corresponding FACS machine used. We routinely use compensation beads. We routinely sort directly into 1.5 ml Eppendorf tubes containing 500 µl MACS buffer. The tube 'MNC unstained' can be used to define the negative unstained control population in all plots. An example of the gating strategy and expected frequencies is shown in Figure 2.

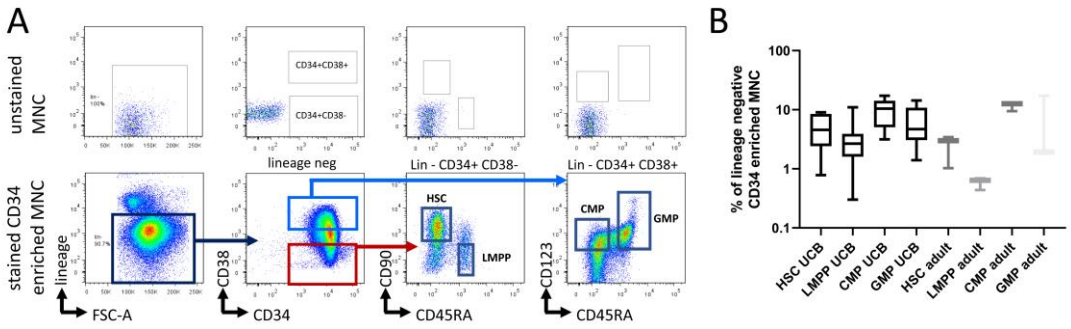


**Figure 2. HSPC subpopulation gating strategy and frequencies.**

A. The gating strategy for HSC, LMPP, CMP and GMP populations is shown. B. The frequencies of each population are shown as % of lineage negative CD34 enriched cells. UCB, umbilical cord blood (n = 12); adult, adult bone marrow or mobilized PBMC (n = 3).

*Note: Different phenotypic definitions for human HSPC populations are described in the literature which may use different lineage marker cocktails and/or different HSPC markers to purify distinct populations (Edvardsson et al., 2006; Doulatov et al., 2012; Pellin et al., 2019).*

14. Count sorted cells and use at least 300 cells to assess post-sort purity, which should be higher than 95%. To do so, transfer at least 300 cells into a new labelled FACS tube. Add FACS buffer to make up 150 µl. Repeat for each sorted population. Assess post-sort purity by running the samples in the FACS sorter.
15. Centrifuge the Eppendorf tubes with the sorted populations at 2,000 × *g* for 2 min. Aspirate supernatant and resuspend in expansion media with cell density of 2,000-20,000 per 100 µl.
16. Transfer up to 200 µl into one well of a U-bottom 96-well plate and mark the identity of the cell population on the cover plate. Repeat for all sorted cell populations. Add 250 µl of MACS buffer to all outer wells of the 96-well plate to avoid evaporation of expansion media. Place in 37°C incubator with 5% $CO_2$ overnight.

C. Viral transduction of HSPC populations

 

1. Take out the 96-well plate with the sorted populations from the incubator.
2. Calculate the appropriate number of wells for each cell population depending on the number of oncogenes to be tested. Add an extra condition as your untransduced control. Resuspend cells in expansion media at concentrations of 1,000-10,000 cells/100 µl and transfer 100 µl into the desired number of wells of a new 96-well plate.
   *Note: As an example, if 2 oncogenes will be tested then prepare 2 wells for each population plus one extra well for each population for untransduced control (A total of 3 wells per sorted population).*
3. For each prepared well, mix the following in a single 1.5 ml Eppendorf tube: 9.6 µl of IMDM, 15% FBS, P/S, 0.12 µl (1.2 ng) of each SCF, TPO, and FLT3L, 0.075 µl (0.75 µg) of Polybrene. Add 10 µl of this mixture to each well containing cells (a master mix should be made for accurate pipetting).
4. The desired multiplicity of infection (MOI) for the viral transduction of HSPC populations is 5-10. Use the determined viral titer (see Step A2h) to calculate the required volume of virus for the prepared cell density (see Step C2) for each population.
5. First transduction: Thaw frozen virus in water bath at 37°C. Prepare the correct amount of virus for each transduction by pipetting the calculated virus volume into a new Eppendorf tube and add D10 media to make a total of 50 µl. Transfer 50 µl of the titrated concentrated virus to the cell populations in the 96-well plate. Add 50 µl warm D10 to the control cells. Now the total volume of cells/virus mix should be 150 µl. Mix cells and virus well by pipetting.
6. Place the 96-well plate in 37°C incubator with 5% $CO_2$ overnight.
7. The next day, take out the 96-well plate from incubator.
8. For each well, prepare the following mix in a single 1.5 ml Eppendorf tube: 9.8 µl IMDM, 15% FBS, 0.1 µl (1 ng) of each SCF, TPO, and FLT3L. Add 10 µl of this mixture to each well containing cells (a master mix should be made for accurate pipetting).
9. Second transduction: Thaw frozen virus. Prepare the correct amount of virus for each transduction by pipetting the calculated virus volume into a new Eppendorf tube and add D10 media to make a total of 50 µl (use the same calculation as in Step C4 and the virus volumes from Step C5). Transfer 50 µl of the concentrated virus to the cell populations as the day before. Add 50 µl of warm D10 to the control cells. Now the total volume of cells/ virus mix should be 200 µl. Mix cells and virus well by pipetting.
   *Note: If multiple different viruses are used, ensure that the same virus will be added to the wells as on the previous day.*
10. Place the 96-well plate in 37°C incubator with 5% $CO_2$ overnight.

D. Liquid culture assay with cytokines
   1. The next day, take out the 96-well plate from incubator and inspect the cells under the microscope using a 10× objective. A cell cluster should be visible in the centre of the well.
   2. Aspirate 100 µl of supernatant from each well containing cells, without disturbing the cells. Add

 

back 100 µl of IMDM, 15% FBS, 0.2 µl (2 ng) of each SCF, TPO, and FLT3L, and 0.4 µl (4 ng) of each IL-3, and IL-6. Mix well by pipetting.

3. Place the 96-well plate in 37°C incubator with 5% $CO_2$ overnight.
4. The next day, take out the 96-well plate from incubator and check under the microscope using a 10× magnification. If cells fill more than 2/3 of the field of view, then transfer cells to a 48-well plate, add 300 µl culture media and return to 37°C incubator with 5% $CO_2$ overnight. If not, then leave cells in 96-well plate, return to 37°C incubator with 5% $CO_2$ overnight and repeat step 4 the next day.
5. Once the cells are in the 48-well plate, monitor cell expansion under the microscope every other day. If the cells cover more than 70% of the surface of the well or if the color of media changes yellowish, add 500 ul fresh culture media once to a total volume of 1ml. Place the 48-well plate in 37°C incubator with 5% $CO_2$.
6. Once the cells are in a total volume of 1 ml and cover the whole well, transfer them to a 12-well plate and add 2 ml of culture media (now total volume is 3 ml). Place the 12-well plate in 37°C incubator with 5% $CO_2$.
7. Monitor the cells in the 12-well plate every other day and count the number of cells using a hemocytometer. Once the cell concentration is within 600K-1 million/ml transfer 400K cells resuspended in 2 ml fresh culture media (*i.e.*, 200K cells/ml) to a new well of a 12-well plate. Place the new 12-well plate in 37°C incubator with 5% $CO_2$.
8. Three days later, add another 2 ml of culture media to the new 12-well plate containing the cells (now total volume is 4 ml). Place the 12-well plate in 37°C incubator with 5% $CO_2$.
9. Another 4 days later (*i.e.*, 1 week after Step D7), count the cells, and transfer 400K cells resuspended in 2 ml of fresh culture media to a new well of a 12-well plate. Place the new 12-well plate in 37°C incubator with 5% $CO_2$.
10. Repeat Steps D8-D9 for up to 15 weeks to monitor the growth of the cells.

*Notes:*

a. *Control cells will usually cease to expand and fully differentiate between week 3-8, while transformed cells continue to proliferate in vitro (Figure 3A and 3B) and retain an immature myeloblast cell morphology (Figure 3C). The time to obtain fully differentiated control cultures varies from sample to sample. While sometimes untransduced HSCs may still show a proportion of immature cells at day 36, other cell populations may have fully differentiated long before day 36.*
b. *Once a difference in proliferation pattern between transduced and control cells from the same cell population is observed, make cytospin preparations (Figure 3C) and perform FACS (Figure 3D) to characterize these early transformed cells. Freeze cells (>1 million cells/vial).*
c. *Perform weekly characterisation of cell morphology and cell surface marker expression. Continue to freeze down cells bi-weekly to build up frozen stock of transformed cells which can be accessed in the future. As soon as the proliferation difference is observed between control and transduced cells, transduced cells can be used for other studies including transplantation*

 

*into immunodeficient mice (protocol Procedure E).*

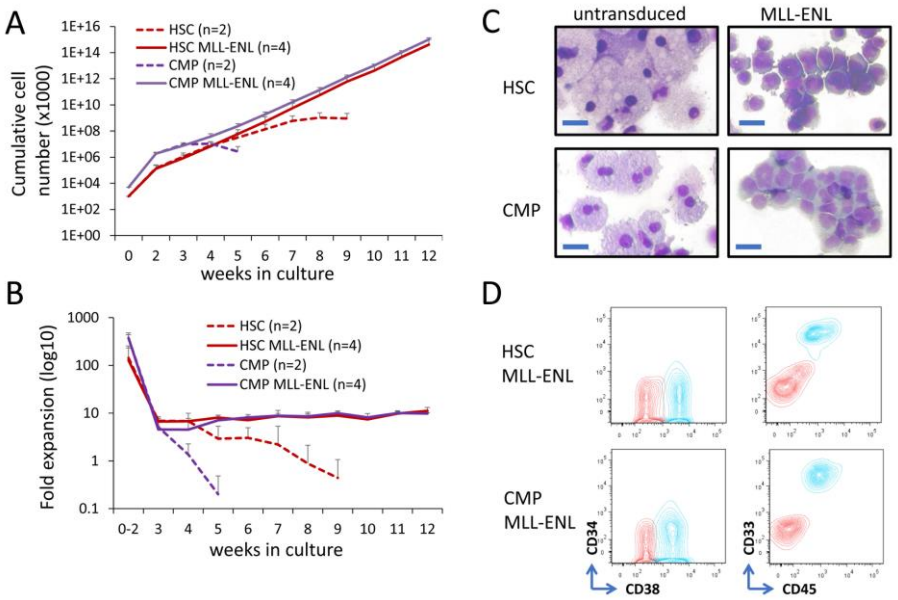


**Figure 3. *In vitro* transformation of MLL-ENL transduced HSPC populations.**

A-B. The cumulative cell number (A) and the fold expansion (B) of MLL-ENL transduced or untransduced HSC and CMP population are shown. C. May-Gruenwald Giemsa stained cell preparations show the typical cell morphology for the indicated cell types at day 36 in culture. D. Typical surface marker expression of transformed cells at day 36 in culture. Red profiles represent unstained cells and blue profiles are cells stained with the indicated surface marker.

E. Transplantation for *in vivo* disease development

Perform sublethal irradiation (200-250 cGy) of immunodeficient NSG mice no more than 24 h before transplantation. The mice should always be housed in independently ventilated cages (IVCs) and all procedures are always performed under the safety hood.

The mice are transplanted intravenously (i.v.) (Video 1) or intraosseously (i.o.) (Video 2) via a hind leg bone (femur is preferred over tibia, as it has a bigger bone marrow cavity). The transplantation route depends on the number of cells:

For i.v., a minimum of 500,000 and no more than 10 million cells should be transplanted.

For i.o., no minimum cell number is set but they should not exceed 1 million.

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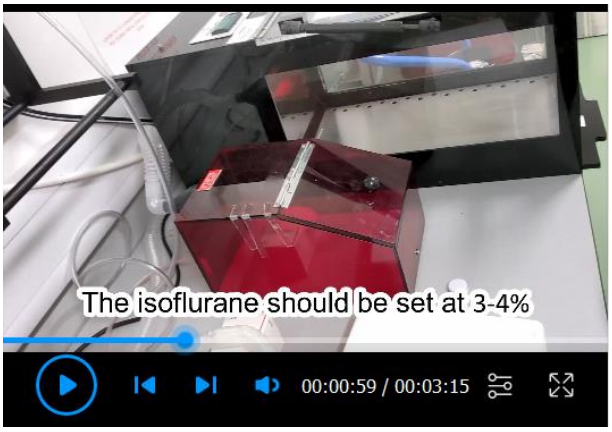
**Video 1. Intravenous transplantation.**

This video was made at King's College London. The experimental procedure shown in this video was approved by King's College London ethics committees and conform to the UK Home Office regulations.




**Video 2. Intraosseous transplantation.**

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1. Preparation of the cells for transplantation.
   Cells should be resuspended in PBS or isotonic saline. The following volumes are injected into each mouse: For i.v. injection, 150-200 µl in a 29G 1 ml needle attached to an insulin syringe. For i.o. injection, amaximum 20 µl in a 29G 1 ml needle attached to an insulin syringe. Cells should always be kept on ice and brought back to RT 5 min before transplantation.
   *Note: Matrigel can also be used as vehicle for i.o. injection. In this case, cells should always be kept on ice before injection.*
2. Transplantation via i.v. injection.
   a. The mice are warmed in their IVCs with an infrared radiator (200-300 W) 15 cm apart for 10 min, or in a Mini-Thermacage set at 37°C for 10 min. The Mini-Thermacage is preferred

 

as the warming is more homogeneous. Warming the mice prior to transplantation allows their tail veins to dilate and become more visible. The mice should be monitored regularly, and moved away from the heat source immediately if they show signs of heat exhaustion *e.g.*, staying away from the heat source and breathing heavily.

b. Place the mouse in a sterile animal holder and keep its tail outside the tube. There are four blood vessels visible at the tail; the two veins are located at the tail's lateral sides. Only use veins for injection.

*Note: Never inject via ventral and dorsal blood vessels of the tail; they are the arteries. It would cause severe bleeding!*

c. Carefully resuspend the cells by tipping the needle several times and ensure that there are no bubbles inside the syringe. Use a sterilizing wipe to clean the injection site. Insert the needle into the vein for about 5mm in the direction from tail to head, push the plunger gently, and inject all the volume slowly for 2-3 s. You should be able to push the plunger smoothly and see the tail vein temporarily turn white for 3-5 s, before turning red/pink again.

*Note: If you feel resistance from pushing the plunger, and the area around the injection site turns white, then the injection failed, i.e., the needle is not inside the vein. Try injecting again, via the other lateral vein 5 to 10 min later, when the vein is visible again.*

d. When all the content in the syringe is injected, hold for 5 sec before withdrawing the needle (Removing the needle immediately after injection would flush some cells out from the injection site). If the injection is successful, there is mild bleeding once the needle is withdrawn. Clean the injection site again using a sterilizing wipe and stop the bleeding by gently pressing the injection site for around 10 s. Place the mouse back in the IVC.

3. Transplantation via i.o. injection into femur.

*Note: All procedures should be performed under the safety hood.*

a. Keep cells on ice. Just before the injection, carefully resuspend the cells by tipping the needle several times and ensuring that there are no bubbles inside the syringe.

*Note: When preparing the cell suspension, resuspend the cells in a volume of 22 µl per mouse, as there is about 1-2 µl dead volume in the insulin syringe. By doing so, you ensure injecting 20 µl of cells.*

b. The mouse is put under anesthesia in a sterile chamber by a light inhalational anesthesia agent such as 2-4% isofluorane by Plenum vaporizer (*e.g.*, VetFlo™ Vaporizer). Observe the breath of the mouse changing from "shallow but rapid" to "deep but slow". When the mouse breaths deeply but slowly, it is in deep anesthesia, and can be transferred to a heat mat set at 37°C (or a prewarmed polystyrene board if heat mat is not available) and maintained under anesthesia by inhaling isoflurane. During the whole procedure, it is vital to keep monitoring the breath of the mouse, if it turns shallow but rapid again, you need to increase the dose of isoflurane. No invasive procedure should be performed when the mouse breaths shallow but rapid. If it turns shallow and slow, remove the mouse from anesthesia immediately, as the dose is too strong and the mouse can be killed.

 

c. Place the mouse dorsal side up. Remove hairs around its knee by plucking. Clean the injection site using sterilizing wipe. Apply pain relief cream (*e.g.*, EMLA cream) on the skin. Use the sharp bevel of a sterile 27G needle (NOT the needle for cell injection) to cut open the skin in a lateral direction (3-4 mm in length) at the knee region, and the patellar ligament (white in color) should now be exposed and visible. Use the same needle to drill a hole via the anterior end of the femur under the patellar ligament. Move the needle back and forth gently in all directions—you should feel scratching in the inner cavity of the bone in each movement.

d. Withdraw the 27G needle gently and slowly, and remember the direction of the needle withdrawal. Insert the cell-containing 29G insulin needle into the same site with the same direction of the 27G needle withdrawn. You should feel no resistance when inserting the needle into the injection site. Adjust the direction of the needle going in slightly if you feel resistance. After the needle is inserted, scratch slightly in all directions to confirm the needle is in the bone marrow cavity. Inject all the content in the syringe and withdraw the needle. Return the mice to the IVC warmed by infrared radiator, where they should recover from anesthesia within 3-5 min (if anesthetized by isoflurane).

e. Monitor the mice transplanted via i.o. daily. Their injected legs should be slightly lamed/dragged for at least 2 days but should recover to normal within one week. A pain relief agent can be administred to the mouse for 1 week according to the veterinarian's instruction.

4. Mouse monitoring

a. After transplantation, the health of the mice should be monitored daily. The engraftment of human cells can be tracked by blood sampling via the tail vein followed by FACS analysis, no more than once a week. Engrafted cells should be human $CD45^+$. The engraftment of human primary *in vitro* transformed cells into NSG mice is highly variable for both tumor burden and time, and sometimes they even fail to engraft at all. From our experience, the human *in vitro* transformed cells could be detected as early as 2 weeks after transplantation.

b. When the transplanted mouse starts to show signs of moderate pain and distress (Burkholder *et al.*, 2012), or reaches humane endpoint (Burkholder *et al.*, 2012), the mouse needs to be culled by humane methods. Harvest blood, bone marrow, spleen, liver, thymus, and relevant organs to check for any human cell engraftment by FACS with appropriate cell surface markers (including but not limited to CD34, CD38, CD45, and CD33), and produce blood/ bone marrow smears to check the cell morphology. Spleen, liver, and/or thymus could be enlarged if leukemia cells infiltrated into those organs, so their weight and size should be recorded (Figure 4A), and their engraftment of human cells can be evaluated by FACS with appropriate cell surface markers (Figure 4B), and hematoxylin and eosin staining, after the tissue is fixed and sectioned (Figure 4C).

 

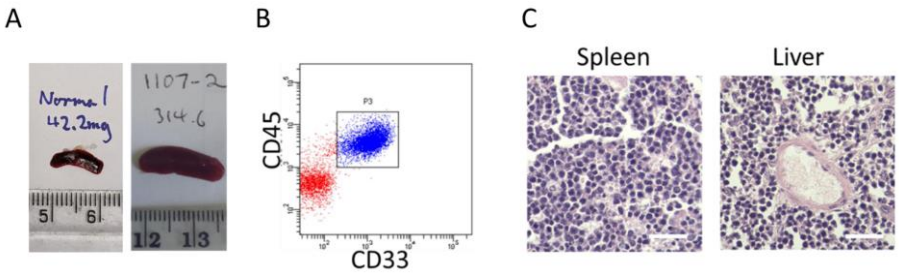


**Figure 4. Analysis of human leukemia engrafted mice.**

A. Photo of the spleen from the control mouse (weight = 42.2 mg) and the sick mouse (weight = 314.6 mg), with ruler in cm. B. Representative FACS plot of engrafted human AML cells (blue), which are positive for both human CD45 and CD33. C. Hematoxylin and eosin staining of fixed spleen and liver of a sick mouse. The human AML cells are stained in dark blue, scale bar = 50 µm.

## Data analysis

It is recommended to perform the *in vitro* transformation assay at least 3 times using different cord blood or adult bone marrow samples, from which defined HSPC populations are sorted. Within each independent experiment, it is recommended to perform each condition at least in duplicates. Typical intra- and inter-assay variability of transformed cells for the weekly fold expansion are as follows:

Intra-assay variability early culture (week 2-4) HSC MLL-ENL (1%-85%), CMP MLL-ENL (3%-25%); mid-term culture (week 5-8): HSC MLL-ENL (1%-12%), CMP MLL-ENL (1%-21%); long term culture (week 8-12): HSC MLL-ENL (2%-10%), CMP MLL-ENL (3%-23%);

Inter-assay variability early culture (week 2-4) HSC MLL-ENL (18%-80%), CMP MLL-ENL (29%-50%); mid-term culture (week 5-8): HSC MLL-ENL (12%-32%), CMP MLL-ENL (6%-29%); long term culture (week 8-12): HSC MLL-ENL (7%-24%), CMP MLL-ENL (5%-21%). While the %CV is relatively high in the early phase of culture (week 2-4), reflecting the highly variable nature of the cord blood sample, the variation reduces over culture time due to MLL-ENL mediated transformation.

In contrast, inter-assay %CV for untransduced HSC and CMP populations range between 13% and 141%, with lower variations observed only at the beginning of the cultures and higher variations from week 3 (CMP) and week 5 (HSC) onwards. For data analysis, we generally use Microsoft Excel for the cumulative growth curves and fold expansion, and GraphPad Prism for the survival curves of the transplanted animals.

## Recipes

1. Culture media
   IMDM, 15% FBS, P/S, 20 ng/ml of each SCF, TPO, FLT3L, IL-3, and IL-6
2. D10
   DMEM, 10% FBS, P/S

3. Expansion media
   IMDM, 15% FBS, P/S, 100 ng/ml of each SCF, TPO, FLT3L
4. FACS Buffer
   PBS, 0.5% FBS, P/S, Propidium iodide
5. MACS buffer
   PBS, 2 mM EDTA, 0.5% FBS, P/S
6. PEI (1 µg/µl)
   a. Dissolve PEI in endotoxin-free $dH_2O$ that has been heated to ~80°C.
   b. Let cool to room temperature.
   c. Neutralize to pH 7.0, filter sterilize (0.22 µm), aliquot and store at -20°C; a working stock can be kept at 4°C.
7. Red cell lysis buffer
   a. 10 mM $KHCO_3$, 150 mM $NH_4Cl$, 0.1 mM EDTA
   b. Make up to 300 ml with endotoxin-free $dH_2O$, and filter sterilize (0.22 µm). Store at 4°C.

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## Competing interests

The authors declare no conflict of interest.

## Ethics

All experimental procedures were approved by King's College London ethics committees and conform to the UK Home Office regulations.

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