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Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol

Generation of hematopoietic lineage cell-specific chimeric mice using retrovirus-transduced fetal liver cells

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SUMMARY

Hematopoietic lineage cell-specific transgenic or knockout mice provide a valuable platform to identify the role of specific genes in hematopoiesis in vivo. Here, we describe protocols for preparation of retroviruses for overexpression or knockdown of a gene of interest, retroviral transduction of fetal liver cells, and generation of hematopoietic lineage cell-specific chimeric mice by transfer of the retrovirus-transduced fetal liver cells. This protocol is applicable for the study of in vivo functionality of a gene of interest in immune cells. For complete details on the use and execution of this protocol, please refer to Chang et al. (2013), Lee et al. (2016), and Hong et al. (2022).

BEFORE YOU BEGIN

Institutional permissions

All animal procedures were approved by the Pohang University of Science and Technology Institutional Animal Care and Use Committee. Embryos were euthanized by decapitation, and adult mice were euthanized by CO₂ inhalation. Male and female mice were used throughout the study. Animals were maintained in a specific pathogen-free animal facility under a standard 12:12 h light/dark cycle and administered standard rodent chow and water ad libitum.

Preparation of retroviral construct for overexpression

© Timing: 4 days–1 week

This manuscript describes the generation of Bhlhe41-overexpressing fetal liver chimeric mice.

1. Sub-clone the coding sequence (CDS) of genes desired for overexpression into a MSCV-based retroviral vector (e.g., MIGR1).

   Note: The vector includes an IRES fluorescent protein cassette (e.g., GFP) that is used to track transduced cells and transduction efficiency.

2. Isolate plasmid DNA using commercially available plasmid prep kit.

   Note: Use a working concentrate of 500–600 ng/μL.
3. Verify the sequence of the clone vector using the appropriate primers.

**Preparation of retroviral construct for knockdown**

© Timing: 1–1.5 weeks

Short hairpin RNAs (shRNAs) were cloned into LMP vectors according to the manufacturer’s protocol (Open BioSystems, Catalog #: EAV4678) (Chang et al., 2013).

This manuscript describes the generation of Bhlhe41-knockdown fetal liver chimeric mice.

4. Design and order shRNA oligonucleotides for knockdown of target genes.
   a. To generate new shRNA clones, go to RNAi Central-RNAi Oligo Retriever (http://katahdin.cshl.org/siRNA/RNAi.cgi?type=shRNA).
   b. Choose the shRNA psm2 Design and enter the accession number of the target gene or its nucleotide sequences (Figure 1A).

   **Note:** The designed template oligonucleotide has a miR-30 loop sequence inserted between the sense and antisense sequences, and flanking sequences of miR-30 miRNA to the 5′ and 3′ ends.

   c. Chemically synthesize the designed template oligonucleotide.

5. Prepare inserts for cloning into the vector.
   a. Amplify the designed template using PCR (Tables 1 and 2).

   **Note:** The amplified PCR products will have XhoI and EcoRI restriction enzyme sites at the 5′ and 3′ ends, respectively.

   **Note:** miR-30 common forward primer: 5′-CAGAAGGCTCGAGAAGGTATATTGCTGTGTA-CAQTGAGCG-3′ and reverse primer: 5′-CTAAAAGTAGCCCTTTGAATCCGAGGCAGTAGGCA-3′. The underlined sequences indicate the XhoI and EcoRI sites, respectively.

   b. To determine the yield of the amplified target, take 10 μL of the PCR product and analyze using electrophoresis through 2%–3% agarose gel.

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Table 1. PCR reaction master mix

| Reagent                  | Volume (μL) |
|--------------------------|-------------|
| Pfu-X DNA polymerase     | 1           |
| 10× buffer               | 10          |
| 10 mM dNTPs              | 2           |
| 10 pmole forward primer  | 5           |
| 10 pmole reverse primer  | 5           |
| ddH₂O                    | 76          |
| 0.02 pmole template      | 1           |
| **Total**                | **100**     |

Table 2. PCR cycling conditions

| Steps          | Temperature | Time  | Cycles |
|----------------|-------------|-------|--------|
| Initial denaturation | 95°C        | 2 min | 1      |
| Denaturation    | 95°C        | 20 s  | 30 cycles |
| Annealing      | 54°C        | 30 s  |        |
| Extension      | 72°C        | 30 s  |        |
| Final extension| 72°C        | 10 min| 1      |
| Hold           | 4°C         | Indefinite |         |
c. Purify the PCR product using a commercially available PCR purification kit.

6. Digest the PCR product and a retroviral vector (e.g., MSCV-LTRmiR30-PIG [LMP]) using EcoRI and XhoI (Figure 1C).

**Note:** The expected size of a single band is 138 bp (Figure 1B).

**Note:** 3 μg of the LMP vector is used for digestion.

7. Purify the vector and insert by either running the DNA on an agarose gel and excising the appropriate bands or using a gel purification kit.

**Note:** Prepare the final volume of the vector and insert to 40 and 14 μL, respectively.

**Note:** For electrophoresis, use 2%–3% agarose gel for PCR products and 1% gel for vectors.

8. Ligate the insert into the vector with T4 DNA ligase (Table 3).

**Note:** Incubate the reaction at 16°C for approximately 15 h.

9. Transform competent bacteria with the recombinant DNA.
   a. Thaw an aliquot of competent bacteria (100 μL) on ice and gently mix with 7.5 μL of the ligation reaction by tapping.

   **Note:** The DH5α strain is used for efficient transformation.

   b. Incubate the reactions sequentially on ice for 5 min, in a heating block at 42°C for 1 min, and on ice for 10 min.

   c. Add 1 mL of LB medium to the reactions and incubate in a shaking incubator for 1 h.

   d. Spin down the cells at 2,000 × g for 1 min and remove 1 mL of the supernatant by pipetting.

   e. Resuspend the cell pellet by vortexing and spread the cells onto the 100 mm agar plate with 15 mL of LB medium and ampicillin (50 μg/mL). Incubate the plate at 37°C for approximately 15 h.

10. Isolate the plasmid and confirm the presence of the desired insert in the vector.
    a. Select and inoculate individual colonies in 4 mL of liquid LB with ampicillin (50 μg/mL) at 37°C for 12–18 h in a shaking incubator.

    b. Isolate plasmid DNA using a commercially available plasmid prep kit.

    c. Digest the plasmid with SacII and XhoI to confirm insertion of the shRNA construct.

**Note:** If the shRNA construct is accurately inserted into the vector, bands of 980 and 7019 bp size will be observed.

d. Verify the shRNA cassette sequences of each clone by sequencing using the recommended primer: 5’-CCCTTGAAACCTCCTCGTTGACC-3’.

| Table 3. Ligation reaction |
|---------------------------|
| Reagent                   | Volume (μL) |
|---------------------------|-------------|
| T4 DNA ligase             | 1           |
| 10× buffer                | 1.5         |
| Vector                    | 1–1.5       |
| PCR product               | 11.0–11.5   |
| **Total**                 | 15          |
11. Western blot analysis can be used to determine the knockdown efficiency of the shRNA (Figure 1D).
   a. How to prepare retrovirus is described in the [Part 1] Preparation of retrovirus section.
   b. For retroviral transduction, seed 5 \times 10^5 NIH3T3 cells onto 6-well plates and incubate in a CO_2 incubator at 37°C for approximately 15 h.
   c. Remove the culture medium and add 1 mL of fresh DMEM media with 10% (v/v) FBS and 1 mL of the filtered retroviral supernatant with 12 μg/mL hexadimethrine bromide (polybrene).
   d. After 72 h, perform western blotting using the virus-transduced cells.

**Setting up timed pregnant mice**

© Timing: 16 days

12. To obtain embryos at embryonic day 15.5 (E15.5), establish the breeding cages for timed mating.

**Note:** Rapid expansion of functional hematopoietic stem cells (HSCs) occurs from E12.5 to E15.5 in mouse fetal liver (Ema and Nakauchi, 2000; Morrison et al., 1995). To obtain as
many HSCs as possible and to increase the probability of successful engraftment, use E15.5 embryos.

Note: Use 8- to 15-week-old mice for effective mating. Select proestrus or estrus females to mate with male mice. The vaginal opening of females during the proestrus or estrus period is swollen, pink, and moist. Cross one female mouse with one male mouse.

13. At 9 am on the morning after mating, determine if a plug has formed in the female’s vagina. In the C57BL/6 strain, the plug is thin and dissolves quickly. Since plug formation does not guarantee pregnancy, separate the male from the female and monitor the female continuously.

14. The morning that a plug is found is considered gestational day 0.5. Prepare the gestational day 15.5 female mice for the experiment.

Note: A pregnant female mouse has 8 fetuses on average. Approximately 3 fetal liver chimeric mice can be generated with fetal liver cells from two E15.5 embryos.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| violetFluor 450 Anti-Human/Mouse CD45R (B220) Antibody (1:300 dilution) | Tonbo Biosciences | 75-0452, RRID: AB_2621948 |
| PE/Cyanine7 anti-mouse IgM Antibody (1:300 dilution) | BioLegend | 406513, RRID: AB_10640069 |
| PE Rat Anti-Mouse CDS (1:300 dilution) | BD Biosciences | 553023, RRID: AB_394561 |
| FITC Rat Anti-Mouse CD43 (1:300 dilution) | BD Biosciences | 561856, RRID: AB_10895376 |
| Anti-mouse BHLHE41 (SHARP1) (1:500 dilution) | abcam | ab82825, RRID: AB_1859984 |
| Anti-mouse GAPDH (1:1000 dilution) | Santa Cruz | sc-32233, RRID: AB_627679 |
| Bacterial and virus strains |        |            |
| DH5alpha competent cells | Sigma-Aldrich | 18265017 |
| Chemicals, peptides, and recombinant proteins |        |            |
| Pfu DNA polymerase | Solgent | SPDP5-E500 |
| 10× Pfu reaction buffer | Solgent | SPD22-B50h |
| 10 mM dNTP | Solgent | N/A |
| HiQ Agarose | BioD | HQ80500_PQ2 |
| Ethidium bromide | Sigma-Aldrich | E7637 |
| DNA Gel Loading Dye (6×) | Thermo Scientific | R0611 |
| 1 kb DNA ladder marker | Enzymomics | dm003 |
| 10× EzBuffer | Enzymomics | RB006 |
| EcoRI restriction enzyme | Enzymomics | R0025 |
| XhoI restriction enzyme | Enzymomics | R0075 |
| T4 DNA Ligase | Promega | M1801 |
| T4 DNA Ligase 10× buffer | Promega | C1263 |
| Agar | Sigma-Aldrich | A6886 |
| LB Broth Miller | BD Difco | 244620 |
| Ampicillin | Sigma-Aldrich | A9393 |
| Sacl restriction enzyme | Enzymomics | R0365 |
| Fetal Bovine Serum (FBS) | Welgene | S001-01 |
| Dulbecco’s Modified Eagle’s Medium (DMEM) (1x) high glucose required) | Welgene | LM001-07 |
| Medium (DMEM) (1x) high glucose required) | BioShop | PBS404 |
| Phosphate buffered saline tablets | BioShop | PBS404 |
| Ammonium chloride | Sigma-Aldrich | A4934 |
| Sodium bicarbonate | Sigma-Aldrich | S5761 |
| UltraPure™ 0.5 M EDTA (pH 8.0) | Invitrogen™ | 15575020 |
| Penicillin-Streptomycin | Gibco | 15140122 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| RPMI 1640 Medium    | Welgene| LM011-60   |
| FuGENE HD Transfection Reagent | Promega | E2311 |
| Retro-X Concentrator | Takara | 631456 |
| Trypan Blue Solution, 0.4% | Gibco | 15250061 |
| Mouse IL-3, research grade | Miltenyi Biotec | 130-096-687 |
| Mouse IL-6, research grade | Miltenyi Biotec | 130-096-683 |
| Mouse SCF, research grade | Miltenyi Biotec | 130-101-741 |
| Sodium Hydroxide    | Sigma-Aldrich | 567530 |
| Tris(hydroxymethyl)aminomethane | Sigma-Aldrich | 252859 |
| Hydrochloric acid   | Sigma-Aldrich | H1758 |
| e-Taq DNA polymerase | Solgent | SET95-E500 |
| 10x e-Taq reaction buffer | Solgent | SET22-850h |
| Hexadimethrine bromide | Sigma-Aldrich | 107689 |
| Ghost Dye™ Violet 510 | Tonbo Biosciences | 13-0870 |
| Clarity Western ECL Substrate | Bio-Rad | BR170-5061 |

**Critical commercial assays**

- ExPrep Plasmid Mini: GeneAll #101-102
- Expin PCR SV: GeneAll #103-102
- Expin CleanUp SV kit: GeneAll #102-102
- QIAprep Spin Miniprep Kit: Qiagen #27104
- QIAquick PCR Purification Kit: Qiagen #28104
- QIAquick Gel Extraction Kit: Qiagen #28706

**Experimental models: Cell lines**

- Platinum E (Plat E) retroviral packaging cell line: Cell Biolabs N/A
- NIH/3T3: ATCC CRL-1658

**Experimental models: Organisms/strains**

- Mouse: Ifitm3
  - Female and male mice; C57BL/6 background; Embryonic day 15.5 fetus and 8- to 15-week-old mice
    - (Lu et al., 2017) N/A
- Mouse: Cd19-Cre
  - Female and male mice; C57BL/6 background; 8- to 15-week-old mice
    - (Rickert et al., 1997) N/A
- Mouse: Cd19-Cre
  - Female and male mice; C57BL/6 background; Embryonic day 15.5 fetus and 8- to 15-week-old mice
    - (Hong et al., 2022) N/A
- Mouse: Rag1+/−
  - Female and male mice; C57BL/6 background, 10- to 12-week-old mice
    - (Mombaerts et al., 1992) N/A
- Mouse: C57BL/6J
  - Female and male mice; Embryonic day 15.5 fetus and 8- to 15-week-old mice
    - The Jackson Laboratory #006664

**Oligonucleotides**

- miR-30 common forward primer; 5'-CAGAAGGCCTCGAGAGGTATATTTGGTCGACAGTGACGCGC-3'
  - Open Biosystems N/A
- miR-30 common reverse primer; 5'-GCTGACGTGACAGCCGCAATATGCTGCTGGACAGGCG-3'
  - Open Biosystems N/A
- LMP sequencing primer; 5'-GCCCTGCAGGCGCCCTGGGAGACGAAATTCAGGGCCTACTTCTTAG-3'
  - Open Biosystems N/A
- shBhlhe41 template oligonucleotide 1; 5'-CAGAAGGCCTGACGTGACAGTGACGCGC-3'
  - This manuscript N/A
- shBhlhe41 template oligonucleotide 2; 5'-CAGAAGGCCTGACGTGACAGTGACGCGC-3'
  - This manuscript N/A
- shBhlhe41 template oligonucleotide 3; 5'-CAGAAGGCCTGACGTGACAGTGACGCGC-3'
  - This manuscript N/A

(Continued on next page)
MATERIALS AND EQUIPMENT

- DMEM 10% (v/v) FBS

| Reagent | Final concentration (v/v) | Volume |
|---------|--------------------------|--------|
| DMEM (high glucose) | 90% | 450 mL |
| FBS | 10% | 50 mL |
| Total | 100% | 500 mL |

**Note:** Store at 4°C for up to one month.

**Note:** FBS should be inactivated by heating at 56°C for 30 min in a water bath with occasional shaking.

**Note:** Do not add antibiotics to the media.

- Phosphate-Buffered Saline (PBS)

| Reagent | Final concentration (mM) | Amount |
|---------|--------------------------|--------|
| NaCl | 137.00 | 0.801 g |
| KCl | 2.70 | 0.020 g |
| Na₂HPO₄ | 10.00 | 0.142 g |
| KH₂PO₄ | 1.47 | 0.020 g |
| ddH₂O | | |
| Total | n/a | 100 mL |

**Note:** pH 7.4 ± 0.05 at 25°C.

**Note:** Store at 25°C up to one month.

- Red blood cell (RBC) lysis buffer
Note: Store at 25°C, discard if solution begins to precipitate.

- DMEM 20% (v/v) FBS supplemented with antibiotics

Note: Store at 4°C for up to one month.

- RPMI-1640 supplemented with antibiotics

**STEP-BY-STEP METHOD DETAILS**

The step-by-step method details are divided into two main parts. The first is to prepare a retrovirus, and the second is to make chimeric mice using fetal liver cells transduced with the retrovirus. If a fresh virus is to be used for transduction, the preparation of fetal liver cells should be performed 48 h after transfection of the packaging cells.

**[Part 1] Preparation of retrovirus**

**Seeding of the retrovirus-producing cell line – Day 1**

- **Timing:** 30 min

Cells are grown and plated for transfection of retroviral vectors.

1. Seed 2.5 × 10⁶ Platinum-E (Plat-E) retroviral packaging cells in 100 mm cell culture plates containing 10 mL of DMEM media with 10% (v/v) FBS.
2. Incubate the cells in a 5% CO₂ incubator at 37°C for approximately 15 h.

**Note:** Cells should be approximately 60% confluent on the day of transfection.

**Note:** The health of the packaging cell line is critical for obtaining high levels of the viral yield. It is recommended to use cells passaged no more than 4 times.
Transfection of the retroviral vectors into the packaging cell line – Day 2

© Timing: 30 min

A retroviral vector and a retrovirus packaging vector are co-transfected into the seeded cells according to manufacturer’s protocol (Promega, Catalog #:E2311, https://www.promega.kr/-/media/files/resources/protcards/fugene-hd-transfection-reagent-quick-protocol.pdf?rev=2b9dfca8e1aa4c53ad63d5f2b7ca340e&sc_lang=ko-kr). Mixing of plasmids and transfection reagents in DMEM medium and transfection of the cells should be performed in a clean bench to avoid microbial contamination.

3. Before working with cells, warm the serum-free DMEM medium in a 37°C water bath for at least 15 min, and allow the vial of FuGENE® HD transfection reagent to reach approximately 25°C.

Alternatives: OPTI-MEM reduced-serum medium may also be used.

4. Add 4.5 µg of the cloned retroviral vector and 1.5 µg of pCL-Eco helper plasmid to the pre-warmed medium to reach a final volume of 600 µL. Vortex the tubes and then spin down the contents.

Note: pCL-Eco helper plasmid contains gag/pol/env genes and is designed to maximize a recombinant-retroviral titer.

5. For a 3:1 FuGENE® HD transfection reagent:DNA ratio, add 18 µL of FuGENE® HD transfection reagent directly to medium and mix immediately by tapping the tube.

Note: Do not allow FuGENE® HD transfection reagent to contact the sides of the tube.

6. Incubate the mixture for 15 min at approximately 25°C.

7. Carefully add the mixture to the plated cells in a dropwise manner and mix gently.

8. Return cells to the incubator for 48 h.

△ CRITICAL: After 24 h, the transfection efficiency is measured using the GFP signal. Transfection efficiency should be at least 80%.

1st harvest of retroviral supernatant – Day 4

© Timing: 30 min

For retrovirus enrichment, harvest the cell supernatant containing the retrovirus.

9. Forty-eight h after transfection, harvest the retrovirus-containing supernatants (approximately 90% of the culture medium). Filter through a 0.45 µm filter.

Note: Use only cellulose acetate or polyethersulfone filters, but not nitrocellulose filters. Nitrocellulose binds surface proteins on the retroviral envelope and destroys the virus.

10. Add 10 mL of fresh DMEM 10% (v/v) FBS medium to the plated cells and return cells to the incubator for 24 h.

11. Transfer clarified supernatant to a sterile 15 mL conical tube and combine 3 mL of Retro-X Concentrator with 9 mL of clarified supernatant. Mix by gentle inversion.

12. Incubate mixture at 4°C for approximately 15 h.
1st concentration of retroviral supernatant – Day 5

- **Timing:** 1 h

The retrovirus-containing supernatant is concentrated according to the manufacturer’s protocol (Takara, Catalog #: 631456, https://www.takarabio.com/documents/User%20Manual/PT5063/PT5063-2.pdf).

13. After an incubation of the retroviral supernatant with Retro-X concentrator (see step 12), centrifuge the mixture at 1,500 \( \times \) g for 45 min at 4°C. After centrifugation, an off-white pellet should be visible.

14. Carefully remove the supernatant.

**Note:** Take care not to disturb the pellet.

15. Gently resuspend the pellet in 1 mL DMEM 20% (v/v) FBS medium supplemented with antibiotics. The pellet can be somewhat sticky at first, but will go into suspension quickly.

-Pause point: The enriched retroviral supernatant can be stored at −80°C in single-use aliquots.

2nd harvest of retroviral supernatant – Day 5

- **Timing:** 30 min

For retrovirus enrichment, harvest the cell supernatant containing the retrovirus.

16. Twenty-four h after adding fresh medium to the packaging cells (see step 10), harvest the retrovirus-containing supernatants. Filter through a 0.45 \( \mu \)m filter.

17. Repeat steps 11 and 12.

2nd concentration of retroviral supernatant – Day 6

- **Timing:** 1 h

The retrovirus-containing supernatant is concentrated according to the manufacturer’s protocol.

18. Repeat steps 13–15.

19. The sequence for retrovirus preparation is shown in Figure 2.

-Pause point: The enriched retroviral supernatant can be stored at −80°C in single-use aliquots.

[Part 2] Generation of chimeric mice using fetal liver cells transduced with retrovirus

Preparation of fetal liver cells – Day 1

- **Timing:** 1–2 h

Fetal liver cells are obtained from an embryonic day 15.5 fetus.

20. Euthanize pregnant mice by CO2 inhalation.

21. Harvest E15.5 embryos and place them in PBS in a 100 mm cell culture dish on ice.
22. Euthanize embryos by decapitation. Dissect liver tissues and place them in a 24-well cell culture plate with 1 mL DMEM 10% (v/v) FBS medium.

**Note:** If necessary, cut a section of the tail and place in an Eppendorf microcentrifuge tube for genotyping (see PCR genotyping (optional) – Day 1 section). Ensure that the liver sample is numbered in the same manner as the genotyping biopsy.

23. To prepare single cell suspensions, squeeze livers through a 45 μm cell strainer using the end of a 1 mL plastic syringe. Resuspend these samples in 10 mL DMEM 10% (v/v) FBS medium.

24. Spin down cells at 300 × g for 5 min at 4°C.

25. Remove the supernatant and resuspend cells in 1 mL RBC lysis buffer. Incubate for 1 min at approximately 25°C and stop the lysis reaction by adding 5 mL DMEM 10% (v/v) FBS medium.

**Note:** Resuspend the cell pellet completely using a 1000 μL pipette.

△ **CRITICAL:** RBC lysis buffer is a hypotonic solution, and if the reaction lasts for a long time, cells other than RBCs are also damaged. Therefore, the reaction times must be strictly observed.

**Alternatives:** Commercially available RBC lysis buffers can also be used. Please follow the manufacturer’s protocol when using commercially available products.

26. Filter 6 mL of the cell suspension through a 40 μm pore nylon mesh and spin down cells at 300 × g for 5 min at 4°C.

27. Remove the supernatant and resuspend cells in 1 mL DMEM 20% (v/v) FBS medium supplemented with antibiotics.

28. Count the cells using a hemocytometer.

29. Plate fetal liver cells at a density of 4 × 10⁷ cells per 150 mm cell culture dish in 15 mL of DMEM 20% (v/v) FBS medium supplemented with antibiotics, IL-3 (20 ng/mL), IL-6 (50 ng/mL), and SCF (50 ng/mL).
It is possible to pool cell suspensions from embryos with the same genotype.

30. Incubate fetal liver cells at 37°C with 5% CO₂ for 24 h.

**PCR genotyping (optional) – Day 1**

- **Timing:** 3–4 h

If the embryos have different genotypes, genotyping is required for each embryo.

31. Add 300 μL of 50 mM NaOH to the tail biopsy and dissolve completely in a heat block at 97°C for 1 h.

  **Note:** Check that the tail is completely dissolved by vortexing.

32. Neutralize by adding 50 μL of 1 M Tris-HCl buffer (pH 8.0).
33. Centrifuge the samples at 11,500 × g at 4°C for 6 min.
34. Prepare a cocktail of PCR reagents for each mouse to be analyzed (Table 4).
35. Add 1 μL of tail DNA to 11 μL of the prepared cocktail, briefly vortex a mixture, and spin down.
36. Perform PCR using the appropriate thermal cycling conditions.
37. Analyze products of the amplification reaction using electrophoresis through an agarose gel to verify the genotype.
38. Figure 3 shows a series of procedures for obtaining E15.5 fetal liver cells.

**Fetal liver cell harvest – Day 2**

- **Timing:** 1 h

Explanted fetal liver cells are harvested for retroviral transduction.

39. Harvest the fetal liver cells cultured for 24 h (see steps 29 and 30) from 150 mm cell culture plates by pipetting and transfer to a sterile 50 mL conical tube.

  **Note:** Wash the plates vigorously with 10 mL of PBS and collect into the 50 mL conical tube. Repeat the washing step to harvest as many cells as possible.

40. Centrifuge the cells at 300 × g for 10 min at 4°C, remove the supernatant, and resuspend the pellet in 1 mL DMEM 20% (v/v) FBS medium supplemented with antibiotics.
41. Count cells and resuspend 3 × 10⁶ cells per 1 mL of DMEM 20% (v/v) FBS medium supplemented with antibiotics, IL-3 (40 ng/mL), IL-6 (100 ng/mL), and SCF (100 ng/mL).

  **Note:** About 70% of the cells seeded the day before are usually harvested.

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**Table 4. Cocktail of PCR reagents (for 1 reaction)**

| Reagent                   | Volume (μL) |
|---------------------------|-------------|
| e-Taq DNA polymerase      | 0.1         |
| 10× buffer                | 1.2         |
| 10 mM dNTPs               | 0.3         |
| 10 pmol forward primer    | 0.4         |
| 10 pmol reverse primer    | 0.4         |
| dH₂O                      | 8.6         |
| **Total**                 | 11.0        |
1st transduction – Day 2

© Timing: 3 h

A retroviral vector is introduced into the fetal liver cells.

42. Add 5 mL of DMEM 20% (v/v) FBS medium supplemented with antibiotics and polybrene (12 μg/mL) to 1 mL of the concentrated retroviral supernatant.

Note: The dilution ratio can be adjusted to increase transduction efficiency.

43. Mix the reconstituted retroviral supernatant and fetal liver cell suspension (see step 41) in a 1:1 (v/v) ratio and transfer 2 mL of the mixture per well to a 6-well tissue culture plate.
44. Spin the wrapped 6-well plates at 1,000 g for 60 min at 37°C.
45. When the spin infection is complete, remove the plastic wrap and place the plates in a 5% CO2 incubator at 37°C for 24 h.

2nd transduction – Day 3

© Timing: 3 h

A retroviral vector is introduced into the fetal liver cells.

46. Add 5 mL of DMEM 20% (v/v) FBS medium supplemented with antibiotics, IL-3 (20 ng/mL), IL-6 (50 ng/mL), SCF (50 ng/mL), and 6 μg/mL polybrene to 1 mL of the concentrated retroviral supernatant.
47. Carefully add 1 mL of the retroviral supernatant mixture to each well of a 6-well plate with fetal liver cells incubated for 24 h (see step 45).
48. Wrap the plate with plastic wrap and repeat the spin infection at 37°C at 1,000 g for 60 min.
49. Unwrap the plate and incubate in a 5% CO2 incubator at 37°C for 24 h.

Addition of fresh supplemented DMEM – Day 4

© Timing: 30 min

A retroviral vector is introduced into the fetal liver cells.

46. Add 5 mL of DMEM 20% (v/v) FBS medium supplemented with antibiotics, IL-3 (20 ng/mL), IL-6 (50 ng/mL), SCF (50 ng/mL), and 6 μg/mL polybrene to 1 mL of the concentrated retroviral supernatant.
47. Carefully add 1 mL of the retroviral supernatant mixture to each well of a 6-well plate with fetal liver cells incubated for 24 h (see step 45).
48. Wrap the plate with plastic wrap and repeat the spin infection at 37°C at 1,000 g for 60 min.
49. Unwrap the plate and incubate in a 5% CO2 incubator at 37°C for 24 h.
Addition of fresh media maintains fetal liver cells in a healthy condition.

50. After 24 h, carefully remove the top 2 mL of medium from each well of the 6-well plate containing the fetal liver cells, leaving only 1 mL of medium.

△ CRITICAL: Cells mostly tend to cluster in the center of the well and are sucked up very easily by pipetting, so place the tip on the edge and carefully aspirate the supernatant. Spin down at 300 × g for 5 min at 4°C to prevent loss of fetal liver cells before removing the top 2 mL of medium.

51. Add 1 mL of fresh DMEM 20% (v/v) FBS medium supplemented with antibiotics, IL-3 (20 ng/mL), IL-6 (50 ng/mL), and SCF (50 ng/mL) to each well of the 6-well plate.

52. Incubate the cells in a 5% CO2 incubator at 37°C for 24 h.

Irradiation of recipient mice – Day 4

© Timing: 1 h

Recipient mice receive systemic lethal irradiation from an X-ray or gamma-irradiator to disrupt their own hematopoiesis.

53. Irradiate mice the day before injection of fetal liver cells.

Note: It may be necessary to empirically establish the optimal radiation dose. We routinely used Rag1−/− mice as recipient mice and irradiated them with 4 Gy.

Alternatives: In certain cases, cells can be injected on the day of irradiation. However, since damage-associated molecular pattern (DAMP) signaling is activated due to irradiation, injection the next day is recommended to avoid damage to the injected fetal liver cells.

Harvest of transduced fetal liver cells – Day 5

© Timing: 3 h

Transduced fetal liver cells are harvested for transplantation into irradiated recipient mice.

54. After 24 h of incubation, collect the fetal liver cells from 6-well plates by pipetting.

Note: Wash the plates vigorously with 2 mL of PBS and collect into 50 mL conical tubes. Repeat the washing step to harvest as many cells as possible.

55. Centrifuge the cells at 300 × g at 4°C for 10 min and remove the supernatant.

56. Resuspend the fetal liver cells in 1 mL RPMI-1640 medium supplemented with antibiotics. Keep the cells on ice.

Alternatives: PBS 0.5% (v/v) FBS may also be used.

57. For each set of fetal liver cells, take a 10 μL sample and count the cells using a hemocytometer.

58. Resuspend the cells in RPMI-1640 medium supplemented with antibiotics at a concentration of 0.5–1 × 10⁷ cells/mL.

Note: We routinely intravenously inject 2 × 10⁶ cells with a transduction efficiency of 40%–50% per mouse. The number of cells to be injected can be adjusted for transduction efficiency, but the injection volume should not exceed 300 μL.
Alternatives: PBS 0.5% (v/v) FBS may also be used. Incubate FBS at 56°C for 30 min to inactivate components of the complement system and other potentially unknown cell growth inhibitors present in FBS.

59. For each set of fetal liver cells, take a 10 μL sample and analyze transduction efficiency using flow cytometry based on GFP expression.

Injection of fetal liver cells and monitoring the mice – Day 5

© Timing: 1 h, 6 weeks

Transduced fetal liver cells are adoptively transferred into recipient mice. Six weeks after cell transfer, the chimeric mice are euthanized, and immune cells are analyzed via flow cytometry.

60. For injection, load the cells into a 1/2 cc short needle insulin syringe (30-gauge).
61. Heat the mice using an infrared lamp and intravenously (via a tail vein) inject 200 μL of fetal liver cells per mouse.

△ CRITICAL: Be careful not to heat the mouse for a long time as it may cause skin burns.

62. Monitor the mice every week to ensure that they remain healthy.

Note: If reconstitution does not occur, the mice usually die within 2 weeks.

63. After 5–6 weeks, collect a small amount of blood from each mouse and confirm reconstitution of immune cells derived from the injected fetal liver cells using flow cytometry based on GFP expression.
64. Ultimately, sacrifice recipient mice and analyze cells from their lymphoid organs using flow cytometry.
65. Figure 4 shows the retroviral transduction of fetal liver cells and the transplantation of retrovirus-transduced fetal liver cells into recipient mice.
EXPECTED OUTCOMES

Basic helix-loop-helix family member E41 (BHLHE41, also known as SHARP1 and DEC2), is an important transcription factor that regulates development, self-renewal, and B cell receptor (BCR) repertoire of B-1a cells (Kreslavsky et al., 2017). The frequency and number of B-1a cells in Bhlhe41−/− mice are significantly reduced compared to those in wild-type mice (Kreslavsky et al., 2017). Capicua (CIC) is a high mobility group (HMG) box-containing transcriptional repressor that regulates lymphocyte development and differentiation (Hong et al., 2022; Kim et al., 2021; Lee, 2020; Park et al., 2017, 2019, 2020). B-cell-specific Cic null (Cic−/−;Cd19-Cre) mice exhibited expansion of the B-1a cell population (Hong et al., 2022). In addition, BHLHE41 levels were significantly increased in splenic B cells from Cic−/−;Cd19-Cre mice (Hong et al., 2022).

As a result of retroviral transduction, GFP-positive cells are observed 24 h after the 2nd spin infection (Figure 5A). Six weeks after cell transfer, the chimeric mice are euthanized, and splenic GFP-positive cells are purified by fluorescence-associated cell sorting (FACS) (Figure 5B). Proteins are extracted from GFP-positive cells and subjected to western blotting to confirm whether the desired chimeric mice were produced successfully (Figure 5B). We generated Bhlhe41-overexpressing fetal liver (FL) chimeric mice to investigate whether increased Bhlhe41 levels promote B-1a cell formation. To generate negative control FL chimeric mice, an empty vector was used for the transduction of FL cells. We confirmed the overexpression of BHLHE41 in GFP-positive B cells from Bhlhe41-overexpressing fetal liver chimeric mice (Figure 5C).

The frequency of GFP-positive cells in splenic lymphocytes was comparable between the negative control and Bhlhe41-overexpressing FL chimeric mice (65% versus 60%; Figures 6A and 6B). BHLHE41 overexpression significantly increased the frequency of splenic B-1a cells (Figures 6A and 6C). An increase in the frequency of B-1a cells was observed in GFP-positive cells among the splenocytes of Bhlhe41-overexpressing FL chimeric mice, suggesting an intrinsic effect of Bhlhe41 on B-1a cell development.

To determine whether the expansion of the B-1a cell population in Cic−/−;Cd19-Cre mice was caused by increased Bhlhe41 levels, we generated FL chimeric mice using wildtype (WT) and Cic−/−;Cd19-Cre FL cells transduced with the control or shBhlhe41-expressing retrovirus. The increased frequency of B-1a
cells was substantially rescued by the knockdown of Bhlhe41 in Cic-deficient B cells (Figure 7). These results indicate that CIC deficiency-induced BHLHE41 overexpression promotes B-1a cell formation.

LIMITATIONS
The efficiency of hematopoietic reconstitution after transplantation of transduced FL cells varies in each set of recipient mice. This may be due to fluctuations in the retroviral yield in cells transfected with viral vectors. Although the same amount of DNA is transfected into the same retroviral packaging cell line, the amount of virus produced can vary according to cell state and culture conditions. The recombinant retroviral titer directly affects transduction efficiency. Consequently, difference in the viral titer used for transduction causes differences in the frequency of newly generated transgenic cells in chimeric mice. In order to produce chimeric mice in which hematopoiesis occurs only with transduced cells, FACS-sorted GFP-positive cells should be transferred to the recipient mice. However, when sorting GFP-positive cells, fewer cells are obtained than predicted arithmetically; therefore, it is almost impossible to use the same number of retrovirus-transduced FL cells for every experiment to generate FL chimeric mice.

Unlike adult bone marrow (BM) HSCs, which are mostly quiescent, FL HSCs are highly proliferative (Zeng et al., 2020). Oxidative phosphorylation-, the citric acid cycle-, cell cycle-, and DNA replication-related genes are upregulated in FL HSCs compared to BM HSCs (Clarke et al., 2018; Manesia et al., 2015). Since retroviral transduction depends on cell proliferation (Bieniasz et al., 1995), it...
is preferable to use FL cells, which are comparatively more active in cell division than BM cells, for effective retroviral transduction. Moreover, as the repopulation potential of FL HSCs exceeds that of BM HSCs more than twofold (Rebel et al., 1996), it is possible to stably produce chimeric mice using fewer cells. However, FL HSCs injected into recipient mice cannot maintain their own properties because they are mainly located in the endosteal region of bone (Arai and Suda, 2007; Nilsson et al., 2001), which is the niche of the BM HSCs. The FL and adult BM are major niches for HSCs and play a crucial role in determining HSC properties. Bowie et al. showed that the characteristics of FL HSCs change after transfer to adult recipient mice (Bowie et al., 2007). Furthermore, cytokines and enzymes, which are expressed differently between fetuses and adults, also influence immune cell differentiation patterns. Terminal deoxynucleotidyl transferase (TdT), the enzyme responsible for the addition of non-templated (N)-nucleotides at the V(D)J junction, exhibits different expression profiles in fetuses and adults. Fetal hematopoiesis-derived B cells and γδ T cells scarcely display N-nucleotide additions to the V(D)J junction because the TdT expression is induced after birth. However, when FL cells were transferred to adult recipient mice, the repopulated cells exhibited similar N-nucleotide additions at the V(D)J junction compared to that in BM-derived cells (Matsuzaki et al., 1993). This result suggests that the recipient’s environment greatly influences hematopoiesis of HSCs, regardless of their origin. Therefore, it would be recommended to use mice of the same age as recipient mice to reduce variation in the results from each set of experiments.

**TROUBLESHOOTING**

**Problem 1**
Low transduction efficiency (steps 42 and 46).

**Potential solution**
In general, GFP expression can be observed through a fluorescence microscope 24 h after transduction, and the transduction efficiency can be determined based on the degree of GFP expression. Since
transduction efficiency is the most important factor that determines the number of transgene-expressing cells in chimeric mice. FL cells with a transduction efficiency of less than 30% are not suitable for use as donor cells. The following factors can affect transduction efficiency: 1) the retroviral vector size, 2) a retrovirus titer, and 3) conditions for activated cell proliferation. Since the retroviral yield decreases as the size of the vector increases, reducing the size of the retroviral vector may help to increase transduction efficiency. Alternatively, the retrovirus titer used for transduction can be increased by reducing the volume of the DMEM medium used for resuspension (step 15) or not performing additional dilution (steps 42 and 46) while concentrating the retroviral supernatant.

Problem 2
Inconsistent retroviral titers (step 15).

Potential solution
Retroviral titers may vary in each experimental set, depending on the transfection efficiency and the conditions of the packaging cell line. To ensure constant transduction efficiency, it is necessary to know the titer of the retrovirus used for transduction. Preliminary experiments can be performed to determine the titer of retroviral stocks. To determine retroviral titers, please refer to the protocol provided by MD Anderson Cancer Center (https://www.mdanderson.org/documents/core-facilities/Functional%20Genomics%20Core/Virus%20titering%20protocols.pdf).

- The day before transduction, seed HEK293T cells in a 96-well tissue culture plate at 2.5–3.0 × 10⁴ cells per well in 100 μL growth medium (i.e., DMEM with 10% FBS and 1% Penicillin-Streptomycin).
- After 24 h, make 2-fold serial dilution of viral stock in a round bottom 96-well plate using serum-free media (Figure 8). Mix the dilution by pipetting the content of the well up and down for 10–15 times and transfer 40 μL of the dilution to the next well. Repeat this until the serial dilutions are complete (Figure 8).
- Gently remove the culture medium from each well of the 96-well plate with HEK293T cells (see the first step), add 30 μL of diluted virus to each well, spin down at 1,000 × g for 2 h, and incubate the plate in a CO₂ incubator at 37°C for 4–6 h.
- Add 170 μL of growth medium to each well and continue to incubate the cells in a CO₂ incubator at 37°C for 72 h.
- Count the GFP-expressing cells under a fluorescence microscope.
- Calculate the Transducing Units per mL (TU/mL) using the follow formula:
GFP-expressing cell number per well \times \text{dilution fold} \times \frac{1 \text{ (mL)}}{\text{Volume of virus used for transduction (mL)}} = \text{TU/mL}

\text{Note: If 30 } \mu\text{L of diluted virus is used for transduction, multiply by } 33.3 \left(\frac{1\text{ (mL)}}{0.03\text{ (mL)}}\right) = 33.3.

\text{Problem 3}
Poor cell survival after transduction (step 52).

\text{Potential solution}
We routinely used 6 \mu\text{g/mL polybrene for infecting FL cells with retroviruses and found no effect on cell viability. The incubation time following the second transduction and the addition of fresh DMEM medium can be adjusted from 24 h to 15 h.}

\text{Problem 4}
Poor survival of mice after X-ray irradiation (step 53).

\text{Potential solution}
Biological irradiation produces undesired low-energy photons, which do not penetrate the mouse deep enough to disrupt hematopoietic function. These undesired photons are delivered to the mouse’s superficial layers, such as skin, potentially causing burns. Inflammation from skin burns can lead to mouse death before reconstitution occurs. With appropriate beam-conditioning filters, these low-energy photons are filtered out and only desired photons can be delivered to the mouse. There are typically three types of filters (Figure 9). Mix the dilution by pipetting the content of the): 1) filter 1 (2 mm Al), 2) filter 2 (1.5 mm Al, 0.25 mm Cu, 0.75 mm Sn), and 3) filter 3 (0.3 mm Cu). Before irradiating the mouse, it is important to note the irradiation dose per minute of each filter to ensure that the correct filter is selected.

\text{Problem 5}
Poor survival of mice after fetal liver cell injection (step 62).
Potential solution
Antibiotics can be added to the drinking water of the mouse before irradiation. The mouse may receive antibiotics for the first 2 weeks after FL cell injection. The daily oral dose of antibiotics commonly used is as follows: amoxicillin (50 mg/kg), doxycycline (10 mg/kg), enrofloxacin (50 mg/kg), and TMS (160 mg/kg) (Marx et al., 2014). If reconstitution does not occur, a maximum of $8 \times 10^6$ FL cells can be injected. When injecting a large number of cells, it is necessary to pipette well before loading the cells into the syringe.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yoontae Lee (yoontaell@postech.ac.kr).

Materials availability
This study did not generate novel reagents. Plasmids generated in this study are available upon request.

Data and code availability
Data reported in this paper will be shared by the lead contact upon request.

This manuscript does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS
Conceptualization: H.H. and Y.L., Writing—original draft: H.H., Writing—review and editing: H.H. and Y.L., Funding acquisition: Y.L.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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