

Chapter 2

Serial Transplantation of Bone Marrow to Test Self-renewal Capacity of Hematopoietic Stem Cells In Vivo

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Abstract

Hematopoietic stem cells (HSCs) have the ability to self-renew and replenish the blood and immune system for the life span of an individual. An age-associated decline in HSC function is responsible for the decreased immune function and increased incidence of myeloid diseases and anemia in the elderly. The changes in HSC function are thought to occur as the result of an intrinsic defect in the self-renewal potential of HSCs as they age. In this chapter, we describe a bone marrow serial transplantation protocol designed to test the self-renewal capacity of HSCs in vivo.

Keywords Hematopoietic stem cells, Serial transplantation, Stem cell self-renewal, Stem cell aging, Bone marrow

1 Introduction

Tissue-specific or adult stem cells are capable of self-renewal to preserve stem cell pools and differentiation into a variety of effector cells. With advancing age, the self-renewal capacity of stem cells invariably declines, eventually leading to the accumulation of unrepaired, damaged tissues in old organisms (1). Hematopoietic stem cells (HSCs), which give rise to various cellular components of blood, are known to exhibit a decline in self-renewal capacity with age. This is thought to contribute to decreased immune function in the elderly. The age-associated changes in HSC function have been extensively studied in mice, which include a strain-dependent increase in HSC number (2–5) and a decrease in their lymphoid differentiation potential (2, 4, 6). These changes are thought to occur due to an intrinsic defect in HSC self-renewal potential as they age (1).

Bone marrow transplantation is used to measure the “stemness” of HSCs, as a single HSC is sufficient to reconstitute the entire hematopoietic system of lethally irradiated recipients (7, 8). The fundamental principle of stem cell self-renewal is established by

the ability of bone marrow from such recipients to reconstitute secondary recipients with cells that are originated from the primary transplanted cells (7, 9). As a gold standard test for long-term self-renewal and multi-lineage potential of HSCs, serial transplantation of bone marrow cells is able to reconstitute lethally irradiated recipients in successive but limited transplants, reflecting the finite potential of HSC self-renewal (10–12). It has been shown that serial transplantation leads to a dose-dependent decrease in self-renewal capacity of HSCs (13–15), and this decline in stem cell function increases with the number of transplantations (10, 11, 16–19). Stem cell exhaustion has been reported in serial transfer experiments when unfractionated bone marrow (12, 18) or purified HSCs (20) are transplanted. The stem cell exhaustion in serial transplantation has been likened to an accelerated aging process, thus making it a powerful system to study HSC aging. Here we describe a protocol to test the long-term HSC (LT-HSC) self-renewal capacity using serial transplantation of unfractionated bone marrow.

2 Materials

2.1 Mouse Strains

1. Donor mice: Mice with the genotype of interest on a C57BL/6 background. Age-matched wild-type C57BL/6 littermates are used as controls.
2. Recipient mice: B6.SJL-*Ptprca*^a *Pepcb*^b/BoyJ (CD45.1⁺) aged 8–10 weeks (The Jackson Laboratory). This congenic strain with an allelic variant CD45.1 antigen can be distinguished from the C57BL/6 (CD45.2⁺) donor by flow cytometric detection of the CD45 antigens. The use of CD45.1⁺ recipient mice allows the determination of the contribution of the donor cells (CD45.2⁺) to reconstitution of bone marrow in lethally irradiated recipients, separate from residual recipient HSCs (CD45.1⁺) that survive radiation and subsequently give rise to hematopoietic cells marked by CD45.1.

2.2 Antibiotics

1. Neomycin (200×): 5 g dissolved in 50 ml distilled water, and sterilized with a 0.45 µm filter.
2. Polymyxin-B (200×): 1 million units dissolved in 50 ml distilled water, and sterilized with a 0.45 µm filter.

2.3 For Irradiation

1. Cesium-137 radiation source.
2. Radiation chamber for mice.

2.4 For Harvesting Bone Marrow

1. Harvesting medium: Biotin, flavin and phenol red-deficient RPMI-1640 medium (Invitrogen) supplemented with 10 mM HEPES (pH 7.2), 1 mM EDTA, and 2% fetal bovine serum (FBS).

2. Viability staining solution (1,000× stock): 3 mg/ml of acridine orange and 5 mg/ml of ethidium bromide dissolved in distilled water. Prepare 100× solution with PBS.
3. 70% Ethanol.
4. Razor blades, dissecting scissors, and forceps.
5. 5 ml syringes with needles (25 G1/2 and 18 G).
6. 60-mm tissue culture plates and 15 ml Falcon tubes.
7. 70 µm nylon mesh (autoclaved).
8. Hemocytometer.
9. Temperature controlled centrifuge.
10. Fluorescence microscope.

2.5 For Injections

1. Isoflurane.
2. Sterile Dulbecco's phosphate buffered saline (PBS).
3. Insulin syringe with fitted needle (29 G1/2).
4. Mouse restrainer.
5. Heat lamp.

2.6 For Flow Cytometry Analysis

1. Antibodies: Lineage cocktail contains biotin-conjugated Ter119 (clone TER-119), CD11b (clone M1/70), Ly-6G (Gr1, clone RB6-8C5), CD45R (B220, clone RA3-6B2), CD19 (clone 1D3), and CD3e (clone 145-2C11). Additional antibodies for HSC analysis include Ly-6A/E (Sca1)-FITC (clone D7), CD117 (c-Kit)-PE-Cy7 (clone 2B8), CD135 (Flt3)-PE (clone A2F10), and CD150-APC (clone mShad150). Other materials include CD45.1-APC-eFluor 780 (clone A20), CD45.2-Alexa Fluor 700 (clone 104), streptavidin-eFluor 450, and Fc block CD16/CD32 (clone 2.4G2, from BioXCell). All except Fc block are purchased from eBioscience.
2. Staining medium: Biotin, flavin and phenol red-deficient RPMI-1640 medium (Invitrogen) supplemented with 10 mM HEPES (pH 7.2), 1 mM EDTA, 2% FBS, and 0.02% sodium azide.
3. 96-well flexible plates and 5 ml polystyrene tubes.
4. LSR II flow cytometry system with 5 lasers and 18 detectors (BD Biosciences).

3 Methods

3.1 Antibiotic Treatment

Recipient mice are treated with antibiotics in drinking water 24 h prior to exposure to radiation. Add 2 ml each of 200× antibiotic stock solutions to 396 ml of autoclaved acidified water. Drinking water with antibiotics must be changed twice weekly until 1 month after transplantation (see Note 1).

3.2 Irradiation

Recipient mice are exposed to a lethal dose of 10 Gy (1,000 Rads) whole body radiation using a Cesium-137 source (see Note 2). At least five recipient mice are needed for each donor. For later (>3) cycles of transplantation, at least ten recipient mice are used per donor.

3.3 Harvesting Donor Bone Marrow

Harvest bone marrow on ice in a laminar flow hood. Using sterile techniques is essential while flushing and preparing bone marrow for injection.

1. Euthanize the donor mouse with isoflurane and cervical dislocation, and immerse the mouse in 70% Ethanol completely.
2. Cut the hind limbs away from the hip joint. Be careful not to break the femur while dissecting the hip joint. Similarly, cut the forelimbs away from the shoulder joint. Place dissected limbs in a 60-mm plate with cold bone marrow harvesting medium on ice.
3. Hold one dissected limb with a pair of forceps, and scrape away skin and muscle with a razor blade until only bone remains. Try to get rid of as much tissue that is attached to the bone as possible. Repeat this procedure with all limbs.
4. In a separate 60-mm plate with cold bone marrow harvesting medium on ice, disarticulate the knee joint by cutting through it with a razor blade. Cut tibia, femur, and humerus bones at both ends so that marrow cavities are open.
5. Fill a 5 ml syringe with bone marrow harvesting medium and fit a 25 G1/2 needle on the syringe. Hold the bone with a pair of forceps. Fit the needle into one of the cut ends of the bone and flush the bone marrow out. Repeat flushing until the color of the bone changes from a pinkish tinge to almost completely white. Repeat this procedure with all bones.
6. When all marrow has been flushed out, change the 25 G1/2 needle to an 18 G needle. The marrow is in large chunks and can be broken up into a single cell suspension by passing it through an 18 G needle several times.
7. Once a single cell suspension has been achieved, filter these cells through a 70 μ m nylon mesh into a 15 ml Falcon tube. Bring the final volume of cells to 10 ml with staining medium. Place cells on ice.

3.4 Cell Counting

1. Mix 10 μ l of cell suspension, 89 μ l of staining medium, and 1 μ l of 100 \times viability stain solution by pipetting. Add 10 μ l of this mixture in the hemocytometer.
2. Count green (viable) cells in a 5 \times 5 grid under fluorescence microscope. Red/orange cells are dead (ethidium bromide-positive) and can be counted in order to determine the ratio of live to dead cells in a sample.

3. Calculate concentration and total number of live cells:

Cells / ml in hemocytometer

$$= \# \text{ green cells in the } 5 \cdot 5 \text{ square} \cdot 10^4$$

Cells / ml in tube = cells / ml in hemocytometer $\cdot 10$

Total live cells in tube = cells / ml in tube $\cdot 10$

3.5 Preparation of Bone Marrow Cells for Injection

1. 2×10^6 bone marrow cells (~ 200 HSCs) per recipient mouse are usually used. Calculate the volume of cells sufficient for required injections plus two extra injections (see Note 3).
2. Spin down cells at 1,500 rpm ($388 \times g$) for 5 min in a centrifuge prechilled to 4°C . Resuspend cell pellet in 10 ml sterile Dulbecco's PBS.
3. Spin down cells again and resuspend cells in Dulbecco's PBS at a concentration of 2×10^6 cells per 200 μl . Place resuspended cells on ice and bring them to mouse facility for injection.
4. The remaining cells are centrifuged and resuspended in staining medium at 6×10^7 cells/ml. They are used to stain for the LT-HSC population in flow cytometry analysis (see Subheading 3.7).

3.6 Bone Marrow Cells Injection

Tail vein or retro-orbital injection can be used to inject donor bone marrow cells into recipient mice. Tail vein injection is commonly used, but it can be difficult to visualize tail veins in C57BL/6 mice. Alternatively, cells are injected into the retro-orbital sinus of the mouse in retro-orbital injection. While this method is technically less challenging, it has the limitation of being able to inject a maximum volume of 200 μl into the sinus. In addition, if the needle scrapes the cornea while injecting, the chances of developing corneal ulcers are high. Both methods of injection require practice, and we recommend practicing injection with PBS a few days before the actual experiment.

3.6.1 Tail Vein Injection

1. Prepare the sample for injection by filling 200 μl of cell suspension into an insulin syringe and keep aside (see Note 4).
2. Place an irradiated recipient mouse in a mouse restrainer. Warm the tail by shining a heat lamp on the tail briefly. This causes vasodilation and enables easy visualization of the tail veins.
3. Hold the tail in one hand, and select the vein you want to inject. There are two veins, lateral and medial, in each tail. Insert the tip of the needle into the vein, and withdraw slightly. If blood is drawn into the syringe, the needle is in the vein. Inject the cells quickly (see Note 5).

3.6.2 Retro-orbital Injection

1. Anesthetize the recipient mouse with isoflurane as follows. Soak a nestlet with isoflurane and place it in an empty cage. Place the mouse in this cage and wait until its respiration slows. This usually takes about 30 s.
2. Once respiration has slowed, take the mouse out and open an eye wide by spreading the lids with one hand. The retro-orbital sinus will be visible as a small opening at the medial corner of the eye.
3. Place the tip of the needle in the sinus, hold the syringe at an angle of 45° to the eye and inject cells into the sinus (see Note 6).
4. Place the animal back in its cage and observe until it awakens from the anesthesia completely.

3.7 Fluorescence-Activated Cell Sorting Analysis of LT-HSCs in Bone Marrow

1. Cells resuspended at 6×10^7 /ml in staining medium are incubated with anti-CD16/CD32 antibody at 1 μ g/ 10^6 cells for 10 min on ice to block the Fc receptors.
2. 25 μ l of these cells are then incubated with 25 μ l of each primary antibody in staining medium for 20 min in a 96-well flexible plate on ice.
3. Spin down cells at 1,500 rpm ($388 \times g$) for 5 min in a centrifuge prechilled to 4°C, and resuspend cell pellets in 100 μ l of staining medium. Repeat the washing step two more times.
4. Cells stained with biotin-labeled antibodies (lineage cocktail) are incubated with streptavidin-eFluor 450 for 15 min on ice and washed three times with staining medium.
5. After the final wash, cells are resuspended in 1 μ g/ml propidium iodide (PI) in staining medium for the exclusion of dead cells.
6. Flow cytometry analysis is performed on a 5-laser, 18-detector LSR II fluorescence-activated cell sorting (FACS) machine using 405, 488, 561, and 633-nm lasers. Data are analyzed using FlowJo software (Treestar). A representative experiment in Fig. 1 shows the progressive gating strategy used to analyze LT-HSCs from live bone marrow cells (PI-negative). Lineage negative (Lin^-) cells are those lacking significant expression of Gr-1, CD11b, Ter119, CD3, B220, and CD19. Long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs), and multipotent progenitors (MPP) are characterized by $\text{Lin}^- \text{Sca1}^+ \text{c-kit}^+ \text{CD150}^+ \text{Flt3}^-$, $\text{Lin}^- \text{Sca1}^+ \text{c-kit}^+ \text{CD150}^- \text{Flt3}^-$, and $\text{Lin}^- \text{Sca1}^+ \text{c-kit}^+ \text{CD150}^- \text{Flt3}^+$, respectively (21, 22).

3.8 Monitoring Mice and Subsequent Transplantations

1. After transplantation, recipient mice must be monitored daily for signs of ill-health including pallor, ruffled fur and lethargy. Typically, recipient mice receiving inadequate injections will become increasingly pale and sick, and die between 10 days and 2 weeks after irradiation due to bone marrow failure.

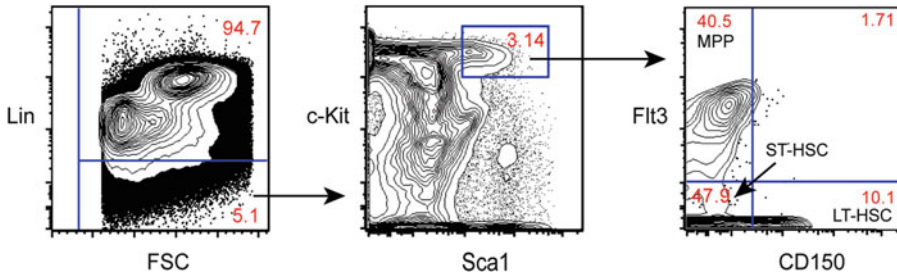


Fig. 1 A representative FACS analysis of LT-HSCs in bone marrow of a wild-type C57BL/6 mouse. Gates used for FACS analysis are displayed as *blue boxes*. The frequency of each gated population as a percent of the displayed cells is shown in *red*. Only live cells (propidium iodide excluding) are displayed. Lineage negative (Lin⁻) cells (*left panel*) are gated and then displayed in the *middle panel*. LSK (Lin⁻Sca1⁺c-Kit⁺, *middle panel*) cells are gated and displayed in the *right panel*

2. When 2 months after the injection have elapsed, the stem cell numbers reach homeostasis and secondary transplants can be performed. These recipients can be used as donors for the next transplantation. With every ensuing transplant, a sequential decrease in the frequency of the LT-HSC population is expected. HSCs from wild-type mice normally can reconstitute recipient bone marrow for 4–5 cycles of transplantation before stem cell exhaustion occurs.
3. Relative contributions of the donor and residual recipient bone marrow to the reconstitution in recipient mice can be determined by staining for CD45.1 and CD45.2 of bone marrow from the first transplantation onward. More than 90% of the cells are usually derived from the donor (CD45.2) bone marrow.

4 Notes

1. Leave a note on cages of recipient mice that they are on antibiotic treatment, so animal care technicians do not change the water.
2. Set up irradiation late in the afternoon, and irradiated mice can be transplanted with fresh bone marrow the next morning. Make sure to check the Cesium-137 source to calculate the radiation dosage every time you irradiate.
3. If injecting five mice, calculate the volume of cells required for 14×10^6 cells (5 plus 2 extra injections, 2×10^6 cells/injection).
4. Before injection, make sure that the cells are not chilled by warming them between your hands briefly.
5. Watch for clearing of the vein lumen; this indicates a successful injection. If you feel any resistance while injecting, it means the

needle has slipped out of the vein. If that happens, withdraw the syringe and reinsert it into the vein proximal to the original injection site.

6. If cells regurgitate back, that means they are not in the sinus. Take out the needle and reinsert. If cells come out through the nose, the procedure is unsuccessful and must be repeated. This process has to be performed quickly, as the effect of isoflurane wears off within 1–2 min.

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