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Adhesion, Migration, and Homing of Murine Hematopoietic Stem Cells and Progenitors

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Abstract

Recent advances in cell biology have demonstrated the role of multiple signaling proteins in the transduction of external signals to cytoplasmic and nuclear effectors, controlling the movement and/or retention of hematopoietic stem cells and progenitors (HSC/P) within the bone marrow, with important clinical implications. Multiple assays have become routine in the analysis of adhesion to the microenvironment, migration toward chemoattractant gradients, and homing of HSC/P in the bone marrow *in vivo*. In this chapter, we analyze some of the most frequently used assays in our laboratory to explore the ability of HSC to migrate, adhere, and home *in vitro* and *in vivo* assays.

Keywords

Hematopoietic stem cells; Migration; Homing

1. Introduction

Hematopoietic stem and progenitor cells (HSC/P) reside in the bone marrow (BM) cavity during postnatal life and may be localized to specific “niches” within the hematopoietic microenvironment (HM). A tiny fraction of HSC, of unknown physiological relevance, is to be found in the blood circulation and the number of these cells in circulation can be increased in a process termed “mobilization.” When infused into the blood during BM transplantation procedures these HSC engraft in the BM space and lead to the subsequent reconstitution of multilineage hematopoiesis. Poor engraftment of HSC/P has been shown as one cause of the failure of many protocols of cord blood transplantation, *ex vivo* HSC/P expansion, and retroviral-mediated gene therapy. Defective homing of HSC/P resulting from decreased adhesion to the extracellular matrix and/or loss of retention in the BM microenvironment may result in decreased HSC/P engraftment.

Fibronectin (FN), a major component of the extracellular matrix protein, is involved in the adhesion of HSC/P to the extracellular matrix of the BM through γ_1 -integrins and CD44 adhesion receptors. CXCL12, the best characterized chemoattractant has also been involved in BM retention and mobilization of HSC/P. Adhesion to FN and migration toward CXCL12 have become the reference surrogate assays to measure the ability of HSC/P to adhere to and/or migrate toward (chemotaxis) a chemoattractant gradient, and the *in vivo* homing

assay of HSC/P has become the gold standard in studying the ability of HSC/P to migrate into and be retained in the BM.

We have used these three assays in our analysis of the role of Rho GTPases in homing, mobilization, and retention, and in controlling the fine equilibrium between the marrow and the circulating pools of HSC/P (1–12).

2. Materials

2.1. Animals, Cells, and Cell Culture Reagents

1. C57B1/6 and B6.SJL-Ptpr^a Pepc^b/BoyJ mice (Jackson Labs, Bar Harbor, ME).
2. Bone marrow (BM) cells are obtained after crunching femorae, tibiae, and iliac crest bones from 8- to 10-week-old mice.
3. Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal calf serum (FCS), 2 mmol/L l-glutamine, and 100 IU/mL penicillin and 0.1 mg/mL streptomycin.
4. RPMI 1640 (Invitrogen, Carlsbad CA).
5. Protease-free bovine serum albumin (BSA).
6. Histopaque 1083 (Invitrogen).
7. Cell dissociation buffer (PBS based).
8. Phosphate-buffered saline (PBS).
9. Nontissue culture, 5- μ m pore, 24-well polycarbonate transwells.
10. Nontissue culture, 24-well plates, polystyrene.
11. 35 $\times$ 10-mm, 2 $\times$ 2-mm, 3-mL, gridded dishes, polystyrene.
12. 40- μ m mesh filters, nylon.
13. 15-mL conical and 4-mL round-bottom polypropylene tubes.
14. Turk's Solution

2.2. Flow Cytometry and Sorting

1. FACS Canto equipped with two lasers (Argon 488 and HeNe 633 nm) and ability to analyze signals from eight detectors (Becton–Dickinson, San Jose, CA).
2. Antibodies: Antimouse lineage antibodies, anti-CD3-FITC (clone 145-2C11), anti-CD4-FITC (clone GK1.5), antiCD8-FITC (clone 53-6.7), anti-CD45R/B220-FITC (clone RA3-6B2), anti-Ly6C/G-FITC (clone RB6-8C5), antiCD11b-FITC (clone M1/70), and anti-TER119-FITC (clone Ly-76). Anti-CD117(c-kit)-PE (clone ACK45), anti-Sca-1 (Ly6A/E)-APC (clone D7), anti-CD45.1-PE (clone A20), and anti-CD45.2-FITC (clone 104). Isotype controls were rat isotype-FITC (IgG2b kappa), rat isotype-PE (IgG2a kappa), and rat isotype-APC (IgG2b kappa). (All BD Biosciences, San Jose, CA).

3. Mouse serum.
4. PKH26 dye (Sigma-Aldrich, St. Louis, MO).

2.3. Recombinant Proteins

1. Recombinant human FN carboxyterminal fragment CH-296, corresponding to a protein of ~63 kDa (574 amino acids) containing a central cell-binding domain (type III repeat, 8–10), a high affinity heparin-binding domain II (type III repeat, 12–14), and a CS1 site within the alternatively spliced IIICS region of human FN (RetroNectin Reagent, Takara Bio USA, Madison, WI).
2. Recombinant murine CXCL12 (stromal cell-derived factor (SDF)-1 $\pm$) (R&D Systems, Minneapolis, MN).

2.4. Colony-Forming Cell (CFU-C) Assay

1. MethoCult[®] GF M3434 complete methylcellulose medium with recombinant cytokines for colony assays of murine cells, with erythropoietin (StemCell Technologies, Vancouver, Canada).

3. Methods

A description of the method of analysis of adhesion, migration, and homing of BM cells is presented. These cells can be preselected in vivo through 5-fluorouracil (5-FU) administration followed by in vitro low-density BM gradient.

3.1. Low-Density Gradient BM Cells

1. Crunched bones and their BM are sieved through 40-mm mesh filters and suspended in PBS/5% FCS at a concentration of $20\text{--}50 \times 10^6/\text{mL}$.
2. 4 mL of Histopaque 1083 solution (room temperature) are deposited into 15-mL conical tubes. A maximum volume per tube of 2 mL of cells is layered onto the Histopaque medium.
3. The tubes are spun down at $500 \times g$ for 30 min at room temperature.
4. Collect interphase cell layer between the upper phase and the Histopaque phase of the gradient.
5. Collected cells (~2 mL) are washed in 40 mL of PBS once at $400 \times g$ for 10 min at room temperature. The supernatant is removed and the cell pellet resuspended in 1 mL of PBS.
6. Cells are counted in a hemocytometer (1:10 dilution with Turk's solution).
7. The cells are resuspended in PBS at a concentration of 20×10^6 cells/mL.

3.2. Input Lineage-Negative/c-kit⁺/Sca-1⁺ BM Cells

1. Three aliquots of 1×10^6 cells per specimen are used for flow cytometry analysis. All the specimens will be stained according to manufacturer's instructions, by

incubating cells with antibodies in a final volume of 100 μ L, containing cells, antibodies, and 2% mouse serum in PBS.

2. The first is stained with rat isotype-FITC, rat isotype-PE, and rat isotype-APC antibodies.
3. The second is stained each with anti-CD3, CD4, CD8, Mac-1, Gr-1, CD45R/B220, Ter119, rat isotype-PE, and rat isotype-APC antibodies.
4. The third is stained with each of the lineage antibodies along with anti-c-kit and Sca-1 antibodies.
5. The thresholds of positivity are determined to 1% of the population. Lineage-negative/c-kit⁺/Sca-1⁺ cells appear as a well-defined population in a dot plot (c-kit vs. Sca-1) gated on lineage-negative low-density BM cells.
6. The percentage of lineage-negative/c-kit⁺/Sca-1⁺ cells is recorded.
7. Cell selection of lineage-negative/c-kit⁺/Sca-1⁺ cells can be performed in a cell sorter, according to established protocols (see under Chapter 3).

3.3. Input CFU Quantitation

1. Input CFUs are enumerated by mixing 80,000 low-density BM cells (in 4 mL of Methocult medium, in a 4-mL round bottom tube).
2. Tubes are vortexed allowed to rest for 10 min, and plated on three 35-mm gridded dishes.
3. Dishes are incubated at 37°C, 5% CO₂ for 10 days.
4. Colony-forming units (CFUs) are identified as clusters with more than 50 cells and counted. All colonies, including BFU-E, CFU-GM, and CFU-Mix are counted. Differential analysis of these three populations is possible but it requires experience. If inexperienced, the count, the total number, of CFU per dish is recommended.
5. The average number of CFU per 20,000 low-density BM cells plated in each of the three dishes is recorded. The average number of CFU per 100,000 plated cells is calculated by multiplying the resulting number by 5 (*input CFU*).

3.4. Adhesion to Fibronectin

1. Nontissue culture, 24-well plates are coated with CH-296 at 8 μ g/cm² or BSA (as control) and kept overnight at 4°C. Assays are performed by triplicate.
2. The plates are subsequently blocked with 2% BSA for 30 min at room temperature.
3. A total of 50,000 low-density BM cells, suspended in RPMI 1640 medium containing 10% FBS, and allowed to adhere to the coated plates for 1 h at 37°C.
4. After incubation, nonadherent cells are collected by carefully rinsing the plates three times with medium. Adherent cells are harvested by vigorously rinsing the

plates with PBS twice and cell dissociation buffer once. Finally, wells are rinsed with PBS and the content is collected into the adherent fraction tube.

5. The adherent fraction is centrifuged at $400 \times g$ for 10 min and the supernatant removed.
6. The cell pellet is resuspended in 200 μ L of PBS/5% FCS and mixed with 3.8 mL of CFU assay medium. 3 mL of CFU medium with cells is plated, incubated, and scored as for the input CFU assay. The output CFU is the average of the three dishes scored on day +10 of incubation (*output CFU*).
7. The percentage of CFU adhesion is calculated as follows: Adhesion of CFU-C to CH-296 (%) = $100 \times [(\text{output CFU} \times 2)/(\text{input CFU})]$. The average of the three adhesion assays is the final result.
8. Expected result: The percentage of CFU-C adhering to CH-296 after 1 h for low-density BM cells from C57Bl/6 mice ranges from approximately 8 to 15%, averaging ~10%.
9. Alternative flow cytometry analysis: Alternatively, the overall volume of the triplicate assay can be pooled, spun down at $400 \times g$ for 10 min, resuspended in 100 μ L of PBS/5% FCS, and stained with lineage antibodies, anti-c-kit, and anti-Sca-1 as mentioned for the third flow cytometry tube in steps 1, 4, and 6 in Subheading 3.2. The acquisition and analysis of this population will be performed using the same gating analysis as in Subheading 3.2.

3.5. Chemotaxis Toward a CXCL12 Gradient

1. Cells from step 4 in Subheading 3.1 are suspended in a cell solution containing RPMI 1640/0.5% BSA at a concentration of 1×10^6 cells/mL.
2. 600 μ L of chemotaxis buffer containing 100 ng/mL CXCL12 is added to the bottom well of the transwell plate.
3. 100 μ L of cell solution is added to upper chamber (100,000 cells).
4. The plate is incubated for 4 h at $37^\circ\text{C}/5\% \text{ CO}_2$.
5. The upper chamber is carefully removed and discarded.
6. The solution from lower chamber is removed and placed into a labeled, sterile 4-mL round-bottomed polypropylene tube.
7. The wells are washed with PBS, the wash added to the tube, and then washed again with cell dissociation buffer once.
8. The percentage of migrating cells are calculated as in steps 5–7 in Subheading 3.4 (see Note 1).
9. Expected result: The percentage of CFU-C from low-density BM of C57Bl/6 mice, adhering to CH-296 after 4 h, ranges from 20 to 40%, averaging ~30%.

¹This experiment can be performed at different doses of CXCL12 if a dose-response analysis of chemotaxis is required.

10. Alternative flow cytometry analysis: Alternatively, the overall volume of the triplicate assay can be pooled, spun down at 400 ×g for 10 min, resuspended in 100 µL of PBS/5% FCS, and stained with lineage antibodies, anti-c-kit, and anti-Sca-1 as mentioned for the third flow cytometry tube in steps 1, 4, and 6 in Subheading 3.2. The acquisition and analysis of this population will be performed using the same gating analysis as in Subheading 3.2.

3.6. Progenitor Homing Assay

1. We recommend that a minimum of three mice (pooled) are used per group to compare different groups of BM cells. BM nucleated cells suspended in PBS/5% FCS will be the starting cell population.
2. Input CFU analysis is done as mentioned earlier (see step 5 in Subheading 3.3).
3. A minimum of eight recipient mice (C57B1/6 females, ages 8–12 weeks) per group is included.
4. Recipient mice are lethally irradiated at a validated dose able to kill all the endogenous CFUs and CFU-S-Day 12. In our institution, this dose is 700 cGy in a first dose followed by a second dose of 475 cGy 3 h apart. The dose rate is 50–100 cGy/min. If the dose that completely destroys any remaining endogenous CFU is unknown, set up a control group with five irradiated mice just transplanted with 0.2 mL of PBS. Find the minimal dose since an excessive irradiation may induce severe mucositis and animal death (13, 14).
5. One hour after irradiation, a total of 50×10^6 donor BM cells are intravenously injected into the tail vein of each of the recipient mice. This is usually done at 5.00 pm.
6. On the following day (9.00 am), mice are sacrificed by CO₂ inhalation and cervical dislocation and their two femurs and tibiae, and each spleen are harvested (see Note 2).
7. All the harvested bones from each mouse are crunched and processed as mentioned in step 1 in Subheading 3.1. Splenocytes are obtained by shearing each individual spleen between two glass slides and flushing them with PBS/5% FCS into 35-mm dishes. Cell suspensions are sieved and processed as described in step 1 in Subheading 3.1.
8. BM and spleen cells are each resuspended in 200 µL of IMDM + 10% FCS and counted.
9. 0.2 mL of BM or spleen cell suspensions are added to 4 mL of CFU medium. Assays are set as indicated in the CFU assay protocol (see Subheading 3.3).

²Other time points can also be analyzed and the progenitor homing kinetics may differ in specific protein deficiencies. In our experience, the time points that can be used are 3, 6, 12, 16, and 24 h. Later time points may be affected by cell division.

10. Colonies are scored on day +10. Specific CFU subset (CFU-GM or BFU-E) homing can be calculated if required. In our experience, it is very difficult to address CFU-Mix homing.
11. Homing in BM (%) is calculated as follows (considering that two femurs and two tibiae contain approximately 20% of the total BM of a mouse)(15): Homing per BM (%) = $100 \times 5 \times [(\text{output CFU per femora and tibiae})/(\text{input CFU} \times 1,000)]$.
12. Homing per spleen (%) is calculated as follows: Homing per spleen (%) = $100 \times [(\text{output CFU per spleen})/(\text{input CFU} \times 1,000)]$.
13. Expected results: Homing of normal CFU in C57B1/6 mice (donors and recipients) is around 10% (5–15%).

3.7. Stem Cell BM Homing Assay

This assay is very similar to the previous one but it uses a competitive repopulation readout on 3-h homing cells. It intends to measure the frequency of homed repopulating stem cells in a cell inoculum.

1. Proceed as in steps 1, 2, 4, and 5 in Subheading 3.6.
2. After 3 h, recipient mice are sacrificed and their bones harvested and processed as in step 1 in Subheading 3.1.
3. BM and spleen cells are each resuspended in 1.1 mL of IMDM + 10% FCS and counted.
4. BM specimens are mixed with 1.1 mL of a cell suspension of freshly harvested B6.SJL-Ptprca^a Pepcb^b/BoyJ BM (prepared as in step 1 in Subheading 3.1) at a concentration of 5×10^6 cells/mL.
5. Fractions of 0.2 mL of mixed BM (corresponding to 1/6 of bone cell suspensions and 5×10^5 competitor BM cells) are transplanted into ten lethally irradiated (same irradiation dose as in primary recipients) secondary B6.SJL-Ptprca^a Pepcb^b/ BoyJ recipient mice intravenously via the tail vein.
6. An input control of repopulation ability of BM from CD45.2+ cells can be performed in parallel if two groups of transgenic mice are compared. Low-density BM are harvested and processed as indicated in Subheading 3.1. A cell suspension made by mixing 15×10^6 BM cells/mL of test (C57B1/6) mice and 15×10^6 BM cells/mL of B6.SJL-Ptprca^a Pepcb^b/ BoyJ mice BM is made.
7. Every month after transplantation, the chimerism of recipient mice in peripheral blood is determined by flow cytometry. This determination is performed by staining peripheral blood with anti-CD45.1 and anti- CD45.2 antibodies, according to manufacturer's instructions.
8. The content of competitive repopulation units (CRU) in the inoculums of homing specimens and input controls are calculated as follows (Harrison's formula): $\text{CRU} = 5 \times [(\text{percentage of CD45.2+ cells})/(1 - \text{percentage of CD45.1+ cells})]$, which assumes that there is 1 CRU per 100,000 BM cells.

9. Homing in BM (%) is calculated as follows (considering that two femurs and two tibiae contain approximately 20% of the total BM of a mouse) (Boggs et al.):
Homing per BM (%) = $100 \times 6 \times 11 \times (\text{homed CRU}/500)$.
10. If a control of CRU content in BM is performed, this formula can be corrected:
Homing per BM (%) = $100 \times 6 \times 11 \times [\text{homed CRU}/(\text{control CRU} \times 500)]$ (see Note 3).

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³We have only validated this assay for BM homing and not for spleen homing, however, it may be applied if a large number of cells are to be transplanted into the primary recipients. We have not done linearity studies beyond 50×10^6 cells transplanted.

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