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Measuring the Aging Process in Stem Cells

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Summary

Stem cells persist in replenishing functional mature cells throughout life by self-renewal and multilineage differentiation. Hematopoietic stem cells (HSCs) are among the best-characterized and understood stem cells, and they are responsible for the life-long production of all lineages of blood cells. HSCs are a heterogeneous population containing lymphoid-biased, myeloid-biased and balanced subsets. HSCs undergo age-associated phenotypic and functional changes, and the composition of the HSC pool alters with aging. HSCs and their lineage-biased subfractions can be identified and analyzed by flow cytometry based on cell surface makers. Fluorescence-activated cell sorting (FACS) enables the isolation and purification of HSCs that greatly facilitates the mechanistic study of HSCs and their aging process at both cellular and molecular levels. The mouse model has been extensively used in HSC aging study. Bone marrow cells are isolated from young and old mice and stained with fluorescence-conjugated antibodies specific for differentiated and stem cells. HSCs are selected based on the negative expression of lineage markers and positive selection for several sets of stem cell markers. Lineage-biased HSCs can be further distinguished by the level of SLAMF7/CD150 expression and the extent of Hoechst efflux.

Keywords

Stem Cells; Hematopoietic Stem Cells; Aging; Flow Cytometry; Fluorescence-activated Cell Sorting

1. Introduction

Stem cells are rare and self-renewing cells that give rise to all types of mature cells. In any tissue or organ with high cell turnover, stem cells should be long lived in order to constantly replenish cells lost throughout the lifetime and to maintain optimal tissue function.

Therefore, stem cells are exposed to the noxious effects of both intrinsic and extrinsic effectors of damage during organismal aging (1). As a result, stem cells may undergo functional decline, and their repair and renewal capacity may be impaired, which in turn contributes to overall organismal aging (2, 3). Because of the unprecedented experimental

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model systems that are available for the exploration of hematopoietic stem cells (HSCs), stem cell aging research in the field of hematology has been the subject of extensive studies and has advanced dramatically in the past several years (4). It is likely that the same broad concepts that define and characterize blood-forming stem cells will apply to stem cell populations found elsewhere.

HSCs reside in the bone marrow and provide life-long production of hematopoietic progenitors (HPCs) and peripheral blood lymphoid and myeloid cells. At the same time, HSCs undergo self-renewal divisions in order to sustain the stem cell pool. Precisely regulated blood cell production is vital for organismal survival; therefore functional failure of HSCs can potentially threaten the longevity of an organism. Accumulating evidence in the study of mouse models has suggested that HSCs undergo age-related changes in phenotype, function and clonal composition. The changes of aged HSCs include: increased HSC number (5–9); reduced self-renewal capacity (10, 11); skewed differentiation towards myeloid lineage at the replacement of lymphoid cells (5, 7, 12); enhanced mobilization from bone marrow to peripheral blood (13); reduced homing back to bone marrow (14); decreased proliferative response to cytokines (9); and loss of cell polarity (15). The HSC population is heterogeneous and is composed of three subfractions with distinct differentiation potentials (16–18). These subfractions are 1) myeloid-biased HSCs with a high myeloid differentiation potential, 2) lymphoid-biased HSCs with a preferred lymphoid differentiation, and 3) balanced HSCs with equal lineage outputs. With aging, myeloid-biased HSCs become dominant in the old bone marrow, resulting in a skewed myeloid output in the circulation. These phenotypic and functional alterations in old HSCs have been ascribed to the age-associated accumulation of a variety of damages that are intrinsic to HSCs as well as extrinsic to their microenvironment (19–21). DNA mutations (22–24), telomere shortening (25), and oxidative stress (26, 27) are among the most significant cellular changes in old HSCs; these changes trigger signaling cascades that lead to cell cycle checkpoint activation (28, 29), apoptosis (30), senescence (31, 32) or differentiation (33). At the molecular level, young and old HSCs demonstrate distinct profiles in both transcriptome and epigenome, resulting in the identification of genes and pathways that correlate with HSC aging (34–37).

Characterization of HSCs and their aging process requires the isolation and purification of HSCs. The advent of flow cytometry has allowed this task to be successfully implemented and enables researchers to isolate HSCs and other types of blood cells from young and old subjects (mice or humans) for further functional analysis. In this procedure, bone marrow cells are stained with fluorescence conjugated monoclonal antibodies that bind specific cell surface proteins. HSCs are analyzed and sorted by fluorescence activated cell sorting (FACS) based on the expression level of these markers. In the mouse model, HSCs and HPCs are enriched in the population negative for the markers of all differentiated lineages cells (Lineage-) and positive for stem cell markers *Sca-1* and *c-Kit* (LSK cells) (38). HSCs are further purified from LSK population by several sets of cell surface proteins, including 1) Flk-2⁻ CD34⁻ LSK (39); 2) CD150⁺ CD48⁻ CD41⁻ LSK (40, 41); 3) SP^{LSK} (Side Population with high Hoechst efflux) (42); and 4) EPCR⁺ CD150⁺ CD48⁻ CD34⁻ LSK (10). In these phenotypically-defined HSCs, lineage-biased HSCs can be distinguished by the expression of CD150 protein or the extent of Hoechst efflux (17, 43, 44). Myeloid-biased HSCs are Flk-2⁻ CD34⁻ LSK CD150^{high} or SP^{LSK} with lower Hoechst efflux (Lower-

SP^{LSK}), whereas Flk-2⁻ CD34⁻ LSK CD150^{negative/low} or SP^{LSK} with higher Hoechst efflux (Upper- SP^{LSK}) population contains a majority of lymphoid-biased HSCs. Balanced HSCs have not been clearly defined, but cells with Flk-2⁻ CD34⁻ LSK CD150^{low} immunophenotype demonstrate a balanced output at both lymphoid and myeloid lineages. Another SLAM maker, CD229, has recently been found to distinguish lymphoid-biased HSCs from myeloid-biased cells (45). Although the functionality of these phenotypically-defined HSCs needs to be confirmed by the gold-standard transplantation assay, flow cytometry-mediated HSC purification greatly facilitates analysis of the cellular and molecular mechanisms of HSCs and their aging process. HSC aging in humans has not been well studied partially due to lack of markers to define highly purified HSCs (46). Despite this limitation, studies have shown that HSCs from older people have some characters similar to those in old mice: increased numbers, myeloid differentiation skewing (47, 48), and accumulation with damages (26, 32, 47, 49), for example. Human HSCs and HPCs are identified by a different set of markers, which are lineage⁻CD34⁺ CD38⁻ CD45RA⁻ CD90⁺. The size of this population has been shown to increase with aging (48). CD49 has been recently discovered to present in a group of highly primitive HSCs, leading to a further purification of human HSCs (50). Since CD150 cannot be used for labeling human HSCs, the clonal composition of HSC population in human and its shift during the aging process is not clear.

Flow cytometry-mediated HSC sorting is a multi-step process (Fig. 1). HSCs are very rare in the adult bone marrow, comprising less than 0.005% of total bone marrow cells. This low frequency requires a large number of cells to be processed in order to obtain sufficient HSCs for experimental uses. For this reason, pre-enrichment steps are typically used to remove differentiated cells and reduce the sample size and subsequent sorting time. Ficoll-mediated density separation and/or red blood cell lysis are two procedures commonly used to remove granulocytes and red blood cells. The most prominent step for pre-enrichment of stem and progenitor cells from the bulk of bone marrow cells is the immunomagnetic depletion of all types of differentiated cells. HSCs are identified based on the antibody-mediated negative and positive selections, in which differentiated cells are antibody-labeled and removed, whereas HSCs are stained with specific makers and recovered. This is a multiple-step cell separation process requiring multiple fluorochromes to be used. Therefore, the precise compensation among different fluorescent signals will ensure that these signals are correctly detected by the flow cytometer and HSCs will be selected by the defined immunophenotype and sorted with a high purity. In this chapter, we will focus on FACS and describe the procedures involved in the HSC staining and sorting from the mouse bone marrow.

2. Materials

2.1 Animals

C57BL/6 mice are the strain commonly used for aging studies. This strain has a mean lifespan of 800 days in males and 750 days in females (52, 53). Typically, young C57BL/6 mice are 6–8 weeks old, and old ones used in aging studies are usually more than 24 months old. If other inbred strains are used, the age of old mice needs to be practically determined based on their actual mean lifespan.

2.2 Reagents and Supplies

1. Medium: Hank's Balanced Salt Solution (HBSS) with 2% heat-inactivated fetal bovine serum, phosphate buffered saline (PBS) without calcium and magnesium.
2. 3-mL syringes with 23G 1-gauge needles to flush marrow out of femurs and tibias of young mice. Marrow cavity in old mice is bigger, therefore the use of bigger needles, such as 20G 1 1/2-gauge, is recommended.
3. Cell strainer with 70 μ m nylon screen to filter bone marrow single cell suspension.
4. Density-separation medium: Ficoll-Paque PREMIUM 1,084; store and use at room temperature.
5. Red blood cell lysis buffer: 8.26 grams of NH_4Cl , 1.19 grams of NaHCO_3 and 200ul of EDTA (0.5M, pH8) in 1 liter distilled H_2O . Adjust pH to 7.3 and filter sterilize through 0.2 μ m filter. Store stock solution at 4°C.
6. 15-mL or 50-mL conical tubes for holding bone marrow cells during antibody staining.
7. 5-mL polystyrene round-bottom tube (facs tube) for holding cells during fluorescence-activated cell sorting.
8. 5-mL polypropylene round-bottom tubes (collection tube) or 1.5-mL eppendorf tubes for collecting sorted cells.
9. Magnetic stand for 15ml and 50ml centrifuge tubes.

2.3 Fluorescence-Conjugated Antibodies

1. Lineage marker antibodies: 53-7.3 (anti-CD5), 53-6.7 (anti-CD8a), RA3-6B2 (anti-B220; CD45R), M1/70 (anti-CD11b; Mac-1), RB6-8C5 (anti-Gr-1; Ly-6G and Ly-6C), and Ter119 (anti-erythroid antigen; Ly76). Note that all these lineage antibodies (used in the author's lab) are biotinylated and anti-mouse antibodies.
2. Streptavidin-conjugated allophycocyanin-Cy7 (APC-Cy7) antibody for the secondary immunofluorescent staining of lineage cells, labeled with biotinylated primary antibodies.
3. Alexa Fluor® 700-conjugated anti-mouse c-Kit (CD117) antibody.
4. Pacific blue (PB)-conjugated anti-mouse Sca-1 (Ly6A/E) antibody.
5. Phycoerythrin (PE)-conjugated anti-mouse Flk-2/Flt3 (CD135) antibody
6. Fluorescein-5-isothiocyanate (FITC)-conjugated anti- mouse CD34 antibody.
7. Alexa Fluor® 647 anti-mouse CD150 (SLAM) antibody.
8. Hoechst 33342 is a DNA dye that can be pumped out by stem and progenitor cells. Stem/progenitor cells thus have a Hoechst low fluorescence in both the blue and red regions of the spectrum.
9. A viability dye, such as propidium iodide (PI) or 7-aminoactinomycin D (7-AAD).

10. 2.4G2 antibody against FcγII/III receptors for blocking non-specific antibody binding.

2.4 Magnetic Beads for Depletion of Differentiated Cells

Magnet beads-mediated depletion of differentiated cells is recommended for HSC sorting, because it will pre-enrich HSCs and HPCs and reduce the sorting time. Two types of magnetic beads are commercially available for this purpose.

1. Dynabeads® Sheep Anti-Rat IgG: 4.5 μm superparamagnetic beads covalently bound with affinity purified polyclonal sheep anti-rat IgG.
2. Streptavidin-conjugated paramagnetic beads used for MACS columns and cell separation unit from Miltenyi Biotec (Auburn, CA).

2.5 FACS Instrument

Flow cytometers/cell sorters are equipped with lasers, filters, and detectors and can simultaneously select for the presence or absence of several cell-surface markers. Several instruments are available from different manufacturers. For the technique described in this chapter, sorting is performed using the BD FACS Aria™ II.

3. Methods

3.1 Preparation of Bone Marrow Cells

3.1.1 Sample—

1. Euthanize young or old mice by either cervical dislocation or isoflurane inhalation.
2. Dissect the femurs and tibias, and cut the ends off the bones.
3. Put 10ml medium (HBSS + 2% FBS) into a 50-mL conical tube, put 23G 1-gauge (young marrow) or 20G 1 1/2-gauge (old marrow) needles onto a 3-mL syringe, and pre-fill the syringe with 1ml medium. Note that the amount of medium used is for collecting bone marrow cells from 10 mice.
4. Poke the needle to one end of the bone and flush the marrow out of the bone cavity with pre-filled medium several times. Repeat the same procedure from the other end of the bone to ensure the maximal collection of marrow cells.
5. Prepare a single-cell suspension by drawing and expelling the marrow and medium through the needle several times; the marrow will tend to dissociate as it passes through the needle.
6. Filter the single cell suspension through a nylon screen (cell strainer, 70 μm).

3.1.2. Density Separation Procedure (Ficoll)—

1. Dilute samples 1:2 in medium, and make the final volume 30mL.
2. Pour 20 mL Ficoll into a 50-mL conical tube, and then slowly layer (tilting tube and running the cells down the side of the tube) 30 mL of diluted marrow cells on top (*see* Note 1).

3. Centrifuge at 600g for 30 min at room temperature with the “Brake Off” setting.
4. Remove half of the top layer and discard.
5. Carefully pipet off “cloudy” interface layer (approximate 10 mL) and transfer into a clean 50-mL tube. Wash these cells with 50 mL medium twice; a pellet should be seen at the bottom of the tube (*see* Note 2).

3.1.3. Red Cell Lysis Procedure (Optional)—

1. Resuspend cells in red blood cell lysis buffer at 3–4 times the original sample volume.
2. Incubate on ice for 10 min.
3. Centrifuge cells at 400g, 4°C for 5 minutes, wash twice, and resuspend cells in medium with a density of 10^8 cells per mL. Meanwhile, aliquot 0.5×10^6 cells into a facs tube and use them as a non-staining control (Control 1) (*see* Note 3). Note that all control cells are resuspended into 50 microliters of medium.

3.2 Staining Bone Marrow Cells with Lineage Antibodies

1. Cells are first stained with 2.4G4 antibody directed against FcγII/III receptors on ice for 15 minutes, washed twice, and resuspended into 3ml medium.
2. Cells are stained with biotinylated antibodies against lineage markers, including CD5, CD8a, B220, Mac-1, Gr-1 and Ter119. All antibodies should be titrated before use and used at dilutions that brightly stain antigen-positive cells without nonspecifically staining antigen-negative cells. The concentrations of antibodies used in the authors’ laboratory are CD5 (1:200), CD8a (1:200), B220 (1:300), Mac-1 (1:320), Gr-1 (1:350) and Ter119 (1:320). All the antibodies are mixed to make lineage antibody cocktail according to these titrations.
3. Add lineage antibody cocktail to bone marrow cells prepared in step 3.1.3 with a concentration of $112.4 \mu\text{L}$ per 1×10^8 cells.
4. Incubate cells with antibody for 30 minutes at 4°C on a rocker.

¹Add the Ficoll-Paque into the tube first, and then put the bone marrow cells on the top. It is extremely important to load the cells slowly – especially the first several millimeters of cell solution. After a correct loading, a clear interface between cells and Ficoll-Paque should be seen, and this will ensure a successful gradient separation.

²All procedures are performed on ice from this step. After washing and spinning, a white pellet containing mononucleated cells should be seen at the bottom of the tube. The presence of a red pellet indicates the incomplete separation of red blood cells from mononucleated cells; red blood cell lysis is recommended. After the gradient separation, around 10% of total bone marrow cells will be recovered.

³Single-color controls are used for the background staining and compensation that are required for any type of flow cytometry. The excitation spectrum of a fluorochrome is a range of light wavelengths. When multiple fluorochromes are used in flow cytometry, the emission spectra from different fluorochromes could be overlapped. As a result, fluorescence could leak to other filters, causing false positive signals. To correct this spectral overlap, a procedure called fluorescence compensation is needed. Cells are stained with a single fluorochrome and run through the flow cytometer. The compensation settings are adjusted to ensure that the signal detected in a particular detector derives solely from the fluorochrome that is being measured. In the chapter described here, 8 tubes of control cells are set up, and each tube contains 0.5×10^6 cells in 50 μL medium. Tube 1 is unstained (background) control. Tube 2 is APC-Cy7 lineage control. Tube 3 is FITC CD34 control. Tube 4 is Pacific Blue Sca1 control. Tube 5 is Alexa Fluor 700 c-Kit control. Tube 6 is PE Flk2 control. Tube 7 is Alexa Fluor 647 CD150 control. Tube 8 is PI control.

5. Wash cells twice and resuspend cells in medium, with a density of 10^8 cells per mL in a 15mL conical tube. In the meanwhile, aliquot 0.5×10^6 cells into a facs tube and use them as a lineage staining control (Control 2).

3.3 Magnetic Beads Depletion of Differentiated Lineage Cells

3.3.1 Dynabeads® Sheep Anti-Rat IgG—

1. Wash Dynabeads® Sheep Anti-Rat IgG before use according to the manufacturer's instruction. Resuspend Dynabeads with a density of 4×10^8 beads per 1ml PBS and store at 4°C .
2. Mix Dynabeads well and add them to cells (stained with lineage antibodies) with a concentration of 1×10^8 cells per 1ml beads solution (4×10^8 beads).
3. Incubate cells with Dynabeads for 20 minutes at 4°C on a rocker.
4. Bring the total volume to 6 ml. Put cells into the magnet stand. The Dynabeads, along with differentiated cells, are attracted and attached to the magnet during a period of 2–3 minutes.
5. Carefully remove the cell suspension without disturbing the attached beads, and transfer it to a new 15ml conical tube for the second round of magnet separation. Repeat the same procedure one more time for the maximal removal of remaining beads from the cell suspension.
6. In order to enhance the cell yield, the attached beads are washed again with 6ml medium and subjected to 3 rounds of magnet separations.
7. After two rounds of washes and three rounds of magnet separations, all cells are collected into a 50mL conical tube, spun down, and resuspend into the medium at the concentration of $10 \times 10^6/\text{ml}$.

3.3.2. Miltenyi Streptavidin-Conjugated Paramagnetic Beads—

1. Miltenyi beads are already in the ready-to-use solution and can be directly added into the cells prepared from step 3.2. For 10^8 cells, use 0.4 mL medium plus 0.1 mL magnetic beads. Incubate for 15 min at 4°C on a rocker.
2. During this incubation period, place the column in the magnet and prepare a miniMACS column (capacity 10^7 cells in the magnetic fraction) by running medium through it. This column size is appropriate for enriching progenitors from up to 2×10^8 bone marrow cells. If larger amounts of bone marrow are being processed, midiMACS columns with a capacity of 10^8 cells in the magnetic fraction can be used.
3. Load the cells to a MACS column and let cells pass through the magnet. Return the cell suspension to the top of the magnet twice, allowing the cells to pass through the column a total of three times to reach the maximal removal of beads. Unbound cells in the fluid phase are lineage-depleted (or stem/progenitor-enriched) cells (*see* Note 4). Meanwhile, take 6 aliquots of 0.5×10^6 cells into 6 facs tubes that will be

used for the following stem cell marker single color controls (Controls 3–7) and for PI viability marker staining (Control 8).

3.4 Stem Cell Marker Staining

1. Add CD34-FITC to lineage-depleted cells (main sample) at a concentration of 1:25. Incubate for 90 minutes at 4°C on a rocker. Add 2 microliter (μl) CD34-FITC into Control 3 tube.
2. Around one hour after the incubation, add other markers for staining stem cells. The panel of markers and the corresponding concentrations used in the authors' laboratory are Sca1-PB (1:100), c-Kit (1:100), Flk2/Flt3 (1:100) and CD150 (1:100). Streptavidin-APC-Cy7 is also added to the cell suspension to bind to biotinylated lineage antibodies (1:150). Meanwhile, add Sca1 (0.5 μl), c-Kit (0.5 μl), Flk2/Flt3 (0.5 μl) and CD150 (0.5 μl) to Control tubes 4–7, respectively, and add Streptavidin-APC-Cy7 (0.33 μl) to Control tube 2. Incubate cells with these antibodies for 30 minutes.
3. Wash the sample and control cells twice.
4. Resuspend sample cells into the medium at a density of $10 \times 10^6/\text{mL}$. Add 2 μl PI (1mg/mL stock concentration) per mL cells. Add 0.1 μl PI into control tube 8 for PI single color control.
5. Cells must be transferred to 5-mL polystyrene round-bottom facs tube before FACS.

3.5 Flow Cytometry and FACS

1. FACS Instrument Alignment Procedure and Instrument Settings

The FACS instrument must be aligned using procedures recommended by the manufacturer. The use of calibration beads that mimic fluorescently labeled cells facilitates selection of instrument settings in the range of the type of cells to be sorted. For the techniques described in the chapter, access to a two-laser (Octagon 488 nm and Trigon 633 nm) instrument capable of sorting cells on the basis of ten independent variables is recommended.

3 FACS Windows

Figure 2 shows typical flow cytometry profiles about serial gating for the selection of HSCs. Cells are gated on the forward- and side-scatter and PI staining (Fig. 2A). PI negative viable cells (PI-) cells are then gated for the negative expression of lineage antibodies-APC Cy7 (Fig. 2B). In lineage- cells, Sc-1 and c-Kit positive cells are selected (Fig. 2C). Lineage- Sca-1+ c-Kit+ cells are further gated for the negative expression of Flk2/flt3 and CD34 (Fig. 2D). Therefore, PI- lineage- Sca1+ c-Kit+ Flk2/flt3⁻ CD34⁻ cells represent a

⁴Around 10% of mononucleated cells will be recovered from the ficoll-gradient separation. Another 10% cells will be recovered from the magnetic lineage depletion. Therefore, only about 1% of whole bone marrow cells will be obtained after ficoll-gradient separation and beads depletion, and bone marrow stem and progenitor cells are significantly enriched.

population of highly purified HSCs, and they comprise 0.0032% of whole bone marrow cells in young mice. In this population, three subfractions are divided based on the level of CD150 expression in which myeloid-biased, balanced, and lymphoid-biased HSCs are enriched in the populations with high, low, and negative expression of CD150, respectively (Fig. 2E). With aging, numbers of HPCs and HSCs, like LSK (Fig. 2F) or more greatly purified LSK Flk2/flt3⁻ CD34⁻ (Fig. 2G) cells, are significantly increased, and the proportion of LSK Flk2/flt3⁻ CD34⁻ cells in the aged bone marrow (0.0331%) are nearly tenfold higher than in young marrow. With aging, myeloid-biased HSCs progressively dominate the old bone marrow, whereas the proportions of balanced and lymphoid-biased HSCs gradually diminish (Fig. 2H).

4 Collection Medium and Vessels

The choice of collection tubes and media will depend on the number of cells needed and the intended use of the sorted cells. It should be noted that medium needs to be added to the collection tubes. If less than 500,000 cells are collected, 1.5mL eppendorf tube pre-filled with 0.5 mL medium will be suitable. 5-mL polypropylene round-bottom tubes containing around 1.5mL medium will be required for collecting 0.5 to 1.5×10⁶ cells. Cells can also be directly sorted into multi-well plates using the program Automated Cell Deposition Unit (ACDU). Suitable collection media includes HBSS plus 2% FBS, long-term-culture (LTC) media, or serum free media.

5 Verify the Purity of Sorted Cells

Even though flow cytometry is expected to reliably sort an HSC product of high purity with an acceptable yield, postsort analysis is necessary to determine the purity of sorted cells. An aliquot of the sorted product should be reanalyzed on the same instrument on which it was sorted. In general, if greater than 90% of cells fall into the original gates, the sorting would be considered to have high fidelity. In addition to purity, cell viability and recovery should also be measured by independent quantitative analysis in sorted cells. For example, a hemocytometer with Trypan blue staining can be used to determine the number and viability of cells recovered from the flow cytometry.

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References

1. Liu L, Rando TA. Manifestations and mechanisms of stem cell aging. *J Cell Biol.* 2011; 193(2): 257–266. [PubMed: 21502357]
2. Beerman I, Maloney WJ, Weissmann IL, et al. Stem cells and the aging hematopoietic system. *Current opinion in immunology.* 2010; 22(4):500–506. [PubMed: 20650622]

3. Rando TA. Stem cells, ageing and the quest for immortality. *Nature*. 2006; 441(7097):1080–1086. [PubMed: 16810243]
4. Geiger H, de Haan G, Florian MC. The ageing haematopoietic stem cell compartment. *Nat Rev Immunol*. 2013; 13(5):376–389. [PubMed: 23584423]
5. Morrison SJ, Wandycz AM, Akashi K, et al. The aging of hematopoietic stem cells. *Nat Med*. 1996; 2(9):1011–1016. [PubMed: 8782459]
6. de Haan G, Van Zant G. Dynamic changes in mouse hematopoietic stem cell numbers during aging. *Blood*. 1999; 93(10):3294–3301. [PubMed: 10233881]
7. Sudo K, Ema H, Morita Y, et al. Age-associated characteristics of murine hematopoietic stem cells. *J Exp Med*. 2000; 192(9):1273–1280. [PubMed: 11067876]
8. Geiger H, True JM, de Haan G, et al. Age- and stage-specific regulation patterns in the hematopoietic stem cell hierarchy. *Blood*. 2001; 98(10):2966–2972. [PubMed: 11698278]
9. Henckaerts E, Geiger H, Langer JC, et al. Genetically determined variation in the number of phenotypically defined hematopoietic progenitor and stem cells and in their response to early-acting cytokines. *Blood*. 2002; 99(11):3947–3954. [PubMed: 12010793]
10. Dykstra B, Olthof S, Schreuder J, et al. Clonal analysis reveals multiple functional defects of aged murine hematopoietic stem cells. *J Exp Med*. 2011; 208(13):2691–2703. [PubMed: 22110168]
11. Levi BP, Morrison SJ. Stem cells use distinct self-renewal programs at different ages. *Cold Spring Harbor symposia on quantitative biology*. 2008; 73:539–553.
12. Kim M, Moon HB, Spangrude GJ. Major age-related changes of mouse hematopoietic stem/progenitor cells. *Ann N Y Acad Sci*. 2003; 996:195–208. [PubMed: 12799297]
13. Xing Z, Ryan MA, Daria D, et al. Increased hematopoietic stem cell mobilization in aged mice. *Blood*. 2006; 108(7):2190–2197. [PubMed: 16741255]
14. Liang Y, Van Zant G, Szilvassy SJ. Effects of aging on the homing and engraftment of murine hematopoietic stem and progenitor cells. *Blood*. 2005; 106(4):1479–1487. [PubMed: 15827136]
15. Florian MC, Dorr K, Niebel A, et al. Cdc42 activity regulates hematopoietic stem cell aging and rejuvenation. *Cell stem cell*. 2012; 10(5):520–530. [PubMed: 22560076]
16. Muller-Sieburg CE, Cho RH, Karlsson L, et al. Myeloid-biased hematopoietic stem cells have extensive self-renewal capacity but generate diminished lymphoid progeny with impaired IL-7 responsiveness. *Blood*. 2004; 103(11):4111–4118. [PubMed: 14976059]
17. Beerman I, Bhattacharya D, Zandi S, et al. Functionally distinct hematopoietic stem cells modulate hematopoietic lineage potential during aging by a mechanism of clonal expansion. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107(12):5465–5470. [PubMed: 20304793]
18. Muller-Sieburg CE, Sieburg HB, Bernitz JM, et al. Stem cell heterogeneity: implications for aging and regenerative medicine. *Blood*. 2012; 119(17):3900–3907. [PubMed: 22408258]
19. Wagner W, Horn P, Bork S, et al. Aging of hematopoietic stem cells is regulated by the stem cell niche. *Experimental gerontology*. 2008; 43(11):974–980. [PubMed: 18504082]
20. Mayack SR, Shadrach JL, Kim FS, et al. Systemic signals regulate ageing and rejuvenation of blood stem cell niches. *Nature*. 2010; 463(7280):495–500. [PubMed: 20110993]
21. Oakley EJ, Van Zant G. Age-related changes in niche cells influence hematopoietic stem cell function. *Cell stem cell*. 2010; 6(2):93–94. [PubMed: 20144780]
22. Rossi DJ, Bryder D, Seita J, et al. Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature*. 2007; 447(7145):725–729. [PubMed: 17554309]
23. Garinis GA, van der Horst GT, Vijn J, et al. DNA damage and ageing: new-age ideas for an age-old problem. *Nat Cell Biol*. 2008; 10(11):1241–1247. [PubMed: 18978832]
24. Rossi DJ, Seita J, Czechowicz A, et al. Hematopoietic stem cell quiescence attenuates DNA damage response and permits DNA damage accumulation during aging. *Cell cycle*. 2007; 6(19):2371–2376. [PubMed: 17700071]
25. Sahin E, Depinho RA. Linking functional decline of telomeres, mitochondria and stem cells during ageing. *Nature*. 2010; 464(7288):520–528. [PubMed: 20336134]

26. Yahata T, Takanashi T, Muguruma Y, et al. Accumulation of oxidative DNA damage restricts the self-renewal capacity of human hematopoietic stem cells. *Blood*. 2011; 118(11):2941–2950. [PubMed: 21734240]
27. Garrison BS, Rossi DJ. Reactive oxygen species resulting from mitochondrial mutation diminishes stem and progenitor cell function. *Cell metabolism*. 2012; 15(1):2–3. [PubMed: 22225868]
28. Janzen V, Forkert R, Fleming HE, et al. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature*. 2006; 443(7110):421–426. [PubMed: 16957735]
29. Xiao N, Jani K, Morgan K, et al. Hematopoietic stem cells lacking Ott1 display aspects associated with aging and are unable to maintain quiescence during proliferative stress. *Blood*. 2012; 119(21):4898–4907. [PubMed: 22490678]
30. Rossi DJ, Bryder D, Weissman IL. Hematopoietic stem cell aging: mechanism and consequence. *Experimental gerontology*. 2007; 42(5):385–390. [PubMed: 17275237]
31. Chen J, Astle CM, Harrison DE. Genetic regulation of primitive hematopoietic stem cell senescence. *Exp Hematol*. 2000; 28(4):442–450. [PubMed: 10781902]
32. Wagner W, Bork S, Horn P, et al. Aging and replicative senescence have related effects on human stem and progenitor cells. *PLoS One*. 2009; 4(6):e5846. [PubMed: 19513108]
33. Wang J, Sun Q, Morita Y, et al. A Differentiation Checkpoint Limits Hematopoietic Stem Cell Self-Renewal in Response to DNA Damage. *Cell*. 2012; 148(5):1001–1014. [PubMed: 22385964]
34. Beerman I, Bock C, Garrison BS, et al. Proliferation-dependent alterations of the DNA methylation landscape underlie hematopoietic stem cell aging. *Cell stem cell*. 2013; 12(4):413–425. [PubMed: 23415915]
35. Rossi DJ, Bryder D, Zahn JM, et al. Cell intrinsic alterations underlie hematopoietic stem cell aging. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102(26):9194–9199. [PubMed: 15967997]
36. Chambers SM, Shaw CA, Gatz C, et al. Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. *PLoS Biol*. 2007; 5(8):e201. [PubMed: 17676974]
37. Tadokoro Y, Ema H, Okano M, et al. De novo DNA methyltransferase is essential for self-renewal, but not for differentiation, in hematopoietic stem cells. *J Exp Med*. 2007; 204(4):715–722. [PubMed: 17420264]
38. Okada S, Nakauchi H, Nagayoshi K, et al. In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. *Blood*. 1992; 80(12):3044–3050. [PubMed: 1281687]
39. Adolfsson J, Borge OJ, Bryder D, et al. Upregulation of Flt3 expression within the bone marrow Lin(–)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. *Immunity*. 2001; 15(4):659–669. [PubMed: 11672547]
40. Kiel MJ, Yilmaz OH, Iwashita T, et al. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell*. 2005; 121(7):1109–1121. [PubMed: 15989959]
41. Yilmaz OH, Kiel MJ, Morrison SJ. SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity. *Blood*. 2006; 107(3):924–930. [PubMed: 16219798]
42. Goodell MA, Brose K, Paradis G, et al. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med*. 1996; 183(4):1797–1806. [PubMed: 8666936]
43. Challen GA, Boles NC, Chambers SM, et al. Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. *Cell stem cell*. 2010; 6(3):265–278. [PubMed: 20207229]
44. Morita Y, Ema H, Nakauchi H. Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. *J Exp Med*. 2010; 207(6):1173–1182. [PubMed: 20421392]
45. Oguro H, Ding L, Morrison SJ. SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. *Cell stem cell*. 2013; 13(1):102–116. [PubMed: 23827712]
46. Doulatov S, Notta F, Laurenti E, et al. Hematopoiesis: a human perspective. *Cell stem cell*. 2012; 10(2):120–136. [PubMed: 22305562]
47. Kuranda K, Vargaftig J, de la Rochere P, et al. Age-related changes in human hematopoietic stem/progenitor cells. *Aging cell*. 2011; 10(3):542–546. [PubMed: 21418508]

48. Pang WW, Price EA, Sahoo D, et al. Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108(50):20012–20017. [PubMed: 22123971]
49. Vaziri H, Dragowska W, Allsopp RC, et al. Evidence for a mitotic clock in human hematopoietic stem cells: loss of telomeric DNA with age. *Proceedings of the National Academy of Sciences of the United States of America*. 1994; 91(21):9857–9860. [PubMed: 7937905]
50. Notta F, Doulatov S, Laurenti E, et al. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science*. 2011; 333(6039):218–221. [PubMed: 21737740]
51. Larochelle A, Savona M, Wiggins M, et al. Human and rhesus macaque hematopoietic stem cells cannot be purified based only on SLAM family markers. *Blood*. 2011; 117(5):1550–1554. [PubMed: 21163926]
52. Goodrick CL. Life-span and the inheritance of longevity of inbred mice. *Journal of gerontology*. 1975; 30(3):257–263. [PubMed: 1120887]
53. Rowlatt C, Chesterman FC, Sheriff MU. Lifespan, age changes and tumour incidence in an ageing C57BL mouse colony. *Laboratory animals*. 1976; 10(10):419–442. [PubMed: 979138]

Figure 1. Flowchart of mouse HSC isolation and purification by flow cytometry

Figure 2. Flow cytometry profiles and gate settings for selection of young and old lineage- Sca1+ c-Kit+ Flk2- CD34- HSCs and lineage-biased subpopulations

(A) Low-density mononucleated cells without PI expression (PI-). (B) Selection of lineage negative cells (lineage-). (C) Positive selection of cells expressing Sca1 and c-Kit antigens (Sca1+ c-Kit+). (D) Selection of cells with undetectable levels of Flk2 and CD34 (Flk2- CD34-) from lineage- Sca1+ c-Kit+ (LSK) cells. LSK Flk2- CD34- cells are highly purified HSCs. (E) Subdivision of LSK Flk2- CD34- cells into myeloid-biased, balanced and lymphoid-biased populations based on the expression level of CD150. (F) LSK cell selection in the bone marrow of old mice (>24 month old). (G) Dramatic increased in the number of

LSK Flk2- CD34- cells in old bone marrow. (H) Dominance of myeloid-biased CD150^{high} LSK Flk2- CD34- cells at the replace of lymphoid-biased and balanced HSCs in old bone marrow.

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