Optimization of pre-enrichment strategies for mouse hematopoietic stem cell isolation and metabolomic analysis

Célina Nielsen[a], Youzhong Liu[a], Fleur Leguay[a], Hernán A. Tirado[a], Nicolas Dauguet[b], and Nick van Gastel[a,c,*]

[a]*Cellular Metabolism and Microenvironment Laboratory, de Duve Institute, UCLouvain, Brussels, Belgium;* [b]*Flow Cytometry and Cell Sorting Facility, de Duve Institute, UCLouvain, Brussels, Belgium;* [c]*WELBIO Department, WEL Research Institute, Wavre, Belgium*

Blood cell production arises from the activity of hematopoietic stem cells (HSCs), defined by their self-renewal capacity and ability to give rise to all mature blood cell types. The mouse remains one of the most studied species in hematological research, and markers to define and isolate mouse HSCs are well-established. Given the very low frequency of HSCs in the bone marrow, stem cell pre-enrichment by red blood cell lysis and magnetic cell separation is often performed as part of the isolation process to reduce sorting times. Several pre-enrichment strategies are available, differing in their speed, degree of enrichment, final cell yield, and cost. In the current study, we performed a side-by-side comparison and provide a decision tree to help researchers select a pre-enrichment strategy for mouse HSC isolation depending on their downstream application. We then compared different pre-enrichment techniques in combination with metabolomics analysis of HSCs, where speed, yield and temperature during pre-enrichment are crucial factors, and found that the choice of pre-enrichment strategy significantly impacts the number of metabolites detected and levels of individual metabolites in HSCs.

HIGHLIGHTS

- Performing a pre-enrichment can increase HSC frequency more than 30-fold.
- Lineage depletion is the fastest strategy, while c-Kit or Sca-1 selection give the highest enrichment.
- Combining two pre-enrichment strategies increases stem cell frequency at the cost of final yield.
- c-Kit enrichment appears optimal for metabolomics analysis of HSCs.

Given their rarity and small size, hematopoietic stem cells (HSCs) can be challenging to work with, yet their unique cell characteristics and clinical relevance makes them widely studied. Able to self-renew and differentiate into any blood cell type, HSCs reside at the apex of the hematopoietic system and are found in a complex niche supporting their maintenance in the bone marrow [1]. Under acute stress conditions such as infection or blood loss, HSCs quickly adapt and expand their output to meet the needs of the host. This capacity to replenish the entire blood system is now used in HSC transplantations (HSCTs) to help blood disorder patients recover after undergoing intensive chemotherapy or irradiation. Although considered a standard of care, HSCTs have drawbacks that researchers are currently addressing to improve the clinical outcome of transplanted patients [2]. Other fields of interest in stem cell research aim at preventing the functional decline of HSCs and the increased risk of hematological malignancies, both associated with a chronic exposure to inflammation caused by infections, inflammatory diseases, and/or aging [3].

Ever since the first identification of phenotypic cell surface markers of mouse HSCs in 1988 [4], the study of these cells has relied heavily on the ability to identify and purify stem cells from whole bone marrow using fluorescence-activated cell sorting (FACS). As no single marker currently exists to characterize HSCs, a combination of cell surface markers and/or vital dyes are used. Except for the side population staining method using Hoechst33342 [5], the different approaches for HSC isolation commonly use the markers lineage-negative (Lin^-), c-Kit^+, and Sca-1^+ to define what is known as the LKS population, onto which additional markers are added to better identify HSCs [6,7]. LKS cells are a heterogeneous population, mainly composed of early progenitor cells with only around 10% of HSCs in young animals at steady state [8,9]. Given the very low frequency of HSCs in the bone marrow (around 0.01% of total nucleated cells) [8], harvesting enough mouse HSCs for analysis is challenging. Consequently, LKS cells are widely used instead as indicators of HSC biology.

Metabolism is a cellular process that is especially difficult to study in rare cell types. The preferred technique used to measure intracellular metabolite levels is metabolomics; however, this method typically requires the use of millions of cells [10]. As one mouse usually does not yield more than 150,000 LKS cells, even performing metabolo-

Address correspondence to Nick van Gastel, Cellular Metabolism and Microenvironment Laboratory, de Duve Institute, UCLouvain, Brussels, Belgium; E-mail: nick.vangastel@uclouvain.be

0301-472X/

https://doi.org/10.1016/j.exphem.2024.104588

mics on LKS cells can be difficult. A study published in 2013 successfully performed metabolomics on over 1 million HSCs by pooling cells from 120 mice [11]. However, this approach lead to a decreased statistical significance as pooling samples can conceal biological variability. Recent studies have managed to detect metabolites in as little as 5,000 or 10,000 HSCs [10,12], removing the need for pooling samples. Nevertheless, whether one intends to perform metabolomics on LKS cells, HSCs, or other hematopoietic populations, isolating as many cells as possible from each mouse is essential to obtain high quality results, as the number of detected metabolites increases with cell number [12,13]. In addition to collecting all easily accessible bones from a mouse and performing FACS with high sort efficiency by limiting cell clumps and cell doublets [14], another element that may impact stem cell yield is the choice of pre-enrichment strategy. When sorting low abundant cells, such a pre-enrichment step is necessary to reduce sorting time, which would otherwise be excessively long [14]. Commonly, stem cell pre-enrichment is performed by magnetic separation, targeting one or several of the three LKS markers. However, if downstream metabolomics analysis is intended, keeping cells between 0°C and 4°C at all times is essential to minimize metabolic changes, and having both the pre-enrichment process and cell sorting time as short as possible is preferable.

A direct comparison of different pre-enrichment strategies, using manual or automated magnetic cell separation, and evaluating them based on speed, degree of enrichment, final cell yield, and impact on the cellular metabolome has not been done. In the current study, we performed this side-by-side comparison and provide a decision tree to help researchers select a pre-enrichment strategy for mouse HSC isolation depending on their downstream application. We further compared different pre-enrichment strategies for downstream metabolomics analysis of HSCs, where speed, yield, and temperature during pre-enrichment are crucial factors.

METHODS

Animals

Bone marrow was obtained from male and female C57BL/6 mice at an age of 10−13 weeks. Mice were obtained from the in-house specific pathogen-free (SPF) animal breeding facility of the UCLouvain, and experiments were performed under approval of the local Animal Ethics Committee (project number 2021/UCL/MD/40).

Sample Preparation

To minimize metabolic changes, bone marrow cells were kept on ice or at 4°C whenever possible from the collection of the bones to the analysis of the pre-enriched cells on the flow cytometer, and buffers were used ice-cold. Mice were euthanized by cervical dislocation and femurs, tibiae, pelves, humeri, sternum, and vertebrae were quickly harvested and stored on ice in phosphate-buffered saline (PBS). Bones were cleaned by rubbing off the muscle tissue with wiping paper and subsequently crushed (using four long bones or all vertebrae at a time) in 5 mL of FACS buffer, composed of PBS (Gibco), 2% fetal bovine serum (FBS) (Gibco) and 5 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich), using a precooled mortar and pestle. The obtained cell suspension was passed through a 40-μM cell strainer into a 50-mL conical tube, and the crushing process was repeated once more with 5 mL of fresh FACS buffer. Once all bones had been crushed, the obtained single-cell suspension was centrifuged at 300 × g for 7 min at 4°C. For the experiments including red blood cell (RBC) lysis, cells were incubated in 5 mL ammonium chloride potassium (ACK) lysing buffer (Fisher Scientific, 11509876) for 5 min on ice. The cells were then washed with PBS and centrifuged at 300 × g for 7 min at 4°C. Cells were resuspended in 10 mL of FACS buffer and counted using the Countess II Automated Cell Counter (Thermo Fisher Scientific).

Magnetic Separation with the MojoSort Magnet

After counting, the cells were divided into two (RBC lysis comparison experiments) or four (pre-enrichment method comparison experiments) 15-mL conical tubes, keeping the cells from each mouse separate, and resuspended at a concentration of 100×10^6 cells/mL FACS buffer.

Lineage depletion. Following the MagniSort Mouse Hematopoietic Lineage Depletion Kit (Life Technologies Europe, 8804-6829-74) protocol, bone marrow cells were incubated with 20 μL of MagniSort Mouse Hematopoietic Lineage Depletion Antibody Cocktail per 100 μL of cells for 10 min on ice. After washing with 4 mL FACS buffer and centrifuging at 300 × g for 5 min at 4°C, the cells were resuspended in FACS buffer to their original volume. MagniSort Negative Selection Beads B were thoroughly vortexed, and 20 μL was added per 100 μL of cells. After 5 min of incubation on ice, cells were transferred to 5-mL polystyrene tubes, and FACS buffer was added to obtain a total volume of 2.5 mL. The tubes were incubated in MojoSort magnets (BioLegend Europe BV, 480019) for 5 min on ice, after which the supernatants were poured into precooled 15-mL conical tubes. The tubes containing bound cells were removed from the magnets, the cells were resuspended using 2.5 mL FACS buffer, and magnetic separation was repeated a second time. The lineage-depleted cells, found in the supernatants, were subsequently analyzed using flow cytometry.

c-Kit enrichment. The MagniSort Mouse CD117 (c-Kit) Positive Selection Kit (Life Technologies Europe, 8802-6838-74) protocol was followed, which is nearly identical to the one for lineage depletion, except the antibody staining was performed with 20 μL of MagniSort Anti-Mouse CD117 Biotin per 100 μL of cells for 10 min on ice, and the bead staining was performed with 10 μL of MagniSort Positive Selection Beads B per 100 μL of cells for 10 min on ice. The cells were incubated a total of three times in the magnet, and the c-Kit-enriched cells were collected by resuspending the tube-bound cells in 1 mL of FACS buffer.

Sca-1 enrichment. The EasySep Mouse SCA1 Positive Selection Kit (STEMCELL Technologies SARL, 18756) protocol was used, which follows the same steps as for the lineage depletion. Cells were first incubated with 50 μL EasySep Mouse SCA1-PE Labeling Reagent per 1 mL of cells for 15 min in the refrigerator, then with 70 μL of EasySep PE Selection Cocktail per 1 mL of cells, and again for 15 min in the refrigerator (no wash between and after the two incubations). The bead staining was performed using 50 μL of EasySep Dextran RapidSpheres 50100 per 1 mL of cells for 10 min in the refrigerator. The cells were incubated a total of four times in the magnet, and the Sca-1-enriched cells were collected by resuspending the tube-bound cells in 1 mL of FACS buffer.

Magnetic Separation with the QuadroMACS Separator or autoMACS Pro Separator

After counting, the cells were split into two 15-mL conical tubes, keeping the cells from each mouse separate, and resuspended at a concentration of 250×10^6 total cells/mL FACS buffer (lineage depletion) or 125×10^6 total cells/mL FACS buffer (c-Kit enrichment).

Lineage depletion. Following the Direct Lineage Cell Depletion Kit protocol (Miltenyi Biotec, 130-110-470), cells were incubated with 20 μL of Direct Lineage Cell Depletion Cocktail per 2×10^7 total cells for 10 min in the refrigerator. The cells were passed through a 35-μm nylon mesh before proceeding to magnetic separation on either the QuadroMACS Separator with LS columns (Miltenyi Biotec, 130-091-051) placed in a cold room, following the supplier's instructions, or the autoMACS Pro Separator (Miltenyi Biotec, 130-092-545) using the depletion program *Deplete*.

c-Kit enrichment. Following the CD117 MicroBeads protocol (Miltenyi Biotec, 130-091-224), cells were incubated with 20 μL of CD117 MicroBeads per 10^7 total cells for 15 min in the refrigerator. After washing with 1−2 mL of FACS buffer per 10^7 total cells and centrifuging at 300 × g for 10 min at 4°C, the cells were resuspended at a concentration of 200×10^6 total cells/mL FACS buffer. The cells were passed through a 35 μm nylon mesh before proceeding to magnetic separation on either the QuadroMACS Separator with LS columns placed in a cold room or the autoMACS Pro Separator using the positive selection program *Posselwb*.

Flow Cytometry

Pre-enriched bone marrow cells were resuspended in 500 μL FACS buffer and stained with fluorochrome-conjugated antibodies against lineage markers, c-Kit, Sca-1, CD150, CD48, and the viability dye 7-AAD (Supplementary Table E1) for 15 min in the refrigerator. A wash was performed by adding 800 μL FACS buffer and centrifuging at 300 × g for 5 min at 4°C. Samples were analyzed on the BD FACSVerse with the FACSSuite software (BD Biosciences) in a volume of 500 μL (*lineage depletion + Sca-1 enrichment* samples) or 1 mL (all other samples) of FACS buffer. Unstained and single antibody-stained cells were used for compensations. Flow cytometry data was analyzed using the FlowJo software (BD Biosciences).

Cell Culture

Lineage-negative bone marrow cells were collected and cultured for one week in RPMI1640 medium (Lonza) supplemented with 10% FBS (Gibco), 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine (all from Corning), 20 ng/mL recombinant mouse stem cell factor, 10 ng/mL recombinant mouse interleukin 3, and 10 ng/mL recombinant mouse interleukin 6 (all from R&D Systems).

FACS

To understand the effect of pre-enrichment and sorting on the cellular metabolome, cultured lineage-negative bone marrow cells were collected, counted, and washed in saline (0.9% NaCl in water). Then, 500,000 cells were used immediately for metabolite extraction. In parallel, cells were "mock" pre-enriched and sorted. For this, culture-expanded lineage-negative bone marrow cells were processed exactly as during RBC lysis, lineage depletion pre-enrichment, and FACS sorting, except that no antibodies were added in any of the steps, thus collecting and sorting all cells, only excluding debris. Cells were sorted into cooled microcentrifuge tubes containing 800 μL saline on a BD FACSAria II (BD Biosciences) with 1× PBS sheath fluid, 45 psi sheath pressure, and a 85 μm nozzle, collecting 500,000 cells per sample.

For HSC metabolomics analysis, bone marrow cells pre-enriched using different methods were resuspended in 500 μL FACS buffer and stained with fluorochrome-conjugated antibodies against lineage markers, including c-Kit, Sca-1, CD150, and CD48, and the viability dye 7-AAD (Supplementary Table E1) for 15 min in the refrigerator. A wash was performed by adding 800 μL FACS buffer and centrifuging at $300 \times g$ for 5 min at 4°C. Cells were sorted into precooled microcentrifuge tubes (kept at −20°C until just before sorting) containing 28 μL LC-MS grade acetonitrile (LICHROSOLV, Merck) on a Cytek Aurora CS (Cytek Biosciences) with 5 g/L NaCl sheath fluid, 69 psi sheath pressure, 70 μm nozzle, and 84 KHz drop frequency. The sample and collection chamber were kept at low temperature. We sorted 5,000 cells from each sample or as many cell as possible using the Multi-Way sort mode. Halfway through each sort, the collection tube was vortexed and briefly put on dry ice to cool the sample down and to quench metabolism. After sorting, the collection tube was vortexed at high speed for 1 min and stored immediately at −20°C for at least 2 hours to precipitate salt and protein. A blank sample was produced by sorting the same number of events from the cell-free suspension buffer, using a very low cutoff for forward and side scatter signals.

Untargeted Metabolomics Analysis of Cultured Lineage-Negative Bone Marrow Cells

Cultured lineage-negative bone marrow cells (FACS-sorted or collected directly from culture) were centrifuged at $300 \times g$ for 3 min at 4°C and the cell pellet was lysed in 100 μL ice-cold methanol and stored at −80°C. Polar metabolites were extracted using methanol−chloroform phase separation (final volumes, 1 mL methanol, 500 μL water, and 1 mL chloroform). After centrifugation at $14{,}000 \times g$ for 15 min at 4°C, the polar top phases were collected and dried under nitrogen flow, resuspended in 25 μL 70% acetonitrile in water containing 2.5 μM of an internal standard (^{13}C-, ^{15}N-labeled amino acid mix; Cambridge Isotope Laboratories) and run on a Thermo Fisher Q−Exactive Orbitrap mass spectrometer coupled to an UltiMate 3000 UHPLC equipped with Zic−pHILIC column (150 × 2.1 mm, 5 μm; Merck). A volume of 5 μL was injected, and metabolites were monitored in full-scan, polarity-switching mode (0−45 min; resolution, 70,000; AGC target, 3×10^6; m/z range, 66.7−1,000). Mobile phase A for chromatography consisted of 20 mM ammonium carbonate, 0.1% ammonium hydroxide, in water and mobile phase B of 97% acetonitrile in water. A pooled sample was created from all samples and used for MS/MS runs. Metabolite measurements were normalized to the internal ^{13}C/^{15}N-labeled amino acid standard. Data were processed with Compound Discoverer 3.0 (Thermo Fisher Scientific) using the mzCloud mass spectral library for compound annotation. For compounds for which an MS/MS spectrum was not available, the Kyoto Encyclopedia of Genes and Genomes (KEGG) Compound database (www.genome.jp/kegg; [15]) and human metabolome database (HMDB; www.hmdb.ca; [16,17]) were used for putative compound annotation based on the monoisotopic mass.

Targeted Metabolomics Analysis of HSCs

Cell debris, protein and salt were removed by centrifugation of samples at 17,000 × g for 30 min at −9°C. The supernatants were filtered through 0.2 μm PTFE filters (Whatman, Merck), transferred to auto-sampler vials (SureSTART, Thermo Scientific), and run on a Vanquish Neo UHPLC (Thermo Scientific) using SeQuant ZIC-HILIC trap column (0.3 × 10 mm, 5 μm particle size; Merck) and SeQuant ZIC-HILIC Capillary separation column (0.3 × 150 mm, 3.5 μm particle size; Merck). Then, 5 μL of sample or blank was injected onto the trap column in 30 sec before gradient elution. Mobile phase A of the HILIC gradient was 5% acetonitrile, 95% water containing 5 mM ammonium acetate; mobile phase B was 90% acetonitrile, 10% water. The separation proceeded as follows: from 0 to 8 min, mobile phase B was kept at 88% to elute nonpolar compounds; from 8 to 33 min, B was ramped linearly from 88% to 42.5%; at 34 min B was decreased to 10%; from 34 to 44 min, B was held at 10% to remove salt from the column; at 44 min, B was increased back to 88%; and from 44 to 51 min, B was held at 88% to regenerate the water layer of the column. Solvent flow rate was set at 3 μL/min for the gradient and at 6 μL/min for the salt elution phase (diverted to waste). The column temperature was maintained at 40°C.

Mass spectrometry data were acquired using an Orbitrap Exploris 240 mass spectrometer (Thermo Scientific) in full-scan MS mode without polarity switching. Each polarity was acquired at a resolution of 60,000 (at m/z = 200); default settings were used for the automatic gain control target and maximum injection time; microscans was set to 2 and the scan range to 80−800 Daltons. MS source parameters were as follows: capillary voltage, 3,400 V for positive ion mode and 2,300 V for negative ion mode; sheath gas, 3; auxiliary gas, 2; sweep gas, 0; and ion transfer tube temperature, 320°C.

Raw data in .raw format was converted to .mzXML format using ProteoWizard [18]. The .mzXML files were imported and processed in MZmine 2.53 [19]. Metabolite detection was performed in targeted feature detection mode by searching exact masses of 140 commonly detected mammalian metabolites (in-house database). The parameters for feature extraction were as follows: mass detection (exact mass, noise 1.0E3); targeted feature detection (intensity tolerance, 80; noise, 1.0E3; m/z tolerance, 2 ppm); smoothing (filter width, 11). The feature lists were then manually inspected to assess peak shape and peak integration before being exported for data analysis. Values of the blank were subtracted from sample values, and only metabolites detected in at least 30% of samples were retained for analysis.

Statistical Analysis

Analysis of the metabolomics data was performed in MetaboAnalyst 5.0 (www.metaboanalyst.ca; [20]), using the "Statistical Analysis" node. Data were normalized by log10 transformation and auto-scaling (mean-centered and divided by the standard deviation of each variable).

All flow cytometry data are reported as mean ± SEM. Statistical significance between different experimental groups was evaluated by two-tailed unpaired Student t test or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test, using the Prism software (GraphPad). Differences were considered statistically significant at $p < 0.05$.

RESULTS

RBC Lysis Increases HSC Enrichment

To confirm stem cell frequencies in the bone marrow at baseline (before any enrichment), we performed flow cytometry on freshly isolated mouse whole bone marrow cells, staining for LKS cells and HSCs, using the LKS-SLAM markers (Lin$^-$ c-Kit$^+$ Sca-1$^+$ CD150$^+$ CD48$^-$) to define the latter (Supplementary Figure E1A). As expected, the frequencies were very low with a mean of 0.12% LKS cells and 0.013% HSCs among viable nucleated cells (Supplementary Figure E1B), in line with previous findings [6,21].

Before comparing different pre-enrichment strategies, we first assessed whether it would be beneficial to add an RBC lysis step before the magnetic separation (Figure 1). Although lysing erythrocytes allows to directly eliminate one type of unwanted mature cells and thereby could increase the efficiency of the magnetic separation, it could potentially be accompanied by a loss of cells of interest. To test the effect of RBC lysis on stem cell enrichment and final cell yield, whole bone marrow cells were either treated with ACK lysing buffer or left untreated before performing a lineage depletion by manual magnetic separation. The obtained Lin$^-$ cells were then stained with antibodies and analyzed by flow cytometry. All quantification experiments were performed using flow cytometry instead of FACS, as it enables to compare the efficiency of each strategy without the added variability of cell sorting. Both for the LKS and HSC populations, we observed a twofold increase in frequency among live cells in the samples treated with RBC lysis (Figure 2A, B). Using the same flow cytometry data, we calculated the estimated cell yield of the different samples based on the number of cells acquired per unit of time, flow rate, and sample volume. The use of RBC lysis did not result in a

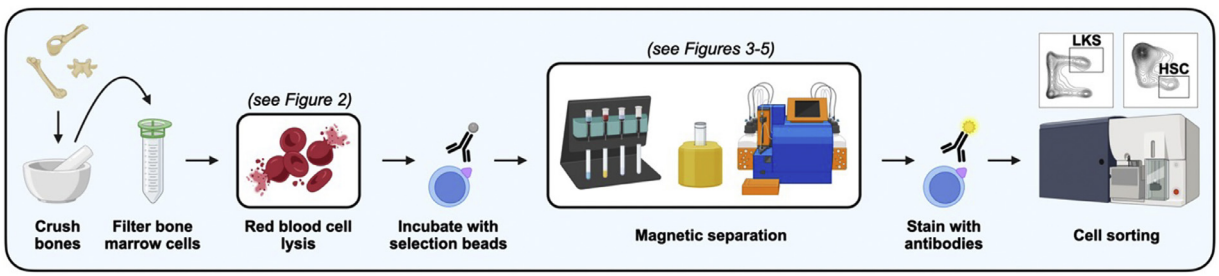
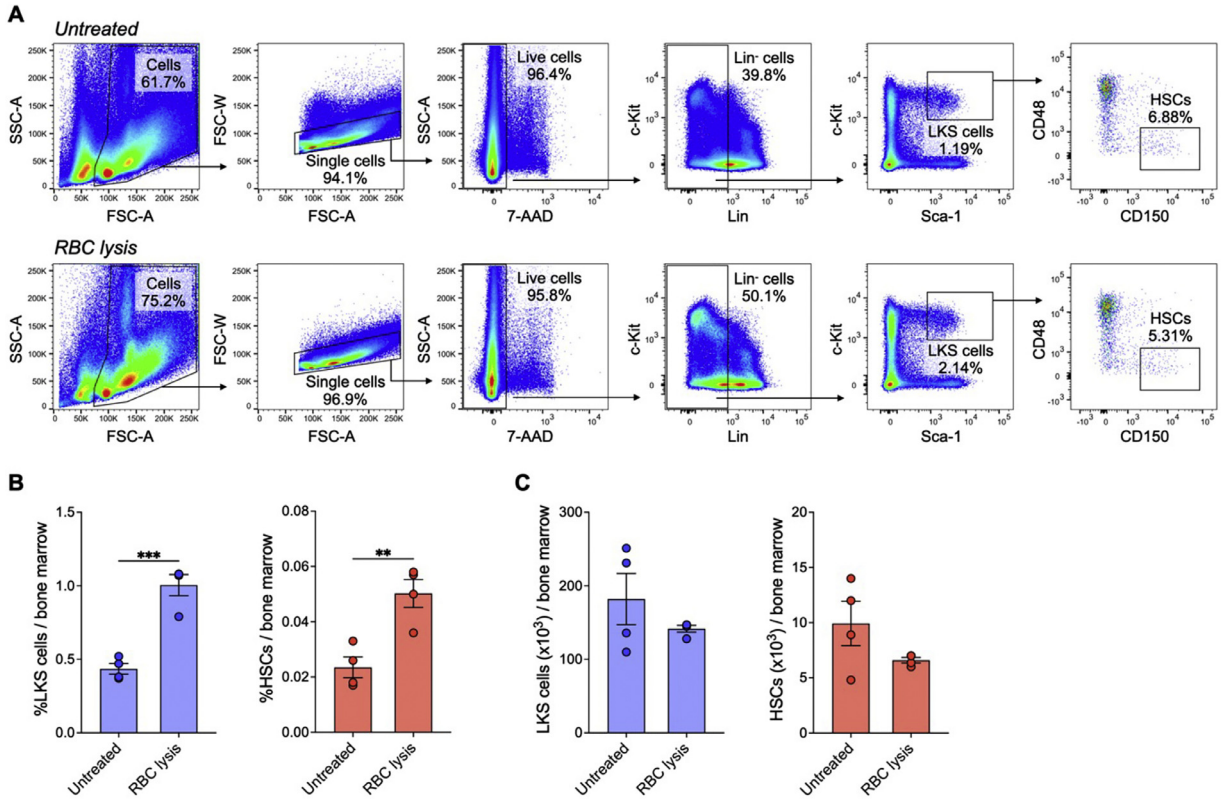


Figure 1 Schematic overview of the protocol used to isolate HSCs from mouse bone marrow.



Q9 Figure 2 RBC lysis increases HSC enrichment without impacting final cell yield. **(A)** Representative flow cytometry plots of lineage-depleted bone marrow cells untreated or treated with ACK lysing buffer followed by lineage depletion. **(B)** Frequency of LKS cells (left) and HSCs (right) among viable nucleated lineage-depleted bone marrow cells in samples untreated or treated with ACK lysing buffer (n = 4 from two independent experiments). **(C)** Estimated yield of LKS cells (left) and HSCs (right) per mouse from lineage-depleted samples untreated or treated with ACK lysing buffer (n = 4 from two independent experiments). Data are represented as mean ± SEM. Significance was evaluated by two-tailed unpaired Student *t* test. $^{**}p < 0.01$; $^{***}p < 0.001$.

lower cell yield (Figure 2C), making it a simple way to increase enrichment without impacting stem cell numbers. Based on those results, we decided to carry out the rest of our experiments using ACK lysing buffer.

Side-by-Side Comparison of Different Pre-enrichment Strategies

Next, we compared the efficiency of different magnetic separation strategies. We first tested a manual free-standing magnet system (Figure 1), which has the advantage of being accessible and simple to use. The manual free-standing magnet separation can also easily be performed on ice, which may be preferred for downstream transcriptomic, proteomic, or metabolomic analyses. In a same experiment, we compared the targeting of each of the three LKS markers by performing lineage depletion (negative selection), c-Kit enrichment, and Sca-1 enrichment (positive selections). We also tested combining lineage depletion with a subsequent Sca-1 enrichment in an attempt to obtain a highly pre-enriched population. We observed a tendency for lineage depletion to yield a lower LKS cell and HSC frequency than the single positive selections (Figure 3A, B). Moreover, enriching for Sca-1 after lineage depletion significantly increased the frequency of HSCs compared with lineage depletion alone. Unfortunately, this high enrichment impacted cell yield, as the final number of both LKS cells and HSCs was significantly lower than for the three other strategies (Figure 3C). The lower stem cell frequency observed with lineage depletion did, however, not seem to affect the final LKS or HSC yield of this method. Taken together, no significant differences were observed between lineage depletion, c-Kit enrichment, and Sca-1 enrichment regarding their degree of stem cell enrichment and final cell yield. Performing a second enrichment step increased purity but also led to a great loss of cells of interest.

In addition to the manual free-standing magnet system, we compared different enrichment methods using the column-based MACS MicroBead Technology (Miltenyi). The combination of nanosize MicroBeads and a strong magnetic field is thought to enable minimal labeling of target cells and better preservation of cellular properties. We first tested the autoMACS Pro Separator (Miltenyi), a fully automated magnetic cell separation instrument known for yielding a high purity and with the advantage of being hands-off. In our search for the best enrichment method, we hypothesized that using this instrument might help enrich further for stem cells without negatively affecting final cell yield. As no directly labeled MicroBeads were available for Sca-1, we only tested lineage depletion and c-Kit enrichment. The best suited separation programs were selected for each enrichment strategy based on the user manual of the machine: *Deplete* for

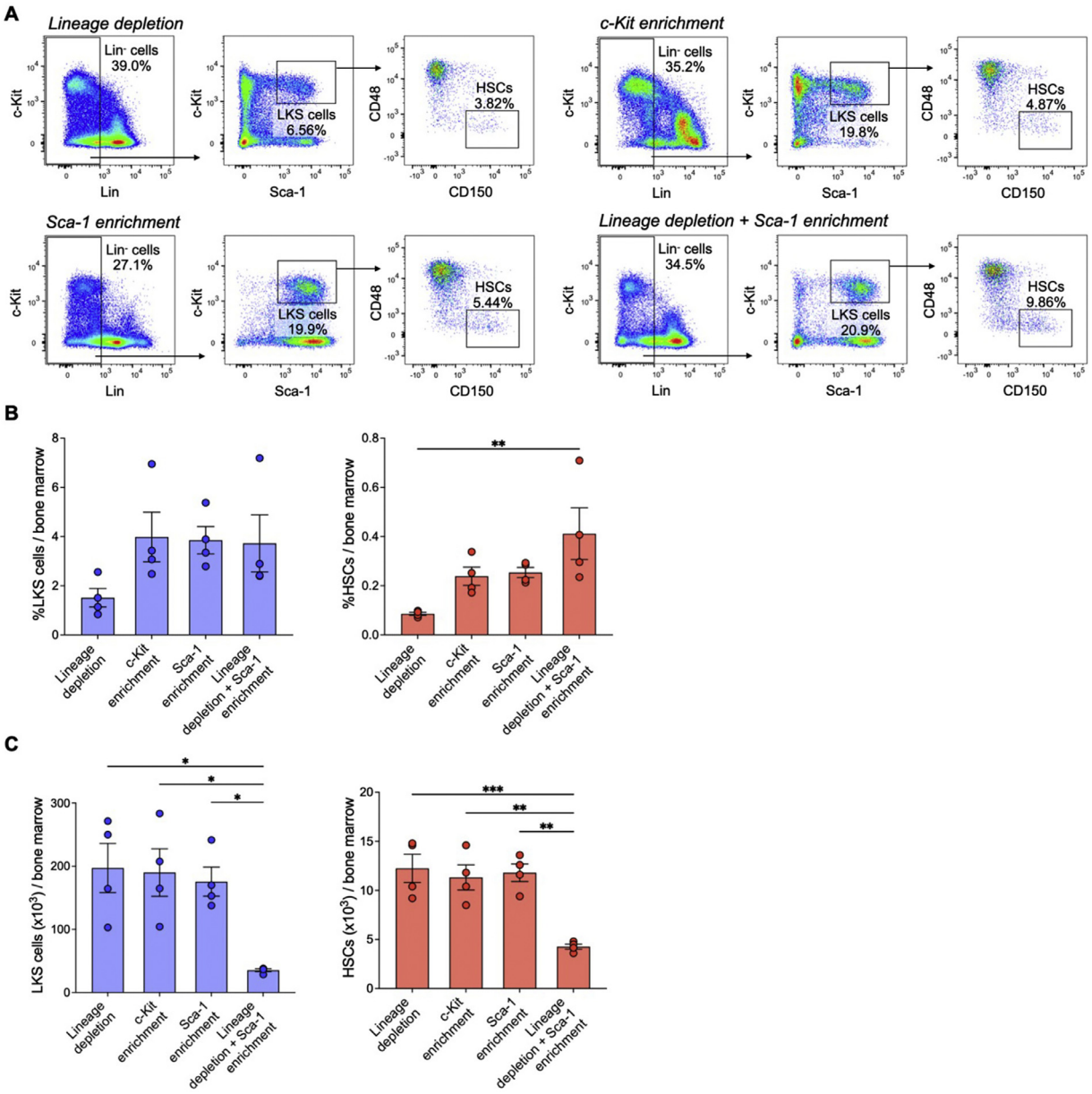


Figure 3 Comparison of pre-enrichment strategies using manual magnetic cell separation. **(A)** Representative flow cytometry plots of bone marrow cells enriched by lineage depletion, c-Kit enrichment, Sca-1 enrichment, or lineage depletion followed by Sca-1 enrichment. **(B)** Frequency of LKS cells (left) and HSCs (right) among viable nucleated bone marrow cells after pre-enrichment (n = 4 from four independent experiments). **(C)** Estimated yield of LKS cells (left) and HSCs (right) per mouse after pre-enrichment (n = 4 from four independent experiments). Data are represented as mean ± SEM. Significance was evaluated using one-way ANOVA followed by Tukey's multiple comparisons test. *$p < 0.05$; **$p < 0.01$.

lineage depletion, recommended for "removal of cells with normal antigen expression, if recovery is the highest priority," and *Posselwb* for c-Kit enrichment, a double-positive selection recommended for bone marrow. Following the same steps as previously for the bone marrow isolation (Figure 1), we again observed a lower degree of enrichment in the lineage-depleted samples. Although it was accompanied by a higher LKS cell yield, no difference in the final number of HSCs was found between both strategies (Figure 4A–C). Compared with the results obtained with the manual magnet, the LKS cell frequencies were on average higher when using automated cell isolation; however, it did not result in greater LKS cell numbers. HSC frequencies were comparable, or slightly lower in the case of c-Kit enrichment, whereas final HSC numbers were slightly lower.

Given that an autoMACS Pro Separator may not be available to all researchers, we also tested pre-enrichment with the MACS MicroBeads using a standard MACS Separator. This column-based magnetic separator is more cost-effective than its automated analog and can easily be moved into a cold room when needed, making it more

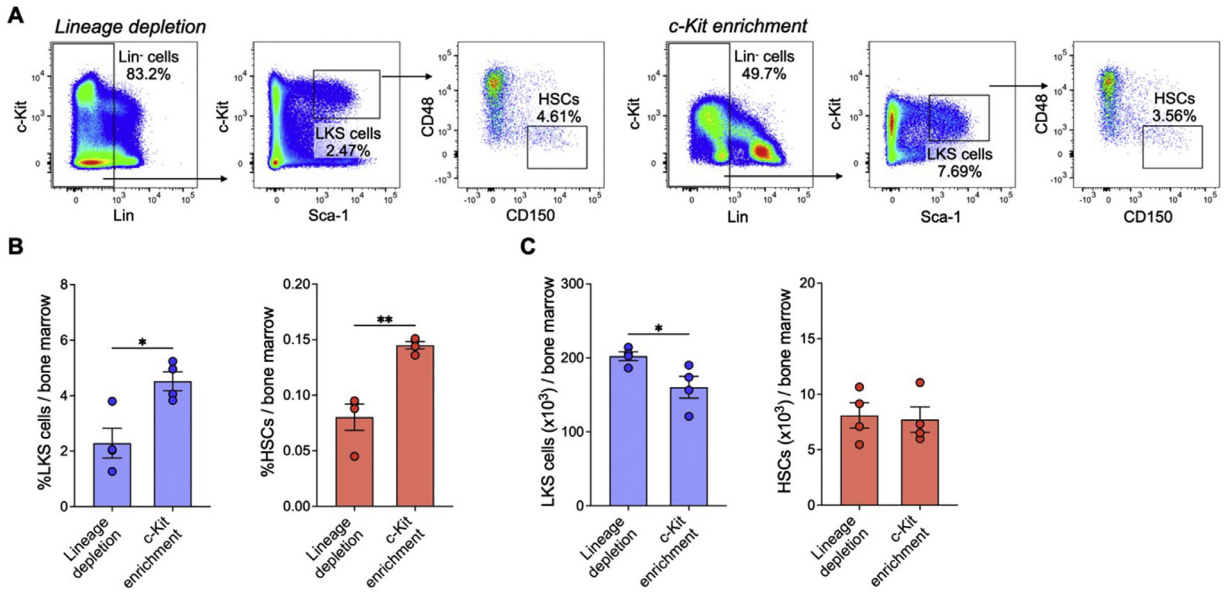


Figure 4 Comparison of pre-enrichment strategies using autoMACS cell separation. **(A)** Representative flow cytometry plots of bone marrow cells enriched by lineage depletion or c-Kit enrichment. **(B)** Frequency of LKS cells (left) and HSCs (right) among viable nucleated bone marrow cells after pre-enrichment (n = 4 from two independent experiments). **(C)** Estimated yield of LKS cells (left) and HSCs (right) per mouse after pre-enrichment (n = 4 from two independent experiments). Data are represented as mean ± SEM. Significance was evaluated using two-tailed unpaired Student *t* test. *$p < 0.05$; **$p < 0.01$.

commonly used. To allow direct comparison, lineage depletion and c-Kit enrichment with the MACS Separator were performed side-by-side with lineage depletion using the manual column-free magnet (Figure 5). For c-Kit enrichment by MACS, the percentages and yield of both LKS cells and HSCs were comparable to those of the manual column-free system (Figure 3) and autoMACS (Figure 4). Surprisingly however, the degree of enrichment in LKS cells and HSCs reached after lineage depletion were 10-fold higher when using the MACS Separator as compared with using the manual magnet (Figure 5A, B). Unfortunately, this increased enrichment was associated with a much lower yield of both LKS cells and HSCs (Figure 5C).

Considering the time needed for each pre-enrichment strategy (from the moment cells have been counted until the end of the enrichment protocol), we found that the fastest method is lineage depletion (± 65 min), followed by c-Kit enrichment (±85 min) and Sca-1 enrichment (±110 min). The absolute slowest being to perform a double enrichment (±185 min). Using the manual free-standing magnet, the MACS Separator or the autoMACS Pro Separator roughly takes the same time when processing a single sample. However, when processing multiple samples, time increases with around 10 min for each extra sample separated on the autoMACS. Indeed, with the latter samples are unavoidably processed one after the other; however, with the other approaches several magnets can be used in parallel.

Pre-enrichment and FACS Change the Levels of Some but not all Metabolites

When analyzing the metabolome of cells, a rapid processing of samples is essential. Because isolation of different cell populations from mouse bone marrow inherently requires lengthy pre-enrichment and cell sorting steps, keeping samples below 4°C can help to minimize metabolic enzyme activity. The third factor to consider is yield, as the number of detected metabolites increases with cell number [12,13]. Whether the type of pre-enrichment strategy itself impacts the cellular metabolome has not yet been studied. Before initiating this, we first investigated how the lengthy process of pre-enrichment and cell sorting influences the cellular metabolome, similar to what we have done previously for acute myeloid leukemia cells, which require cell sorting but not pre-enrichment [13]. To allow comparison of the full metabolome, a source of easily accessible and abundant cells is required, and we therefore decided to use culture-expanded lineage-negative mouse bone marrow cells that were either lysed directly from culture or "mock" pre-enriched and sorted, using 500,000 cells per sample. For the "mock" pre-enrichment strategy we chose the manual lineage depletion protocol (without antibodies) as this is the fastest protocol. We used an untargeted metabolomics pipeline, resulting in the detection of 1,437 compounds, of which 230 could be putatively identified as known metabolites based on MS/MS fragmentation spectra and/or chemical formula. When considering all compounds, no difference was observed in the overall metabolite abundance, represented by the peak area distribution, between directly lysed and pre-enriched/FACS-sorted samples (Figure 6A). However, when only considering the identified metabolites, a small but significant decrease in overall peak areas was noted in the pre-enriched/FACS-sorted samples, indicating that cell processing does negatively impact the abundance of some intracellular metabolites. When comparing the pre- and post-processing levels of individual metabolites, we noted that levels of several glycolytic intermediates (glucose, dihydroxyacetone phosphate [DHAP], lactate), metabolites related to glutathione metabolism (reduced glutathione [GSH], 2-S-glutathionyl acetate) and vitamin metabolism-linked metabolites (threonate, 1-

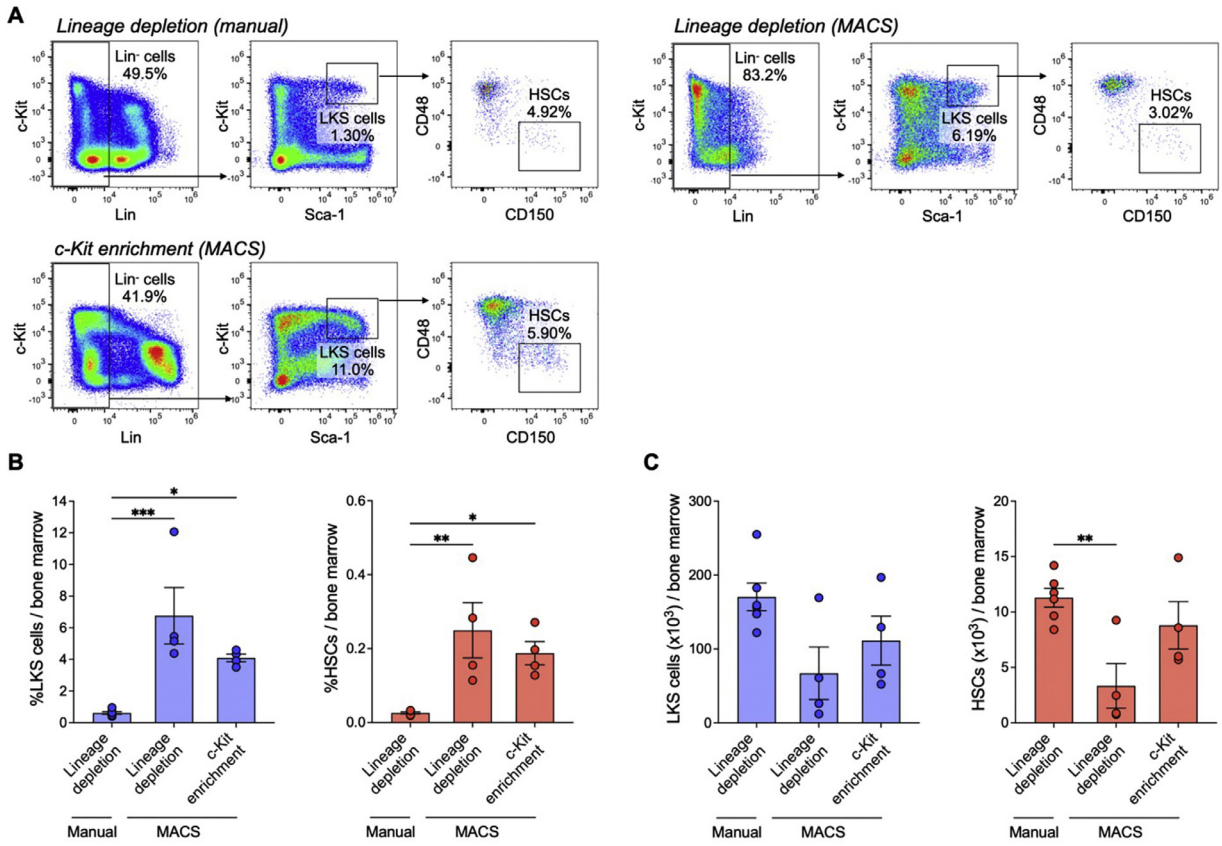


Figure 5 Comparison of pre-enrichment strategies using MACS magnetic cell separation. **(A)** Representative flow cytometry plots of bone marrow cells enriched by lineage depletion using a manual magnet, or by lineage depletion or c-Kit enrichment using MACS technology. **(B)** Frequency of LKS cells (left) and HSCs (right) among viable nucleated bone marrow cells after pre-enrichment (manual: n = 6 from three independent experiments; MACS: n = 4 from two independent experiments). **(C)** Estimated yield of LKS cells (left) and HSCs (right) per mouse after pre-enrichment (manual: n = 6 from three independent experiments; MACS: n = 4 from two independent experiments). Data are represented as mean ± SEM. Significance was evaluated by one-way ANOVA followed by Tukey's multiple comparisons test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

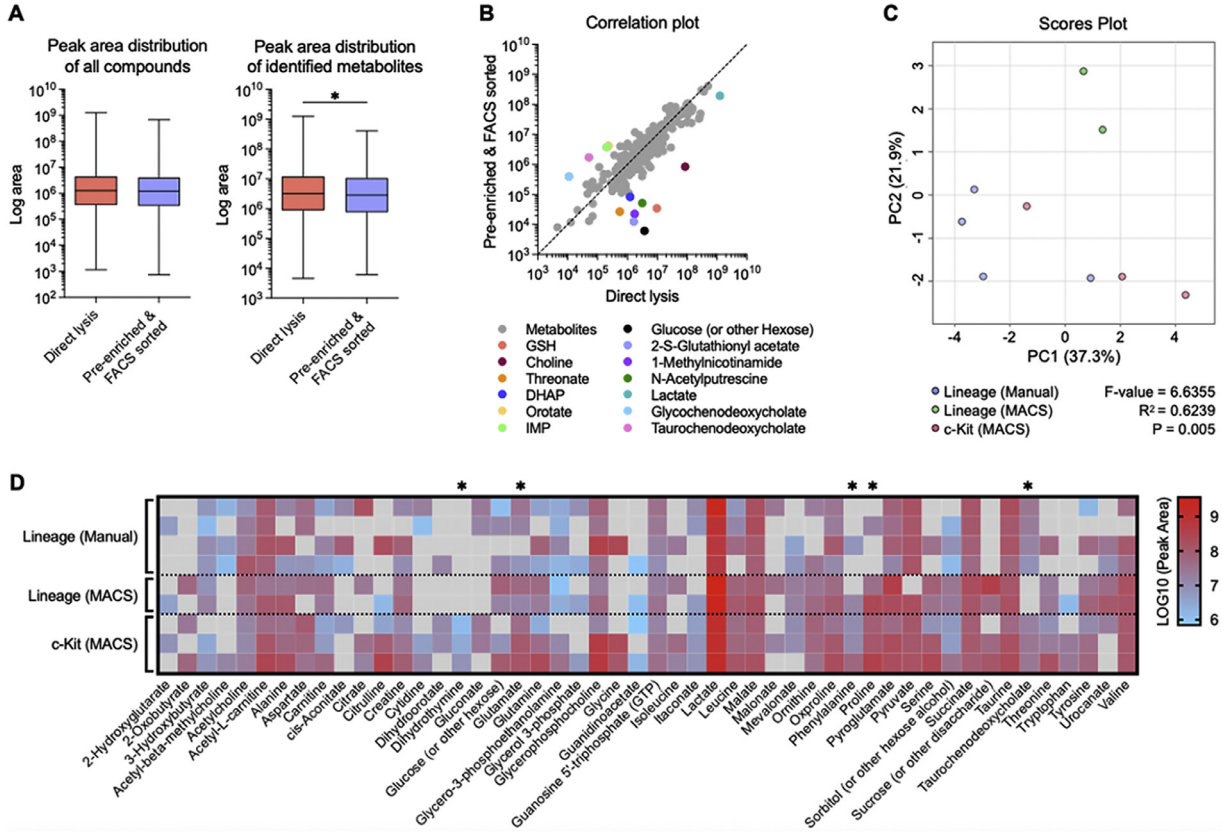
methylnicotinamide, choline) were decreased in the pre-enriched/FACS-sorted samples (Figure 6B). In contrast, bile acids (glycochenodeoxycholate, taurochenodeoxycholate) and nucleotide intermediates (orotate, inosine 5′-monophosphate [IMP]) were more abundant after cell processing and sorting.

Taken together, despite the evident changes in the levels of certain metabolites, cell processing by pre-enrichment and FACS cell sorting had relatively mild effects on most intercellular metabolites.

The Choice of Pre-enrichment Strategy Affects the HSC Metabolome

To understand whether and how different pre-enrichment strategies alter HSC metabolism, we pre-enriched fresh mouse bone marrow cells by manual column-free lineage depletion (fastest protocol, highest yield, low enrichment), MACS-based lineage depletion (highest enrichment, low yield) and MACS-based c-Kit enrichment (high enrichment, high yield) (see Figure 5). We did not include the autoMACS approaches as the autoMACS Pro Separator in our institute is maintained at room temperature, preventing us from always keeping samples at 4°C. After pre-enrichment, 5,000 HSCs (as defined by LKS-SLAM markers) from individual mice were sorted directly into acetonitrile lysis buffer and processed for metabolomics analysis. For MACS-based lineage depletion, we were only able to obtain 5,000 cells for one out of four samples, 2,500 for another sample (volume corrected before injection), and less then 1,000 cells for the remaining two samples (excluded from metabolomics analysis), underscoring the lower yield of this strategy.

Using our HILIC-based LC-MS setup we were able to detect and quantify 50 metabolites in at least 30% of samples, of which on average 30 were detected above background in mouse HSCs pre-enriched by manual column-free lineage depletion, 36 in mouse HSCs pre-enriched by MACS-based lineage depletion, and 42 in mouse HSCs pre-enriched by MACS-based c-Kit enrichment. Clustering of samples using principal component analysis based on their metabolome separated the three groups and indicated significant differences between them (Figure 6B). When comparing the levels of individual metabolites, we found that dihydrothymine, glutamate, and phenylalanine were significantly higher in MACS-based c-Kit enriched cells compared with the lineage depletion methods, whereas other metabolites were consistently detected in all samples for MACS-based c-Kit enrichment but not in the other two methods,



Q11 **Figure 6** Impact of pre-enrichment on the HSC metabolome. **(A, B)** Analysis of the effect of magnetic pre-enrichment and FACS sorting on the metabolic profile of culture-expanded lineage-negative bone marrow cells, shown by **(A)** peak area distributions of all compounds and putatively identified metabolites, and **(B)** correlation of individual metabolite levels between directly lysed and pre-enriched/FACS-sorted cells (n = 2 from one experiment). **(C, D)** Principal component analysis **(C)** and heat map–based visualization **(D)** of metabolomics results obtained from 5,000 HSCs pre-enriched using different strategies from mouse bone marrow and FACS sorted (in D, each row represents an individual mouse, gray = metabolite not detected) (n = 2–4 from two independent experiments). Significance was evaluated by two-tailed paired Student *t* test in A (*$p < 0.05$), by PERMANOVA in C, and by one-way ANOVA in D (*FDR, <0.05).

including aspartate, citrulline, and itaconate (Figure 6C). Several metabolites, including alanine, carnitine, creatine, glutamine, leucine, ornithine, proline, serine, tyrosine, and valine were consistently detected in both MACS-based methods but not the manual column-free method. Levels of acetylcholine, acetyl-L-carnitine, glycerophosphocholine, GTP, lactate, malate, oxoproline, succinate, and taurine were comparable in all samples, whereas other metabolites, often at the limit of detection, were found only in a few samples across groups. The choice of pre-enrichment strategy thus clearly impacts metabolomics results, with MACS-based c-Kit enrichment giving the most consistent results, and manual column-free lineage depletion performing the poorest.

DISCUSSION

Given the importance of a pre-enrichment step before the sorting of HSC populations, we compared different strategies to pinpoint the advantages and disadvantages of each. As shown by our results, performing a pre-enrichment can increase the frequency of LKS cells and HSCs more than 30-fold, significantly reducing the potential cell sorting time. Regarding the calculated cell yields, we estimated these based on the number of cells acquired by flow cytometry per unit of time, flow rate, and sample volume. We found that an alternative way of calculating potential cell yields based on the number of total cells counted after pre-enrichment and percentages of LKS/HSCs measured using flow cytometry gave similar results. Nevertheless, these are theoretical values that overestimate the number of cells that can be isolated per mouse. Indeed, cells of interest are inevitably lost when performing FACS, due to the presence of a dead volume when aspirating the sample and the sorting efficiency rarely being equal to 100%. The number of stem cells that can be isolated from a mouse further depends on age, sex, and strain [8]. One thing to be noted is that all our experiments were performed at 4°C or on ice to minimize transcriptional, proteomic, and metabolic changes. Therefore, it cannot be excluded that slightly different cell numbers would be obtained if performing the whole cell isolation procedure at room temperature.

Whether planning to pre-enrich bone marrow cells for downstream omics analysis or for another purpose, there are several factors

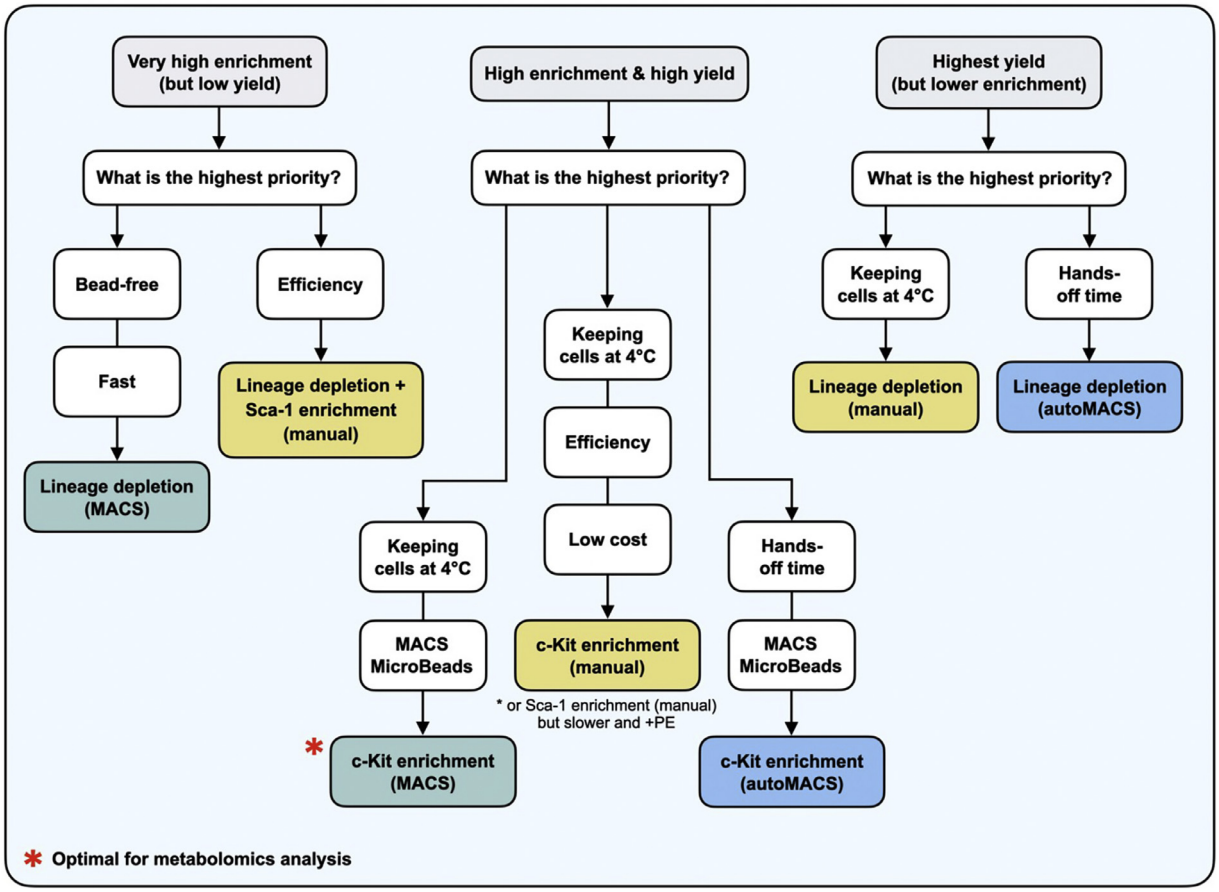


Figure 7 Decision tree to select the optimal pre-enrichment strategy based on downstream application.

to consider before deciding on a pre-enrichment strategy (Figure 7). First, one must choose among three main categories: very high enrichment (but low yield), high enrichment and high yield, and highest yield (but low enrichment). A high degree of enrichment is beneficial if planning on studying HSC-enriched cells without any further isolation or if wanting to decrease sorting time and only a small number of cells is needed. In this category, the options are lineage depletion with the MACS column and lineage depletion combined with Sca-1 enrichment using the manual magnet. The MACS column method is bead-free and fast, whereas the double enrichment is more efficient, providing higher HSC enrichment and yield.

The second category likely represents the preferred strategy for most researchers, as it offers a good balance between enrichment and yield. Each method involves performing a c-Kit enrichment, with the choice of magnet depending on one's specific priorities or availability. If cells need to be kept cold during processing, we advise to go for either the MACS column or the free-standing magnet. Keeping cells cold when using the automated separation can be problematic as buffers used for operating the autoMACS Pro Separator are required to have a temperature of ≥10°C, and the instrument is not specified for use in a cold room. If temperature is not a concern, opting for automated separation can gain the user some hands-off time and if concerned about preserving cell properties, using MACS MicroBeads may offer an advantage over the beads required for the manual magnet. Nonetheless, if choosing to use the autoMACS Pro Separator, the price of the machine as well as its cost of use and maintenance must be considered. When having to choose between the MACS column and the manual magnet, one may want to consider the slightly increased LKS and HSC yield provided by the manual magnet, as wells as its lower cost. When testing the free-standing magnet, Sca-1 enrichment came out as being nearly identical to c-Kit enrichment; however, the EasySep Sca-1 kit requires a slightly longer processing time and the PE-labeling of Sca-1$^+$ cells (included in the protocol of the kit) may be an advantage or disadvantage depending on the downstream experimental design. As a side note, it is worth noting that although we did not test Sca-1 enrichment on the autoMACS system, it has been performed by others using anti-mouse Sca-1-biotin followed by magnetic anti-biotin MicroBeads [8]. Finally, if planning to perform metabolomics analysis, we recommend using c-Kit enrichment with the MACS column, as based on our results this gives the highest and most consistent metabolite signals. However, we did not have the opportunity to assess the metabolic profile of cells c-Kit enriched with the manual magnet, so this method may also be suitable for metabolomics analysis.

The last category of the decision tree includes the remaining two lineage depletion methods. Both give very high cell yields but lower enrichment. Here we recommend to choose the manual magnet if the cells need to be cold or cost is an important factor, or the autoMACS Pro Separator to gain some hands-off time. The main advantage of using the lineage depletion protocols is that they are fast and

leave the target cells unstained by antibodies, which may be preferred for certain downstream applications.

When comparing the different pre-enrichment strategies, some interesting observations can be made. The MACS-based lineage depletion approach gives very high enrichment but low yield of both HSCs and LKS cells compared with autoMACS-based lineage depletion, even though we used the same Lineage Cell Depletion Kit and staining protocol for both. Both approaches achieve an enrichment of lineage-negative cells above 80%, which is far superior to that of the manual kit which only achieves 40%–50% enrichment. The use of the standard MACS Separator however leads to much greater cell loss even though we used LS columns, which are recommended by the supplier for lineage depletion using this kit and which should theoretically only deplete strongly magnetically labeled cells. The lower yield of the MACS-based lineage depletion strategy can create downstream issues, as we encountered during our metabolomics analysis.

For metabolomics analysis of HSCs, we found that MACS-based c-Kit pre-enrichment outperformed the two other tested approaches. Although most highly abundant metabolites were adequately detected across all samples independent of pre-enrichment method, we detected significantly more metabolites in the MACS-based c-Kit pre-enriched samples, especially compared with those processed by manual column-free lineage depletion, and found much less variability between individual samples. There was overall not that much difference in metabolic profile between HSC enriched using both MACS-based methods, although the lower HSC number obtained per mouse using the MACS-based lineage depletion strategy argues against its use for metabolomics analysis, as discussed above. The reason for the superior performance of the MACS-based methods compared with the manual column-free method is currently not clear. The higher stem cell enrichment obtained by both MACS methods led to a shorter FACS sorting time on average, although this is nearly fully compensated by the longer time needed for pre-enrichment itself. In addition, given that the total processing time between mouse sacrifice and cell lysis is around 3.5 hours, a slightly longer sorting time may not be that consequential. Other possibilities are that a different composition of the buffers in which the beads are provided somehow influences HSC metabolism, or that passing through the MACS column itself can influence the metabolic profile of the cells. Overall, our detection of 40–45 metabolites for 5,000 HSCs for MACS-based methods is very comparable to recent findings from others [12]. Based on these results we consider MACS-based c-Kit pre-enrichment to be the optimal strategy for HSC metabolomics analysis. It may be worthwhile for future studies to also test other pre-enrichment strategies, such as EPCR positive selection, which is not yet commonly used in the field but which according to the manufacturer can lead to very high HSC enrichment.

Although we find MACS-based c-Kit pre-enrichment to be the best strategy for HSC metabolomics analysis, the removal of HSCs from their natural bone marrow environment and exposure to supra-physiological oxygen levels and nutrient-poor buffers inherently alters their metabolism. In accordance, we found that cell processing by magnetic pre-enrichment and FACS-based cell sorting had a significant, although overall minor, impact on the metabolome of cultured hematopoietic progenitor cells. Although the metabolic state of these cells (which are in a different nutritional environment compared with the bone marrow and have undergone activation and differentiated induced by the cytokines in the medium) is likely very different than that of HSCs, it shows that as long as samples are kept at 4°C throughout the process the impact of processing on cell metabolism can be limited, with the exception of some metabolites. This is comparable to our previous results from the metabolomics analysis of acute myeloid leukemia cells, which during isolation require RBC lysis and FACS sorting, but no pre-enrichment [13]. The metabolites that did change during cell processing are in line with what can be expected. Glycolysis for example is known to be a very dynamic pathway where fluxes can change acutely, and it is thus not surprising that levels of glycolytic metabolites decrease rapidly in cells maintained in FACS buffer that contains only low levels of glucose coming from the serum, even when cells are kept at 4°C. This aspect has previously been noted also by others, as Schönberger et al. [22] mentioned that "For data interpretation, it has to be taken into account that sample processing is expected to induce a starvation-like phenotype. Of note, we do not drive any conclusions on high-turnover metabolites such as glycolytic intermediates or adenosine triphosphate (ATP)". Processing cells in a buffer with physiological glucose levels, such as Hanks' balanced salt solution, was shown to limit this effect [10]. In a similar manner, the metabolism of glutathione and certain vitamins may be rapidly activated in cells exposed to atmospheric oxygen concentrations to prevent oxidative damage, given that physiological oxygen levels in the bone marrow are very low [23]. A previous study showed that this oxidative stress can be prevented by harvesting and processing HSCs under low (native) oxygen conditions or by adding a mitochondrial permeability transition pore inhibitor during cell collection [24]. Another element to consider during sample processing is light, as some metabolites (such as retinoic acid that is highly abundant in HSCs [25]) are light sensitive. Processing of samples in the dark, which we did not perform in the current study, may improve the detection of this type of metabolites. In the end, as long as samples from different groups are processed together, we anticipate that these processing-induced changes will not prevent the acquisition of useful metabolomics data and insights into HSC metabolism, although different factors need to be taken into account depending on the metabolite(s) of interest.

A final element to consider during cell sorting for metabolomics is the nozzle size and sheath fluid used. A previous study compared the metabolism of HSCs sorted using a 100 μm or 70 μm nozzle and found that the smaller nozzle size, which reduces sheath fluid volume in the lysis buffer, increased the number of metabolites detected likely by avoiding ion suppression due to higher salt introduced by the sheath fluid [10]. We had similar experiences, and further performed sorts with 5 g/L NaCl in water as sheath fluid instead of PBS, like others have done [12], as the presence of phosphate in the samples increased the chance of clogging our capillary HILIC-LC setup.

In conclusion, we show in the current study that pre-enrichment strategies for the isolation of LKS cells or HSCs from mouse bone marrow differ in their stem cell enrichment, yield, processing time, cost, and impact on the cellular metabolome. We hope these findings will help researchers in the selection of an ideal pre-enrichment strategy for their envisioned application, and can promote further exploration of the role of HSC metabolism in hematopoietic development, homeostasis, and disease.

Conflict of Interest Disclosure

The authors do not have any conflicts of interest to declare in relation to this work.

Acknowledgments

The authors would like to thank Charles Vidoudez and Sunia A. Trauger from the FAS Small Molecule Mass Spectrometry Facility of Harvard University (Cambridge, Massachusetts) for the untargeted metabolomics analysis of culture-expanded cells. We also thank all members of the Cellular Metabolism and Microenvironment Laboratory for constructive discussions and help with sample processing. This work was supported by the Walloon Region as part of the FRFS-WELBIO strategic axis (WELBIO-CR-2022 S-08), and by the King Baudouin Foundation (2021-J1990112-222061; Funds André Vander Stricht, Emile Carpentier, Van Damme, Yvonne & Jacques François - De Meurs, José Plé - Albert Declercq, Arlette Lema tre, M. Waeyenborghs, Julia Ertryckx, Madeleine Wigand, Paulette Capitaine, Georgette & Suzanne Batta, Gaston Gérard - Blanche Franquet, and Van der Poorten - Vermeiren) and by the Fondation Contre le Cancer, under Agreements #2022-045 and #2022-046. HAT. holds a doctoral research fellowship from the Fund for Research Training in Industry and Agriculture (FRIA) (1.E.027.22). Schematics were created with BioRender.com.

Author Contributions

CN performed experiments, analyzed the data, and wrote the manuscript. YL helped with experiments and data analysis. FL helped with experiments. HAT helped with experiments. ND helped with experiments and data analysis. NvG planned and directed the study, analyzed data, and wrote the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.exphem.2024.104588.

REFERENCES

1. Laurenti E, Göttgens B. From haematopoietic stem cells to complex differentiation landscapes. Nature 2018;553:418–26.
2. Granot N, Storb R. History of hematopoietic cell transplantation: challenges and progress. Haematologica 2020;105:2716–29.
3. Caiado F, Pietras EM, Manz MG. Inflammation as a regulator of hematopoietic stem cell function in disease, aging, and clonal selection. J Exp Med 2021;218:e20201541.
4. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science 1988;241:58–62.
5. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 1996;183:1797–806.
6. Kiel MJ, Yilmaz OH, Iwashita T, Yilmaz OH, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 2005;121:1109–21.
7. Osawa M, Hanada K, Hamada H, Nakauchi H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 1996;273:242–5.
8. Challen GA, Boles N, Lin KK, Goodell MA. Mouse hematopoietic stem cell identification and analysis. Cytometry A 2009;75:14–24.
9. Purton LE. Adult murine hematopoietic stem cells and progenitors: an update on their identities, functions, and assays. Exp Hematol 2022;116:1–14.
10. DeVilbiss AW, Zhao Z, Martin-Sandoval MS, et al. Metabolomic profiling of rare cell populations isolated by flow cytometry from tissues. Elife 2021;10:e61980.
11. Takubo K, Nagamatsu G, Kobayashi CI, et al. Regulation of glycolysis by Pdk functions as a metabolic checkpoint for cell cycle quiescence in hematopoietic stem cells. Cell Stem Cell 2013;12:49–61.
12. Schönberger K, Mitterer M, Glaser K, et al. LC-MS-based targeted metabolomics for FACS-purified rare cells. Anal Chem 2023;95:4325–34.
13. van Gastel N, Spinelli JB, Sharda A, et al. Induction of a timed metabolic collapse to overcome cancer chemoresistance. Cell Metab 2020;32:391–403.e6.
14. Cossarizza A, Chang HD, Radbruch A, et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). Eur J Immunol 2019;49:1457–973.
15. Kanehisa M, Goto SKEGG. Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res 2000;28:27–30.
16. Wishart DS, Tzur D, Knox C, et al. HMDB: the human metabolome database. Nucleic Acids Res 2007;35:D521–6.
17. Wishart DS, Feunang YD, Marcu A, et al. HMDB 4.0: the human metabolome database for 2018. Nucleic Acids Res 2018;46:D608–17.
18. Chambers MC, Maclean B, Burke R, et al. A cross-platform toolkit for mass spectrometry and proteomics. Nat Biotechnol 2012;30:918–20.
19. Schmid R, Heuckeroth S, Korf A, et al. Integrative analysis of multimodal mass spectrometry data in MZmine 3. Nat Biotechnol 2023;41:447–9.
20. Chong J, Soufan O, Li C, et al. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. Nucleic Acids Res 2018;46:W486–94.
21. Courties G, Herisson F, Sager HB, et al. Ischemic stroke activates hematopoietic bone marrow stem cells. Circ Res 2015;116:407–17.
22. Schönberger K, Mitterer M, Buescher JM. Cabezas-Wallscheid N. Targeted LC-MS/MS-based metabolomics and lipidomics on limited hematopoietic stem cell numbers. STAR Protoc 2022;3:101408.
23. Spencer JA, Ferraro F, Roussakis E, et al. Direct measurement of local oxygen concentration in the bone marrow of live animals. Nature 2014;508:269–73.
24. Mantel CR, O'Leary HA, Chitteti BR, et al. Enhancing hematopoietic stem cell transplantation efficacy by mitigating oxygen shock. Cell 2015;161:1553–65.
25. Schönberger K, Obier N, Romero-Mulero MC, et al. Multilayer omics analysis reveals a non-classical retinoic acid signaling axis that regulates hematopoietic stem cell identity. Cell Stem Cell 2022;29:131–148.e10.