A Novel Method for Generating Non-myeloablative Bone Marrow Chimeric Mice

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Maiko MORITA

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Maiko MORITA

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General Abstract

Hematopoietic stem cells (HSCs) are one of the tissue stem cells that reside in the bone marrow (BM) and are capable of self-renewal and multipotent differentiation, providing a continuous supply of hematopoietic cells throughout the life of the organism. HSCs are used in hematopoietic stem cell transplantation (HSCT) for the treatment of leukemia and other hematological diseases. HSCT involves the transplantation of hematopoietic stem cells from a healthy donor after the pretreatment of the patient with irradiation or chemotherapy. This method is also being applied in research to restore normal hematopoiesis by expanding HSCs from patients with immunodeficiency or other hematological diseases and treating them with gene therapy. However, the functions and properties of HSCs remain to be elucidated, and further research is needed. Although transgenic mice are sometimes used for functional analysis in basic hematology research, they require a long period and advanced techniques to produce. As an alternative, bone marrow chimeric mice have been routinely used in hematological studies because they are simpler and can be produced in a shorter period than transgenic mouse models. By transplanting HSCs and hematopoietic stem progenitor cells (HSPCs) from donor mice into lethally irradiated recipient mice, bone marrow chimeric mice allow us to observe the reconstruction of the hematopoietic and immune systems of the donor cells. Combined with gene transfer methods to donor cells *ex vivo*, this is a useful approach to investigate a wider range of determinants of hematopoietic and immune system development and function.

Many methods for culturing and transplanting HSCs *ex vivo* have been developed, however, various problems with the culture conditions and the competitive

repopulation assay remains. For culture conditions of HSCs, low reproducibility due to bovine serum albumin (BSA) lot variants and using an undefined medium (S-clone) was controversial. For the competitive repopulation assay, it needs fluorescence-activated cell sorting (FACS) of HSCs and myeloablative conditioning such as irradiation. FACS equipment is not universally available and the operation of FACS sorting requires special skills. Also, myeloablative conditioning occurs significant damage to the BM microenvironment, resulting in the observation of inflammatory hematopoiesis.

In Chapter 1, I examined basic medium and supplements which are universally available and fully defined using recombinant human serum albumin (rHSA), which have fewer lot variants. In a comparative study of basic culture medium, Ham's F-12 was the best to maintain HSCs *ex vivo*. In the comparison of supplements, ITS-X supplement showed equal engraftment to S-clone attached supplement. When compared to conventional medium (S-clone, BSA, S-clone attached supplement) and optimized medium (Ham's F-12, rHSA, ITS-X supplement) by FACS analysis of cultured HSCs, HSCs cultured with the optimized medium contained significantly higher population of c-Kit^{+}Sca-1^{-}Lineage^{-} (KSL) fraction which represents undifferentiated cells. Therefore, I succeeded in the optimization of the HSC culture medium. In a subsequent study, my colleagues and I found that poly-vinyl alcohol (PVA) was the better substitute for rHSA. And it has shown that the HSC medium using PVA could support HSC proliferation for 28 days *ex vivo*. Now this PVA-based HSC medium is currently the most optimal combination.

In Chapter 2, I aimed to purify HSCs/HSPCs without FACS equipment and to generate BM chimeric mice without myeloablative conditioning such as radiation. At first, I hypothesized that the PVA-based HSC culture medium developed in chapter 1

could be used to culture BM cells to selectively amplify only HSCs, without maintaining differentiated cells. By culturing c-Kit$^+$ Whole BM (WBM) cells with a newly optimized HSC culture medium for 28 days, total cell numbers and CD150$^+$KSL fraction were increased. In competitive repopulation assay, cultured c-Kit WBM cells showed long-term engraftment, which implies that these cells contained highly purified HSCs and HSPCs. Thus, the purification of HSCs and HSPCs without FACS was succeeded. When these cultured c-Kit WBM cells were transplanted into non-irradiated mice, long and high peripheral blood (PB) chimerism was observed until secondary transplantation. These results suggested that non-myeloablative BM chimeric mice have been developed without the use of FACS equipment. As *ex vivo* gene manipulation of HSCs has often been used to investigate the gene function in the field of hematopoietic research, I also applied *ex vivo* gene transduction with lentiviral vectors for non-irradiated transplantation. By using the lentiviral vector harboring Tet3G Doxycycline (Dox) inducing target gene system, I transduced EGFP or two oncogenes to HSPCs. When transplanted into non-irradiated mice, these cells showed high engraftment, and administration of Dox to these non-conditioned BM chimeric mice enabled control target gene expression. This is the first report that HSC/HSPC purification without FACS, non-conditioned BM chimeric mice model which can control target gene in vivo by Dox. By utilizing this mouse model, various genetic determinants can be observed in steady-state hematopoiesis at any desired time. Also, the application for the gene therapy model which doesn't require myeloablative conditioning becomes possible. This model will provide a useful and practical experimental approach for the fields of immunology and hematology.

Abbreviations

AML, Acute Myeloid Leukemia

BM, Bone Marrow

BSA, Bovine Serum Albumin

CML, Chronic Myeloid Leukemia

DMEM, Dulbecco Modified Eagle Medium

DOX, Doxycycline

EGFP, Enhanced Green Fluorescent Protein

ES cells, Embryonic Stem cells

FACS, Fluorescence-activated cell sorting

FBS, Fetal Bovine Serum

GVHD, Graft versus Host Disease

HSC, Hematopoietic stem cell

HSCT, Hematopoietic stem cell transplant

HSPC, Hematopoietic stem progenitor cell

IRES, Internal ribosome entry sites

ITS, Insulin-Transferrin-Selenium

KL, c-Kit^{+} Lineage^{-}

KSL, c-Kit^{+} Sca-1^{+} Lineage^{-}

LT, Long-term

NADPH, Nicotinamide adenine dinucleotide phosphate oxidase.

PB, peripheral blood

PSG, Penicillin-Streptomycin-L-Glutamine

PVA, poly-vinyl-alcohol

rHSA, recombinant human serum albumin

ROS, Reactive Oxygen species

RPMI, Roswell Park Memorial Institute 1640 medium

SCF, stem cell factor

SCID-X1, X-linked severe combined immunodeficiency SCID-X1

ST, Short-term

TPO, thrombopoietin

WBM, Whole Bone Marrow

General Introduction

Hematopoietic stem cells (HSCs) are one of the oldest studied tissue stem cells[1]. HSCs are defined as cells that have the ability for self-renewal and multipotency and have the capacity to continue to supply all blood cells in the peripheral blood throughout the lifetime of the organism. This ability and medical utility of HSCs have long been demonstrated by bone marrow transplantation, which has been practiced clinically for many years[1]. Currently, they are also being targeted for regenerative medical applications and gene therapy[2]. Mice have been used in these pre-clinical studies and in the search for genetic determinants of hematopoietic stem cell function and characteristics.

The first bone marrow (BM) transplantation in mice was reported in 1951 by Lorenz et al. who demonstrated that transplantation of bone marrow cells into lethally irradiated mice could avoid radiation-induced death[3]. After that, the study by Ford et al. revealed that the "rescue" was by the engraftment of the cells[4]. In the early 1960s, Till and McCulloch showed that transplantation of BM cells into lethally irradiated mice resulted in the formation of visually observable spleen colonies containing a variety of blood cells (pluripotency) on the spleen surface after 8-12 days after transplantation[5,6]. When these splenic colonies were transplanted into other mice, splenic colonies were detected again (self-renewal ability). This series of studies proposed the concept of hematopoietic stem cells[5–8]. HSC research was then greatly advanced by the development of multicolor fluorescent cell sorters (FACS)[9], monoclonal antibodies[10], and the congenic mice with CD45[11]. Using these techniques, the competitive repopulation assay was developed. In this assay, HSCs are transplanted into lethally irradiated mice

and evaluated for their ability to provide the multilineage cells necessary for survival in the mouse for the long term. In primary transplantation, the engraftment indicates the existence of short-term HSCs (ST-HSC), which have the ability of transient engraftment (Fig 1A). The observation of the engraftment from the transplanted cells at 12 to 16 weeks after the primary transplantation may suggest long-term bone marrow reconstitution. In addition to it, the observation of the engraftment in the secondary transplantation is a powerful way of evaluating the existence of long-term HSCs (LT-HSCs) (Fig 1B)[12,13]. This evaluation assay also enabled the investigation of the fraction of HSCs using the antibodies to multicolor cell surface markers (Fig 2A, B). Currently, it is demonstrated that $CD34^-$, c-Kit^+, Sca-1^+, and $Lineage^-$ ($CD34^-$KSL) [13–16] fraction contains highly purified HSCs. Osawa et al. showed that even a single $CD34^-$KSL cell transplantation to a lethally irradiated mouse made possible long-term reconstitution in the BM in one out of three mice[14]. Other reports that indicate the cell surface marker of HSCs are $CD150^-$, $CD41^-$, and $CD48^-$ fractions, which are membrane protein receptors belonging to the signaling lymphocyte activation molecule (SLAM) family[17]. These markers are widely used as characteristic surface markers for HSC fractions, together with $CD34^-$KSL.

The ability of HSCs is regulated by external factors in the BM microenvironment *in vivo* and by genes expressed within HSCs[18,19]. These functions have often been evaluated using genetically engineered mice for the target genes. Mutant genes or loss of function of target genes can be assessed by transplantation of HSCs from genetically engineered mice. For example, external factors such as Stem Cell Factor (SCF) and Thrombopoietin (TPO) are crucial humoral factors that regulate HSC self-renewal in the bone marrow microenvironment[20–22]. These cytokines are necessary to

maintain long-term HSCs during *ex vivo* culture[23]. Such function was also found using a genetically engineered model[24]. However, the production of genetically engineered mice takes a long time to obtain and requires a highly specialized technique[25]. By combining *ex vivo* gene manipulation of HSCs (e.g., by viral vectors, RNAi, or gene editing techniques) and transplantation, it is possible to generate BM chimeric mice which have mutant hematopoietic cells[26]. This kind of BM chimeric mice is commonly used because it requires less time to produce compared to transgenic mice. Moreover, BM chimeric mice are frequently used as gene therapy models for genetic hematological diseases such as X-linked severe combined immunodeficiency (SCID-X1), Fanconi anemia, and sickle cell disease[2]. For these hematological disorders, transplanting *ex vivo* gene manipulated autologous HSCs is preferable to transplanting allogeneic HSCs from healthy donors as allogeneic HSCT is likely to cause graft-versus-host disease (GVHD)[27,28]. While both HSC *ex vivo* cultures and transplantation into a mouse that underwent myeloablative procedures are necessary for HSC analysis using BM chimeric mice, either *ex vivo* culture medium condition or myeloablative procedures have various disadvantages[29–32]. Therefore, I studied to improve the problems of these HSC research methods.

In Chapter 1, I developed a fully defined HSC medium using rHSA and a basic medium or supplement that is easily available and fully-defined. In Chapter 2, I aimed to develop a new BM chimeric mouse model in which FACS equipment and myeloablative conditioning are not required. Also, I applied *ex vivo* genetic manipulation to these non-myeloablative BM chimeric mice. When combined with a Dox-inducible vector, the target gene expression on these cells would be controlled by addition of Dox. Thus, non-conditioned transplantation of these cells would enable control of target gene expression *in vivo*.

Figures

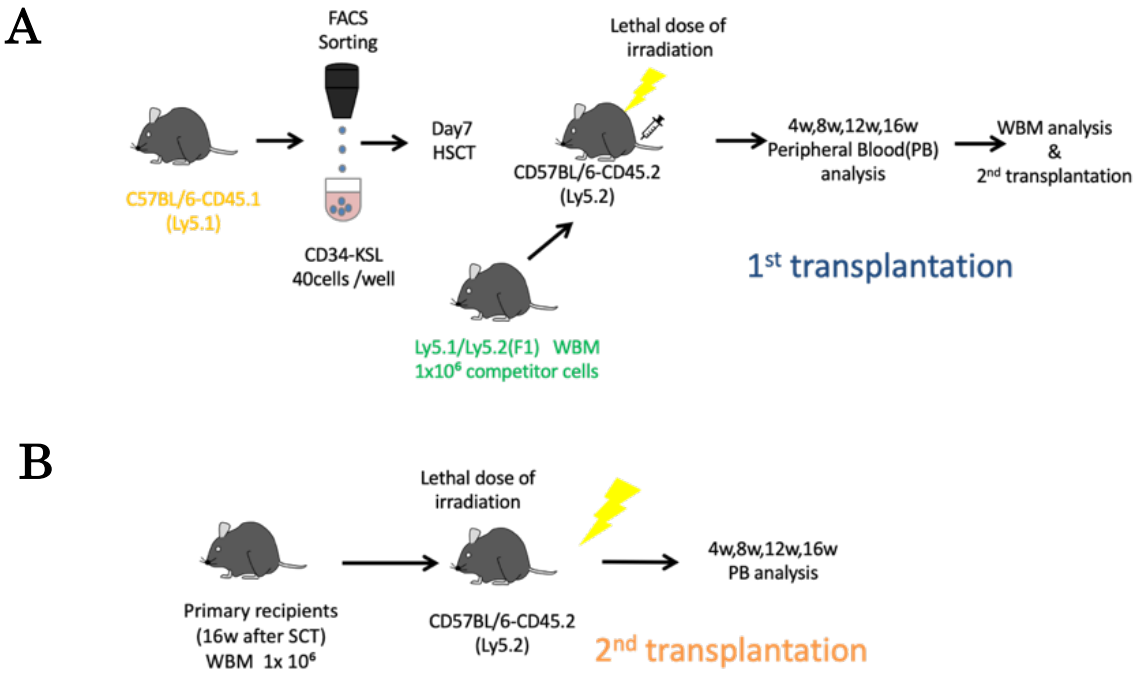


Figure 1 Competitive repopulation assay

(A) Schematic image of the 1st transplantation. FACS sorted 40 $CD34^{-}$KSL cells from Ly5.1 mouse (test cells) and 1×10^6 WBM cells from Ly5.1/Ly5.2 (F1) mouse (competitor/ rescue cells) are transplanted into lethally irradiated mice. PB chimerism is measured with a FACS analyzer every 4 weeks after transplantation.

(B) Schematic image of the 2nd transplantation. 1×10^6 WBM cells from the primary recipient mouse are transplanted into lethally irradiated mice. PB chimerism is measured as indicated in the 1st transplantation.

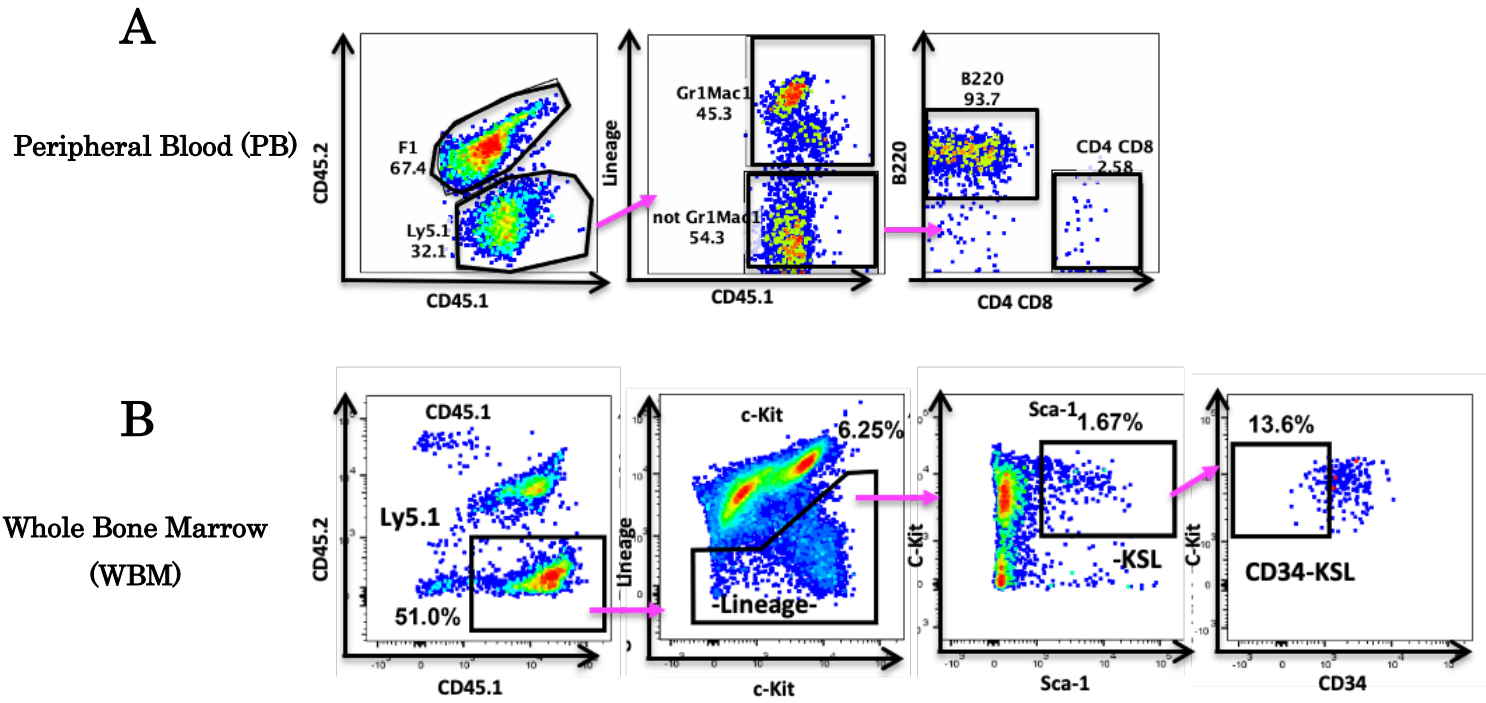


Figure 2 Representative gating strategy of FACS analysis

(A) Representative gating strategy of PB analysis by FACS. CD45.1 single positive cells are test cells and represent the chimerism which indicates the engraftment of test cells. CD45.1 and CD45.2 double-positive cells are competitor cells. Gr-1 Mac-1 positive cells are myeloid cells, B220 positive cells are B cells, and CD4 CD8 positive cells are T cells.

(B) Representative gating strategy of WBM analysis by FACS. Linage marker includes CD4/CD8 (T cells), Gr- 1/Mac-1 (Myeloid cells), B220 (B cells), Ter119 (erythrocyte), and CD127/IL-7R α (lympho-myeloid primed progenitor). $CD34^{-}$, $Linage^{-}$, $c\text{-}Kit^{+}$, and $Sca\text{-}1^{+}$ cells are a highly purified HSC fraction.

Chapter 1: Optimization of HSC *ex vivo* culture medium

Abstract

Hematopoietic stem cells (HSCs) are regarded as one of the most potential therapeutic targets for a variety of blood diseases. However, HSCs are difficult to maintain and proliferate stably *in vitro*, and their limited supply is a major limitation for transplantation research. To culture HSCs, many researchers routinely use the commercially available serum-free medium. Most of these contain bovine serum albumin (BSA), resulting in batch-to-batch variation. Also, these mediums are not chemically defined. To address these issues, I aimed to develop a medium that does not contain BSA, and all components are fully defined. Through collaborative research, it has been shown that recombinant human serum albumin (rHSA) could be a better substitute for BSA because of its reproducibility. Therefore, I decided to use rHSA for a comparative study. When I examined the basic culture medium compared to conventional S-clone, cultured HSCs in Ham's F-12 medium showed significantly higher engraftment to control. In the experiment which compared the supplements, the ITS-X supplement demonstrated the ability to maintain HSCs equivalent to conventional S-clone. After culturing HSCs in the optimized Ham's F-12, rHSA, and ITS-X combined medium, the percentage of HSC fraction was significantly higher than in the conventional BSA mixed S-clone. Thus, I have established a new HSC culture medium that is free from BSA and whose components of the medium are fully defined.

Introduction

Most adult bone marrow HSCs reside in a quiescent state at any given time and infrequently cycle for self-renewal or differentiation into blood lineages. In clinical, HSCs have been utilized Hematopoietic Stem Cell Transplantation (HSCT) for decades, but the function of HSCs and the system to maintain HSCs are still largely unknown. In the field of basic research, murine HSCs have been studied for a long time[1]. To analyze the function of HSCs, *ex vivo* HSC culture and transplantation into lethally irradiated mice are required[12]. However, there are several problems with the *ex vivo* culture medium. One of them is that bovine serum albumin (BSA) which is used for HSC culture has lot to lot differences[29]. Early hematopoietic stem cell culture media used fetal bovine serum (FBS), which is used to culture cell lines. However, the use of FBS had the problem that the biological effects of general "nutritional" serum factors and those of particular regulatory factors are difficult to distinguish[33]. The influence of unknown substances could be excluded by using BSA in the culture medium[33]. Recently, our group has shown that lots differences of BSA also have significant effects on HSC proliferation and engraftment, resulting in the lack of reproducibility between laboratories[29]. When focusing on recombinant serum albumin, recombinant human serum albumin (rHSA) became a candidate as a substitute for BSA. We found that rHSA produced in yeast could proliferate HSCs *ex vivo* in culture and HSCs cultured in an rHSA-based medium showed stable PB chimerism irrespective of lots. That chimerism was twice that of fleshly isolated HSCs[29]. Another problem is that the commercial HSC culture medium is not fully defined. Conventionally, researchers use HSC medium called S-clone which is chemically undefined. Therefore, using this rHSA, I compared the basic medium and the supplement which is universally available and fully defined.

Materials and Methods

Mice

C57BL/6-CD45.1 (CD45.1) and C57BL/6-CD45.1/CD45.2 (CD45.1/CD45.2) mice were purchased from Sankyo-Laboratory Service (Tsukuba, Japan), C57BL/6- CD45.2 (CD45.2) mice were purchased from Japan SLC (Shizuoka, Japan), respectively. Male mice aged 8 to 12 weeks were utilized as both donors and recipients. All mice were kept in SPF (specific pathogen-free) environments with unrestricted access to food and water. All animal protocols were approved by the Animal Care and Use Committee of the Institute of Medical Science, the University of Tokyo. The mouse room has a temperature range of 23–25 °C, a humidity of approximately 50%, and 12-hour light cycles.

Serum-free culture components

S-clone (Sanko Junyaku, Japan) had previously been provided in a package of S-clone medium; the supplement contained insulin, transferrin, bovine serum albumin (BSA), and other factors. Recently, BSA was excluded from the manufacturer's package. Thus, we used rHSA purchased from Albumin Bioscience (Huntsville, AL) or Sigma-Aldrich. Dulbecco Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, Ham's F-12 medium, Insulin-Transferrin-Selenium with ethanolamine supplement (ITS-X, 51500- 056) were purchased from Thermo Fisher Scientific (Waltham, MA).

New standard serum-free medium

I optimized all of my serum-free culture conditions. the new standard serum-

free medium consisted of 0.5mg/mL rHSA (Albumin Bioscience, 1001), 1 x ITS-X (Thermo Fisher Scientific, 51500-056), 10mM HEPES (Sigma Aldrich, H0887), and 0.5mg/mL streptomycin/penicillin (Thermo Fisher Scientific, 10378016), mouse TPO (100 ng/ml; PeproTech), and mouse SCF (10 ng/ml; PeproTech) in Ham F-12 medium (Thermo Fisher Scientific, 21700026)

Cell counting and sample preparation for flow cytometry

An automatic cell counter was used for cell counting (Countess II Automated Cell Counter, Invitrogen). PB samples were collected from the retro-orbital venous plexus into capillary tubes filled with powdered EDTA. PB samples were stained with PE- labeled anti-Gr-1, PE-labeled anti-Mac-1, APC-labeled anti-CD4, APC-labeled anti-CD8a, APC/Cy7-labeled anti- B220, PE/Cy7-labeled anti-CD45.1, and eFluor450- labeled anti-CD45.2 antibodies. BM samples were stained with PE/Cy7-labeled anti-CD45.1, eFluor450-labeled anti-CD45.2, PE-labeled anti-Sca-1, APC-labeled anti-c-Kit, APC/eFluor780- labeled anti-CD4, APC/eFluor780- labeled anti- CD8, APC/eFluor780-labeled anti-Mac-1, APC/eFluor780-labeled anti-Gr-1, APC/eFluor780-labeled anti-B220, APC/eFluor780-labeled anti-Ter119, APC/eFluor780-labeled anti-CD127/IL-7R α, and FITC-labeled anti-CD34 or PE/Cy5-labeled anti-CD34antibodies. Cultured HSC samples were stained with APC-labeled anti-c-Kit, PE/Cy7-labeled anti- Sca-1, APC/eFluor780-labeled anti-CD4, APC/eFluor780-labeled anti- CD8, APC/eFluor780-labeled anti-Mac-1, APC/eFluor780-labeled anti-Gr- 1, APC/eFluor780-labeled anti-B220, APC/eFluor780-labeled anti-Ter119, and APC/eFluor780-labeled anti-CD127/IL-7R α antibodies. Data were acquired on a FACSAriaIII or FACSVerse (BD) and analyzed with FlowJo (v10.5.3) Software (FlowJo, LLC).

Competitive transplantation assays

Cultured 40 $CD34^{-}$KSL cells were transplanted via single intravenous injection into irradiated (9.5 Gy) male C57BL/6-CD45.2 recipient mice along with 1×10^{6} male C57BL/6-CD45.1/ CD45.2 WBM competitor cells. Peripheral Blood (PB) analysis was performed every 4 weeks for 12 to 16 weeks. As a second transplantation, 1×10^{6} WBM cells of primary transplanted mice were transplanted into irradiated (9.5 Gy) male C57BL/6-CD45.2 recipient mice. PB analysis is performed as same as primary transplant.

Comparison with the conventional and the optimized medium

40 $CD34^{-}$KSL cells were cultured in each the conventional and the optimized medium. After 7 days of culture, cells were stained with antibodies and analyzed as mentioned in Cell counting and sample preparation for flow cytometry.

Cell counting and sample preparation for flow cytometry

The components of the conventional medium are 1% S-clone supplement (Sanko Jyunyaku), 1% BSA (Gibco), mouse TPO (100 ng/ml; PeproTech), and mouse SCF (10 ng/ml; PeproTech) in S-Clone medium (sanko jyunyaku). The optimized medium components are 0.5mg/mL rHSA (Albumin Bioscience, 1001), 1 × ITS-X (Thermo Fisher Scientific, 51500-056), 10mM HEPES (Sigma Aldrich, H0887), and 0.5 mg/mL streptomycin/penicillin (Thermo Fisher Scientific, 10378016) mouse TPO (100 ng/ml; PeproTech), and mouse SCF (10 ng/ml; PeproTech) in Ham's F-12 medium (Thermo Fisher Scientific, 21700026).

Results

A serum-free medium called S-clone, consisting of SF-O3 medium, its supplement, BSA, and other components, has regularly been used as one of the best formulations of serum-free media[23,34–36]. The BSA previously included in the S-clone package has recently been removed and is now provided separately by the company. Recently, my colleagues and I have attempted to replace BSA with recombinant human serum albumin (rHSA)[29]. In this study, four different batches of rHSA were tested with competitive repopulation assays. Although some S-clone culture components are known, their detailed concentrations are unknown. Hence, I searched for a substitute for S-clone among universally available reagents and with known detailed concentration compositions.

First, I compared the following basic media formulations and their mixtures: S-clone, DMEM, RPMI1640 (RPMI), Ham's F-12 (F-12), a 1:1 mixture of DMEM and F-12 (DF), a 1:1 mixture of DMEM and RPMI 1640 (DR), and a 1:1:1 mixture of DMEM, F-12, and RPMI 1640 (DFR), since these media are known to be mixed in S-clone medium[37]. To compare these media, $CD34^{-}$KSL cells were cultured in each medium and transplanted into lethally irradiated mice. After 7 days of culture, the proliferation of the cells was observed in each culture (Fig. 3A). In competitive repopulation assay, F-12 medium supported HSC activity to a significantly greater extent than DMEM or RPMI 1640 medium ($P < 0.05$), supporting previous observations that the balance in amino acid components plays an important role in HSC maintenance[38,39] (Fig.3B). However, significant differences were not observed among S-clone, F-12, DF, DR, and DFR media (Fig.3B). To investigate which medium is best for HSC culture, I performed secondary transplantation assays. As a result, the F-12 medium showed much higher chimerism

than other media. (Fig. 3C) These results showed that the F-12 medium could support HSCs better than the S-clone medium. Therefore, the F-12 medium was decided to be used as the basic medium in subsequent experiments.

Next, I compared supplements. In the S-clone supplement, recombinant insulin, transferrin, selenium, and ethanolamine were known to be included. Therefore, I focused on the ITS-X supplement which is used in ES cell culture[40], and compared it with S-clone attached supplement. As with the basic medium experiment, $CD34^{-}KSL$ cells were cultured in F-12 medium with each supplement. After 7 days of culture, these cells were assessed in competitive repopulation assay. As a result, the proliferation of the cells was observed in each culture. In competitive repopulation assay, the chimerism of ITS-X supplement was significantly higher than S-clone supplement ($P<0.05$) after 4w transplantation. After 8w to 16w of transplantation, the chimerism of the ITS-X supplement was as same as the S-clone supplement (Fig. 4A, B). Thus, the ITS-X supplement was shown to be as effective as the S-clone supplement in HSC maintenance. Finally, I compared the ability to support HSC undifferentiated state by the surface marker of Lin^{-}, c-$Kit^{+}Lin^{-}$(KL), and c-$Kit^{+}Sca\text{-}1^{+}Lin^{-}$(KSL) of the conventional (S-clone, S-clone attached supplement, BSA) and the optimized (Ham's F-12, ITS-X supplement, rHSA) medium. After 7 days of culture in each medium, the fraction of Lin^{-}, KL, KSL was significantly higher in the cells cultured in the optimized medium than in the cells cultured in the conventional medium. (Fig. 5A-C). This data represents that the optimized medium has the ability to sustain HSC at an undifferentiated state.

Discussion

Serum-free culture conditions are essential in controlling *ex vivo* proliferation and differentiation of HSCs. In this study, I developed a simple serum-free culture medium completely composed of defined compounds to serve as a standard serum-free culture medium. Conventionally used cell culture protocols often require FBS as a nutrient source. However, the serum contains a variety of proteins that have a positive and negative influence on cells in culture. This issue remains in commercial serum-free culture because serum albumin fractionated from animal blood, such as BSA, is utilized in most serum-free media. Lipids, growth factors, hormones, and many other unknown factors are impurities in BSA[29]. Consequently, this type of serum-free media frequently displays batch-to-batch variations. Furthermore, several components are unstable when stored at 4°C for unidentified reasons, which likely causes difficulties in reproducing data between different laboratories. In this study, I found that Ham's F-12 supplemented with rHSA, and ITS-X can efficiently support the self-renewal of *ex vivo* HSC culture. These new standard serum-free culture conditions may enable us to reproduce and evaluate *ex vivo* expansion data from various laboratories.

As my colleagues and I had been aiming for HSC *ex vivo* culture for a longer term, whether an rHSA-based HSC medium could support HSCs for 14 days was examined. However, after 14 days of culture, several chemokines and cytokines (e.g. IL-6 and CCL2, CCL3, and CCL4) were observed in the supernatant, indicating that rHSA also has a negative effect on HSCs[41]. Therefore, my colleagues and I continued to search for a better component to support for HSCs. As a result, our group discovered that polyvinyl alcohol (PVA) is a candidate to replace rHSA[41,42]. PVA is a degradable semicrystalline type of polymer that has been used for medical applications for several

years. PVA has been widely utilized in the pharmaceutical industry to create solid dispersions that increase the solubility of medicines[43]. Also, it has been reported that the addition of PVA to cell culture media can maintain the survival of pluripotent cells such as embryos[44] and embryonic stem (ES) cells[40]. Focusing on such nature of PVA, my colleagues and I investigated whether PVA can also maintain HSCs. Surprisingly, HSCs cultured with 0.1% PVA supplement with ITS-X in F-12 medium exhibited far better chimerism than HSCs cultured with rHSA supplement with ITS-X in F-12 medium in competitive repopulation assay. In addition to that, PVA-cultured HSCs proliferated for 28 days sustaining pluripotency and its proliferation was about 8000-fold prior to culture[41]. With this discovery, a fully-defined reproducible HSC culture medium was developed. It may progress HSC research more than ever.

Figures

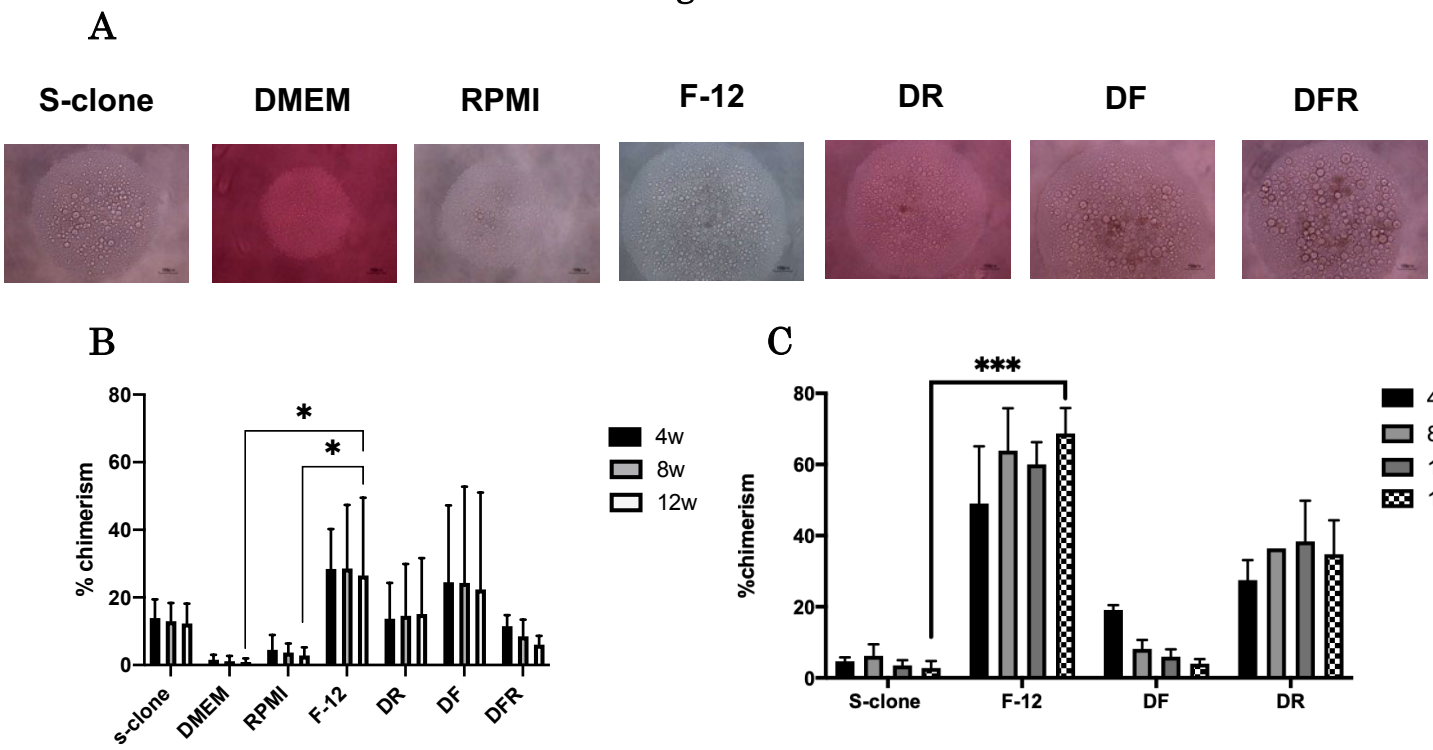


Figure 3 Comparison of basic media

Several types of culture media were tested in the presence of rHSA, SCF, and TPO. DMEM, RPMI 1640, Ham F-12 (F-12), a 1:1 mixture of DMEM and F-12 (DF), a 1:1 mixture of DMEM and RPMI 1640 (DR), and a 1:1:1 mixture of DMEM, F-12, and RPMI 1640 (DFR) media were compared with S-clone medium. $CD34^{-}KSL$ cells were cultured with each medium for 7 days. The maintenance of HSCs was evaluated 12-16 weeks after transplantation by competitive repopulation assay. (n=5 mice per group)

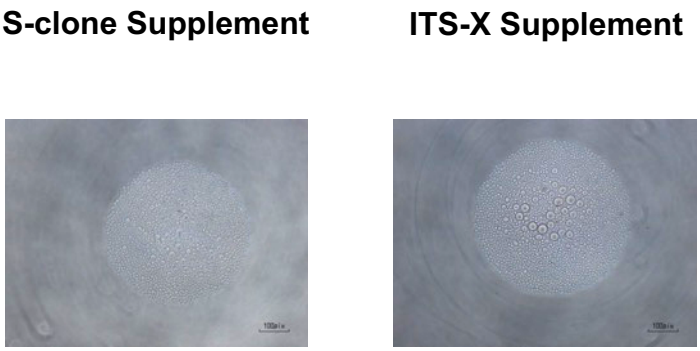
(A) The pictures of 40 $CD34^{-}KSL$ cells cultured with each medium for 7 days.

(B) Changes in donor chimerism after 1st transplantation from 4 weeks to 12 weeks.

(C)Changes in chimerism after 2nd transplantation from 4 weeks to 16 weeks.

Statistical significance was calculated using an unpaired two-tailed *t*-test, (***, P = 0.0002)

A



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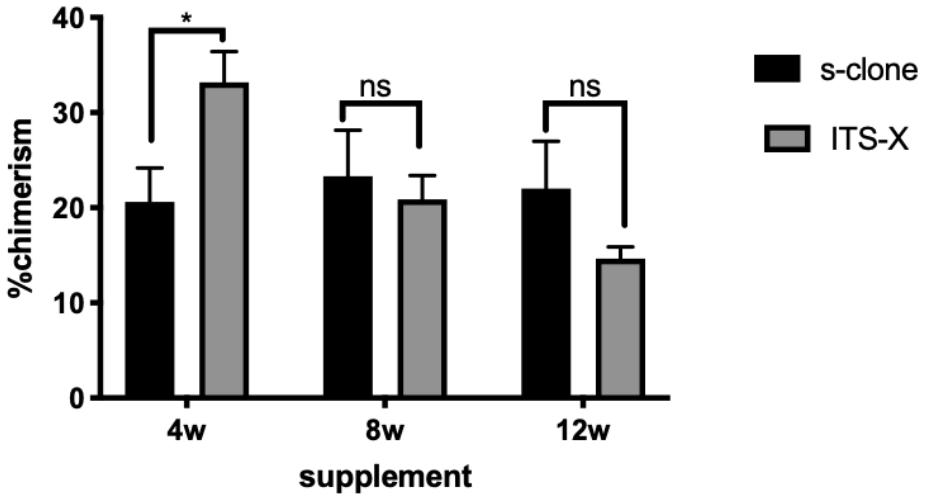


Figure 4 Comparison of S-clone supplement and ITS-X supplement

ITS-X supplement was tested with the S-clone attached supplement using Ham's F-12 medium in the presence of rHSA, SCF, and TPO. $CD34^{-}$KSL cells were cultured with each medium for 7 days. The maintenance of HSCs was evaluated 12-16 weeks after transplantation by competitive repopulation assay. (n=5 mice per group)

(A) The pictures of $CD34^{-}$KSL cells cultured with each supplement for 7 days.

(B) Changes in donor chimerism after 1st transplantation from 4 weeks to 12 weeks. Statistical significance was calculated using an unpaired two-tailed *t*-test, (*P = 0.03, ns: not significant)

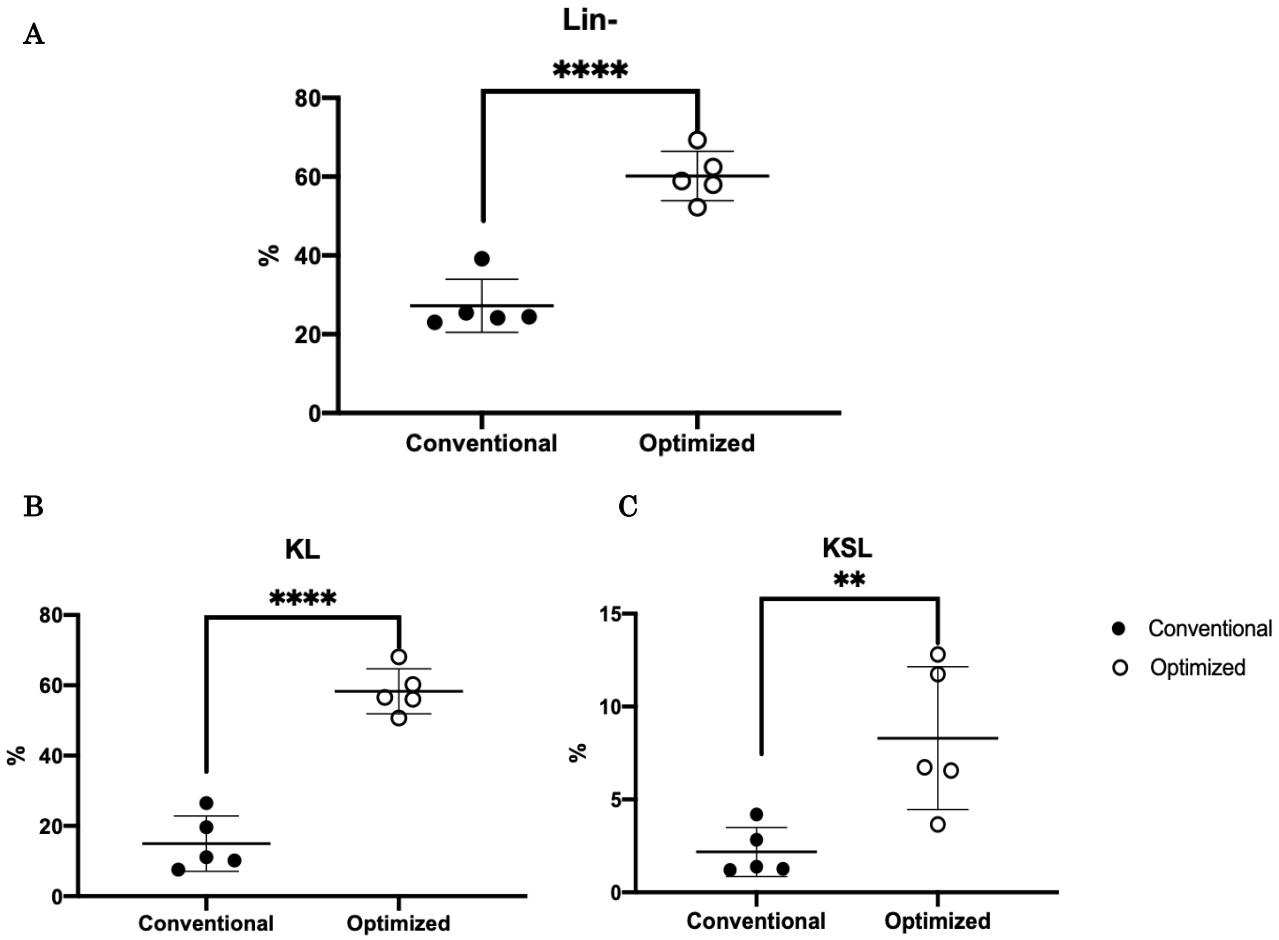


Figure 5 Comparison of conventional formulation and optimized formulation

40 $CD34^{-}$KSL cells were cultured with the conventional formulation or optimized formulation. The conventional formulation was supplemented with lot-checked BSA, the S-clone attached supplement, SCF, and TPO based in S-clone medium. Optimized formulation was supplemented with rHSA, ITS-X supplement, SCF, and TPO based in Ham's F-12 medium. Mean ± SD of independent triplicate cultures. Statistical significance was calculated using an unpaired two-tailed *t*-test.

(A) The percentage of Lin^{-} cells in each culture. (****P < 0.0001)

(B) The percentage of KL cells in each culture. (****P < 0.0001)

(C) The percentage of KSL cells in each culture. (**P = 0.0098)

Chapter 2: Generation of non-myeloablative bone marrow chimeric mice

Abstract

Bone marrow (BM) chimeric mice are an important tool in the field of immunology, and genetic engineering of donor cells is widely used to study gene function in physiological and pathological settings. However, up to date, BM chimeric mouse protocols require myeloablative conditioning of recipient mice, dramatically altering steady-state hematopoiesis. In addition, most protocols use fluorescence-activated cell sorting (FACS) of hematopoietic stem/progenitor cells (HSPCs) for *ex vivo* genetic manipulation. Here, I describe the development of the cell culture method to purify functional HSPCs from mouse BMs without using FACS. Furthermore, the large number of HSPCs derived from these cultures generated BM chimeric mice without irradiation. To apply for *ex vivo* gene manipulation, I constructed a doxycycline (Dox)-inducible lentiviral vector and examined each EGFP and two types of oncogenes. This vector also harbored puromycin-resistant genes so that the selection of gene-transduced cells was possible by adding puromycin. By transducing these vectors to culture-purified HSPCs, gene-transduced HSPCs were effectively selected without FACS equipment. When transplanted these cells into non-myeloablative mice, Dox induction of target genes in donor-derived immune cells was demonstrated. This is the first report of that completely FACS-free, non-myeloablative BM chimeric mouse model that enables target gene transduction. Therefore, it is anticipated that this method will get over the present mice transplanting model limitations.

Introduction

Genetically modified mice are frequently used to study the genetic determinants of mammalian immunity. Although we can generate transgenic mice for the phenotypic and functional analysis of immune cells in vivo, the process to generate and characterize these mice models takes a long time and requires highly specialized and expensive techniques[45–48]. This hinders the advancement of immunology and hematological research. BM chimeras are often used in immunological studies to reconstitute the immune system by transplanting donor hematopoietic stem/progenitor cells (HSPCs) into recipient mice, as a quick alternative to creating transgenic mice[49–51]. When used in combination with *ex vivo* genetic manipulation (such as lentiviral transduction), BM chimeras can be a useful tool for examining the determinants of hematopoietic and immune system development and function[52].

While simple and inexpensive, BM chimera assays currently have several disadvantages. These include the requirement to treat the recipient animals by utilizing radiation or chemotherapy to facilitate donor cell engraftment within the BM[53–56]. Such conditioning damages endogenous hematopoietic tissues such as the BM microenvironment and can irreversibly alter steady-state hematopoiesis[57]. In addition, most protocols use complex multicolor "fluorescence-activated cell sorting (FACS) to purify donor HSPCs for transduction and transplantation, requiring expensive equipment and technical expertise. Recently it was reported that a polyvinyl alcohol (PVA)-based media could expand FACS-purified $CD150^{+}CD34^{-}c\text{-}Kit^{+}Sca1^{+}Lineage^{-}$ hematopoietic stem cells (HSCs) for over a month[41,42]. Undifferentiated HSCs were stimulated to proliferate in these cultures containing a combination of recombinant stem cell factor (SCF) and thrombopoietin (TPO) cytokines, whereas differentiated cell

types were poorly supported.

Here, I demonstrate the application of this media to purify HSPCs derived from BM without using FACS. Also, these enriched HSPCs showed robust engraftment even in non-conditioned immunocompetent recipients. For *ex vivo* gene manipulation, I investigated EGFP which is a fluorescence protein, and two types of oncogenes: AE9a and BCR-ABL. AE9a is an AML1/ETO isoform that results from the t (8;21)(q22;q22) translocation, which is known to cause Acute Myeloid Leukemia (AML)[58]. BCR-ABL results from the t (9;22) chromosomal translocation and is known to cause Chronic Myeloid Leukemia (CML)[59,60]. These genes both were previously reported to develop the myeloproliferative disease in mouse model[58,61]. When EGFP, AE9a, and BCR-ABL were each cloned to Tet-On 3G inducible lentiviral vectors and generated non-conditioned BM chimeric mice, control of gene expression by administration of Doxycycline (Dox) become possible. As this established BM chimeric mouse model will consequently offer an effective and valuable experimental strategy for the fields of immunology and hematology.

Materials and Methods

Mice

Same as described in Chapter 1.

PVA-based serum-free culture

C57BL/6-CD45.1 mouse BM cells, unfractionated or fractionated following magnetic c-Kit$^+$ cell enrichment, were each cultured utilizing Ham's F-12 medium (Wako), supplemented with 0.1% PVA (Sigma, Cat# P8136), 1% Insulin-Transferrin-Selenium-Ethanolamine (ITS-X) (100X) (Thermo Fisher Scientific), 1% Penicillin-Streptomycin-L-Glutamine (PSG) Solution (100X) (Wako), N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) (10 mM; Gibco), mouse TPO (100 ng/ml; PeproTech), and mouse SCF (10 ng/ml; PeproTech) for 28 days, incubated at 37 °C in a humidified 5% CO_2 incubator. The medium was changed every other day[41,42]. Anti-mouse c-Kit MicroBeads (Miltenyi Biotech, Cat# 130-091-224) were used for magnetic cell separation according to the manufacturer's instructions. Unpurified whole BM cells were seeded onto a 100 mm dish in a 10 mL culture medium (Day 0–14) or a 60 mm dish in 4 mL culture media at a density of 2×10^6/mL (Day 15–28). Magnetic column-enriched c-Kit$^+$ BM cells were seeded onto 48-well plates in 1 mL culture media at 1×10^6. For long-term cultures, complete medium changes were made every 2 days and cell cultures were passaged at a ratio of 1:2-3 when cells exhibited 80–90% confluency.

Cell counting and sample preparation for flow cytometry

An automatic cell counter was used for cell counting (Countess II Automated Cell Counter, Invitrogen). PB samples were collected from the retro-orbital venous

plexus into capillary tubes filled with powdered EDTA. PB samples were stained with PE- labeled anti-Gr-1, PE-labeled anti-Mac-1, APC-labeled anti-CD4, APC-labeled anti-CD8a, APC/Cy7-labeled anti- B220, PE/Cy7-labeled anti-CD45.1, and eFluor450- labeled anti-CD45.2 antibodies. BM samples were stained with PE/Cy7-labeled anti-CD45.1, eFluor450-labeled anti-CD45.2, PE- labeled anti-Sca-1, APC- labeled anti-c-Kit, APC/eFluor780- labeled anti-CD4, APC/eFluor780- labeled anti- CD8, APC/eFluor780-labeled anti-Mac-1, APC/eFluor780-labeled anti-Gr-1, APC/eFluor780-labeled anti-B220, APC/eFluor780-labeled anti-Ter119, APC/eFluor780-labeled anti-CD127/IL-7Rα, and FITC-labeled anti-CD34 or PE/Cy5-labeled anti-CD34antibodies. Cultured BM samples were stained with PE-labeled anti-CD150, APC-labeled anti-c-Kit, PE/Cy7-labeled anti-Sca-1, APC/eFluor780-labeled anti-CD4, APC/eFluor780-labeled anti- CD8, APC/eFluor780-labeled anti-Mac-1, APC/eFluor780-labeled anti-Gr- 1, APC/eFluor780-labeled anti-B220, APC/eFluor780-labeled anti-Ter119, and APC/eFluor780-labeled anti-CD127/IL-7R α antibodies. Details of the dilutions used for staining antibodies are described in Table 1. Data were acquired on a FACSAriaIII or FACSVerse (BD) and analyzed with FlowJo (v10.5.3) Software (FlowJo, LLC).

Competitive transplantation assays

1×10^6 fresh or cultured cells were transplanted via single intravenous injection into irradiated (9.5 Gy) male C57BL/6-CD45.2 recipient mice along with 1×10^6 male C57BL/6-CD45.1/ CD45.2 whole BM competitor cells. PB analysis was performed every 4 weeks for 16 weeks. Utilizing ELDA software, the limiting dilution assay was performed with a 1% PB multilineage chimerism as the cutoff for positive engraftment.

Non-conditioned transplantation assays

1×10^6 fresh or cultured cells were transplanted via a single intravenous injection into non-irradiated male C57BL/6-CD45.1/CD45.2 recipient mice. PB analysis was performed every 4 weeks for 16 weeks.

Secondary BM transplantation assays

Secondary BM transplantation assays were performed after 16 weeks by transferring 1×10^6 whole BM cells from the primary recipient mice into irradiated (9.5 Gy) male C57BL/6-CD45.2 mice by intravenous injection.

Vector construct and lentivirus production.

The DOX-inducible lentiviral vector was constructed from an all-in-one inducible lentiviral vector (Ai-LV)[62] provided by Dr. T. Yamaguchi (The University of Tokyo) and was cloned to carry a Tet-responsive promoter driving EGFP, AE9a or BCR-ABL. and a downstream rtTA gene and puromycin gene driven by a human elongation factor -1 alpha (EF-1a) promoter. The AE9a and BCR-ABL transgenes were constructed using the MigR1-AE9a and P210 pcDNA3 plasmids as templates. Using a polyethyleneimine (PEI) transfection protocol, lentiviral vectors were transfected into Lenti-X 293 T Cells to produce viral supernatant. For each 10 cm culture dish, the lentiviral transfer vector plasmid, packaging plasmid (psPAX2), and envelope plasmid (pMD2G) DNA (6:3:1.5 μg, respectively) were mixed in 500 μL of 10 mM HEPES/150 mM NaCl (pH 7.05). Then 42 ul of 1 μg/μL PEI MAX (Polysciences Inc) were added into the diluted DNA. DNA/PEI mixtures were incubated at room temperature for 10 min. Lenti-X 293 T Cells were trypsinized, washed twice with 1X phosphate buffered saline, and

resuspended (2×10^6 cells/ ml) in serum-free Opti-MEM (Invitrogen Life Technologies, Inc.). The DNA/PEI mixture was added to 7.5 ml suspended cells, immediately plated onto a 10 cm dish and incubated at 37 °C in a 5% CO_2 humidified atmosphere. At day 5, the supernatants were collected and concentrated by using a Lenti-X concentrator (Takara Bio Inc.) and stored at -80 °C. MigR1-AE9a was a gift from Dong-Er Zhang (Addgene plasmid #12433)[58]. P210 pcDNA3 was a gift from Warren Pear (Addgene plasmid #27481)[61]. psPAX2 and pMD2.G were a gift from Didier Trono (Addgene plasmid #12260 and #12259) (unpublished).

Viral transfection and puromycin selection

Mouse c-Kit$^+$ BM cells were transduced in the RetroNectin-bound virus (RBV) method according to the manufacturer's instructions (RetroNectin, Takara Bio Inc.). Briefly, before cell seeding, 48-well tissue culture plates were coated with 20 μg/cm^2 of RetroNectin Reagent. After 2 days in culture, cells were transduced by spinoculation (2 h, 2000 g, 32 °C) at a concentration of 5000–100,000 cells/mL at multiplicities of infection of 500, and then selected using puromycin for 48 hours at a final concentration of 1μg/ml on day four of culture. Where indicated, EGFP expression was induced with 1 μg/ml DOX (Clontech) for 48 hours. Induced cells were analyzed for EGFP expression by flow cytometry. Non-transduced cells and transduced cells without puromycin selection were used as a control.

Administration of DOX to mice

Non-conditioned transplant male C57BL/6- CD45.1/CD45.2 mice at 16 weeks post-transplantation were administered DOX via the drinking water (2 mg/mL DOX +

1% sucrose) ad libitum for 48 hours.

Apoptosis assays

Apoptosis assays were performed as previously reported[63]. Expanded whole BM or c-Kit$^+$ BM cells were stained (1×10^6 cells/sample) with 5 µL of Brilliant Violet 421 Annexin V (Biolegend) and 10 ul of 0.5 mg/ml of propidium iodide (Biolegend) at day 7 and day 14. Staining patterns were collected using a FACSAriaIII or FACSVerse (BD) and analyzed with FlowJo (v10.5.3) Software (FlowJo, LLC).

Gene expression analysis

RNA was isolated utilizing the NucleoSpin® RNA Plus XS (MACHERY-NAGEL) or NucleoSpin® RNA Plus (MACHERY-NAGEL). RNA was reverse-transcribed utilizing the PrimeScriptTM RT Master Mix (Perfect Real Time). For *p16^{Ink4a}*, *p19^{Arf}*, *Trp53*, *EGFP*, *AE9a,* and *BCR-ABL* quantitative PCR was carried out on a Quantstudio7 (Applied Biosystems) using THUNDERBIRD® SYBR qPCR Mix (Takara Bio Inc.) and the primer sets described in Table 2[60,58,41]. Gene expression was normalized relative to *Gapdh* expression.

Statistical analysis

Statistical significance was calculated using an unpaired Student's *t*-test (two-tailed), $*p < 0.05$, $**p < 0.01$. Results are shown as arithmetic means ± SD. GraphPad Prism 8 was used for statistical analysis.

Results

Culture-based enrichment of HSPCs from c-Kit$^+$ BM cells.

Based on the selective expansion of HSCs in our recently described PVA-based media[41,42], I hypothesized that I may be able to use *ex vivo* culture to enrich functional HSCs from more differentiated cell types. To test this hypothesis, I collected c-Kit$^+$ BM cells from C57BL/6-CD45.1 mice (via magnetic column enrichment) and cultured them for 28 days *ex vivo* in a PVA-based HSC medium (Fig.6A). In these experiments, cell cultures were initiated with 3.5 × 10^6 cells, where Lin$^-$ and c-Kit$^+$Sca1$^+$Lineage$^-$(KSL) HSPC fractions were at initial frequencies of ~71% and ~5.9%, respectively (meaning each culture was initiated with ~2.5 × 10^6 Lin$^-$ cells including ~2.1 × 10^5 KSL cells). A progressive increase in total cell number was observed throughout these 28-day cultures (Fig.6B) and flow cytometric analysis detected a high percentage of c-Kit$^+$ cells (88% at day 28) (Fig.6C, D). This represented a 5.8-fold increase in c-Kit$^+$ cell numbers over 28 days (Fig.6B, C). A more detailed analysis of phenotypic KSL and CD150$^+$KSL HSPC populations also identified progressive increases in the frequency of these populations during the culture (Fig.6C, D). These results supported my hypothesis that PVA-based HSC media could enrich for and support expansion of HSPCs *ex vivo*.

To evaluate the function of these culture-enriched HSPCs, I performed competitive BM transplantation using the congenic C57BL/6 system, where irradiated C57BL/6-CD45.2 recipient mice are reconstituted with C57BL/6-CD45.1 donor cells and C57BL/6-CD45.1/CD45.2 competitor BM cells, allowing for donor reconstitution to be tracked and quantified using allele- specific CD45 antibodies (Fig 14B). In this study, 1 × 10^6 expanded cultured cells at day 28 (approximately ~1/25 of the culture, equivalent to ~1 ×10^5 Lin$^-$ cells containing ~8.4 ×10^3 KSL cells at day 0) or 1 ×10^6 fresh BM cells

derived from C57BL/6-CD45.1 BM were transplanted against 1×10^6 C57BL/6-CD45.1/CD45.2 BM competitors. Expanded cells displayed stable high-level and multilineage peripheral blood (PB) reconstitution in primary recipients, similar to fresh BM cells (average of 73% vs 61% donor chimerism at 16-weeks, respectively) (Fig.7A). In addition, there were no significant differences in multilineage differentiation within PB myeloid cells (Mac-1^+/Gr-1^+), T cells (CD4^+/CD$8a^+$), and B cells (B220^+) as compared with the fresh BM cells (Fig.7B). Endpoint BM analysis of these primary recipients also revealed no significant differences in donor chimerism within the BM Lin$^-$, KSL, or CD34^-KSL population at 16 weeks post-transplant between 28-day expanded and fresh donor cells (Fig.7C, D).

To further confirm long-term (LT)-HSC function within the expanded cells, I performed secondary transplantation assays. The expanded cells from c-Kit$^+$ BM displayed higher engraftment in secondary recipients than the fresh controls throughout the 16-week assay (average 77% vs 55% donor chimerism at 16 weeks, respectively) (Fig.8A). Finally, BM analysis of these secondary recipients after 16-weeks confirmed robust contributions of the expanded cells to the phenotypic KSL HSPC and CD34^-KSL HSC populations (Fig.8B). Together, these results suggested that I could selectively expand functional HSCs *ex vivo* using PVA-based media.

To further assess the expansion of engraftable HSCs in these c- Kit$^+$ BM cultures, I performed limiting dilution transplantation assays comparing fresh c-Kit$^+$ cells and expanded c-Kit$^+$ cells. Doses of 1×10^3, 1×10^4, and 1×10^5 cells were transplanted into lethally irradiated C57BL/6-CD45.2 recipient mice against 1×10^6 competitor BM cells. At 12 weeks post-transplantation, the average chimerism of the cultured cells was 1.3, 28, and 83%, respectively, while the chimerism of fresh cells

averaged 0.6, 8.7, and 67%, respectively (Fig.9). Using extreme limiting dilution analysis[64] (with 1% donor chimerism threshold cutoff), I estimated the engraftable HSC frequency in the expanded cells as 1:2164 cells whereas fresh c-Kit$^+$cells had an HSC frequency of 1:3971. These data suggest an ~8-fold expansion in HSPC potency can be achieved via this approach. I, therefore, conclude that the expansion culture from c-Kit $^+$ BM cells is a practical approach for efficiently generating functional HSPCs without either expensive equipment or time-intensive methods such as generating transgenic mice.

Given the ability of PVA-based media to enrich for HSCs in culture, I also evaluated whole (unfractionated) BM in our *ex vivo* culture system, with cultures initiated with 2 $\times 10^7$ BM cells (Fig.10A). Within the starting whole BM, the Lin$^-$ and KSL fractions were at initial frequencies of ~2.3% and ~0.1%, respectively (meaning each cell culture was initiated with approximately 4.7 $\times$ 10^5 Lin$^-$ cells including 2.4 $\times 10^4$ KSL cells). Although c-Kit$^+$ cells were enriched over the 28-day cultures, the purity of phenotypic KSL and CD150$^+$KSL remained lower than in the c-Kit$^+$ BM-derived cultures (Fig.10B–D). Primary and secondary transplantation assays were also performed. In these experiments, cell cultures contained ~1 $\times 10^7$ cells by day 28, and primary recipients received 1 $\times$ 10^6 of these cultured cells (approximately ~1/10 of the culture, equivalent to ~4.7 $\times$ 10^4 Lin$^-$ cells with ~2.4 $\times 10^3$ KSL cells at day 0). The functional assays confirmed that hematopoietic progenitors and short-term HSCs expanded in whole BM cell cultures (Fig.11A, B), but low BM chimerism in primary recipients and reduced engraftment in secondary recipients suggested that LT-HSCs were poorly supported (Fig.11C, Fig.12A, B).

Given that total live cell numbers rapidly dropped over 80% in the first 7 days,

to ~3.8×10^6 cells (Fig.10B), I hypothesized that toxicities from the cell death may affect HSC activity in whole BM cultures. To evaluate this further, my colleagues and I compared whole BM and c-Kit$^+$ BM cell cultures by performing Annexin V apoptosis assays and evaluating the expression of senescence-related genes (*p16^{Ink4a}*, *p19^{Arf}*, and *Trp53*). The frequency of apoptotic cells was significantly higher in whole BM cell cultures (Fig.13A), and expression of *p16^{Ink4a}* and *p19^{Arf}* was also higher within both bulk culture cells as well as phenotypic KSL cells in the whole BM cultures (Fig.13B). Additionally, expression of *Trp53* was higher in the whole BM-derived bulk cell cultures (Fig.13B). Together, these results suggested that cellular stress may be the negative regulator of HSC activity *ex vivo*.

Cultured HSPCs efficiently engraft non-conditioned mice.

Generation of mice chimeric BM without conditioning represents an important approach to study immune system development and function without the toxicities associated with irradiation or chemotherapy. Unfortunately, the routine use of the approach has been essentially unfeasible due to the large numbers of HSPCs required for engraftment; donor HSPCs engraft very inefficiently without recipient conditioning[65]. In our previous study, the HSPCs expanded from FACS-purified CD150$^+$CD34$^-$KSL cells were able to engraft in immunocompetent autologous mice without irradiation or other conditioning[41].

My colleague and I, therefore, sought to evaluate the capacity of my culture-enriched HSPCs from c-Kit$^+$ BM cells to engraft and reconstitute non-conditioned recipients by transplanting 1×10^6 culture- enriched cells (from C57BL/6-CD45.1 c-Kit$^+$ BM) into C57BL/ 6-CD45.1/CD45.2 recipient mice (Fig.14A). Donor engraftment and

lineage contribution was tracked by PB chimerism and compared with endogenous CD45.1/CD45.2 lineage contribution (Fig.14B). Robust PB chimerism from culture-enriched HSPCs was observed in non-conditioned recipients, averaging 30–35% between 4 and 16-weeks post- transplantation (Fig.15A). Consistent with immune lineage differentiation kinetics, 4-week engraftment was dominated by B cells, while T cells took longer to be generated (Fig.15B). Additionally, when compared to endogenous CD45.1/CD45.2 hematopoiesis, no significant differences were observed within the BM Lin^-, KSL, or $CD34^-KSL$ populations at 16 weeks post- transplantation (Fig.15C). These results suggested that culture-enriched HSPCs could robustly engraft in the BM of non-conditioned recipients and contribute to immune system formation.

To further validate the function of these engrafted HSPCs in primary recipients, secondary transplantation analysis was performed on C57BL/6-CD45.2 recipients. Stable donor chimerism was observed for the entire 16-week analysis, with mean PB chimerism at 30–31% (Fig.17A). Additionally, consistent donor chimerism was also observed within the BM Lin^-, KSL, and $CD34^-KSL$ cell fractions (Fig.17B). These results confirmed that non-conditioned transplantation of expanded cells resulted in the engraftment of functional LT-HSCs.

Finally, to determine the potential of *ex vivo* expanded c-Kit^+ BM, my colleagues and I performed limiting dilution transplantation assays, comparing fresh c-Kit^+ BM cells and 28-day cultured cells in non-conditioned recipients. Doses of 1×10^4, 1×10^5, and 1×10^6 cells were transplanted into C57BL/6-CD45.1/CD45.2 recipients. After 12 weeks of transplantation, the average chimerism of fresh c-Kit^+ cells was 0.1, 0.2, and 0.8%, respectively, while the average chimerism of cultured cells was 0.1, 2.6, and 20%, respectively (Fig.16A). These results confirmed that culture-enriched HSPCs have high

engraftment and reconstitution potential, and at least 10-fold higher engraftment potential than fresh c-Kit$^+$ cells. It is worth noting the remarkable advantages of these non-conditioned BM chimeric mice; as this procedure does not involve radiation or chemotherapy, the immune system and function can be studied under steady-state conditions, and the lack of undesirable morbidity/mortality from cytotoxic chemotherapy and irradiation-based conditioning seemed that recipients have the possibility to survive long-term (more than a year) post-transplantation (Fig.16B).

Genetically engineered immune cells from cultured HSPCs.

As demonstrated above, these 28-day *ex vivo* cultures enriched and expanded HSPCs with long-term reconstitution activity and immune cell repopulation potential *in vivo*. My colleague and I, therefore, evaluated the possibility of genetic manipulation and *ex vivo* selection using lentiviral vectors in this culture platform. As a proof-of-concept, my colleague and I developed a puromycin-selectable doxycycline (DOX) inducible vector system for inducible transgene expression (Fig.18A) and evaluated expression of enhanced green fluorescent protein (EGFP). As a functional gene, oncogenes such as AML1/ETO9a (AE9a)[58], and BCR-ABL[60,59] were also evaluated. Lentiviral transduction was first evaluated *ex vivo* by EGFP expression and drug selection efficiencies (Fig.18B). In these experiments, c-Kit$^+$ BM cells were seeded in PVA-based HSC medium, transduced with lentivirus particles on day 2, and then treated with puromycin for 48h at day 4. After puromycin selection, cultured supernatants were replaced with fresh medium without puromycin. The 28- day cultures maintained a high percentage of c-Kit positive cells (average 92%) (Fig. 20A) and displayed a 4.8-fold increase in total CD150$^+$ KSL cell numbers (Fig. 20B). After a total of 28 days of culture, the cells were split into

media with or without DOX for 48 h, and then analyzed by flow cytometry and RT-PCR. In terms of the frequencies of phenotypic Lin^-, c-$Kit^+Lineage^-$ (KL), and KSL cell fractions, transduced cells showed no difference from non-transduced (mock) cells (Fig.19A). Quantitative PCR confirmed higher EGFP expression in the presence of DOX (Fig.19B), which was also confirmed by flow cytometry (Fig.20C, D). As compared to cells without puromycin treatment, puromycin selection significantly increased the frequency of $EGFP^+$ cells (average of ~23% vs 98% within the KSL cell population) (Fig.20C, D).

Having validated the stable transduction of culture-enriched HSPCs, my colleague and I next evaluated the reconstitution and EGFP expression *in vivo* (Fig.18B). I injected 1×10^6 cells into each non-conditioned immunocompetent and tracked donor chimerism over 16 weeks; stable multilineage PB chimerism at 22–26% was observed during this time (Fig.21A). To evaluate inducible transgene expression in vivo, recipient mice were administrated with DOX via the drinking water ad libitum at 16 weeks post-transplantation[66,67]. After DOX administration, >90% of $CD45.1^+$ donor-derived cells displayed EGFP expression, and large increases in EGFP gene expression were observed (Fig.21B-D). By contrast, minimal leaky EGFP expression (1–2%) was observed before DOX administration (Fig.21B-D).

These experiments were also repeated with AE9a and BCR- ABL transgenes. Inducible expression of AE9a and BCR-ABL was confirmed in *ex vivo* cell cultures and $CD45.1^+$ PB cells following non-conditioned transplantation (Fig.19A, B, Fif.23A-C). Finally, my colleagues and I evaluated the consequences of lentiviral transduction on the engraftment potential of culture-enriched HSPCs using the EGFP-transduced HSPC recipient cohort. By flow cytometry, no significant differences were observed in the frequency of phenotypic Lin^-, KSL, and $CD34^-$KSL BM cell populations (Fig.21E).

Additionally, stable long-term PB and BM chimerism was observed in secondary transplantation assays (Fig.22A, B). Together, these results confirm that these HSPC-enrichment cultures can be used to efficiently produce genetically engineered LT-HSCs and immune cells in vivo.

Discussion

While hematopoietic and immune system reconstituting HSCs remain the most well-characterized somatic stem cell population, the isolation of HSCs usually involves complex and expensive methodologies[67,68,69]. Here, I have demonstrated that functional HSCs can be enriched by simply culturing magnetic column-enriched c-Kit$^+$ BM cells in PVA-based HSC medium[41]. This culture-based enrichment retains functional LT-HSCs for at least 28 days *ex vivo*. As demonstrated here, these cell culture-enriched HSPCs are also amenable to viral transduction and further selection prior to transplantation. This approach, therefore, has broad and important applications in the study of immune system function. Furthermore, the large numbers of HSPCs that can be generated by this approach allow for the engraftment of genetically modified HSPCs in non-conditioned immunocompetent mice, affording studies to be undertaken without tissue toxicities associated with chemotherapy or irradiation conditioning.

In conclusion, I have developed a simple and useful approach for generating irradiation-free BM chimeric mice for the study of steady-state hematopoiesis and immune system development. The approach improves experimental animal welfare and does not require any specialized equipment (e.g., multicolor FACS). I, therefore, expect that this platform has numerous applications for the investigation of hematopoietic system development and immune response in health and disease.

Figures and Tables

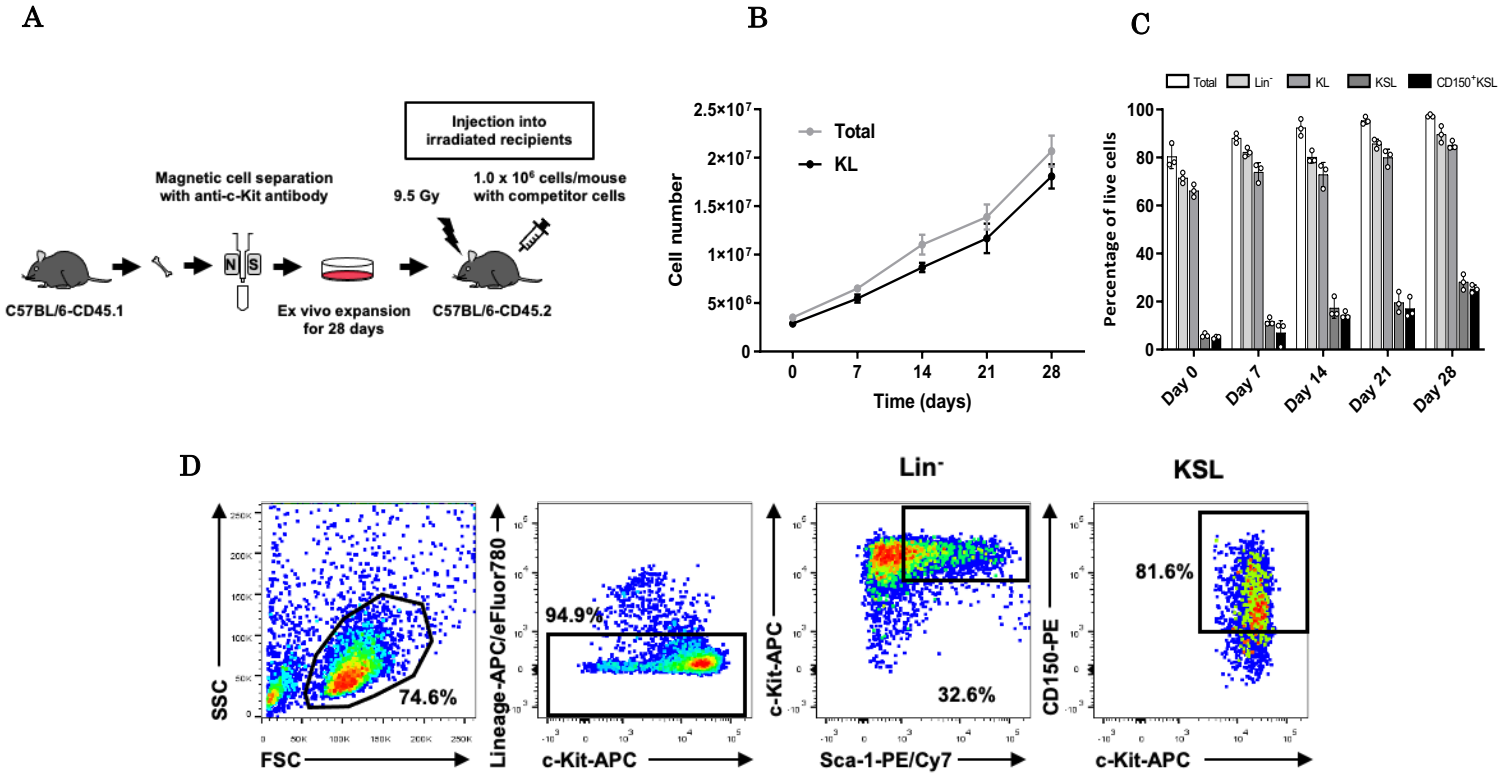


Figure 6 Purification of HSCs and HSPCs from c-Kit$^+$ BM cells

3.5×10^6 c-Kit$^+$ BM cells were plated in PVA-based HSC media and cultured for 28 days. Cell cultures were analyzed by cell counting and flow cytometry every 7 days.

(A) Schematic image of c-Kit$^+$ BM culture cell isolation, culture, and competitive repopulation assay.

(B) Cell number of total and KL cells during *ex vivo* culture. Mean ± SD of independent triplicate cultures.

(C) Percentage of Lin$^-$, KL, KSL, and CD150$^+$KSL phenotypic cell populations during the *ex vivo* culture. Mean ± SD of independent triplicate cultures.

(D) Representative gating strategy for the identification of CD150$^+$KSL cells in cultures derived from c-Kit$^+$ BM cells.

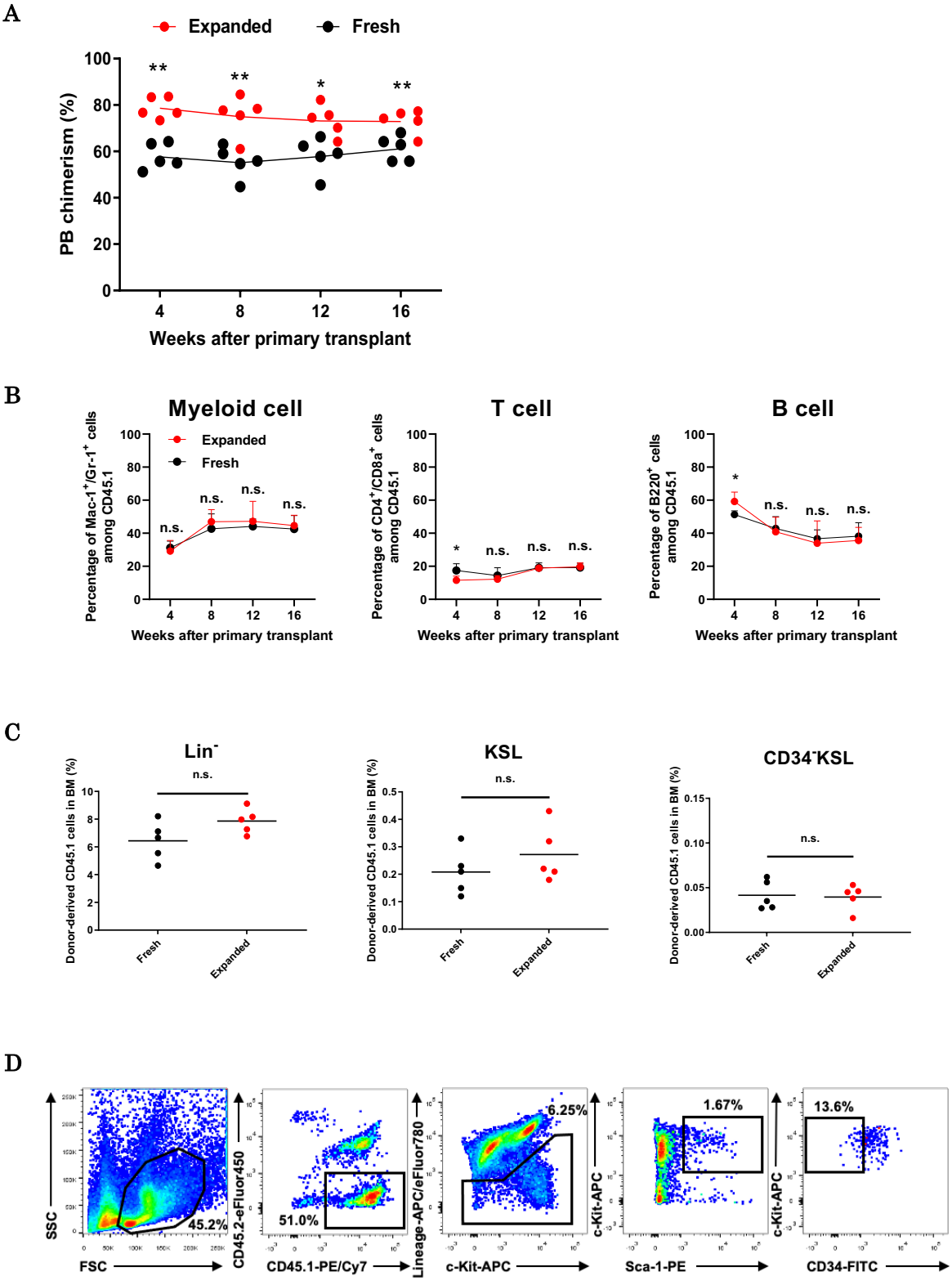

A
Expanded
Fresh
PB chimerism (%)
Weeks after primary transplant
B
Myeloid cell
T cell
B cell
Percentage of Mac-1+/Gr-1+ cells among CD45.1
Percentage of CD4+/CD8a+ cells among CD45.1
Percentage of B220+ cells among CD45.1
n.s.
C
Lin-
KSL
CD34-KSL
Donor-derived CD45.1 cells in BM (%)
Fresh
Expanded
D
SSC
FSC
CD45.2-eFluor450
CD45.1-PE/Cy7
45.2%
51.0%
Lineage-APC/eFluor780
c-Kit-APC
6.25%
Sca-1-PE
1.67%
CD34-FITC
13.6%

Figure 7 Competitive repopulation assay of c-Kit$^+$ BM cells (1st transplantation)

After 28 days of culture, 1 × 10^6 c-Kit$^+$ BM expanded cells were transplanted into irradiated C57BL/6-CD45.2 (Ly5.2) recipients (n = 5 mice per group) with 1 × 10^6 C57BL/6-CD45.1/CD45.2 (F1) whole BM competitor cells. Donor PB chimerism was observed every 4 weeks after transplantation. At 16 weeks after 1st transplantation, BM chimerism was analyzed.

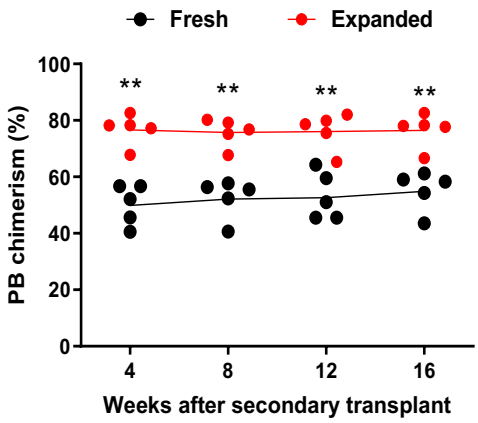
(A) Changes in donor chimerism after 1st transplantation from 4 weeks to 16 weeks. Statistical significance was calculated using an unpaired two-tailed *t*-test, **p = 0.0002 at week 4, **p = 0.0038 at week 8, *p = 0.0111 at week 12, **p = 0.0082 at week 16.

(B) Changes in the percentage of Mac-1$^+$/Gr-1$^+$ (myeloid cells), CD4$^+$/CD8a$^+$ (T cells), and B220$^+$ (B cells) among donor cells in primary transplant recipients over time. Mean ± SD from 5 primary recipients. *p = 0.0262 at week 4 in T cell, *p = 0.0208 at week 4 in B cell.

(C) Percentage of Lin$^-$, KSL, and CD34$^-$KSL cells in BM cells among donor cells at 16 weeks after primary transplantation

(D) Representative gating strategy for the identification of CD34$^-$KSL cells among the CD45.1$^+$CD45.2$^-$ population in BM samples.

A



B

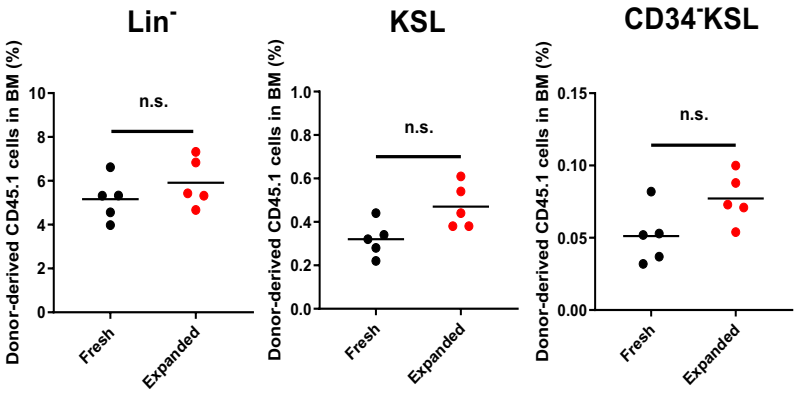


Figure 8 Competitive repopulation assay of c-Kit$^+$ BM cells (2nd transplantation)

At 16 weeks after 1st transplantation, 1 × 10^6 WBM cells from primary recipients were transplanted into irradiated Ly5.2 recipients.

(A) Changes in donor chimerism after 1st transplantation from 4 weeks to 12 weeks. Statistical significance was calculated using an unpaired two-tailed *t*-test, **p = 0.0002 at week 4, **p = 0.0003 at week 8, **p = 0.0013 at week 12, **p = 0.0008 at week 16.

(B) Percentage of Lin$^-$, KSL, and CD34$^-$KSL cells in BM cells among donor cells at 16 weeks after primary transplantation.

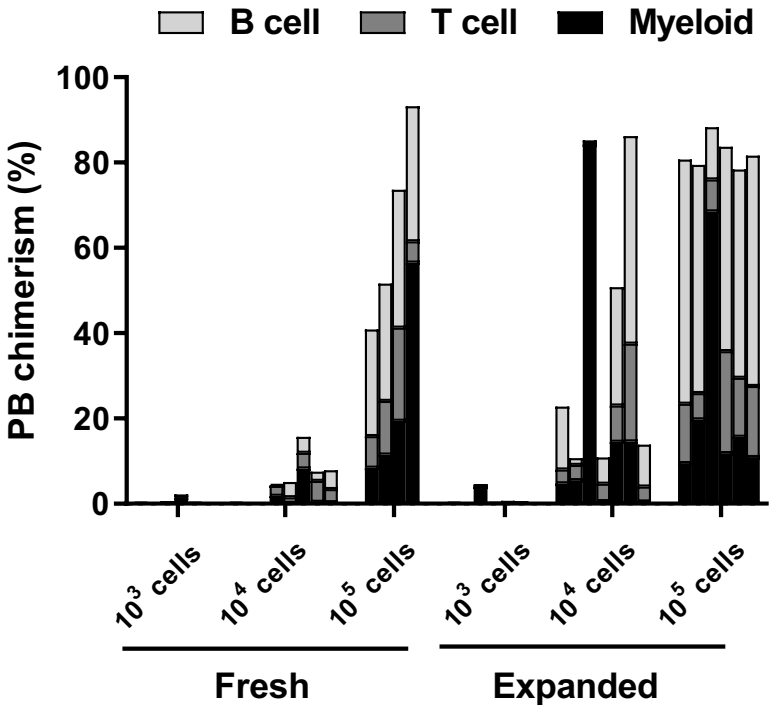


Figure 9 Limiting dilution assay

Limiting dilution assay in conditioned recipients. 1×10^3, 1×10^4, 1×10^5 expanded c-Kit$^+$ BM cells at day 28 of culture were transplanted into lethally irradiated Ly5.2 recipient mice with 1×10^6 F1 WBM competitor cells. 1×10^3, 1×10^4, 1×10^5 fresh c-Kit$^+$ cells are transplanted as a control. Donor PB chimerism at week 12 in primary recipient mice (n = 8 mice per group) with percentages of Mac-1$^+$/Gr-1$^+$ (myeloid cells), CD4$^+$/CD8a$^+$ (T cells), and B220$^+$ (B cells) displayed. Statistical significance was calculated using an unpaired two-tailed *t*-test, *p < 0.05, **p < 0.01; n.s. not significant.

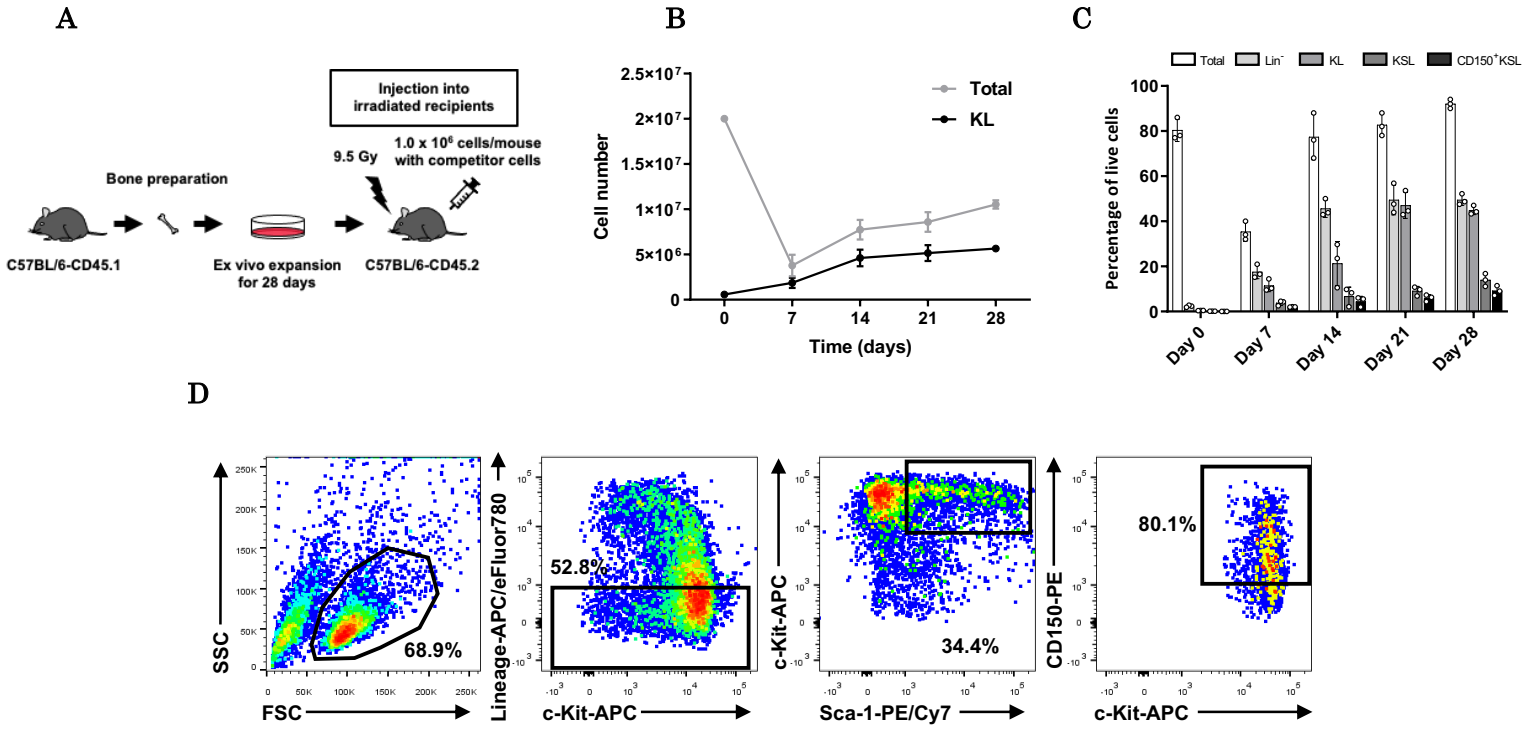


Figure 10 Purification of HSCs and HSPCs from whole BM cells.

2×10^7 whole BM cells were plated in PVA-based HSC media and cultured for 28 days. Cell cultures were analyzed by cell counting and flow cytometry every 7 days.

(A) Schematic image of c-Kit$^+$ BM culture cell isolation, culture, and competitive repopulation assay.

(B) Cell number of total and KL cells during *ex vivo* culture. Mean ± SD of independent triplicate cultures.

(C) Percentage of Lin$^-$, KL, KSL, and CD150$^+$KSL phenotypic cell populations during the *ex vivo* culture. Mean ± SD of independent triplicate cultures.

(D) Representative gating strategy for the identification of CD150$^+$KSL cells in cultures derived from c-Kit$^+$ BM cells.

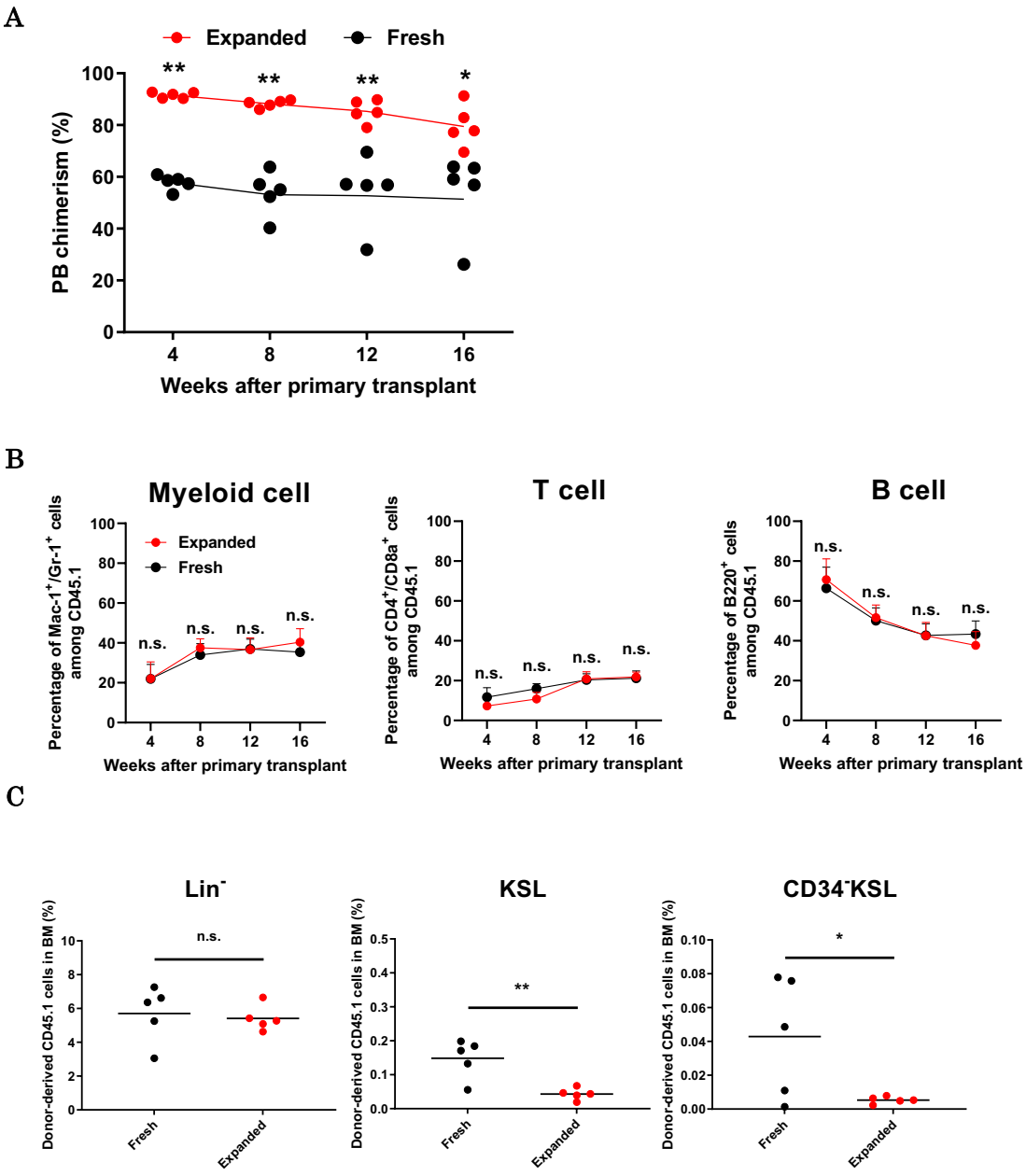


Figure 11 Competitive repopulation assay of whole BM cells (1st transplantation)

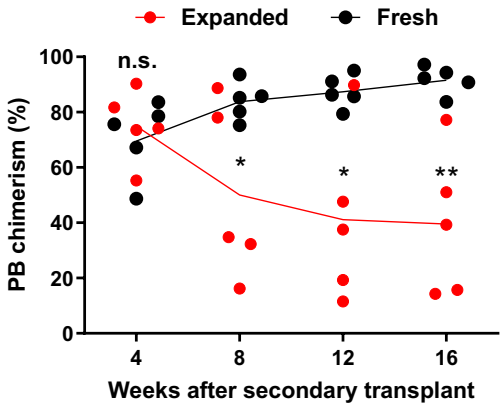
After 28 days of culture, 1 × 10^6 whole BM expanded cells were transplanted into irradiated C57BL/6-CD45.2 (Ly5.2) recipients (n = 5 mice per group) with 1 × 10^6 C57BL/6-CD45.1/CD45.2 (F1) whole BM competitor cells. Donor PB chimerism was observed every 4 weeks after transplantation. At 16 weeks after 1st transplantation, BM chimerism was analyzed.

(A) Changes in donor chimerism after 1st transplantation from 4 weeks to 16 weeks. Statistical significance was calculated using an unpaired two-tailed t-test, **p < 0.0001 at week 4, **p < 0.0001 at week 8, **p = 0.0013 at week 12, *p = 0.0114 at week 16.

(B) Changes in the percentage of Mac-1^+/Gr-1^+ (myeloid cells), $CD4^+$/$CD8a^+$ (T cells), and $B220^+$ (B cells) among donor cells in primary transplant recipients over time. Mean ± SD from 5 primary recipients.

(C) Percentage of Lin^-, KSL, and $CD34^-$KSL cells in BM cells among donor cells at 16 weeks after primary transplantation. Mean ± SD from 5 primary recipients.

A



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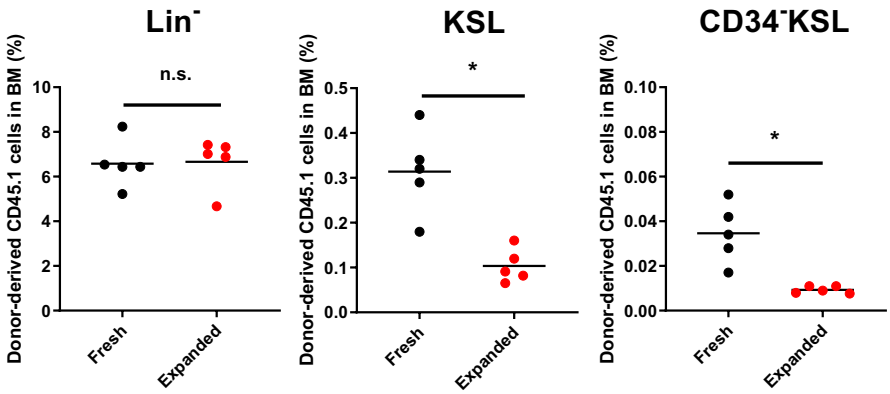


Figure 12 Competitive repopulation assay of whole BM cells (2nd transplantation)

At 16 weeks after 1st transplantation, 1 × 10^6 WBM cells from primary recipients were transplanted into irradiated Ly5.2 recipients.

(A) Changes in donor chimerism after 1st transplantation from 4 weeks to 16 weeks. Statistical significance was calculated using an unpaired two-tailed *t*-test, **p = 0.0002 at week 4, **p = 0.0003 at week 8, **p = 0.0013 at week 12, **p = 0.0008 at week 16.

(B) Percentage of Lin^-, KSL, and $CD34^-$KSL cells in BM cells among donor cells at 16 weeks after secondary transplantation. Mean ± SD from 5 primary recipients. **p = 0.0016 in KSL, **p = 0.0030 in $CD34^-$KSL. Statistical significance was calculated using an unpaired two-tailed *t*-test, *p < 0.05, **p < 0.01; n.s., not significant.

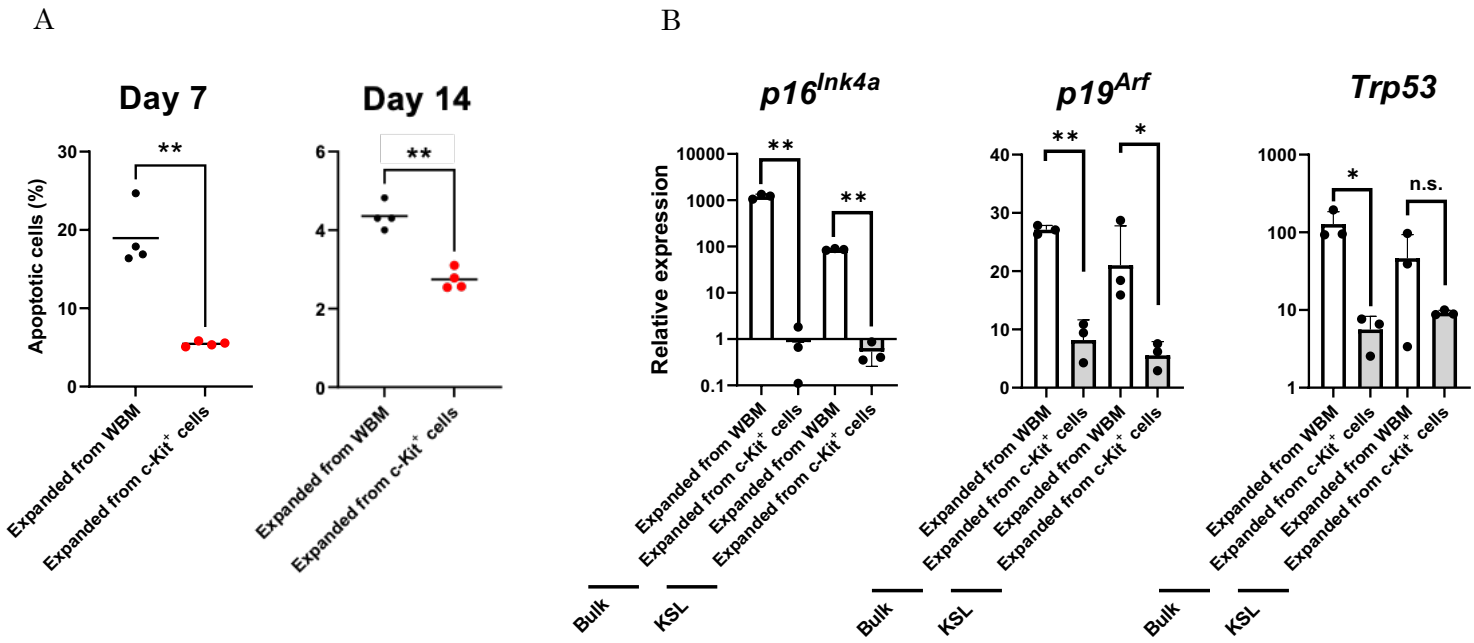
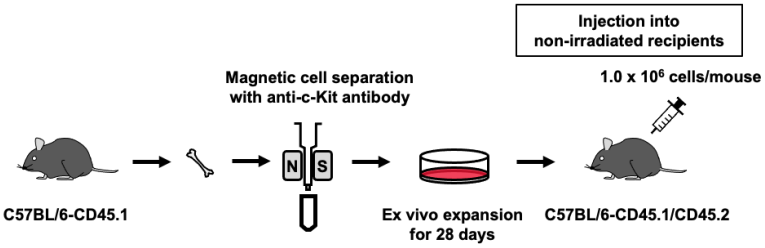


Figure 13 The difference between expanded whole BM cells and expanded c-Kit$^+$ cells.

(A) Percentages of Annexin V$^+$/Propidium iodide (PI)$^+$ from day 7 and day 14 cultured whole BM or c-Kit$^+$ BM cells. Mean ± SD of 4 independent cultures **p = 0.0004 at Day7, **p = 0.0003 at Day 14.

(B) Evaluation of gene *p16^{Ink4a}*, *p19^{Arf}*, and *Trp53* expression in 7-day cultured whole BM or c-Kit$^+$ BM cells (RNA isolated from bulk and phenotypic KSL cells). Mean of three independent cultures, with gene expression normalized to Gapdh expression. Error bars denote SD. *p16^{Ink4a}*: **p = 0.0001 (bulk cells expanded whole BM vs c-Kit$^+$ cells), **p < 0.0001 (KSL expanded from whole BM vs c-Kit$^+$ cells), *p19^{Arf}*:**p = 0.0008 (bulk cells expanded from whole BM vs c-Kit$^+$ cells), *p = 0.0203 (KSL expanded from whole BM vs c-Kit$^+$ cells), *Trp53*: *p = 0.0218 (bulk cell expanded from whole BM vs c-Kit$^+$ cells), p = 0.2424 (KSL expanded from whole BM vs c-Kit$^+$ cells). Statistical significance was calculated using an unpaired two-tailed *t*-test, *p < 0.05, **p < 0.01; n.s. not significant.

A



B

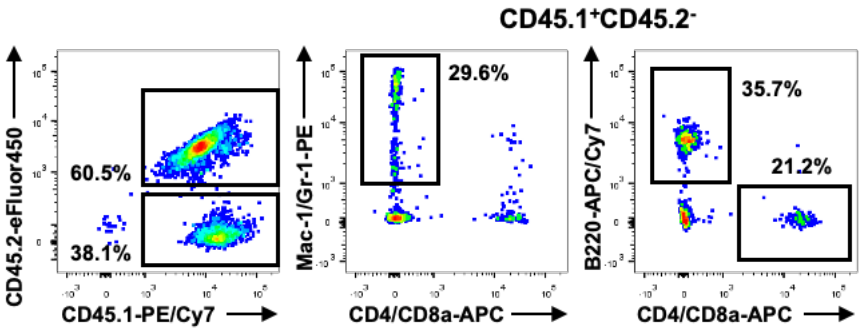


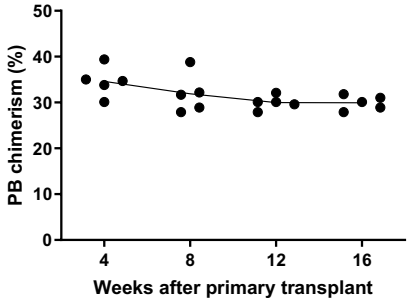
Figure 14　The images of non-conditioned transplantation

After 28 days of culture, 1×10^6 c-Kit$^+$ BM expanded cells were transplanted into non-conditioned C57BL/6-CD45.1/CD45.2 (F1) recipients (n = 5 mice per group). Donor PB chimerism was observed every 4 weeks after transplantation. At 16 weeks after 1st transplantation, BM chimerism was analyzed.

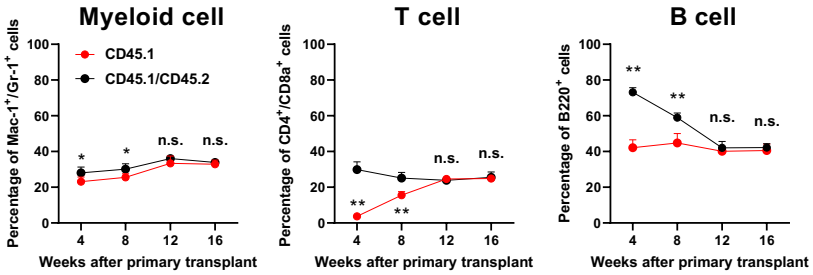
(A) Experimental schematic for the non-conditioned transplantation of culture-enriched HSPC cultures derived from c-Kit$^+$ BM cells.

(B) Gating strategy used to detect donor-derived CD45.1$^+$ PB chimerism in non-conditioned C57BL/6-CD45.1/CD45.2 recipients

A



B



C

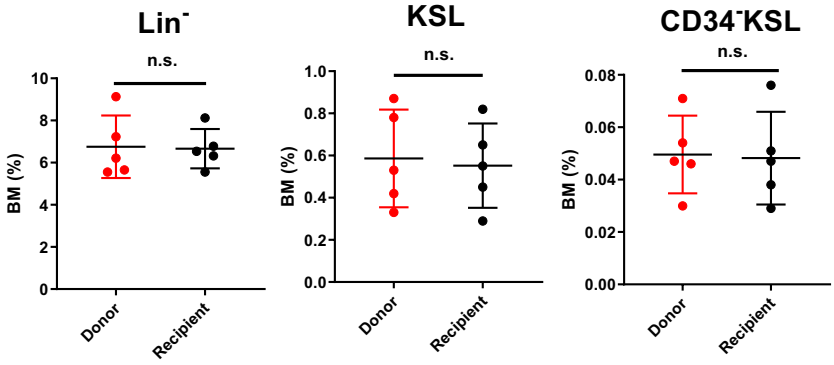


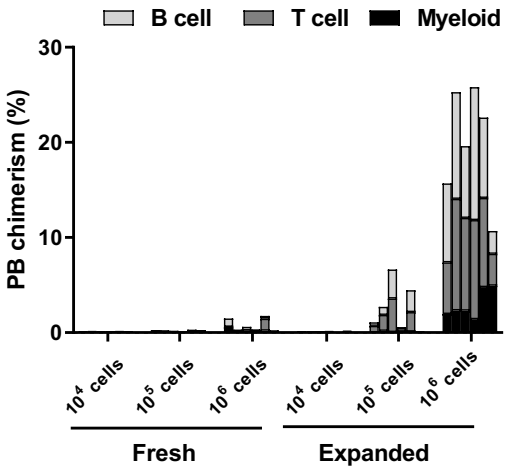
Figure 15 Non-conditioned transplantation (1st transplantation)

(A) Percentage of donor cells after primary transplant of 1×10^6 28-day cultured c-Kit$^+$ BM cells (n = 5 mice).

(B) Percentage of Mac-1$^+$/Gr-1$^+$ (myeloid cells), CD4$^+$/CD8a$^+$ (T cells), and B220$^+$ (B cells) among donor- and recipient-derived cells in primary transplant recipients.

(C) Percentage of Lin$^-$, KSL, and CD34$^-$KSL cells in BM cells among donor-derived or recipient-derived cells at 16 weeks in non-conditioned primary recipients. Mean±SD from 5 primary recipients (n = 5 mice per group). Statistical significance was calculated using an unpaired two-tailed *t*-test; n.s., not significant.

A



B

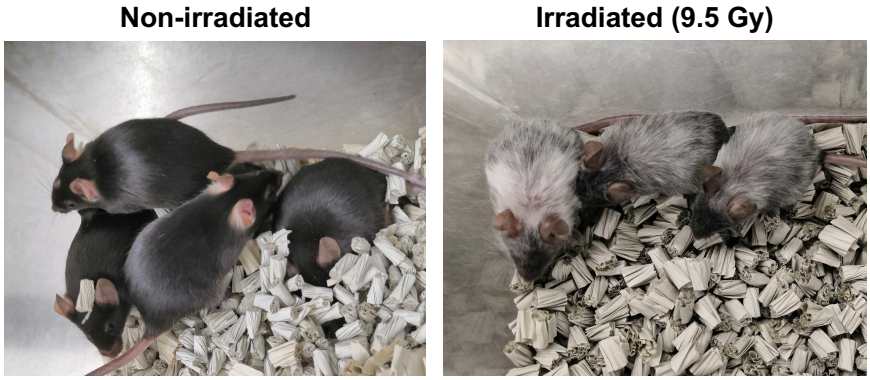
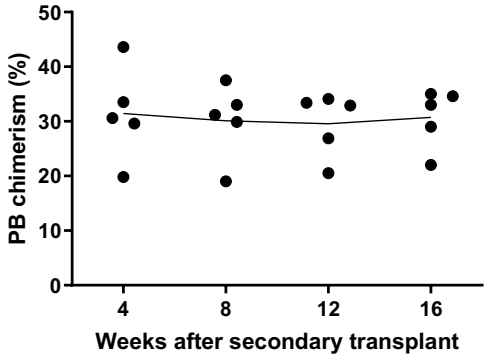


Figure 16 Limiting dilution assay of non-conditioned transplant

(A) Limiting dilution assay in non-conditioned recipients. 1×10^3, 1×10^4, 1×10^5 expanded c-Kit$^+$ BM cells at day 28 of culture were transplanted into recipient F1 mice. 1×10^3, 1×10^4, 1×10^5 Fresh c-Kit$^+$ cells are transplanted as a control. Donor PB chimerism at week 12 in primary recipient mice (n = 6 mice per group) with percentages of Mac-1$^+$/Gr-1$^+$ (myeloid cells), CD4$^+$/CD8a$^+$ (T cells), and B220$^+$ (B cells) displayed.

(B) Gross appearances of representative recipient mice at 16 weeks after primary transplant. Recipient mice transplanted without irradiation (left), and mice transplanted with doses of 9.5 Gy irradiation (right).

A



B

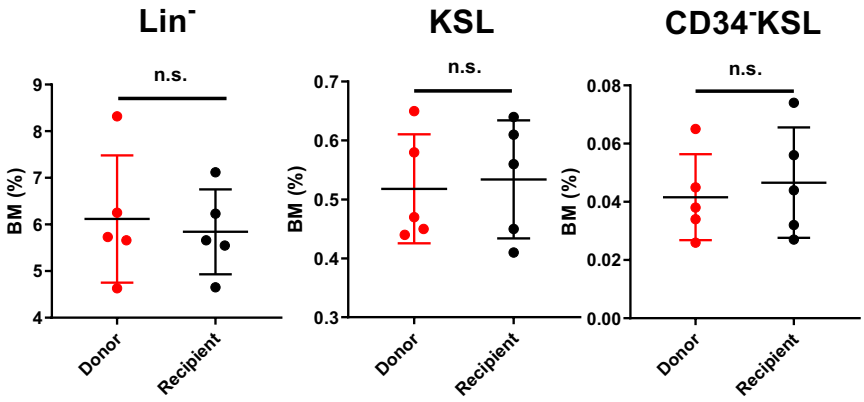


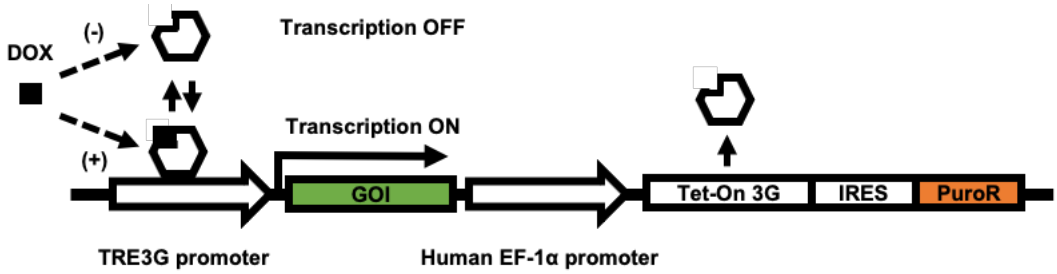
Figure 17 Non-conditioned transplantation (2nd transplantation)

At 16 weeks after 1st transplantation, 1 × 10^6 WBM cells from primary recipients were transplanted into irradiated Ly5.2 recipients.

(A) Changes in donor chimerism after 1st transplantation from 4 weeks to 16 weeks.

(B) Percentage of Lin^-, KSL, and $CD34^-$KSL cells in BM cells among donor cells at 16 weeks after secondary transplantation. Statistical significance was calculated using an unpaired two-tailed t-test; n.s., not significant.

A



B

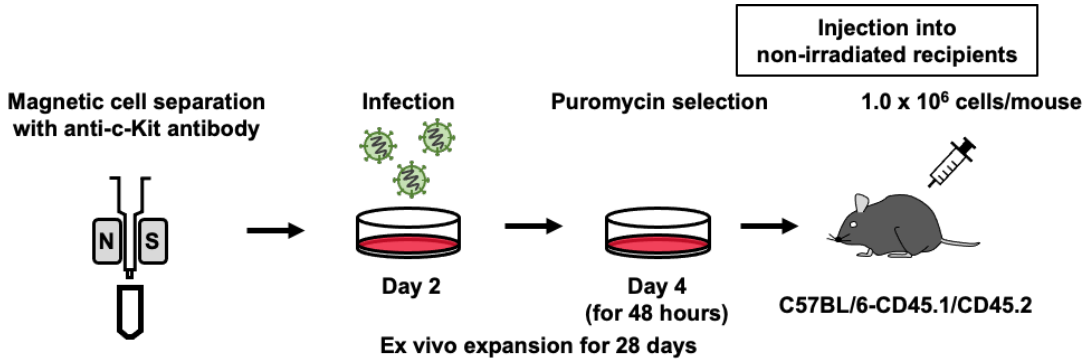


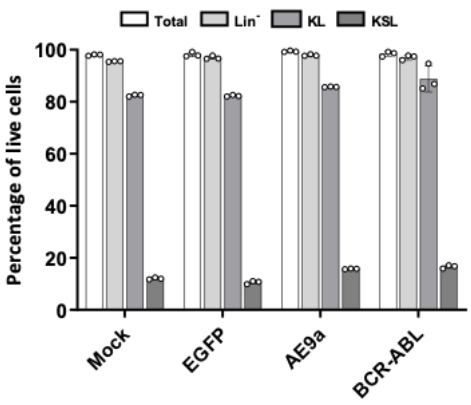
Figure 18 Images of transduction of culture-enriched HSPCs

Cell cultures were initiated with 3.5×10^6 c- Kit^+ BM cells. Lentiviral transduction was performed on day 2, puromycin treatment was performed on day 4 for 48 h, DOX treatment was performed on day 28 for 48 h, and flow cytometric analysis on day 30. 1×10^6 day-28 cells were injected into non-conditioned C57BL/6-CD45.1/CD45.2 recipient mice.

(A) Schematic of the DOX-inducible target gene expression vector including a puromycin selection marker. DOX: doxycycline, EF-1α promoter: elongation factor-1 alpha promoter, GOI: gene of interest, IRES: Internal ribosome entry sites, PuroR: puromycin-resistant gene.

(B) Experimental schematic for non-conditioned transplantation of culture-enriched HSPC cultures derived from c-Kit^+ BM cells following lentiviral transduction and puromycin selection.

A



B

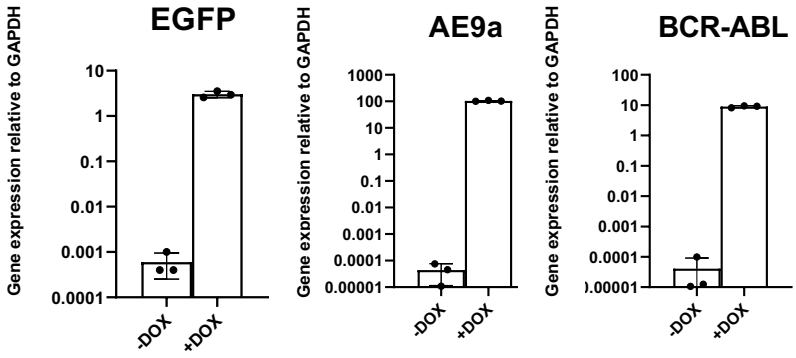


Figure19 Target gene transduction in cultured HSCs and HSPCs

EGFP, AE9a, and BCR-ABL were each transduced with a lentiviral vector and validated their expression and phenotype.

(A) Percentage of phenotypic Lin$^-$, KL, and KSL cell populations at culture day 28. Mean ± SD of independent triplicate cultures.

(B) Quantitative-PCR for expression of EGFP, AE9a, and BCR-ABL in cells from DOX on or off HSPC cultures. Mean ± SD of independent triplicate cultures.

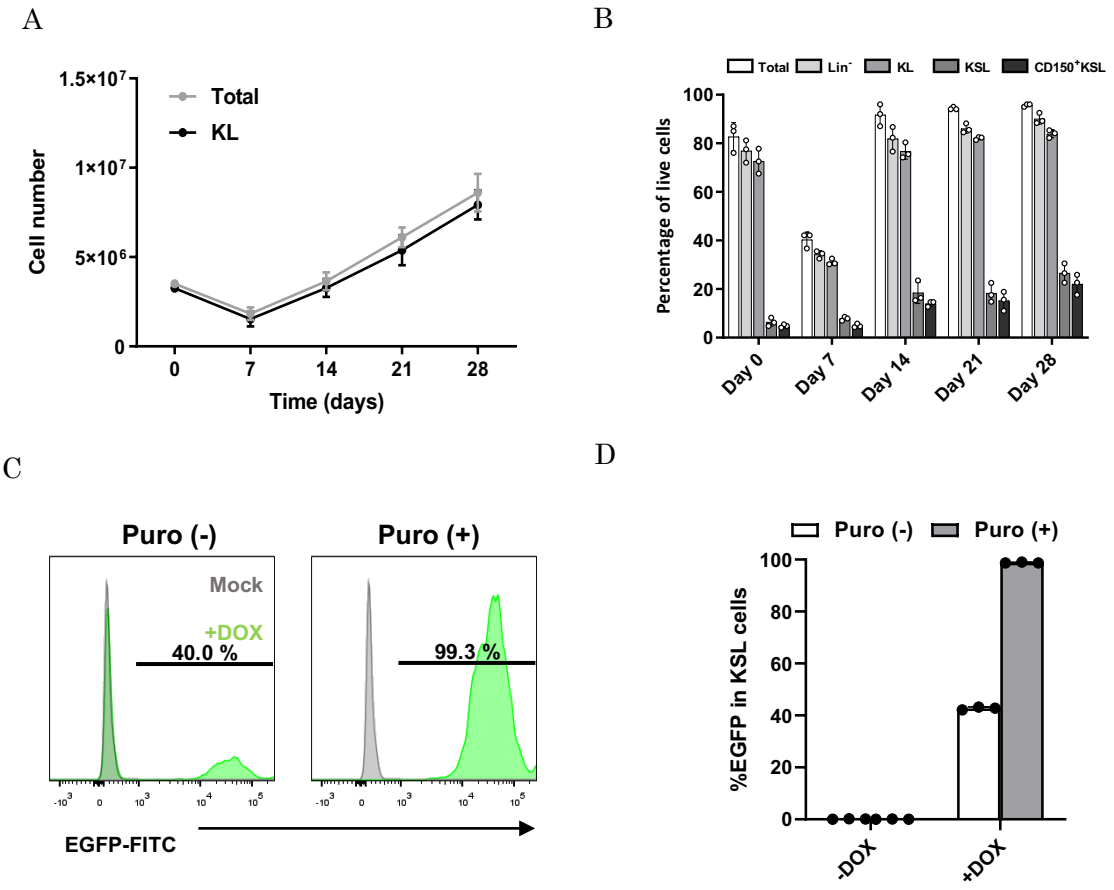


Figure20 Cell proliferation and expression of EGFP-transduced culture-enriched HSPCs

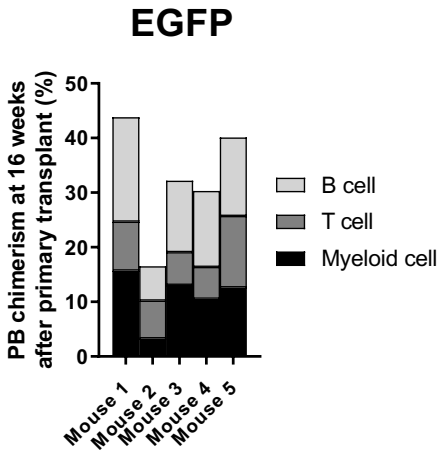
(A) Total and KL cell numbers during the cell culture described in B. Mean ± SD of triplicate cultures.

(B) Mean percentage of the total, Lin^-, KL, KSL, and $CD150^+KSL$ cells. Mean ± SD of triplicate cultures.

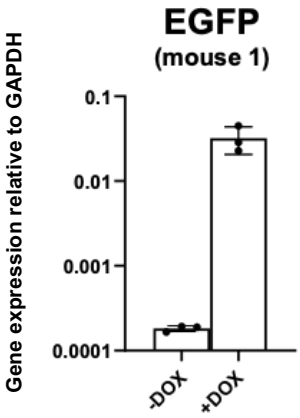
(C) Representative flow cytometric histograms for EGFP expression in HSPC cultures with or without puromycin selection.

(D) Percentages of $EGFP^+$ cells in KSL cells with or without puromycin selection. Mean ± SD of independent triplicate cultures.

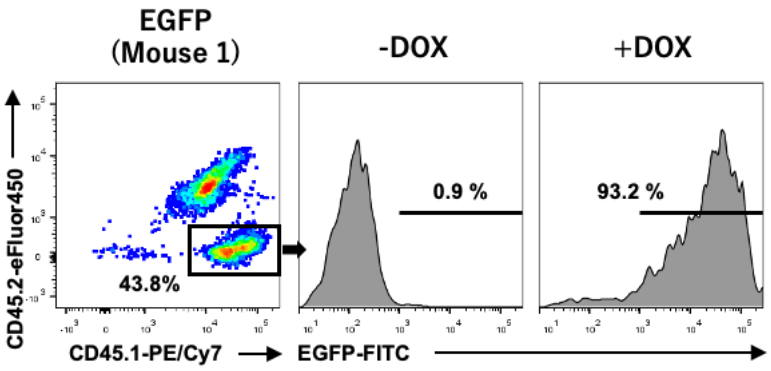
A


EGFP
PB chimerism at 16 weeks after primary transplant (%)
50
40
30
20
10
0
B cell
T cell
Myeloid cell
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5

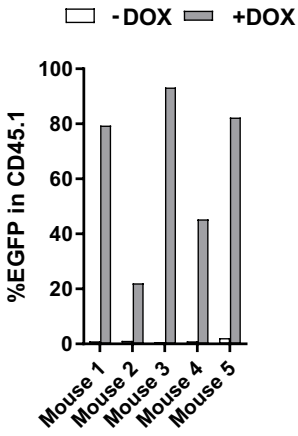
B


EGFP
(mouse 1)
Gene expression relative to GAPDH
0.1
0.01
0.001
0.0001
-DOX
+DOX

C


EGFP
(Mouse 1)
-DOX
+DOX
CD45.2-eFluor450
43.8%
0.9 %
93.2 %
CD45.1-PE/Cy7
EGFP-FITC

D


-DOX
+DOX
%EGFP in CD45.1
100
80
60
40
20
0
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5

E

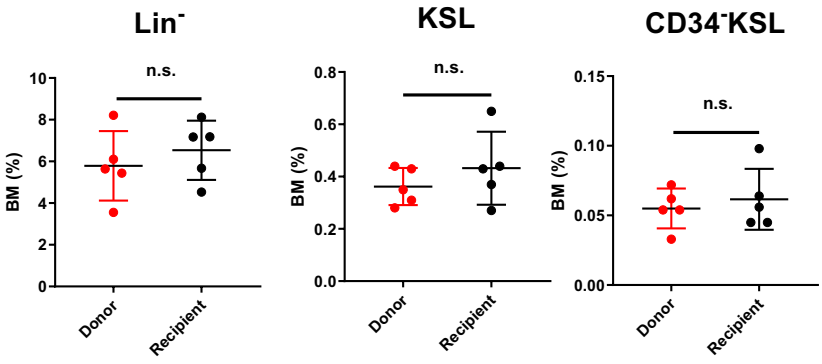

Lin-
KSL
CD34-KSL
n.s.
n.s.
n.s.
BM (%)
BM (%)
BM (%)
Donor
Recipient
Donor
Recipient
Donor
Recipient

Figure 21 Non-conditioned transplantation of EGFP-transduced culture-enriched HSPCs (1st transplantation)

(A) PB chimerism and multilineage differentiation of $CD45.1^+$ cells at 16 weeks post-transplant (n = 5 mice) for the non-conditioned recipients.

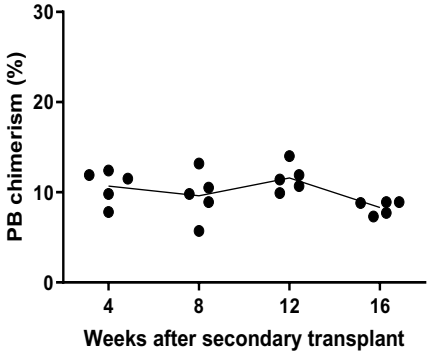
(B) Relative gene expression of EGFP in $CD45.1^+$ PB cells before and after DOX administration (EGFP, mouse 1 as a representative). Mean of three independent experiments, with gene expression normalized to *Gapdh* expression. Error bars denote SD.

(C) Representative flow cytometric plots of $CD45.1^+$ cells and EGFP expression in non-conditioned recipients (described in A) before and after DOX administration at 16-weeks post-transplantation.

(D) Percentage of EGFP in $CD45.1^+$ PB cells in individual mice (described in A) before and after DOX administration at 16-weeks post-transplantation (n = 5 mice).

(E) Percentage of Lin^-, KSL, and $CD34^-$KSL cells in BM cells among donor-derived $CD45.1^+$ or recipient-derived $CD45.1^+CD45.2^+$ cells at 16 weeks in primary recipients following transplantation of EGFP-transduced and selected c-Kit^+ BM cells. Mean of 5 recipients (n = 5 mice per group).

A



B

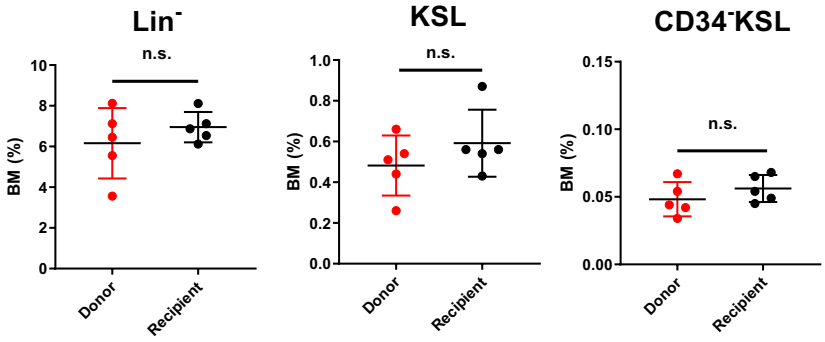


Figure 22 Non-conditioned transplantation of EGFP-transduced culture-enriched HSPCs (2nd transplantation)

(A) Percentage of donor-derived CD45.1$^+$ cells in secondary recipients over time, derived from EGFP-transduced and selected c-Kit$^+$ BM cells. (n = 5 mice)

(B) Percentage of Lin$^-$, KSL, and CD34$^-$KSL cells in BM cells among donor-derived CD45.1$^+$ or recipient-derived CD45.1$^+$CD45.2$^+$ cells at 16 weeks after secondary transplantation, derived from EGFP-transduced and selected c-Kit$^+$ BM cells. Mean ±SD of 5 recipients (n = 5 mice per group).

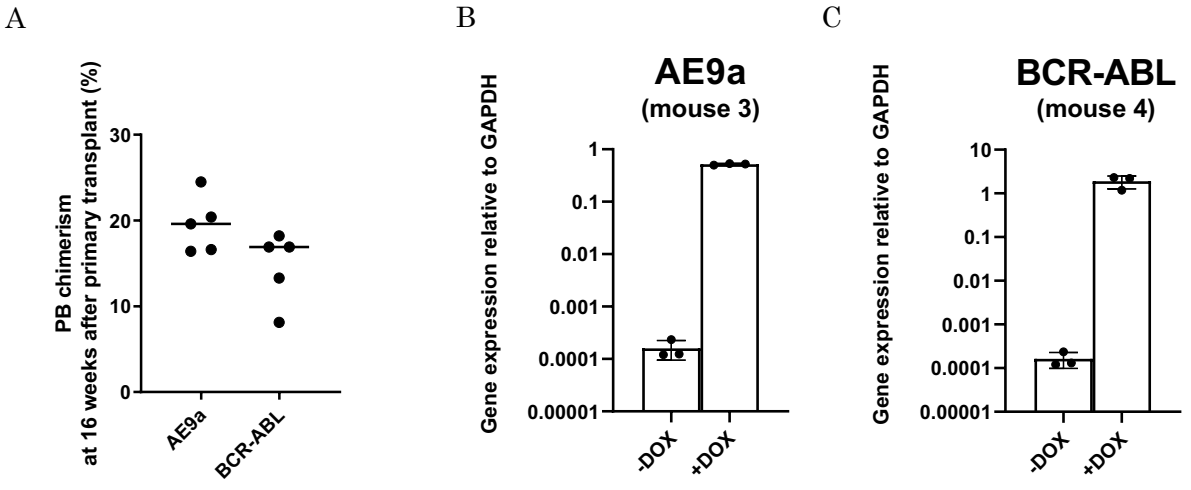


Figure 23 Non-conditioned transplantation of AE9a or BCR-ABL -transduced culture-enriched HSPCs

(A) PB chimerism following non-conditioned transplantation of AE9a and BCR-ABL transduced/selected and expanded c-Kit$^+$ BM cells at 16-week post-transplantation (n = 5 mice per group).

(B) Relative expression of AE9a in CD45.1$^+$ PB cells before and after DOX administration (AE9a, mouse 3 as a representative). Mean ±SD of three independent experiments, with gene expression normalized to *Gapdh* expression. Error bars denote SD.

(C) Relative expression of BCR-ABL in CD45.1$^+$ PB cells before and after DOX administration (BCR-ABL, mouse 4 as a representative). Mean of three independent experiments, with gene expression normalized to *Gapdh* expression. Error bars denote SD.

Table 1 Antibodies used in this study

Antibody	Dilution	Source	Identifier
APC anti-c-Kit (2B8)	1:100	eBioscience	Cat# 17-1171-83
PE anti-CD150 (SLAM) (TC15-12F12.2)	1:350	BioLegend	Cat# 115904
FITC anti-CD34 (RAM34)	1:100	eBioscience	Cat# 11-0341-85
PE/Cy5 anti-CD34 (MEC14.7)	1:100	BioLegend	Cat# 119312
PE/Cy7 anti- Ly-6A/E (Sca-1) (D7)	1:700	eBioscience	Cat # 25-5981-82
PE anti-Ly-6A/E (Sca-1) (D7)	1:700	BioLegend	Cat# 108108
APC-eFluor 780 Ly-6G/Ly-6C (RB6-8C5)	1:1400	eBioscience	Cat# 47-5931-82
APC-eFluor 780 CD11b (M1/70)	1:1400	eBioscience	Cat# 47-0112-82
APC-eFluor 780 CD4 (RM4-5)	1:1400	eBioscience	Cat# 47-0042-82
APC-eFluor 780 CD8a (53-6.7)	1:700	eBioscience	Cat# 47-0081-82
APC-eFluor 780 CD45R (B220)	1:700	eBioscience	Cat# 47-0452-82
APC-eFluor 780 CD127 (A7R34)	1:350	eBioscience	Cat# 47-1271-82
APC-eFluor 780 TER-119 (TER-119)	1:350	eBioscience	Cat# 47-5921-82
PE-Cy7 anti-CD45.1(A20)	1:500	Tonbo Biosciences	Cat# 60-0453-U025
eFluor450 anti-CD45.2 (104)	1:500	eBioscience	Cat# 48-0454-82
PE anti-Ly-6G/Ly-6C (RB6-8C5)	1:2000	eBioscience	Cat# 12-5931-82
PE anti-CD11b (M1/70)	1:2000	eBioscience	Cat# 12-0112-82
APC-eFluor780 anti-CD45R (RA3-6B2)	1:1000	eBioscience	Cat# 17-0452-83
APC anti-CD4 (RM4-5)	1:2000	BioLegend	Cat# 100516
APC anti-CD8 (53-6.7)	1:2000	eBioscience	Cat# 17-0081-83

Table 2 Primers used in this study

Primer name	Primer sequence
p16^{Ink4a} forward	5'-GAACTCTTTCGGTCGTACCC-3'
p16^{Ink4a} reverse	5'-CGAATCTGCACCGTAGTTGA-3'
p19^{Arf} forward	5'-GGGTTTTCTTGGTGAAGTTCG-3'
p19^{Arf} reverse	5'-TTGCCCATCATCATCACCT-3'
Trp53 forward	5'-CAGTCTACTTCCCGCCATAA-3'
Trp53 reverse	5'-GTCTCAGCCCTGAAGTCATAAG-3'
EGFP forward	5'-AAGTTCATCTGCACCACCG-3'
EGFP reverse	5'-TCCTTGAAGAAGATGGTGCG-3'
AE9a forward	5'-CCACCTACCACAGAGCCATCA-3'
AE9a reverse	5'-AGCCTAGATTGCGTCTTCACATC-3'
BCR-ABL forward	5'-CCGCTGACCATCAATAAGGAA-3'
BCR-ABL reverse	5'-CTGAGGCTCAAAGTCAGATGCTACT-3'
Gapdh forward	5'-AACTTTGGCATTGTGGAAGG-3'
Gapdh reverse	5'-ACACATTGGGGGTAGGAACA-3'

General Discussion

In Chapter 1, I have developed a new serum-free HSC culture medium that consists of all chemically-defined reagents. By utilizing rHSA, Ham's F-12 medium exhibited stronger support of HSCs than conventionally used S-clone medium in competitive repopulation assay. Interestingly, DMEM and RPMI1640 media didn't support HSCs even in primary transplantation. According to the product information of each medium, Ham's F-12 contains fewer amino acids compared to DMEM and RPMI1640 medium. It's consistent with the reports from our group which indicated that balance in amino acids has a crucial role in HSC maintenance[38,39]. In comparison to supplements, the commercial ITS-X supplement have enough potential to support HSCs. The ITS-X supplement contains insulin, transferrin, selenium, and ethanolamine. So far, the ITS-X supplement has been added to culture media to reduce the use of FBS in culture medium[70]. Also, several reports that ITS-X supplement was used in the culture of pluripotent cells such as the blastocyst[71] or ES cells[40,72] exist. Thus, it is presumed that the ITS-X supplement is suitable for sustaining pluripotency. In the continuous search for reagents of HSC culture, my colleagues and I have found PVA has better potential to sustain HSCs than rHSA[41]. Although HSCs cultured with PVA sustained *ex vivo* for even 28 days and showed robust and high chimerism in competitive repopulation assay, the system in which PVA mediate HSCs remained to be elucidated. Recently, Kobayashi et al. have shown that HSCs need fatty acids for proliferation, from either endogenous biosynthetic or exogenous sources and that BSA serves as a source of fatty acids for hematopoietic stem cell cultures[73]. Considering that PVA is a substitute for BSA, it is possible that PVA acts like a carrier protein. In addition to it, it has been reported that when PVA was added to ES cells in suspension culture, PVA inhibited ES

cell adhesion to the dish and induced genes related to cell-cell adhesion to form spherical cell masses, which contributed to the proliferation of undifferentiated ES cells[40]. Although no cell mass formation was observed when culturing HSCs in a PVA-based medium, this observation is potentially indicative of a function to maintain stem cells. Far more investigation is required for the PVA function on HSCs.

In Chapter 2, I developed the BM chimeric mouse model which doesn't require FACS equipment and pre-conditioning treatment to generate.

By culturing c-Kit$^+$ BM cells with PVA-based HSC medium for 28 days, HSCs and HSPCs that were engraftable to even in non-conditioned mouse BM was purified. For *ex vivo* gene transduction to HSCs and HSPCs, harboring puromycin-resistant genes in lentiviral vector enabled FACS-independent selection of gene-transduced cells. Therefore, this BM chimeric mouse model is completely free from using expensive FACS equipment which requires high technique to operate. For methods of purifying the target cells utilizing surface proteins, four ways have been listed: FACS cell sorting, magnetic cell sorting, adhesion-based cell sorting, and complement depletion[74]. FACS sorting employs phenotype-specific surface markers together with fluorescent antibodies to those markers. Multicolor staining of various antibodies enables the separation of distinct cell populations. While the accuracy and efficiency are very high, the expenses involved with FACS equipment often restrict individual labs from possessing their own FACS equipment. Magnetic cell sorting also makes use of antibody specificity to separate distinct cellular subpopulations from larger mixtures. This method is also widely used because it is much more affordable than FACS equipment. However, it can only apply to one marker at a time. Adhesion-based cell sorting is utilized in certain cells (e.g., mesenchymal stem cells) to distinguish adherent cells from non-adherent cells. This

method is fast and inexpensive, but due to the cell-cell interactions between cells of interest and contaminating cells in the mixture, false positives occur. Complement depletion is using monoclonal antibodies to eliminate specific cell types in a mixed population. Through a proteolytic cascade, active plasma proteins are used to produce pores on the surfaces of targeted cells. Although the efficiency is relatively high, the negative impact of cell lysis on other cells may remain. Of all, FACS cell sorting is the most widely used because of its significant accuracy and efficiency. Other methods are relatively less accurate than FACS sorting, but their affordability and availability are enough to be the options[74]. In my study, in combination with magnetic cell sorting and long-term culture, HSCs and HSPCs are well purified (Figure 6-8). Since it has not been possible to culture HSCs for long periods of time over 28 days, such a way is totally new to purify stem cells.

For non-conditional transplantation, Shimoto et al. first reported that transplanting a large number of HSCs into mice enabled non-myeloablative transplantation[65]. To conduct this experiment, they isolated HSCs from more than 200 mice. While that experiment was difficult to reproduce, PVA-cultured HSCs can pave the way to non-conditioned transplantation assay. It is known that myeloablative conditions such as irradiation and chemotherapy using Busulfan injure the BM microenvironment by reactive oxygen species (ROS) production via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase[31,32]. Mice models using BM transplantation have always been affected by this effect, but the new BM chimeric mouse model can exclude it. In addition to it, when combined with a Dox-induced expression lentiviral vector, control of gene expression in hematopoietic cells in the body at any desired time is possible. This model allows us to examine the behavior of genes at steady-state, which

has been previously impossible to observe in traditional BM chimeric mice models. In the investigation of non-conditioned transplantation which transplanted HSPCs with oncogenes, the mice did not develop leukemia. These results differed from transplantation with radiation[58,61]. Additionally, the average chimerism was slightly lower than that of no gene-transduced cells and EGFP (Fig 15A, Fig 21A, Fig 23A). From these results, I suspect that endogenous immune cells may have eliminated cells expressing high levels of oncogenes, resulting in no onset of leukemia. Thus, analysis of the gene function in steady-state may provide new findings in hematological research. When focusing on gene therapy similar to inherited blood disorders and immunodeficiency diseases , a variety of clinical studies are currently in progress[2,75]. Most of them perform myeloablative conditioning and use lentiviral vectors, which have currently concerns about the possibility of semi-random gene insertion into the genome [75]. To solve these disadvantages, a combination of the development of gene editing platforms that allow site-specific manipulation of the genome such as CRISPR-Cas9[76] and non-conditioned mice would be a powerful tool. Therefore, I anticipate that this platform will have a wide range of applications in hematologic and immunological research.

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