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Identification of the minimum requirements for successful haematopoietic stem cell transplantation

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Summary

Historically, defining haematopoietic subsets, including self-renewal, differentiation and lineage restriction, has been elucidated by transplanting a small number of candidate cells with many supporting bone marrow (BM) cells. While this approach has been invaluable in characterising numerous distinct subsets in haematopoiesis, this approach is arguably flawed. The haematopoietic stem cell (HSC) has been proposed as the critical haematopoietic subset necessary for transplantation. However, due to the presence of supporting cells, the HSC has never demonstrated sufficiency. Utilising the homeobox B5 (Hoxb5)-reporter system, we found that neither long-term (LT) HSCs nor short-term (ST) HSCs alone were sufficient for long-term haematopoietic reconstitution. Critically, reconstitution can be rescued by transplanting combined LT- and ST-HSCs, without supporting cells; a fraction we term the 'Minimum Subset for Transplantation' (MST). The MST accounts for only 0·005% of nucleated cells within mouse BM, and this MST can be cultured, expanded and genetically modified while preserving its rapid haematopoietic engraftment potential. These results support the consideration of an MST approach for clinical translation, especially for gene therapy approaches that require HSC compartment modification.

Keywords: long-term haematopoietic stem cell, short-term haematopoietic stem cell, homeobox B5 (Hoxb5), purified haematopoietic stem cell transplantation, gene therapy.

Introduction

Haematopoietic stem cell transplantation (HSCT) has been primarily developed as a treatment for haematopoietic malignancies and has been performed in >1 million patients.[1] In recent years, gene therapy approaches, wherein a patient's haematopoietic cells are genetically engineered and transplanted back to normalise the malfunctioning haematopoietic system, have been performed for monogenic diseases, including β-thalassaemia[2,3] and severe combined immunodeficiency.[4,5] Given that autologous HSCT can be applied to many more diseases, including other primary immunodeficiency disorders,[6] inherited metabolic disorders[7] and autoimmune diseases,[8,9] further development of the technology is desirable.

Currently, autologous HSCT with genetic modification has only been performed in a few hundred cases worldwide limited in part to the technical hurdles of HSC genetic modification,[10] lack of persistent gene-modified cell engraftment[11,12] and genotoxicity by random insertion of the vectors throughout the genome of targeted haematopoietic cells.[13] Although genetic engineering challenges of HSCs to solve the genotoxicity have been previously described,[14] efforts aimed at optimising genetic modification and improving insufficient therapeutic efficacy should logically utilise a purified cell fraction containing only the necessary and minimally sufficient cells for HSCT.

While the HSC field has been able to ascribe the fundamental stem cell and haematopoiesis properties of self-renewal,[15,16] multi-potency[17] and lineage restriction[18] to >23 distinct subsets of the haematopoietic hierarchy, these studies have all depended on a transplantation assay in which a small number of candidate cells are co-transplanted with a large number of unfractionated bone marrow (BM) cells,

termed supporting cells (also known as competitor cells).[19] From these studies the field has concluded that the long-term (LT) HSCs, even as a single cell transplant,[20,21] is both necessary and sufficient for successful HSCT. However, this conclusion relies on the assumption that there is a lack of interaction and co-dependence of the LT-HSCs on the unfractionated BM.

Therefore, we sought to identify the cell fractions that are minimally necessary and critically sufficient for safe and long-term haematopoiesis after transplantation, a subset we term the 'Minimum Subset for Transplantation' (MST). We systematically re-evaluated the distinct cell types in the haematopoietic hierarchy for MST but were unable to identify a single fraction meeting these criteria. Only by utilising the Hoxb5 mouse model, which enabled us to isolate the LT-HSCs and short-term (ST)-HSCs with high purity,[22] were we able to identify the MST required for long-term and rapid haematopoietic recovery in transplantation.

Methods

Mice

Homeobox B5 (*Hoxb5*)-tri-mCherry (C57BL/6J background) mice derived from our previous work[22] were used as donors for transplantation assays. CAG-enhanced green fluorescent protein (EGFP) mice (Japan SLC, Shizuoka, Japan) were bred with *Hoxb5*-tri-mCherry mice for transplantation experiments. The C57BL/6-Ly5·1 mice (Sankyo Labo Service, Tokyo, Japan) were used as recipients for transplantation assays. The 10–18-week-old mice were used as donors and 8–11-week-old mice were used as recipients. As an exception to the above, in Fig 4F, C57BL/6-Ly5·1 mice were used as donor cells, and C57BL/6J mice (CLEA Japan, Tokyo, Japan) were used as recipient mice. All mice were housed in pathogen-free conditions. Mice were bred accordingly with RIKEN guidelines. All animal protocols were approved by RIKEN Centre for Biosystems Dynamics Research.

More detailed information of BM analyses, sorting and transplantation without supporting cells, peripheral blood (PB) analyses, Sca-1$^+$ depletion, lentiviral gene transduction, *ex vivo* expansion of haematopoietic cells, fluorouracil (5-FU) conditioning, quantification and statistical analyses are described in the supplementary materials and methods.

Results

Non-lineage marker$^-$/c-Kit$^+$/stem cell antigen 1 (Sca-1)$^+$ (LKS) fraction alone does not rescue lethally irradiated mice

Several reports have shown that the LKS fraction (Figure S1A), which accounts for 0·1% of the mouse BM, enables rapid haematopoietic recovery after myeloablative conditioning.[23,24] On the other hand, it has been reported that the non-LKS

fraction, which is believed to be free of undifferentiated cells, may also contribute to continuous haematopoiesis, albeit to a dramatically lesser extent.[25] Therefore, we first tried to confirm whether the non-LKS fraction, which accounts for 99% of the BM cells, could rescue lethally irradiated recipient mice. Our previous report[22] showed that 2×10^5 whole BM (WBM) cells were enough to rescue lethally irradiated mice. Therefore, we compared the haematopoietic reconstitution between the group transplanted with 2×10^5 WBM cells and the group transplanted with 2×10^5 non-LKS cells (Fig 1A).

Non-LKS BM cells were prepared by depleting Sca-1$^+$ cells from the WBM by magnetic separation (Figs 1B,C). The survival rate of the WBM-transplanted group was 83%. In contrast, none of the mice transplanted with the same number of the non-LKS cells survived (Fig 1D). No donor cells were detected in the PB, even in the survived recipient mouse receiving three doses of non-LKS cells (Fig 1E). These results indicated that non-LKS fraction alone were insufficient for rescuing recipient mice.

Transplantation with different doses of LKS cells revealed a threshold for rapid haematopoietic recovery after HSCT

Although LKS cells were considered necessary for rapid haematopoietic recovery based on the above results, it is desirable to establish a sufficiently safe transplantation dose using fewer donor cells to improve the efficiency of genetic modification.[26,27] Therefore, we transplanted different numbers of LKS cells into lethally irradiated mice to determine the minimum cell dose (Fig 2A). The groups that received $>1 \times 10^3$ cells had an average survival rate of 95·8%. In contrast, the groups that received $<3 \times 10^2$ cells had a 50% survival rate (Fig 2B). The PB analysis revealed that the groups receiving $<1 \times 10^3$ cells had significantly reduced donor chimerism and delayed lymphocyte recovery (Figs 2C–E). On the other hand, the groups receiving $>3 \times 10^3$ cells had stable donor chimerism and rapid lymphocyte recovery 8 weeks after transplantation (Figs 2C–E). These results suggested that the cell dose should be determined based not only on the survival rate but also on the rate of haematopoietic reconstitution to ensure safe transplantation.

Non-HSCs in the LKS fraction do not play a pivotal role in post-transplant haematopoietic reconstitution

Although we demonstrated that the LKS fraction alone was necessary for successful transplantation, we have not determined what is minimally necessary and sufficient as this fraction consists of multiple cell types, including HSCs and multipotent progenitors (MPPs), the latter which have lost their self-renewal capability, and other differentiated cells. The next question is whether all of the constituents are necessary for haematopoiesis after transplantation or not. Then, we sought to identify the minimum subsets that were truly responsible for post-transplant
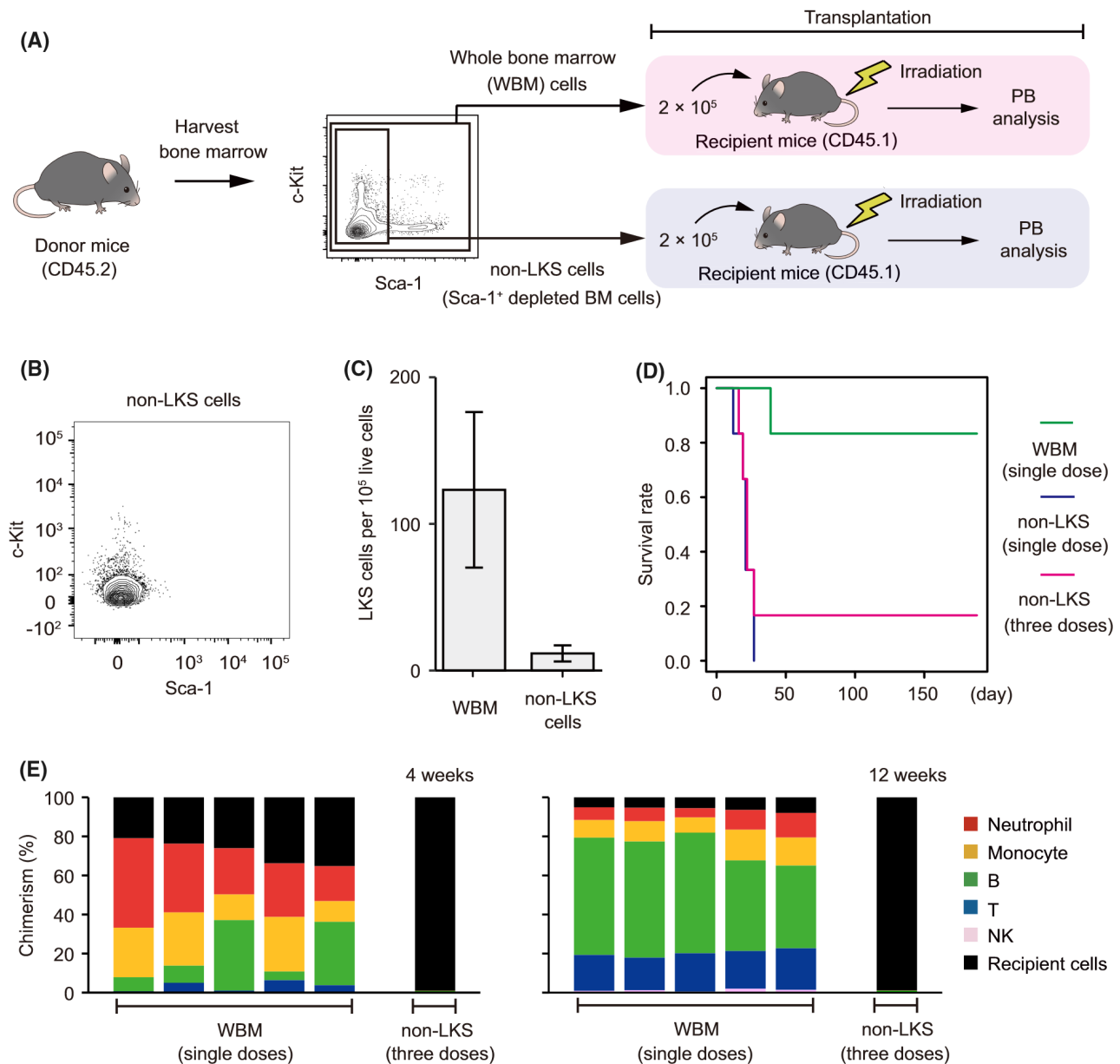
Fig 1. Transplantation of non-LKS cells does not rescue lethally irradiated mice. (A) Experimental design for assessing the haematopoietic reconstitution capacity. In all, 200 000 WBM cells or non-LKS cells (Sca-1$^+$-depleted BM cells) from CD45.2 mice were transplanted into lethally irradiated CD45.1 recipient mice. (B) Representative flow cytometry plots of non-LKS cells after exclusion of doublets and dead cells. (C) Number of LKS cells per 1×10^5 live cells in WBM or non-LKS cells ($n = 3$). (D) Survival rate of recipient mice receiving single dose of 2×10^5 WBM cells or single dose of 2×10^5 non-LKS cells at day 0 or three doses of 2×10^5 non-LKS cells at day 0, 3, and 7 after irradiation ($n = 6$). (E) PB chimerism in each group at 4 and 12 weeks after transplantation. Each column represents an individual mouse. [Colour figure can be viewed at wileyonlinelibrary.com]

haematopoiesis. For this, we analysed the numerical contribution of the LT-HSC (Hoxb5$^+$Lineage$^-$c-Kit$^+$Sca-1$^+$CD150$^+$CD34$^{-/lo}$Flt3/Flk2$^-$), ST-HSC (Hoxb5$^-$Lineage$^-$c-Kit$^+$Sca-1$^+$CD150$^+$CD34$^{-/lo}$Flt3/Flk2$^-$), MPP subset A (MPPa; Lineage$^-$c-Kit$^+$Sca-1$^+$CD150$^+$CD34$^+$Flt3Flk2$^-$), MPP subset B (MPPb; Lineage$^-$c-Kit$^+$Sca-1$^+$CD150$^-$CD34$^+$Flt3/Flk2$^-$) and Flt3/Flk2-positive cells (Flt3/Flk2$^+$; Lineage$^-$c-Kit$^+$Sca-1$^+$Flt3/Flk2$^+$) from the LKS population along the haematopoietic hierarchy (Figure S1A). In our analyses, 1000 LKS cells consists of 500 Flt3/

Flk2$^+$ cells, 300 MPPb cells, 75 MPPa cells, 38 ST-HSCs, and 12 LT-HSCs, excluding outlier cells (Figure S1A).

To determine the sufficiency of each of these fractions to the MST, we first prepared four different cell mixtures starting from the most differentiated population (Flt3/Flk2$^+$) and adding back the upstream population one by one according to the haematopoietic hierarchy (Fig 3A). We used 1000 LKSs as a positive control, which is the minimum number of cells to ensure recipient survival (Fig 2B). All mice receiving non-HSC
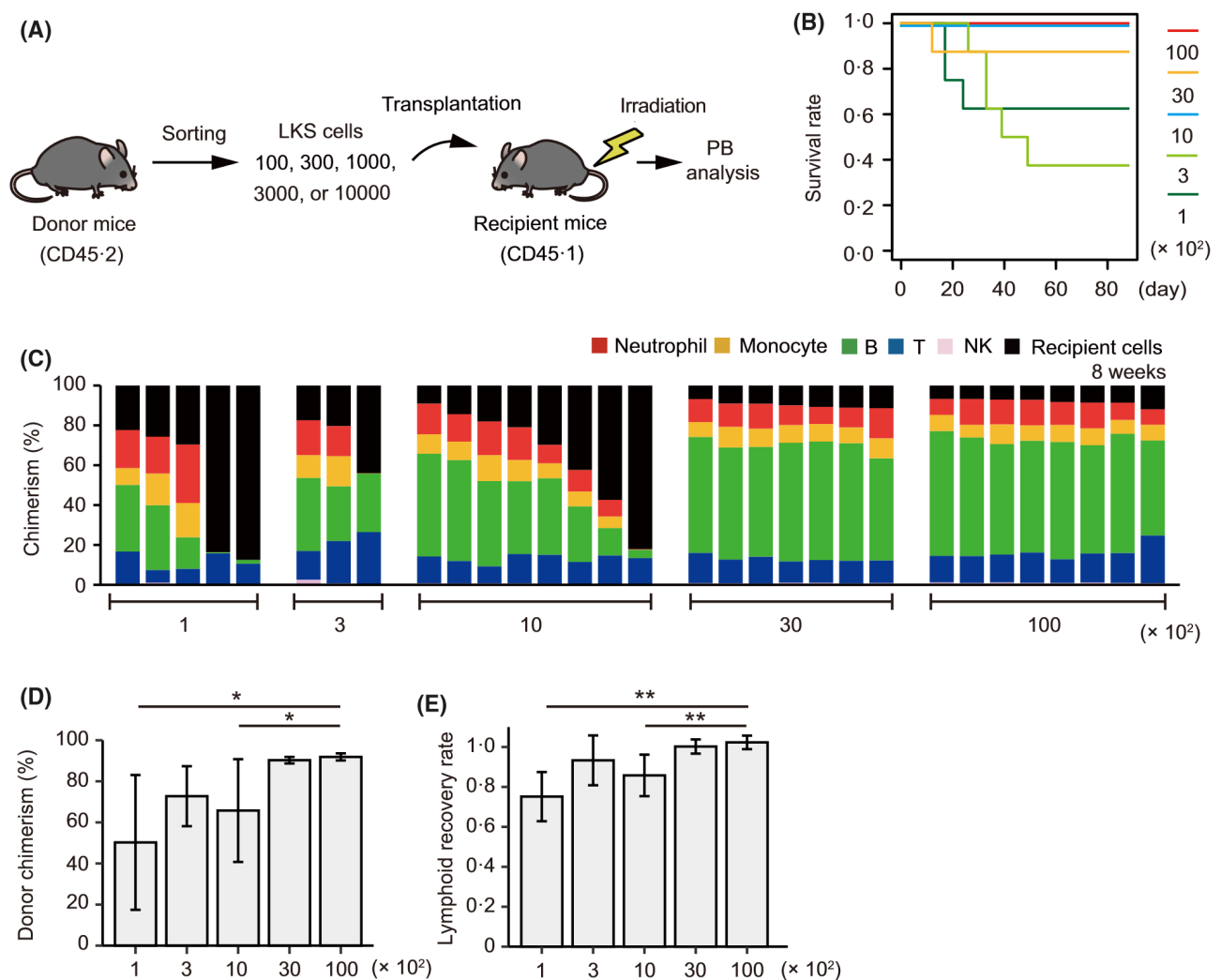
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Fig 2. Transplantation with different numbers of LKS cells revealed a threshold for rapid haematopoietic recovery after LKS transplantation. (A) Experimental design for determining the cell number required for successful transplantation. Different number of LKS cells (100, 300, 1000, 3000, or 10 000 cells) from CD45.2 mice were transplanted into lethally irradiated CD45.1 recipient mice ($n = 8$). (B) Survival rate of each group after LKS transplantation. (C) PB chimerism in each group at 8 weeks after LKS transplantation. Each bar represents an individual mouse. (D) Average donor chimerism in each group at 8 weeks after LKS transplantation. (E) Average of lymphoid recovery rate in each group at 8 weeks after LKS transplantation. The lymphoid recovery rate was defined as the ratio of the lymphocyte percentage in the PB of recipient mice to the average percentage of untreated 3-month-old *Hoxb5*-tri-mCherry mice ($n = 5$). All error bars denote SD. *$P < 0.05$. **$P < 0.01$. [Colour figure can be viewed at wileyonlinelibrary.com]

containing fractions (▼Flt3/Flk2⁺, ▼MPPb, and ▼MPPa) died within a short period of time except for one case in the ▼MPPb group (Fig 3B). Even in this case, no donor myeloid cells were detected in the PB, representing typical graft failure (Fig 3C). On the other hand, adding 38 ST-HSCs to the mixture of Flt3/Flk2⁺, MPPb, and MPPa (▼ST-HSC) dramatically improved the survival rate and myeloid cells output 4 weeks after transplantation (Figs 3B,C). However, compared to the LKS group which critically contains LT-HSCs, the survival outcomes were inferior, suggesting that the LT-HSCs, while only contributing a miniscule cell number to the LKS, were crucial for safer outcomes. These results suggested that >90% of the LKS fraction, which consists of MPPa, MPPb, and Flt3/

Flk2⁺, did not play a critical role in haematopoietic reconstitution after transplantation.

A combined fraction is the minimum necessary and sufficient subset for haematopoietic recovery after transplantation

As the field has concluded that the LT-HSC is both necessary and sufficient for successful transplantation, the results above strongly suggest that the LT-HSC is the key fraction. However, the recipient mice showed significant improvement of survival by adding ST-HSCs. Therefore, we sought to clarify whether either or both fractions were required to achieve

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(A)

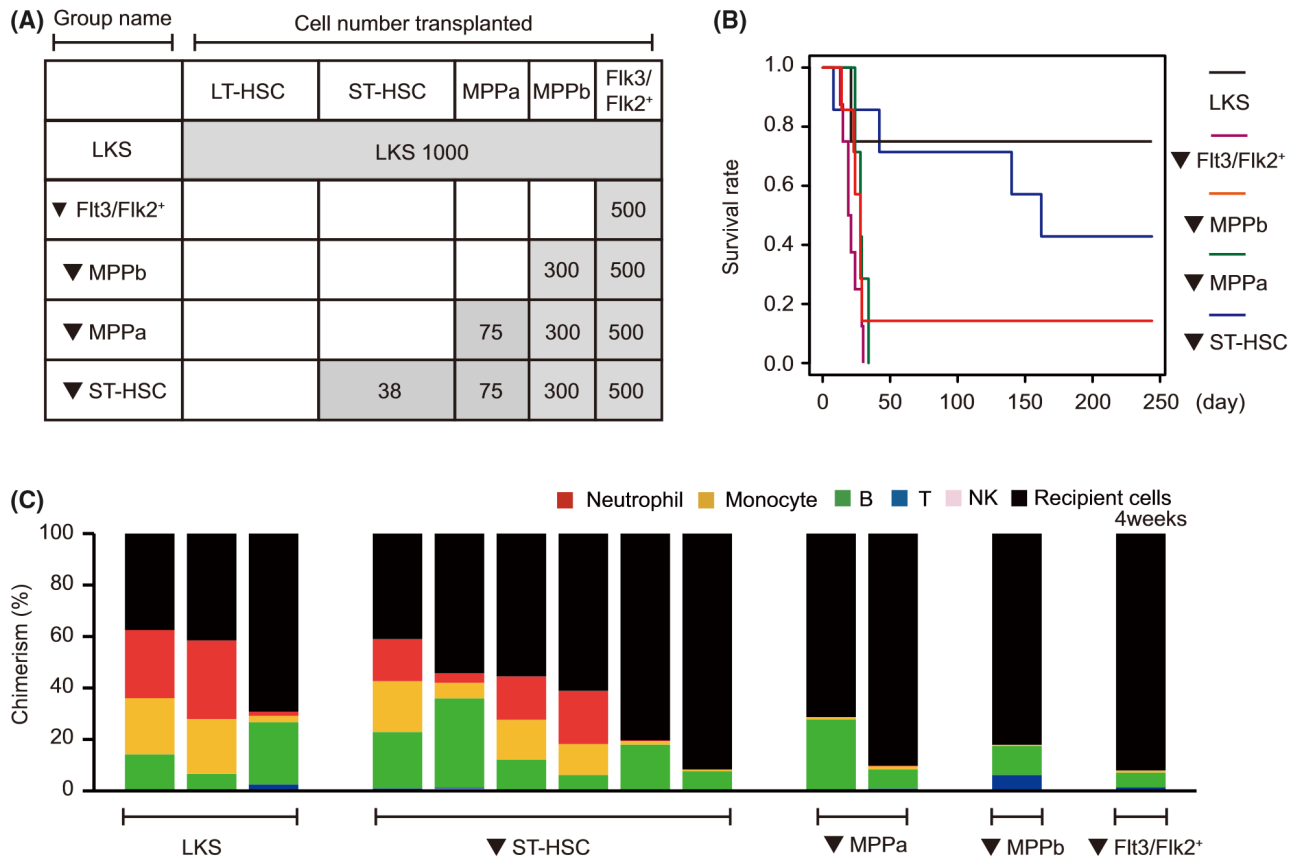| Group name	LT-HSC	ST-HSC	MPPa	MPPb	Flk3/Flk2+
		Cell number transplanted			
LKS	LKS 1000				
▼ Flt3/Flk2+					500
▼ MPPb				300	500
▼ MPPa			75	300	500
▼ ST-HSC		38	75	300	500

(B)

(C)

Fig 3. Non-HSC fractions do not play a critical role in haematopoietic reconstitution after transplantation. (A) Summary of cell types and numbers transplanted in each group. The first column represents group names, and the first row represents cell types. The total number of cells shown in each row were transplanted (LKS, $n = 4$; ▼Flt3/Flk2+, $n = 8$; ▼MPPb, $n = 7$; ▼MPPa, $n = 7$; ▼ST-HSC, $n = 7$). (B) Survival rate of each group. (C) PB chimerism at 4 weeks after transplanting each fraction. Each bar represents an individual mouse. [Colour figure can be viewed at wileyonlinelibrary.com]

successful transplantation. We transplanted isolated 12 LT-HSCs, 38 ST-HSCs, 50 combined HSCs, and 1000 LKSs respectively (Fig 4A). Surprisingly, neither 12 LT-HSCs alone nor 38 ST-HSCs alone rescued recipient mice (Fig 4B). In contrast, 50 combined HSCs had nearly equivalent rescue potential to 1000 LKSs (Fig 4B).

Next, we tried to determine the sufficient dose of the combined HSCs for rapid haematopoietic recovery and long-term survival after transplantation by titrating the combined HSCs dose (Fig 4C). The mice receiving >300 combined HSCs showed rapid haematopoietic recovery 4 weeks after transplantation (Fig 4D), resulting in a high survival rate (Fig 4E). Another group receiving 300 combined HSCs confirmed 100% survival and a year-long stable haematopoietic reconstitution deriving from the donor cells with very high chimerism (Figs 4F,G). In contrast, >90% of the LKS fraction, which consists of MPPa, MPPb, and Flt3/Flk2+, contributed to haematopoiesis only in the very short term and did not play a critical role in the survival of the recipient mice after transplantation. In summary, the combined fraction, LT- and ST-HSCs, was the minimal necessary and sufficient subset to ensure both rapid haematopoietic recovery and long-term haematopoietic reconstitution, i.e. the MST.

LT- and ST-HSCs work complimentarily in rapid haematopoietic recovery and long-term haematopoiesis

Although we found that LT- or ST-HSCs alone were insufficient for rescuing irradiated mice, this may be due to the scarcity of transplanted cells. To confirm this, we transplanted approximately 10-times as many LT- or ST-HSCs compared to the previous experiments (Figures S2A, C). While the group receiving 100 LT-HSCs showed little improvement in survival, the group receiving 380 ST-HSCs showed a significant improvement in survival and haematopoiesis, not only in the short term but also in the long term (Figures S2B, D, and E). These results indicated that LT- and ST-HSCs played different roles in transplantation.

To properly understand the differences, we performed a co-transplantation assay in which we sorted LT-HSCs from *Hoxb5*-tri-mCherry mice and ST-HSCs from CAG-EGFP; *Hoxb5*-tri-mCherry mice, or vice versa. These cells were then co-transplanted into the same recipients in a 25 LT-HSC: 75 ST-HSC ratio, as seen in healthy young mouse BM (Figs 5A, and S1A). Except for one case, all mice survived the transplantation with 300 MST cells, which included 75 LT-HSCs
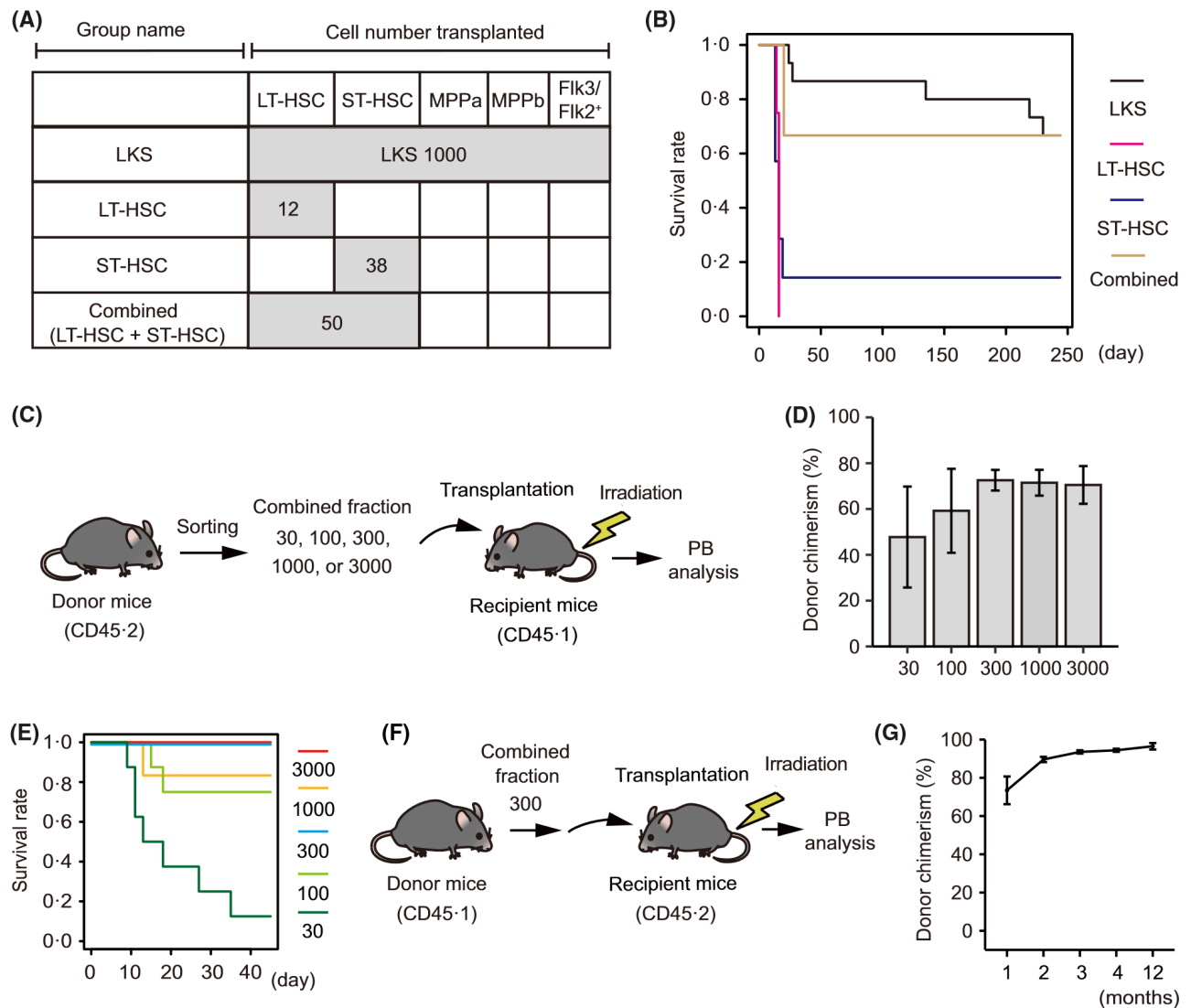
Fig 4. A combined HSC fraction is sufficient for haematopoietic recovery after transplantation. (A) Summary of cell types and numbers transplanted in each group (LKS, *n* = 15; LT-HSC, *n* = 4; ST-HSC, *n* = 7; combined, *n* = 6). (B) Survival rate of each group. (C) Experimental design for determining the cell number required for successful transplantation. Different cell number of combined HSCs (30, 100, 300, 1000, or 3000 cells) from CD45.2 mice were transplanted into lethally irradiated CD45.1 recipient mice. (*n* = 6–8 except for *n* = 4 in the 3×10^3 group). (D) Average donor chimerism in each group at 4 weeks after combined HSC transplantation. (E) Survival rate of each group. (F) Experimental design for assessing the long-term haematopoiesis after combined HSC transplantation. In all, 300 combined HSCs from CD45.1 mice were transplanted into lethally irradiated CD45.2 recipient mice (*n* = 7). (G) Kinetics of average donor chimerism at 1, 2, 3, 4 and 12 months after combined HSC transplantation. All error bars denote SD. [Colour figure can be viewed at wileyonlinelibrary.com]

and 225 ST-HSCs. PB analysis revealed that both LT- and ST-HSCs in the donor fraction produced myeloid lineage cells predominantly in the first 4 weeks after transplantation. However, ST-HSCs started losing the ability to produce myeloid lineage cells 8 weeks after transplantation (Figs 5B,C). On the other hand, LT-HSCs maintained multilineage haematopoietic reconstitution throughout the observation period (Figs 5B,C). A direct comparison of the haematopoietic reconstitution ability between LT- and ST-HSCs within the donor fraction revealed that haematopoiesis by ST-HSCs is predominant in the early post-transplant period, but LT-HSCs are primary source for all the blood lineage over time

(Figs 5D,E). Taken together with the results showing insufficiency of the LT-HSCs to rescue, these findings suggested that rapid haematopoietic recovery by ST-HSCs was essential in the early post-transplant period, while LT-HSCs were essential to ensuring robust long-term multilineage haematopoiesis and survival.

Gene-modified MST represents persistent reporter expression in recipient mice

Next, we examined whether MST was applicable for gene-modified HSCT. For this, we used lentivirus to genetically
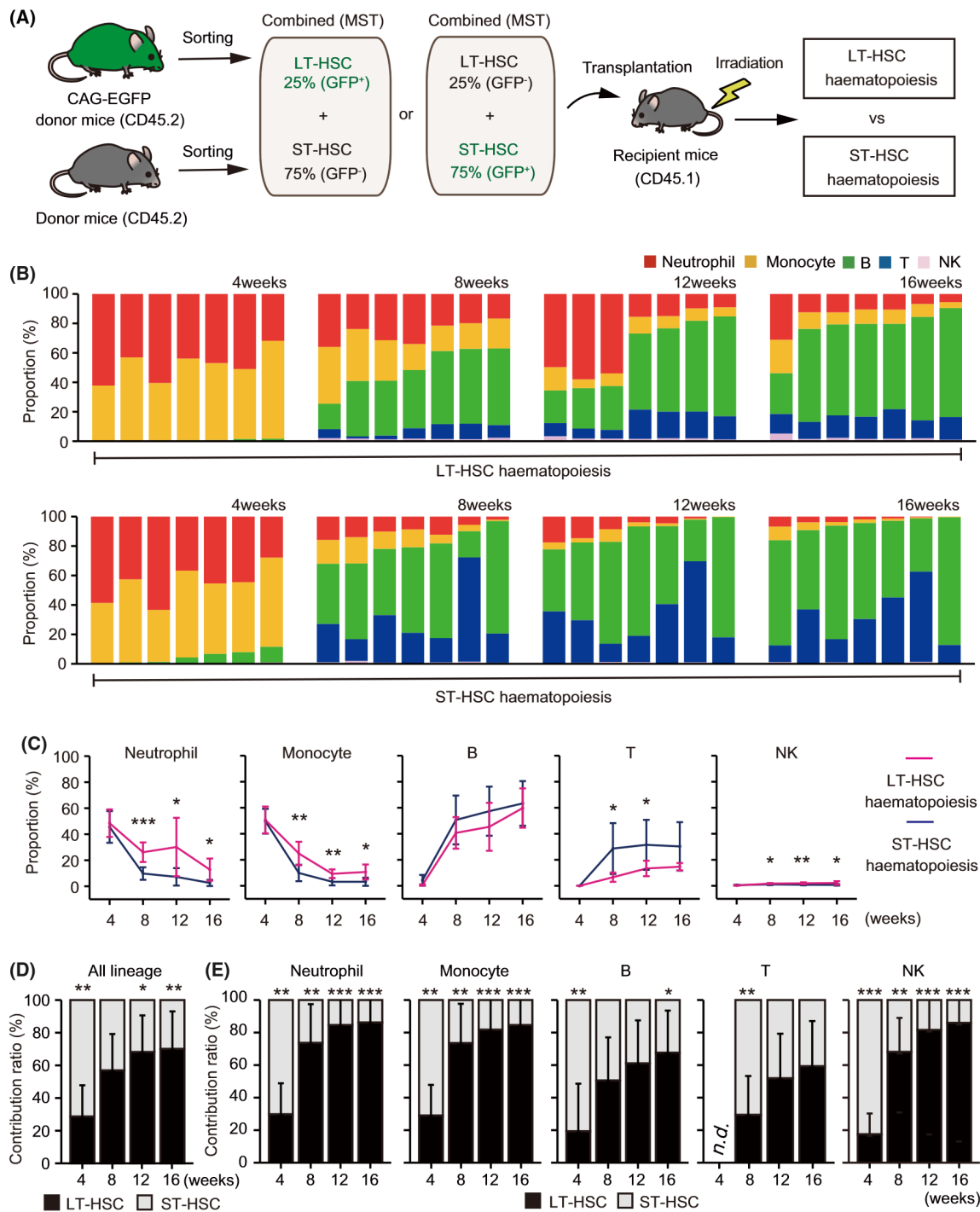
Fig 5. Fig Co-transplantation reveals complimentary roles of LT- and ST-HSCs in rapid haematopoietic recovery and long-term haematopoiesis. (A) Experimental design for co-transplantation assay. In all, 75 LT-HSCs from *Hoxb5*-tri-mCherry GFP mice or *Hoxb5*-tri-mCherry mice and 225 ST-HSCs from *Hoxb5*-tri-mCherry mice or *Hoxb5*-tri-mCherry GFP mice were co-transplanted into lethally irradiated CD45.1 recipient mice ($n = 8$). (B) Individual lineage proportion from LT- and ST-HSCs in the donor PB at 4, 8, 12 and 16 weeks after MST transplantation. Each bar represents an individual mouse. (C) Kinetics of average individual lineage proportion from LT- and ST-HSCs at 4, 8, 12 and 16 weeks after MST transplantation. (D) Average contribution ratio from LT- and ST-HSCs in the donor fraction at 4, 8, 12 and 16 weeks after MST transplantation. The contribution ratio was calculated as the proportion of LT-HSC-derived cells vs. ST-HSC-derived cells in the donor fraction. Statistical significance was calculated using Welch's *t*-test between the proportion of LT-HSC-derived cells vs. ST-HSC-derived cells in the donor fraction. (E) Average contribution ratio from LT- and ST-HSCs in each lineage at 4, 8, 12 and 16 weeks after MST transplantation. All error bars denote SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n.d., not detected. [Colour figure can be viewed at wileyonlinelibrary.com]

modify the minimum subset and confirmed their transduction efficiency and persistence by monitoring the ZsGreen reporter expression (Figs 6A,B). We achieved a mean (SD) 59·6 (15·2)% of infection efficiency with 4×10^3 multiplicity of infection (MOI) (Fig 6C). Under these conditions, transplanting 300 genetically-modified MST cells, we detected stable donor cell contribution and persistent reporter expression for up to 16 weeks without decline (Fig 6D).

Ex vivo *expansion of MST allows rapid and robust multilineage reconstitution*

Although we have shown that transplantation using MST is feasible, as several previous studies have noted, graft failure and delayed haematopoietic recovery are the major impediments to expand the applicability of purified HSCT to various diseases.[28] Recent studies have shown that *ex vivo* expansion of umbilical cord blood stem cells promoted rapid haematopoietic recovery,[29] while maintaining the capacity for long-term haematopoiesis. Taking this into account, we sought to evaluate the effect of *ex vivo* expansion of MST on haematopoietic recovery.

We cultured 300 MST cells for 7 days in the F12-based culture medium with thrombopoietin[30] and stem cell factor.[31,32] After the incubation, we collected the cultured cells and transplanted all expanded cells into lethally irradiated

mice. For comparison, we transplanted freshly isolated 300 MST cells into lethally irradiated mice (Fig 7A). PB analysis showed that donor chimerism at 4 weeks was significantly higher in the group receiving cultured cells than in the group receiving freshly isolated cells (Fig 7B). We also observed faster recovery of lymphocytes in the PB in the group receiving cultured cells than in the group receiving freshly isolated cells (Figs 7C,D). To directly compare haematopoietic reconstitution potential between freshly isolated MST and expanded MST, we co-transplanted freshly isolated 300 GFP+ MST cells and 7-day cultured cells derived from 300 MST cells (Fig 7E). We found that cultured cells outcompeted freshly isolated cells in haematopoietic reconstitution throughout the observation period (Figs 7F,G). In conclusion, these results indicated that transplantation using *ex vivo* expansion of the MST (*ex vivo* MST) improved short-term haematopoietic recovery while maintaining long-term haematopoietic capacity.

5-FU *conditioning followed by MST transplantation enables long-term donor haematopoiesis*

Based on the aforementioned results, we have concluded that the combined HSC fraction consisting of LT- and ST-HSCs is the minimum subset sufficient for successful transplantation. The MST can contribute to expand the applicability of
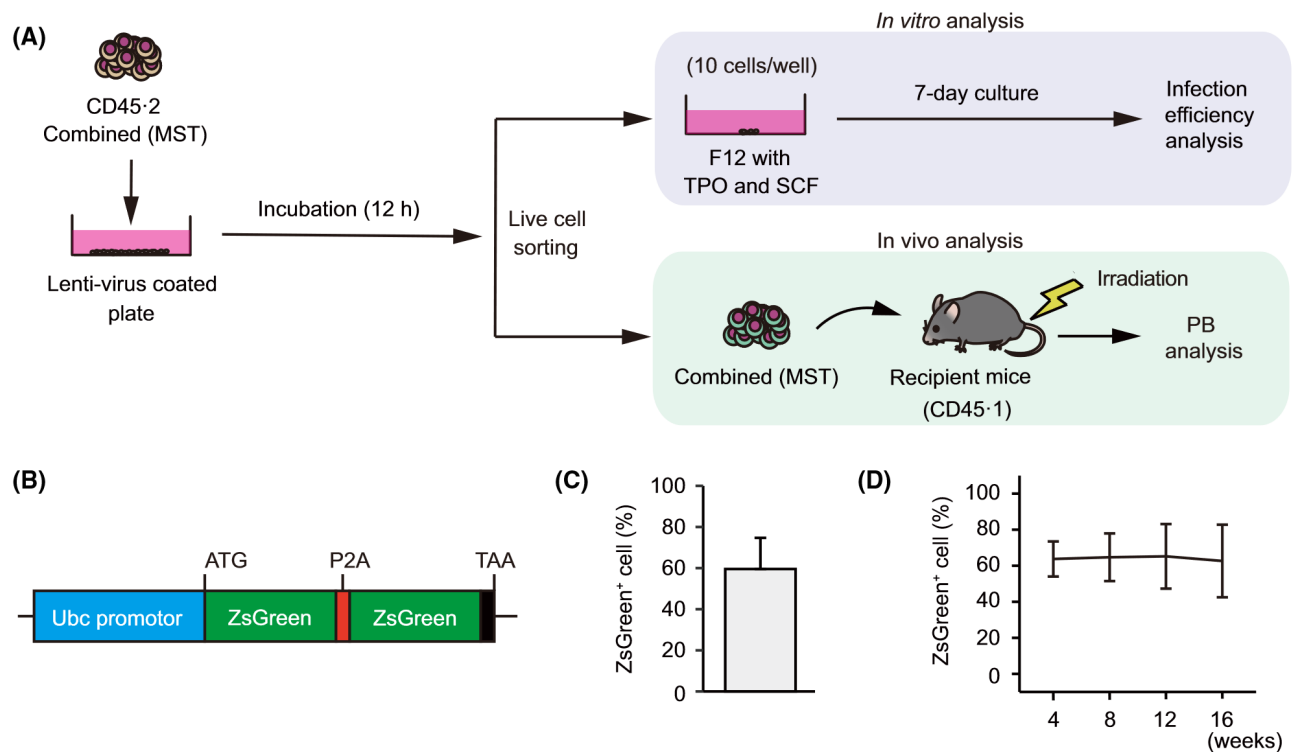

Fig 6. Gene transduction into the minimum subset by the lentiviral system. (A) Experimental design for assessing the gene transduction efficiency into the minimum subset (*in vitro* analysis, *n* = 5; *in vivo* analysis, *n* = 8). (B) Schematic of viral construct. Ubc: Ubiquitin C promoter. (C) Average percentage of ZsGreen-positive cells after 7-day culture. (D) Kinetics of average percentage of ZsGreen-positive cells in donor fraction at 4, 8, 12 and 16 weeks after MST transplantation. All error bars denote SD. [Colour figure can be viewed at wileyonlinelibrary.com]
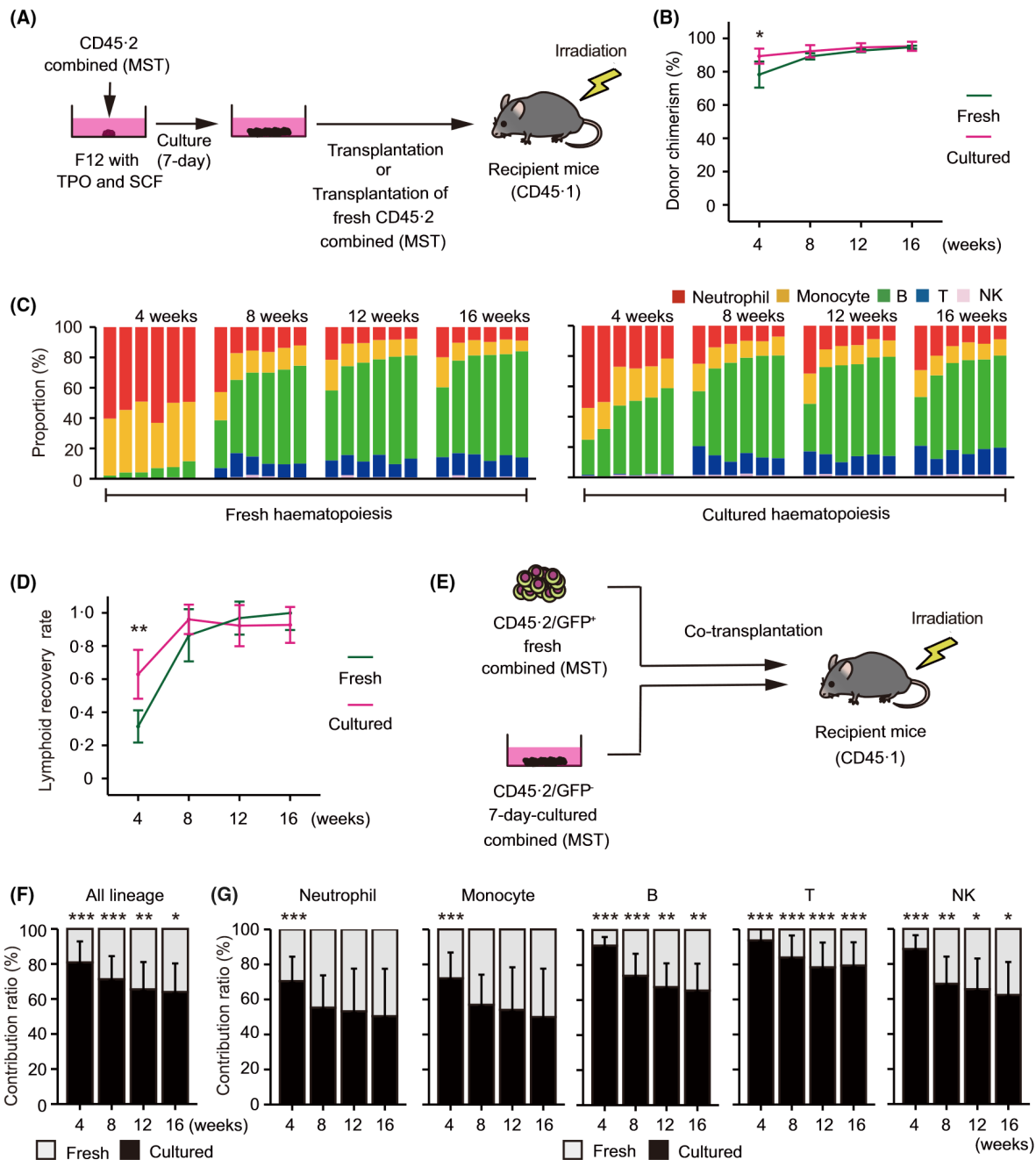
Fig 7. *Ex vivo* expansion of MST accelerates rapid and robust multilineage reconstitution. (A) Experimental design for assessing the haematopoietic reconstitution ability of fresh or cultured MST. Fresh MST cells from CD45.2 mice or 7-day-cultured MST cells from CD45.2 mice were transplanted into lethally irradiated CD45.1 recipient mice ($n = 6$). (B) Kinetics of average donor chimerism in each group at 4, 8, 12 and 16 weeks after MST transplantation. (C) Lineage proportion in the donor PB from fresh or cultured MST cells at 4, 8, 12 and 16 weeks after MST transplantation. (D) Kinetics of average lymphoid recovery rate in each group at 4, 8, 12 and 16 weeks after MST transplantation. (E) Experimental design for co-transplantation assay. Fresh MST (CD45.2/GFP$^+$) and cultured MST (CD45.2/GFP$^-$) cells were co-transplanted into lethally irradiated CD45.1 recipient mice ($n = 6$). (F) Average contribution ratio from fresh and cultured MST cells at 4, 8, 12 and 16 weeks after MST transplantation. The contribution ratio was calculated as the proportion of cultured-MST-derived cells vs. fresh-MST-derived cells in the donor fraction. Statistical significance was calculated using Welch's *t*-test between the proportion of cultured-MST-derived cells vs. fresh-MST-derived cells in the donor fraction. (G) Average contribution ratio from cultured-MST-derived cells vs. fresh-MST-derived cells in each lineage at 4, 8, 12 and 16 weeks after MST transplantation. All error bars denote SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. [Colour figure can be viewed at wileyonlinelibrary.com]
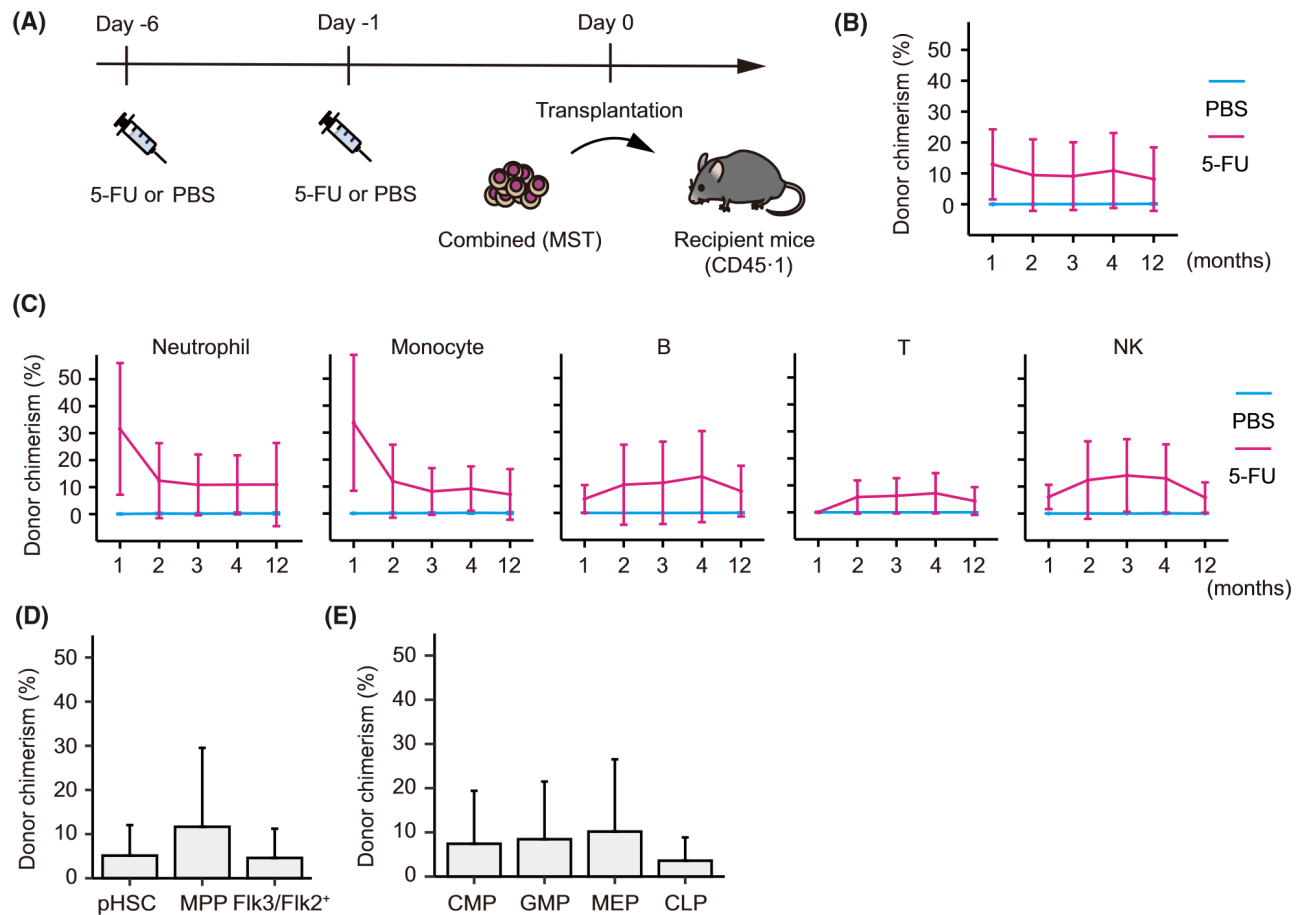
Fig 8. 5-FU conditioning followed by MST transplantation enables long-term donor haematopoiesis. (A) Experimental design for the 5-FU conditioning regimen. In all, 3000 MST cells from CD45.2 mice were transplanted into 5-FU-treated or phosphate-buffered saline (PBS)-treated CD45.1 recipient mice (5-FU-treated, $n = 6$; PBS-treated, $n = 5$). (B) Kinetics of average donor chimerism in the PB at 1, 2, 3, 4 and 12 months after MST transplantation. (C) Kinetics of average donor chimerism in each lineage fractions at 1, 2, 3, 4 and 12 months after MST transplantation. (D) Average donor chimerism in the haematopoietic stem and progenitor fractions at 12 months after MST transplantation. (E) Average donor chimerism in the oligopotent progenitor fractions at 12 months after MST transplantation. All error bars denote SD. [Colour figure can be viewed at wileyonlinelibrary.com]

purified HSCT by improving gene modification efficiency. However, due to life-threatening events caused by myeloablative conditioning, HSCT has not been commonly practised. To potentially address this issue, we sought to explore the utility of the MST to non-myeloablative conditioning.

5-FU has been widely used in various cancer treatments,[33] partially due to 5-FU's low-cost and less toxicity,[34] contrary to other reagents such as busulfan, cyclophosphamide, and fludarabine.[35–39] In addition, a previous report[40] demonstrated that 5-FU conditioning followed by transplantation of 5×10^6 WBM cells achieved 10% donor chimerism. Therefore, we attempted to evaluate the same 5-FU regimen followed by transplanting 3×10^3 MST cells instead of WBM cells, which represent <0·1% of 5×10^6 WBM cell dose (Fig 8A). We observed approximately the same donor chimerism as in the previous report (Fig 8B).[40] As expected, no donor cell engraftment was observed in the non-conditioned group throughout the observation period (Fig 8B). Donor

chimerism analysis in each lineage showed that the MST demonstrated persistent contribution to all cell lineages in the PB (Fig 8C). We also critically detected persistent a MST contribution to all haematopoietic stem and progenitor cell fractions in the BM (Figs 8D,E and S1B), suggesting that the MST maintained long-term self-renewal capacity and multipotency. These data demonstrated that MST transplantation following 5-FU conditioning could provide an efficient therapeutic platform including gene therapy without fatal complications while maintaining long-term haematopoiesis.

Discussion

In the present study, using the Hoxb5-reporter system, we have shown that the minimum subset consisting of LT- and ST-HSCs, which account for only 0·005% of nucleated cells within mouse BM, is critically sufficient for post-transplant haematopoiesis and that MST transplantation is practically

feasible without graft failure and delayed haematopoietic recovery by transplanting a sufficient amount of MST cells.

In conventional HSCT, unpurified WBM cells, which are comprised of mostly differentiated cells, have been intentionally used as donor sources to ensure high engraftment by preventing loss of effective cells and anti-tumour effect (called the graft-*versus*-tumour effect) by transfusing donor mature T cells.[41] On the other hand, recent innovations including sequencing technology, gene modification and big data analytics have significantly impacted on selecting treatment strategies.[42] For instance, molecular targeted drugs,[43] chimeric antigen receptor T-cell therapy[44] and immune checkpoint inhibitors[45] have the potential to replace HSCT as the standard curative therapy for haematopoietic malignancies in the near future. These changes in clinical practise indicate the need to reconsider the use of HSCT in various situations.

Recently, clinical trials have shown that autologous HSCT is very effective in treating autoimmune diseases.[46] Furthermore, genetic engineering technology is expected to expand the applicability of autologous HSCT to many diseases, including primary immunodeficiencies[4] and inborn errors of metabolism.[7] On the other hand, *ex vivo* manipulations of HSCs, such as cell sorting, genetic engineering and cell culture, are likely to lose or abnormalise significantly HSC functions, resulting in the lack of persistent therapeutic effect[11,12] or the increased risk of leukaemogenesis.[13] To solve these issues, starting from purified cell populations is a practical solution to minimise risks around *ex vivo* modifications.

Furthermore, to explore the applicability of MST to humans, current myeloablative conditioning regimens need to be revised due to acute and chronic fatal effects. Although several groups have developed non-intensive conditioning regimens that could be implemented into clinical practice in the future, such as anti-c-Kit antibody treatment,[47] dietary valine restriction[48] and non-conditioned transplantation,[49] we hope that 5-FU conditioning will be an optimal option because of not only low myeloablative toxicity but also low-cost and being very mildly gonadotoxic for young patients. Using a single MST (3000 cells) transplantation following 5-FU conditioning, we achieved ~10% donor chimerism comparable to that of the non-conditioned transplantation using the massive HSC expansion *ex vivo* previously reported.[50] For severe combined immunodeficiency and chronic granulomatous disease, 5–10% myeloid chimerism is high enough to improve their clinical symptom.[51,52] However, given that donor chimerism depends on the dose of transplanted cells,[49] increased cell number of the MST or combination with *ex vivo* expanded MST will be expected to achieve even higher chimerism, which can further expand the application of the MST for gene therapy.[53]

The MST transplantation has also brought new insights into basic research. When assessing the function of HSCs, WBM cells, in addition to donor cells, were commonly co-transplanted as a supporting fraction to ensure the survival of recipient mice. However, the supporting cell fraction also contains functional HSCs. As we reported recently that the cell fate of transplanted HSCs could be flexibly changed depending on the intensity of haematopoietic stress,[54] the outcome of donor cells after transplantation are potentially skewed by the supporting cells, especially when a low dose of donor cells is used. Using MST transplantation, which does not require any supporting cells, we can evaluate the function of the transplanted cell fraction specifically. Based on this approach, we found that LT-HSCs did not improve early haematopoietic recovery even with increasing numbers of transplanted cells, indicating that LT-HSCs are specialised for late and long-term haematopoiesis after transplantation. On the other hand, we also demonstrated that ST-HSCs could maintain long-term haematopoiesis by transplanting a large number of ST-HSCs, although their efficiency was lower than that of the minimum subset. This result strongly supports our recent finding that ST-HSCs can behave like LT-HSCs after transplantation by reducing the haematopoietic stress per transplanted cell.[54] This may also explain why transplanting large numbers of HSCs grown *ex vivo*, including our result, show improved haematopoietic recovery despite a dramatic decrease in the frequency of HSC fractions. Furthermore, we showed that the modified MST, in which the minimum subset was the target for genetic modification, could largely reduce development costs and sustain therapeutic effects.

As we have utilised only a mouse model to identify the MST, further research is necessary to transfer this knowledge into a human context. *In vivo* tracking of human haematopoietic reconstitution after transplantation revealed bimodal peaks in the early and late stages,[55] strongly suggesting the existence of LT- and ST-HSCs in humans as well. The establishment of a purification method of these fractions will solve this in the future.

Finally, through detailed analysis of purified cell fractions within BM, we identified the minimum subset that was minimally necessary and critically sufficient for post-transplant haematopoiesis over time. Based on these findings, we have shown that MST transplantation can be a feasible method, which can be developed as a safer transplantation method by combining minimally invasive pre-treatment and *ex vivo* cell expansion, further applying to genetic disease by high-efficiency gene modification in the minimum subset.

Acknowledgements

Authorship contributions

Katsuyuki Nishi conceived, performed, analysed the experiments. Taro Sakamaki and Kay Sadaoka performed transplantation experiments. Kay Sadaoka and Momo Fujii provided technical supports for the experiments. Masanori Miyanishi conceived, performed, analysed, and oversaw the experiments. Katsuyuki Nishi, Masanori Miyanishi, James Y. Chen wrote the manuscript. Kay Sadaoka, Taro Sakamaki, Momo Fujii, Akifumi Takaori-Kondo, James Y. Chen provided comments on the manuscript.

Conflicts of interest

The authors declare no conflicts of interest in association with the present study.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. Gating scheme for haematopoietic cells.

Fig S2. Either LT- or ST-HSCs are not sufficient for the rapid haematopoietic recovery and long-term haematopoiesis.

Table SI. Antibodies used for flow cytometry.

Data S1. Supplemental materials and methods.

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