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Hoxb5 defines the heterogeneity of self-renewal capacity in the hematopoietic stem cell compartment

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A B S T R A C T

Self-renewal and multipotency are essential functions of hematopoietic stem cells (HSCs). To maintain homeostatic hematopoiesis, functionally uniform HSCs have been thought to be an ideal cell-of-origin. Recent technological advances in the field have allowed us to analyze HSCs with single cell resolution and implicate that functional heterogeneity may exist even within the highly purified HSC compartment. However, due in part to the technical limitations of analyzing extremely rare populations and our incomplete understanding of HSC biology, neither the biological meaning of why heterogeneity exists nor the precise mechanism of how heterogeneity is determined within the HSC compartment is entirely known. Here we show the first evidence that self-renewal capacity varies with the degree of replication stress dose and results in heterogeneity within the HSC compartment. Using the *Hoxb5*-reporter mouse line which enables us to distinguish between long-term (LT)-HSCs and short-term (ST)-HSCs, we have found that ST-HSCs quickly lose self-renewal capacity under high stress environments but can maintain self-renewal under low stress environments for long periods of time. Critically, exogeneous Hoxb5 expression confers protection against loss of self-renewal to Hoxb5-negative HSCs and can partially alter the cell fate of ST-HSCs to that of LT-HSCs. Our results demonstrate that Hoxb5 imparts functional heterogeneity in the HSC compartment by regulating self-renewal capacity. Additionally, Hoxb5-positive HSCs may exist as fail-safe system to protect from the exhaustion of HSCs throughout an organism's lifespan.

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1. Introduction

The fundamental question in stem cell biology of how stem cells can persist throughout the lifespan of an organism has yet to be fully understood. Among the possible factors, self-renewal capacity is arguably the most important function for stem cells to maintain their "stem-ness". Numerous studies have shown that tissue stem cells in various organs, including blood, intestines, and mammary glands exist [1] and are the cells responsible for the cellular homeostasis of their respective organs. However, the mechanisms behind how they do so while maintaining self-renewal capacity remains largely unknown. Determination of factors regulating self-renewal capacity necessitates isolation of the LT-HSC along with its most proximal progeny. However, a longstanding roadblock in the field has been the lack of a methodology to accurately do so. As a result, many experiments have compared impure fractions which contain HSCs along with many of their progenitors, contributing to noise and incongruent results.

HSCs have historically been one of the most purified tissue stem cells [2,3]. Using prospective isolation by immunostaining followed by transplantation, functional HSCs have been shown to be located within cell fraction defined as Lineage^{-}c-Kit^{+}Sca-1^{+}CD150^{+}CD34$^{-/lo}$Flk2^{-} (hereafter referred to as pHSCs) [4–6]. However, attempts to specifically isolate HSCs with long-term self-renewal capacity

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termed long-term hematopoietic stem cells (LT-HSCs) have had challenges [7]. To overcome this, in our previous work, we conducted multi-step screening to identify a single gene enabling us to isolate LT-HSCs—*Hoxb5*—that is exclusively expressed within the LT-HSC fraction [3]. Conventional immunostaining-based isolation along with separation by Hoxb5 expression revealed by transplantation that Hoxb5^{+} pHSCs behave as LT-HSCs and Hoxb5^{-} pHSCs behave as short-term hematopoietic stem cells (ST-HSCs), which only possess limited self-renewal capacity in the setting of transplantation [7]. Utilizing this technical advance—a tool to compare the LT-HSC along with its direct progeny, the ST-HSC—we sought to understand the mechanisms underlying self-renewal capacity and reveal how they contribute to the physiology of pHSCs.

2. Materials and methods

2.1. Animals

Mice were bred at our animal facility according to NIH or RIKEN guideline respectively. All animal protocols were approved by the Stanford University Administrative Panel on Laboratory Animal Care and the relevant committees of RIKEN Center for Biosystems Dynamics Research.

More detailed information of the materials and methods are described in the Supplementary materials and methods.

3. Results

3.1. Hoxb5 does not correlate with dormancy

To obtain insights into how Hoxb5^{+} pHSCs can persist and continue to produce mature blood cells after transplantation, we began by investigating global gene expression by sorting Hoxb5^{+} pHSCs and Hoxb5^{-} pHSCs respectively and conducting bulk RNA-seq. Bulk pHSCs and MPPs were utilized for comparison (Fig. 1A). We confirmed differential expression between the two populations by TMM normalized read counts of *Hoxb5*. To understand overall changes between fractions, a scatter plot of $\log_2$(TMM normalized read counts) of all genes in Hoxb5^{+} pHSCs vs. Hoxb5^{-} pHSCs and pHSC vs. MPPa were plotted (Fig. 1B). While 325 genes with significant differences were identified between pHSCs vs. multipotent progenitors (MPPs), only 22 genes were detected between Hoxb5^{+} pHSCs and Hoxb5^{-} pHSCs, with 7 genes shared between these two sets (Fig. 1C and D), again demonstrating the challenges faced by past studies attempting to study self-renewal comparing pHSCs as a whole to fractions like the MPP. Within the 340 genes, we conducted gene ontology (GO) analysis which revealed that genes differentially expressed in pHSCs vs. MPPa primarily consisted of cell cycle processes and cell proliferation (Fig. 1E). However, few differences between Hoxb5^{+} pHSCs vs Hoxb5^{-} pHSCs were found (Fig. 1F).

Previous studies have shown that dormancy plays an integral role in the hematopoietic hierarchy, with dormant HSCs residing at the very top, entering cell cycle only when necessary [8,9]. In our previous work we demonstrated that Hoxb5^{+} pHSCs give rise to their Hoxb5^{-} counterparts and that Hoxb5^{-} pHSCs behave as ST-HSCs in the setting of transplantation [3]. Based on these findings, it would be expected that Hoxb5^{+} pHSCs are the “dormant” HSC pool, and that Hoxb5^{-} pHSCs are not. To confirm whether this was true, we analyzed the cell-cycle kinetics of Hoxb5^{+} and Hoxb5^{-} pHSC by Hoechst and Ki-67 staining using downstream progenitors (namely Lineage^{-}c-Kit^{+}Sca-1^{+}, LKS) for comparison. Consistent with previous reports, within the LKS fraction we observed a decline in dormancy [10] (Supplementary Fig. 1A). However, interestingly, we found only minor changes in dormancy within the pHSC compartment regardless of Hoxb5 expression, indicating that the function of Hoxb5 is not correlated to dormancy status (Supplementary Fig. 1B). From classical models one might also expect pHSCs to less frequently enter cell cycle, allowing for shorter-lived progenitors like MPPs to instead replenish the blood supply. To investigate whether Hoxb5 expression may instead be involved in entering cell cycle we utilized an EdU (5-ethynyl-2′-deoxyuridine) *in vivo* label retention assay. From this, we demonstrated that while the uptake of EdU is delayed in Hoxb5^{+} pHSCs at one week, they quickly catch up with Hoxb5^{-} pHSCs and even MPPs by the two-week mark, suggesting that all the cells within pHSC compartment almost equally contribute to daily hematopoiesis and respond to external stimuli (Supplementary Figs. 1C and 1D).

Taken together, these data suggest that dormancy and cell cycle status may not account for the self-renewal differences between Hoxb5^{+} and Hoxb5^{-} pHSCs.

3.2. Dose dependent replication stress selectively enriches Hoxb5^{+} pHSC in bone marrow

Despite the lack of significant differences in gene expression and cell cycle status between Hoxb5^{+} vs Hoxb5^{-} pHSCs under homeostasis, our previous work revealed that these two populations behave vastly differently in the setting of transplantation—with only Hoxb5^{+} pHSCs capable of self-renewal as measured by reconstitution in the setting of serial transplantation [3]. Thus, we hypothesized that biological differences between Hoxb5^{+} and Hoxb5^{-} pHSCs may emerge under stress-inducing conditions (e.g., transplantation) rather than under homeostasis. To confirm this, first, we analyzed the bone marrow compartment after transplantation. Mice transplanted with 10 Hoxb5^{+} pHSCs post transplantation when compared to non-treated controls exhibited a marked selection for Hoxb5^{+} pHSCs (Fig. 2A). To exclude the possibility that conditioning with total body irradiation may pose widespread non-specific cytotoxicity which could have contributed to selection, we sought to stress the hematopoietic system with a more moderate stimulus, 5-FU (5-Fluorouracil), which largely only poses cytotoxicity to cells in cell-cycle [11]. Mice treated with 5-FU demonstrated a similar enrichment of Hoxb5^{+} pHSCs, confirming that replication stress is key to revealing the differences between Hoxb5^{+} and Hoxb5^{-} pHSCs (Fig. 2B and C). Transplantation into a lethally irradiated host represents perhaps the harshest level of artificial stress that a pHSC can undergo, as a handful of cells must repopulate an entire hematopoietic system. While we witnessed differences in Hoxb5^{+} and Hoxb5^{-} pHSCs in this high stress setting, we wondered whether differences would emerge in low stress settings over long periods of time. One of the most physiologically relevant and widespread models of low levels of stress is the act of aging. Thus, we sought to investigate whether we could still observe these changes even under physiological stress. For this, we examined the bone marrow of 2-year-old aged mice. When compared to 12-week-old controls, 2-year-old mice also demonstrated a significant enrichment of Hoxb5^{+} pHSCs (Fig. 2D). Given these findings, we postulated whether the degree of stress undergone by an HSC (imparted by natural or artificial means) could modulate the differences in self-renewal capacity of Hoxb5^{+} and Hoxb5^{-} pHSCs, resulting in selection for Hoxb5^{+} pHSCs (Fig. 2E).

3.3. Reciprocal HSC cell fate under low stress

To more directly analyze the impact of varying replication stress on the physiological differences between Hoxb5^{+} and Hoxb5^{-} pHSCs, we proposed to modulate replication stress via transplantation. From our previous work, we have shown that 10 Hoxb5^{+} pHSCs co-transplanted with 2×10^5 supporting bone

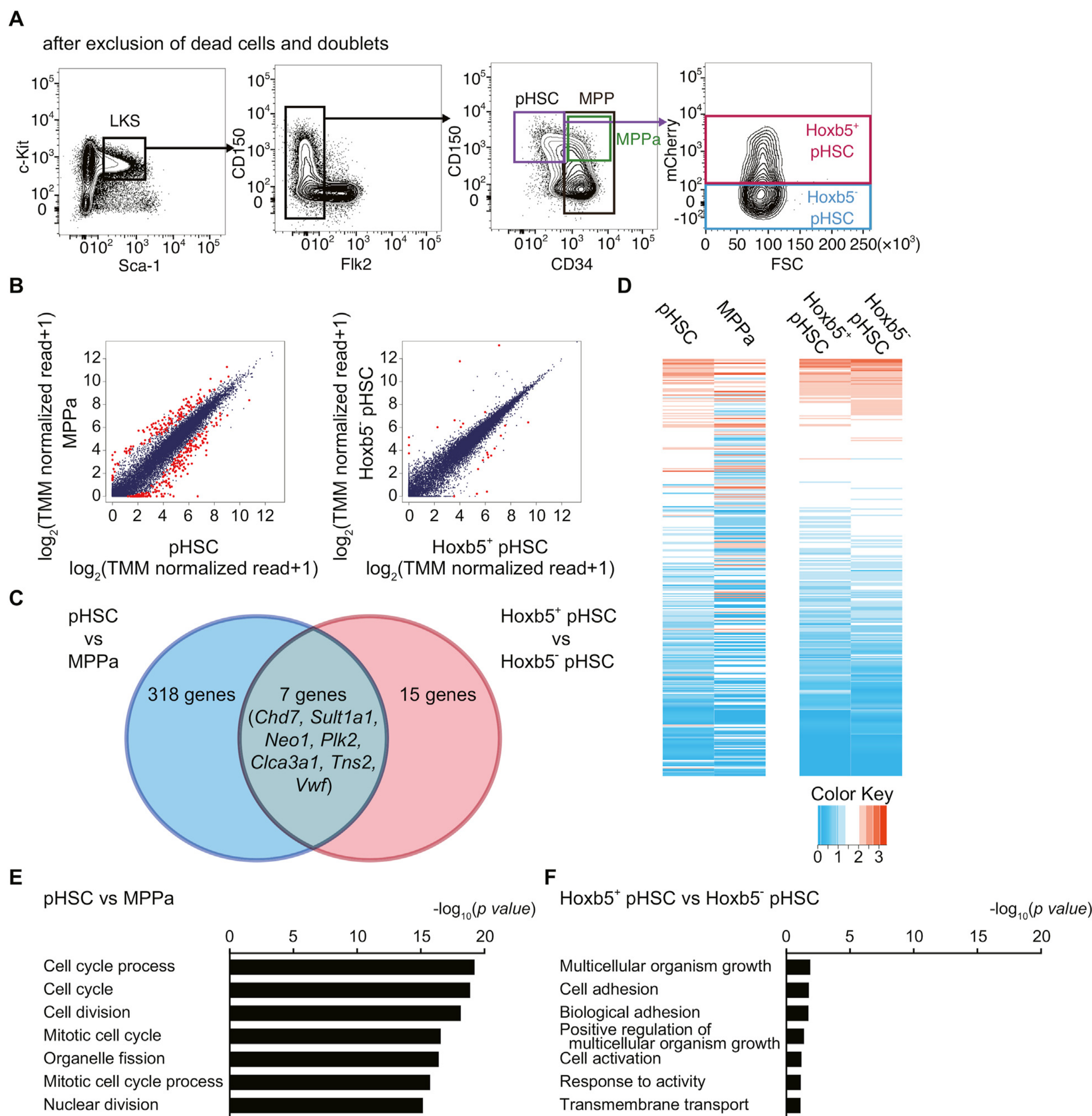

Fig. 1. Minimal biological difference between Hoxb5$^+$ and Hoxb5$^-$ pHSCs. (A) Gating scheme for MPPa, Hoxb5$^+$ pHSCs and Hoxb5$^-$ pHSCs of bone marrow from 12-week-old *Hoxb5*-reporter mice. (B) Scatter plot presenting the values of $\log_2$(TMM normalized read + 1) for each gene in pHSC versus MPPa and LT-HSC versus ST-HSC ($n = 3$). TMM, Trimmed means of M component. (C) Proportional Venn diagram of the differentially expressed genes (FDR < 0.05) in both scatter plot shown are shown. (D) A heat map representing the differentially expressed genes earned from (C) (FDR < 0.05). Each column shows the average $\log_{10}$(TMM normalized read + 1) ($n = 3$). (E, F) Gene ontology analysis by DAVID with the differentially expressed genes in each comparison (FDR < 0.05). Top 7 significantly enriched GO term are represented.

marrow cells are sufficient to provide multilineage reconstitution of the hematopoietic system of a lethally irradiated host. In contrast, 10 Hoxb5$^-$ pHSCs fail to produce long-term multilineage reconstitution [3]. Therefore, keeping the number of pHSCs transplanted constant, we decided to vary the amount of supporting bone marrow cells given during transplant instead, ultimately varying the demand on each donor cell. All recipients received either 10 Hoxb5$^+$ or Hoxb5$^-$ pHSCs with either 5×10^5 or 25×10^5 supporting bone marrow cells (Fig. 3A and B, and Supplementary Fig. 2). As shown in our previous work [3], recipients receiving 10 Hoxb5$^+$ pHSCs with either 5×10^5 or 25×10^5 supporting bone marrow cells demonstrated robust granulocyte chimerism at 16 weeks. Recipients receiving 10 Hoxb5$^-$ pHSCs with 5×10^5 supporting bone marrow cells exhibited little to no granulocyte chimerism at 16 weeks. However, recipients receiving 10 Hoxb5$^-$ pHSCs with an increased dose of supporting cells behaved similarly to their Hoxb5$^+$ counterparts, producing robust granulocyte chimerism with multilineage reconstitution (Fig. 3C and D and Supplementary Figs. 3A–B). This data suggests that Hoxb5$^-$ pHSCs behave like LT-HSCs under lower replication stress conditions. The behavior of Hoxb5$^-$ pHSCs and the loss of self-renewal capacity of pHSCs is regulated by the dose of replication stress.

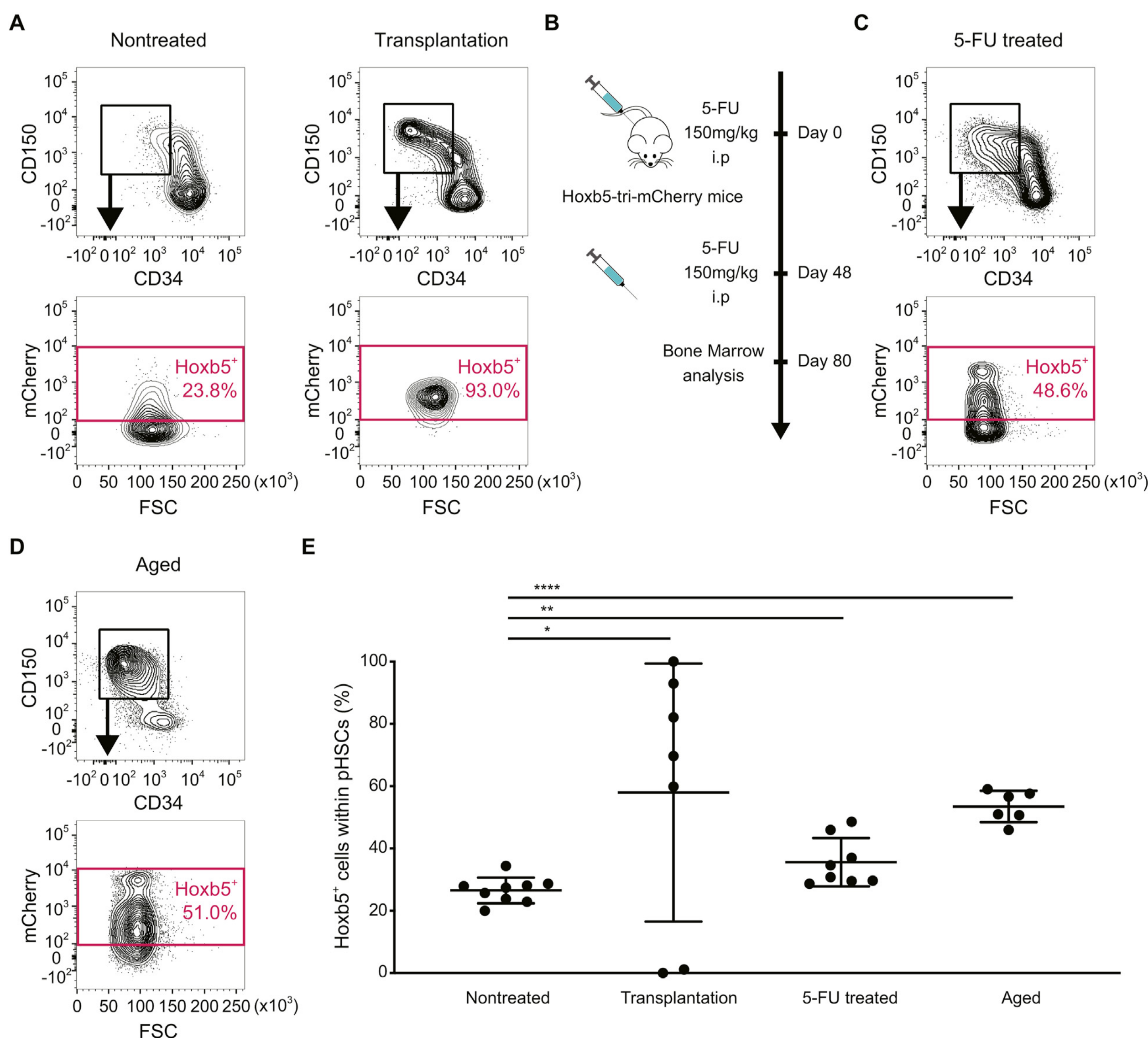

Fig. 2. Selection of Hoxb5$^+$ pHSC in settings of varying stress. (A) Flow cytometry plots of bone marrow from 24-week-old mice transplanted with 10 Hoxb5$^+$ pHSCs (CD45.2$^+$) and 2×10^5 supporting cells (CD45.1$^+$/45.2$^+$) 16 weeks before ($n = 7$) compared with that from 12-week-old *Hoxb5*-reporter mice. (B) Experimental schematic for 5-FU treatment. After intraperitoneal 5-FU injection (3mg/time; 150 mg/kg) at Day 0 and Day 48, analyze bone marrow at Day 80. (C) Flow cytometry analysis for frequency of Hoxb5$^+$ (mCherry$^+$) pHSCs in the bone marrow from 20-week-old mice 12 weeks after 5-FU treatment ($n = 8$). (D) Flow cytometry analysis for frequency of Hoxb5$^+$ (mCherry$^+$) pHSCs bone marrow from 2-year-old mice. ($n = 6$). (E) Summarized data about frequencies of Hoxb5 positive cells within pHSCs ($n = 9$ for untreated, $n = 7$ for transplantation, $n = 8$ for 5-FU, $n = 6$ for aged).

3.4. Exogenous Hoxb5 can protect HSCs under high stress

Given our results demonstrating that Hoxb5$^-$ pHSCs have the capacity to behave similarly to Hoxb5$^+$ pHSCs in settings of lower stress, we next questioned whether Hoxb5 itself plays an essential role. To test this, we utilized a lentiviral system to overexpress either a ZsGreen control or a fusion of *Hoxb5*-ZsGreen. Hoxb5$^+$ and Hoxb5$^-$ pHSCs were isolated by flow cytometry and transduced *ex vivo* with either ZsGreen control or *Hoxb5*-ZsGreen fusion lentivirus. After one day of transduction, live cells were re-sorted and either analyzed *in vitro* after 1-week incubation or 10 cells were transplanted into lethally irradiated recipients along with 2×10^5 supporting bone marrow cells (Fig. 4A and Supplementary Figs. 4A–E). Analysis of transduced Hoxb5$^-$ pHSCs and MPPa *in vitro* confirmed again that Hoxb5 did not affect cell cycle dynamics, despite previous claims on related *Hoxb* family members (Supplementary Fig. 5A) [12,13]. Colony size of control transduced Hoxb5$^-$ pHSCs were comparable to their control counterparts (Supplementary Fig. 4F). Additionally, transduction of *Hoxb5* lentivirus was able to slow down differentiation, with the colonies of transduced HSCs containing a higher percentage of LKS than their control counterparts, indicating that Hoxb5 overexpression may aid in preserving self-renewal (Supplementary Fig. 4G and Supplementary Fig. 5B). To examine whether this effect persisted *in vivo* we analyzed the peripheral blood chimerism of transplanted mice. Exogenous Hoxb5 expression in Hoxb5$^-$ pHSCs was able to significantly preserve self-renewal capacity, with primary recipients demonstrating stable multilineage reconstitution at 16 weeks and limited reconstitution in the secondary recipients (Fig. 4B–D, Supplementary Fig. 6, and Supplementary Figs. 7A–B). Hoxb5$^+$ or Hoxb5$^-$ pHSCs transduced with ZsGreen control lentivirus behaved as expected, with Hoxb5$^+$ pHSC recipients exhibiting

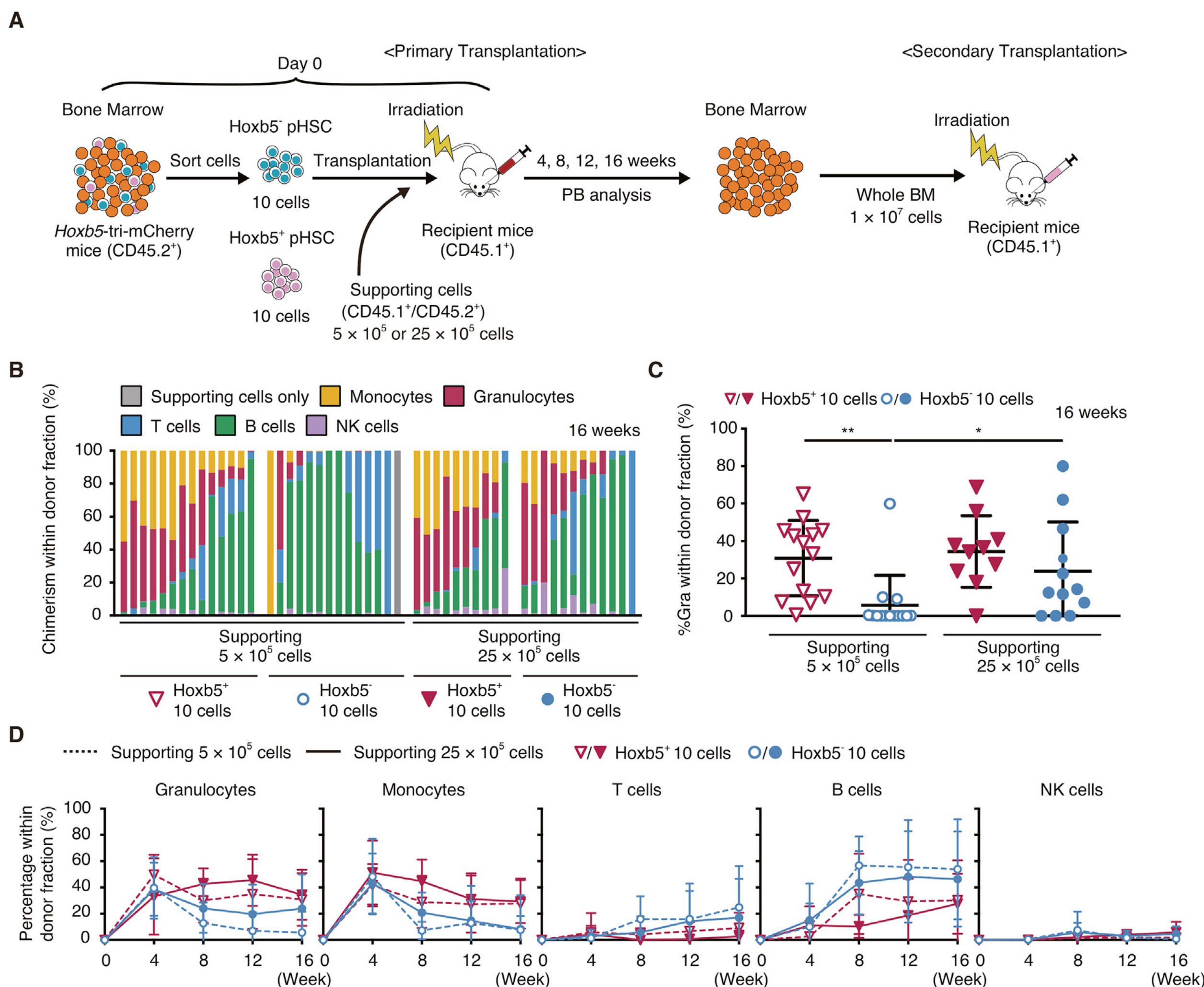

Fig. 3. Replication stress alters cell fate of Hoxb5⁻ pHSCs. (A) Experimental schematic for the stress titration assay. Ten donor cells (Hoxb5$^+$ or Hoxb5$^-$ pHSCs) were transplanted along with variable numbers of whole bone marrow (5×10^5 or 25×10^5 cells) from CD45.1/CD45.2 mice into lethally irradiated CD45.1 recipient mice. Peripheral blood was analyzed every four weeks. For secondary transplants, 1×10^7 whole bone marrow cells were transferred from the primary recipient mice. Peripheral blood was analyzed at 4, 12, and 33 weeks after secondary transplantation. (B) Reconstitution kinetics at 16 weeks in primary recipient mice. Each bar represents an individual mouse. ($n = 14$ for 10 Hoxb5$^+$ pHSCs with 5×10^5 supporting cells; $n = 14$ for 10 Hoxb5$^-$ pHSCs with 5×10^5 supporting cells; $n = 10$ for 10 Hoxb5$^+$ pHSCs with 25×10^5 supporting cells; and $n = 12$ for 10 Hoxb5$^-$ pHSCs with 25×10^5 supporting cells). (C) Granulocyte production at 16 weeks discussed in (B). (D) Average donor lineage kinetics in each group discussed in (A).

stable peripheral blood chimerism at 16 weeks and Hoxb5$^-$ pHSC recipients exhibiting no peripheral blood chimerism at 16 weeks (Fig. 4B–D and Supplementary Fig. 6).

This protective effect on self-renewal capacity can be observed only in the pHSC fraction and not in downstream fractions such as the MPPa compartment. No other effects such as skewing of lineage output were found (Supplementary Fig. 5). Taken together with our previous findings, we concluded that one of the physiological roles of Hoxb5 in the HSC compartment is to prepare a fail-safe system to protect from exhaustion throughout the lifespan by conferring tolerance against cell replication stress and that heterogeneity within the HSC compartment will vary depending on extrinsic environments.

4. Discussion

For the past few decades, the classical model of "stem-ness" has largely revolved around a one-directional model—a model where LT-HSCs reside at the top of the hematopoietic tree and produce their downstream progeny in a stepwise fashion [14,15]. Thus, current consensus in the field is that in homeostasis, a pool of LT-HSCs persists to perpetuate the hematopoietic system of an organism throughout its lifetime; all progenitors, including ST-HSCs, further differentiate, never staying at the same level of cell status for extended periods of time [16]. For this to occur, the maintenance of self-renewal within the HSC compartment is crucial. Numerous studies have strongly supported the notion that HSCs stay in dormancy to minimize the risk of losing self-renewal capacity during cell replication [17]. On the other hand, the empiric finding that only LT-HSCs can survive in the setting of transplantation [7]—an environment which requires rapid and repeated cell replication—suggests that some active mechanisms protecting self-renewal capacity during cell replication must exist [18]. In this study, we have shown that Hoxb5$^-$ pHSCs, which behave as ST-HSCs in the setting of transplantation, have almost identical cell cycle kinetics as Hoxb5$^+$ pHSCs. Furthermore, both Hoxb5$^+$ and Hoxb5$^-$ pHSCs almost equally contribute to hematopoiesis with external stimulation, calling into question whether dormancy truly is the

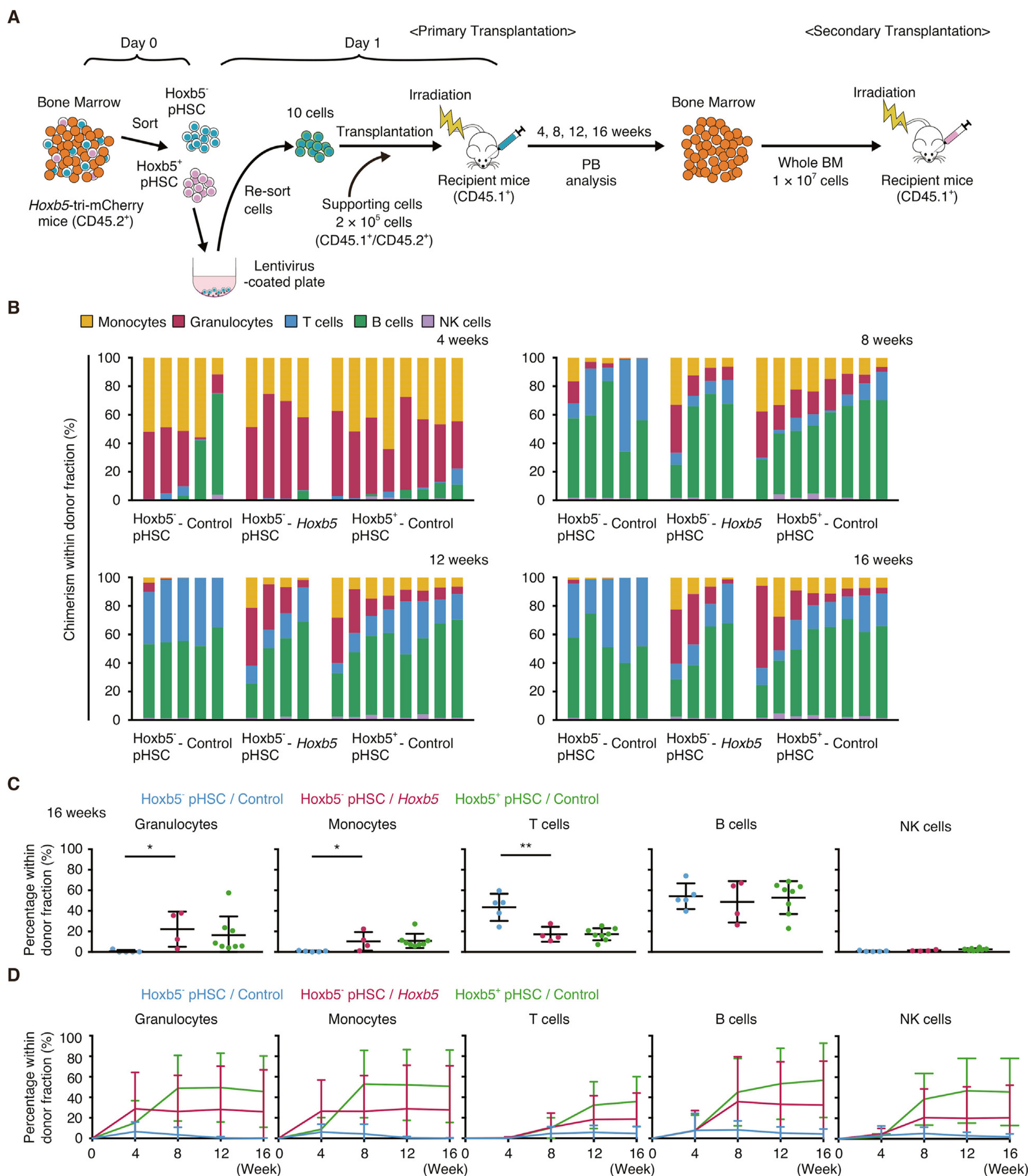

Fig. 4. Exogenous Hoxb5 expression reinforces self-renewal capacity. (A) Experimental schematic for lentiviral transduction of Hoxb5⁻ pHSCs. Transduced cells were used for transplantation assays. Ten donor cells (Hoxb5⁺ or Hoxb5⁻ pHSCs) were transplanted along with 2×10^5 whole bone marrow cells from CD45.1/CD45.2 mice into lethally irradiated CD45.1 recipient mice. Peripheral blood was analyzed every four weeks. For secondary transplants, 1×10^7 whole bone marrow cells were transferred from the primary recipient mice. (B) Peripheral blood chimerism at 4, 8, 12, and 16 weeks in recipients receiving 10 transduced Hoxb5⁻ or Hoxb5⁺ pHSCs along with 2×10^5 whole bone marrow cells from CD45.1/CD45.2 mice. ($n = 5$ for Hoxb5⁻ pHSCs transduced with ZsGreen control; $n = 4$ for Hoxb5⁻ pHSCs transduced with *Hoxb5*-ZsGreen; and $n = 8$ for Hoxb5⁺ pHSCs transduced with ZsGreen control). Each bar represents an individual mouse. (C) Sixteen-week peripheral blood chimerism in each individual lineage (granulocytes, monocytes, T cells, B cells, and NK cells) within total donor cells ($n = 5$ for Hoxb5⁻ pHSCs transduced with ZsGreen control; $n = 4$ for Hoxb5⁻ pHSCs transduced with *Hoxb5*-ZsGreen; and $n = 8$ for Hoxb5⁺ pHSCs transduced with ZsGreen control). Scatter plots summarize each group. (D) Average donor lineage contribution kinetics in 10 cell primary transplants at 4, 8, 12, and 16 weeks ($n = 5$ for Hoxb5⁻ pHSCs transduced with ZsGreen control; $n = 4$ for Hoxb5⁻ pHSCs transduced with *Hoxb5*-ZsGreen; and $n = 8$ for Hoxb5⁺ pHSCs transduced with ZsGreen control).

underlying regulator of self-renewal. Our transplantation experiments in this study demonstrate that Hoxb5⁻ pHSCs behave differently depending on replication stress. In settings of high stress (low numbers of supporting bone marrow cells), Hoxb5⁻ pHSCs behave like ST-HSCs, failing to give rise to multi-lineage reconstitution, and eventually lose their capacity for self-renewal. In the process of losing self-renewal, Hoxb5⁻ pHSCs disappear from the bone marrow, resulting in the disappearance of short-lived cell fractions, such as granulocytes from the peripheral blood, while long-lived cell fractions, such as memory B and T cells, remain in the peripheral blood for an extended period. However, if the level of stress that these cells are exposed to is modified or reduced, these cells have the capacity to behave like LT-HSCs, retaining their capacity for multipotency and self-renewal. Perhaps most importantly, the cellular fate of Hoxb5⁻ pHSCs can be partially reversed to that of LT-HSCs by exogeneous overexpression of Hoxb5. $Hoxb5^+$ pHSCs are located at the top of the hierarchy and as they lose Hoxb5 expression, they lose tolerance to replication stress. Directly downstream, Hoxb5⁻ pHSCs behave as ST-HSCs under high stress conditions but can behave as LT-HSC under low stress conditions.

These behavioral differences between $Hoxb5^+$ and Hoxb5⁻ pHSCs result in a functional heterogeneity within the HSC compartment. To maintain hematopoietic homeostasis, HSCs must leave their niche and differentiate. Under high stress conditions (e.g., total body irradiation), the whole niche must be replaced. To do so, newly transplanted HSCs need to rapidly proliferate, a process which selectively enriches for $Hoxb5^+$ pHSCs which are “stress tolerant”, ultimately resulting in homogeneity in the HSC compartment.

On the other hand, under low stress conditions (e.g., aging) only a small number of pHSCs must be mobilized to restore blood loss, resulting in little selection and a more heterogenous HSC pool. However, when this process is repeated over an individual’s lifetime, the result is similar to that of high stress conditions—a homogeneous HSC pool in which $Hoxb5^+$ pHSCs are selectively enriched. Previous studies have implicated HSCs or their direct downstream progeny as the main players in native hematopoiesis [18–21]. Given our findings that both $Hoxb5^+$ and Hoxb5⁻ pHSC possess the physiologic potential to behave as LT-HSCs in the appropriate settings, it is possible that both play a role in native hematopoiesis, and that the inconsistent conclusions of previous studies were simply due to insufficient labeling techniques.

Additionally, the model that we have provided may also serve as a conceptual answer as to why some HSCT ultimately result in graft failure, as they lack insufficient cell dosages to attenuate pHSC stress [22,23]. If our model holds true to other tissue stem cells, settings of high or repeated stress (e.g., chronic inflammation or repeated tissue damage) may accelerate selection or depletion of tissue stem cells, potentially leading to irreversible changes and dysfunction [24]. Thus, if we can reveal the mechanisms behind how stress tolerance is regulated, we may be able to control or even rejuvenate tissue stem cells in the future.

Author contributions

T.S. and K.S.K. contributed equally to this work, and either has the right to list himself first in bibliographic documents. M.M. conceived, performed, analyzed, and oversaw the experiments. T.S. performed transplantation and *in vivo* experiments and analyzed data. K.S.K. performed transplantation and analyzed data. K.N. performed and analyzed RNA sequencing experiments. J.Y.C supported the design and analysis of the experiments. K.S. and M.F. provided technical supports for the experiments and helped to generate figures. M.M., K.S.K., T.S., J.Y.C., K.N., K.S. wrote the manuscript. A.T. and I.L.W. provided comments on the manuscript.

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Declaration of competing interest

We have no competing interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.12.077.

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