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Maintenance and enhancement of human peripheral blood mobilized stem/progenitor cell engraftment after ex vivo culture via an HDACi/SALL4 axis (3465)

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Currently, there is a growing need for culturing hematopoietic stem/progenitor cells (HSPCs) in vitro for various clinical applications including gene therapy. Compared with cord blood (CB) CD34$^+$ HSPCs, it is more challenging to maintain or expand CD34$^+$ peripheral blood mobilized stem/progenitor cells (PBSCs) ex vivo. To fill this knowledge gap, we have systematically surveyed 466 small-molecule drug compounds for their potential in cytokine-dependent expansion of human CD34$^+$CD90$^+$ HSPCs. We found that epigenetic modifiers, especially histone deacetylase inhibitors (HDACis), could preferentially maintain and expand these cells. In particular, treatment of CD34$^+$ PBSCs with a single dose of HDACi trichostatin A (TSA) at a concentration of 50 nmol/L ex vivo yielded the greatest expansion (11.7-fold) of CD34$^+$CD90$^+$ cells when compared with the control (dimethyl sulfoxide [DMSO] plus cytokines) group. Additionally, TSA-treated PBSC CD34$^+$ cells had a statistically significant higher engraftment rate than the control-treated group in xenotransplantation experiments. Mechanistically, TSA treatment was associated with increased expression of HSPC-related genes such as *GATA2* and *SALL4*. Furthermore, TSA-mediated CD34$^+$CD90$^+$ expansion was reduced by downregulation of SALL4 but not GATA2. Overall, we have developed a robust, short-term (5-day), PBSC ex vivo maintenance/expansion culture technique and found that the HDACi–TSA/*SALL4* axis is important for the biological process.

Hematopoietic stem cells (HSCs) possess the unique capacity to self-renew and give rise to all types of mature cells within the blood and immune systems. HSC self-renewal is regulated by both intrinsic and extrinsic signals [1–3]. Genes and pathways that are functionally linked to self-renewal of HSCs include CEBPα [4], Notch ligands [5,6], Angiopoietin-like proteins [7], SALL4 [8], homeobox protein B4 (HOXB4) [9], and c-MPL [10]. Although self-renewal divisions of HSCs clearly occur in vivo, induction of such events ex vivo has been difficult. This is due to the fact that despite our progress in understanding the molecular factors that support self-renewal and differentiation of the hematopoietic system in vivo, not much is known about the modulation of these factors ex vivo [11].

Currently, there is a growing need for culturing peripheral blood mobilized stem/progenitor cells (PBSCs) in vitro for transplant-related applications such as gene therapy [12] and genome editing via TALENs or CRISPR/Cas9 [13]. Furthermore, the same PBSC in vitro culture technique has the potential to be used for HSCP expansion for poor autologous mobilizations to avoid additional collections [14].

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Unlike embryonic stem (ES) cells, expansion of human $CD34^+$ HSPCs ex vivo in culture is associated with differentiation and loss of "stemness." This is, at least in part, due to the effects of the cytokines used under the culture conditions, which induce HSPCs to proliferate and differentiate. Several approaches have been reported to modify the cytokine-based culture conditions to achieve HSPC expansion ex vivo. These include the use of prostaglandin E2 [15,16], pleiotrophin [17], SR1 [18], UNC0638 [19], pyrimidoindole derivatives [20] and tetraethylenepentamine (TEPA) [21,22]. However, even after several decades of research, the quest for condition(s) that are able to stimulate self-renewal ex vivo continues [23]. Compared with cord blood (CB) $CD34^+$ cells, it is more challenging to maintain and expand $CD34^+$ PBSCs ex vivo [24,25].

In this study, we searched for robust and short-term ex vivo culture conditions that can maintain or expand PBSCs without the loss of their "stemness." We utilized a short-term assay (5 days) that can be easily modified for use in the current clinical HSPC transplantation setting and co-expression of CD34 and CD90 to identify compounds with the potential for PBSC expansion. After surveying 466 compounds, including multiple chromatin modifiers, we found that a single dose of trichostatin A (TSA) treatment led to the greatest expansion of these cells. We further characterized the TSA-mediated PBSC maintenance/expansion functionally and mechanistically. Moreover, we propose a model of an HDACi–TSA/SALL4 axis in the maintenance and expansion of HSPC ex vivo.

Methods

Isolation of PBSCs and CB $CD34^+$ cells and ex vivo culture

PBSCs were collected after granulocyte colony-stimulating factor (G-CSF) mobilization and enriched by $CD34^+$ immunoselection. Fresh CB collections were obtained from the Cell Manipulation Core Facility at the Dana-Farber Cancer Institute (DF/HCC, Boston, MA) according to guidelines established by the DF/HCC institutional review board. CB cells were isolated by density centrifugation on Ficoll–Paque (Stem Cell Technologies, Vancouver, BC, Canada) and enriched using the CD34-positive cell isolation kit (Stem Cell Technologies). We allotted 2×10^4 cells per well, which were incubated in Iscove's modified Dulbecco's medium (IMDM) containing 30% fetal bovine serum (FBS; GIBCO) supplemented with $1 \times$ CC100 cytokine mix (stem cell factor [SCF], Flt3 ligand [FL], interleukin-3 [IL-3], and IL-6; Stem Cell Technologies) or a serum-free expansion system (StemSpan SFEM II, SCF, FL, IL3, and IL6; Stem Cell Technologies) supplemented with $1 \times$ CC100 cytokine mix for 5 to 7 days without changing medium.

Engraftment of $CD34^+$ cells in NSG mice

NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, The Jackson Laboratory, ME, USA) mice were bred and maintained in the Children's Hospital Boston animal facility. All animal work was approved by and done according to the guidelines of the institutional animal care and use committee under Protocol 10-10-1832. The CB or PBSC $CD34^+$ cells treated with TSA or DMSO were injected intravenously via the tail vein into sublethally irradiated (220 rad) 8- to 16-week-old NSG mice. Transplantation or intravenous administration was performed within 24 h after irradiation. Peripheral blood (PB) chimerism was monitored at 8 weeks posttransplantation. Bone marrow (BM) chimerism was monitored at 8 and 18 weeks posttransplantation. These samples were subsequently subjected to flow cytometry analysis utilizing fluorescein isothiocyanate (FITC)-conjugated anti-human CD45 antibody and allophycocyanin (APC)-conjugated anti-mouse CD45 antibody (eBioscience, San Diego, CA). The percentage of human CD45+ cells was calculated as follows: % human CD45+ cells = No. of human CD45+ cells/(No. of human CD45+ cells + No. of murine CD45+ cells) × 100. A threshold of 0.2% human CD45+ cells was established as a reliable predictor of positive engraftment. BM cells from primary recipients were reinfused into sublethally irradiated (220 rad) secondary recipient mice. Mice were sacrificed 8 weeks after transplantation, and a threshold of 0.025% human CD45+ cells was established as a reliable predictor of positive engraftment. For limiting dilution analysis, a threshold of 2.8% human CD45+ cells was established as a reliable predictor of positive engraftment. The frequency of human severe combined immunodeficiency (SCID)-repopulating cells (SRCs) was calculated using L-Calc software (StemCell Technologies Inc.)

Statistical analysis

Results are expressed as the mean ± standard deviation (SD) or standard error (SE) when appropriate. Statistical differences were evaluated using Student's t test, with significance at p values ≤ 0.05.

Additional materials and methods are listed supplemental material.

Results

HTS approach to identifying small-molecule compounds including chromatin modifiers for the maintenance/expansion of $CD34^+CD90^+$ PBSCs ex vivo

We first reviewed HSPC assays. The xenotransplantation model provides a direct quantitative in vivo assay to measure human HSPC functional activity, and HSPCs are therefore also called SRCs. Among the published studies, ex vivo cultured $CD34^+CD90^+$ cells have been well established as being SRCs or having marrow-repopulating potential [26]. Therefore, in our study, we used co-expression of CD34 and CD90 to identify compounds with the potential for PBSC maintenance/expansion. We developed a high-throughput screening (HTS) assay based on the co-expression of these two surface markers. Primary human PBSC $CD34^+$ cells were cultured in 96-well plates with the addition of cytokines and a drug panel for 3 to 5 days. The cells were then evaluated for expression of CD34 and CD90 by flow cytometry. Using this approach, we

surveyed 446 U.S. Food and Drug Administration (FDA)-approved compounds and 20 additional small-molecule drugs, including a panel of chromatin modifiers (Supplementary Table E1, online only, available at www.exphem.org).

Ten compounds were considered to be positive hits based on the increased percentage of $CD34^+CD90^+$ cells. Among them, five were histone deacetylase inhibitors, namely, TSA, DLS3, valproic acid (VPA), SAHA, and Merk60. The others were the H3K9me2 methyltransferase inhibitor UNC0638 and Dot1 inhibitors EPZ4777 and EPZ5676 (Figure 1A). A mild increase in the $CD34^+CD90^+$ percentage was also observed with treatment of the antagonist of the aryl hydrocarbon receptor (SR1) and lysine-specific demethylase 1 (LSD1) inhibitor tranylcypromine (TCP) (Figure 1A). None of the 446 FDA-approved drug compounds were positive in the screen. In addition, epigenetic modifiers such as the BET inhibitor JQ1-S and others were all negative.

By measuring the absolute $CD34^+CD90^+$ cell number, we noted that although most of the positive hits could expand the $CD34^+CD90^+$ population compared with control (DMSO plus cytokines) $CD34^+$ cells, there was a range of ability. Among the 10 positive hits, treatment with TSA, VPA, EPZ4777, and EPZ5676 resulted in the greatest (11.7-, 11.6-, 8.7-, and 10.9-fold) expansion of $CD34^+CD90^+$ cells compared with the control based on absolute cell number (Figure 1B) and expansion fold (Supplementary Figure E1A, online only, available at www.exphem.org). These compounds did not markedly expand $CD34^+$ and $CD34^+CD90^-$ cells in 5 days (Supplementary Figure E1B). To further enhance expansion, we combined the treatment of SR1 with TSA (Supplementary Figure E2A, online only, available at www.exphem.org).

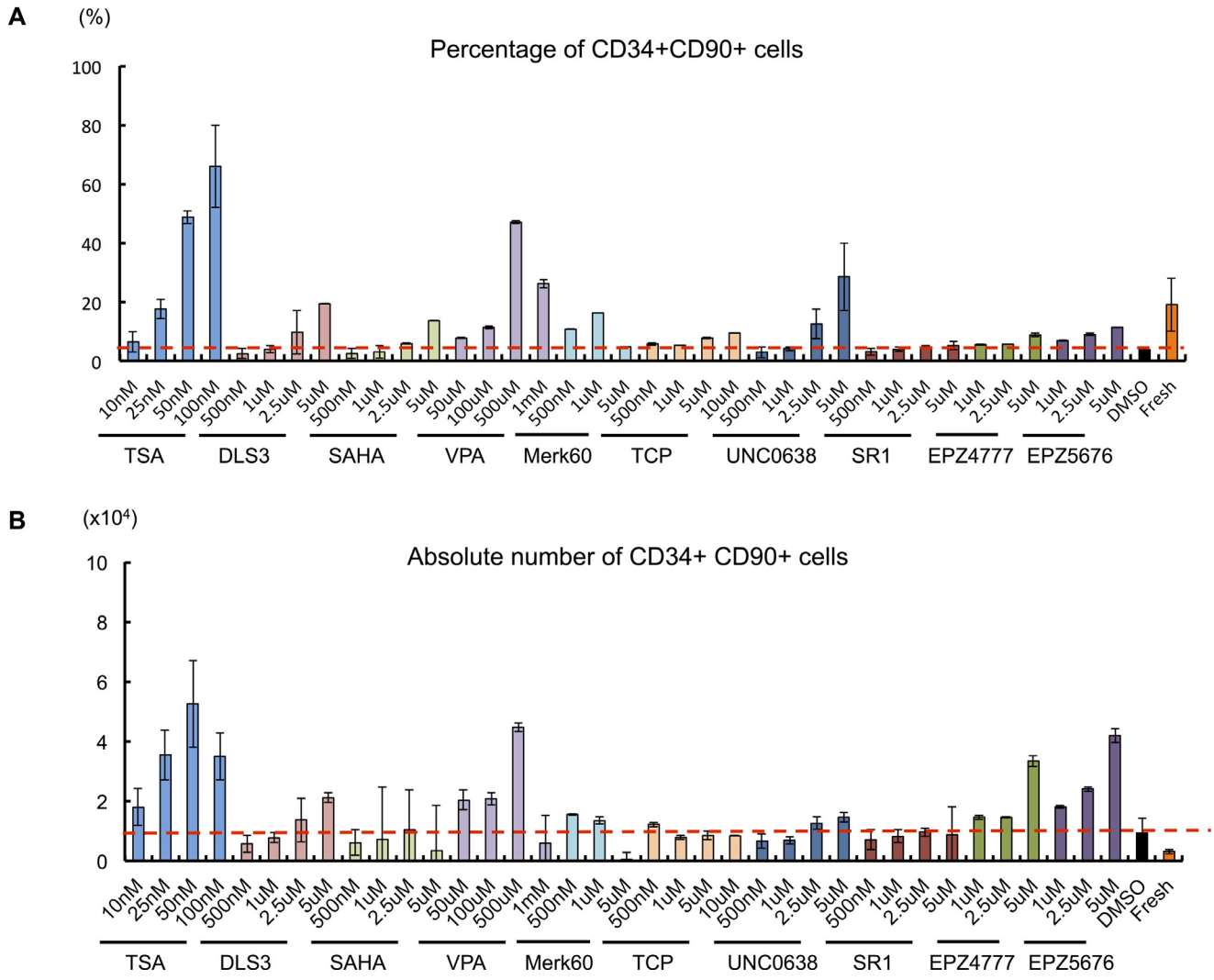

Figure 1. Screening for compounds that increase $CD34^+CD90^+$ cells from PBSCs. **(A)** Effect of the compounds on the percentage of $CD34^+CD90^+$ expression after 5 days of culture. **(B)** Absolute number of $CD34^+CD90^+$cells after 5 days of culture with the compounds. Each value represents the mean ± SE of two or three independent experiments.

However, this combination treatment only mildly improved the expansion of $CD34^+CD90^+$ cells. In addition, we observed that HDACis such as TSA and MS275 can also expand the $CD34^+CD90^+$ population from cord blood (Supplementary Figure E3, online only, available at www.exphem.org).

Overall, our findings suggest that epigenetic modifiers, especially HDAC inhibitors, have the potential to expand $CD34^+CD90^+$ cells under our short-term culture conditions.

TSA treatment of $CD34^+$ PBSCs ex vivo leads to preferential expansion of $CD34^+CD90^+$ cells

From our screening, TSA demonstrated the highest $CD34^+CD90^+$ ex vivo expansion potential at a single dose with the lowest functional concentration of 50 nmol/L, as higher concentrations are toxic to cells (Figure 1, Supplementary Figure E1A). We then focused on TSA to investigate its role in PBSC maintenance/expansion in greater detail. Serial-time-point studies were carried out. We observed that TSA treatment led to a preferential expansion of $CD34^+CD90^+$ cells on days 3, 5, and 7 compared with control cells (Figure 2B, C; $p < 0.05$). There was a significant increase in $CD34^+CD90^+$ cells 24 hours post-TSA treatment (Supplementary Figure E2B). To examine whether TSA is required for further expansion of this population during the subsequent culture period, we performed the following experiments. Because the half-life of TSA in culture is 3 days, we washed out TSA after 24 hours of treatment, which resulted in decreased $CD34^+CD90^+$ cells (Supplementary Figure E2B). This suggests that the TSA-mediated expansion of $CD34^+CD90^+$ cells is reversible if TSA treatment is terminated prematurely. We next examined the effects of TSA treatment on cell growth. We found that the overall cell growth was about 2.5 times lower with TSA treatment than with control treatment (Figure 2A; TSA $10.7 \times 10^4 \pm 1.12$/well vs. TSA $28.3 \times 10^4 \pm 0.49$/well, $p < 0.05$). We further quantitated the absolute number of $CD34^+$ cells (Supplementary Figure E4A, B, online only, available at www.exphem.org) and $CD34^+CD90^+$ subpopulations (Figure 2D). We observed that the absolute number of $CD34^+CD90^+$ cells was significantly increased by TSA treatment, especially from days 5 to 7.

Next, we asked whether the smaller total number of nucleated cells observed after TSA treatment was due to a delay in cell proliferation. We performed a cell division-monitoring assay using the carboxyfluorescein succinimidyl ester (CFSE) fluorescent dye on $CD34^+$ cells during the culture period. Although 66.0% of control $CD34^+$ cells divided more than four times by day 5, only 9.02% of TSA-treated cells went through a similar number of cell divisions (Figure 2E). Comparable results were observed on day 7. In addition, there was no significant difference in cell cycle progression between TSA-treated and control cells on propidium iodide (PI) staining (Supplementary Figure E4C). To investigate whether the smaller total number of nucleated cells observed after TSA treatment was due to increased apoptosis, we performed annexin V and PI staining with TSA-treated or control cells on days 3, 5, and 7. No difference in apoptosis was observed between the two treatment groups (Supplementary Figure E4D). We then asked whether TSA treatment could lead to increased numbers of $CD34^+CD90^+$ cells through proliferation. Using the CFSE assay, we observed that $CD34^+CD90^+$ cells divided on TSA treatment. Because $CD34^+CD90^+$ is the dominant population in TSA treatment, and these cells divide more slowly than the $CD34^+CD90^-$ cells (Supplementary Figure E4E), these observations could explain why there are fewer cell divisions in the TSA-treated group (Figure 2E).

In addition, when we tested the TSA-mediated protocol in a serum-free StemSpan SFEM II medium, we observed a similar result with respect to expansion of the $CD34^+CD90^+$ population (Supplementary Figure E5, online only, available at www.exphem.org).

In summary, the lesser expansion of TSA-treated CD34 cells was due to slower cell division rather than apoptosis.

Treatment of $CD34^+$ PBSCs with TSA enhances marrow-repopulating potential in vivo

Compared with CB cells, $CD34^+$ PBSCs have reduced marrow-repopulation potential (Supplementary Figure E6, online only, available at www.exphem.org) (24,25). To validate whether TSA-treated PBSC $CD34^+$ cells have enhanced function in vivo, we performed xenotransplantation experiments. During the ex vivo course of $CD34^+$ PBSC culture, we noted that on day 5, the majority of control cells were $CD34^+CD90^-$, whereas a large portion of TSA-treated cells were still $CD34^+CD90^+$ (Figure 2B). To compare the marrow-repopulation potential of control and TSA treatments, after culture for 5 days we transplanted control cells and TSA-treated cells into NSG mice to evaluate HSPC function. Eight weeks after transplantation, the mice were sacrificed and their peripheral blood (PB) and bone marrow (BM) samples were analyzed (Supplementary Figure E7A, online only, available at www.exphem.org; Figure 3A). Mice transplanted with 3×10^6 TSA-treated day 5 progeny exhibited a higher level of human hematopoietic cell engraftment (average engraftment of 32.74%), whereas transplantation of the same number of control cells resulted in a lower level of BM engraftment (average engraftment of 18.43%, $p < 0.005$).

To assess the degree of HSPC maintenance/expansion, we used limiting dilution analysis to compare the frequency of SRCs in the progeny of TSA-treated and control cells.

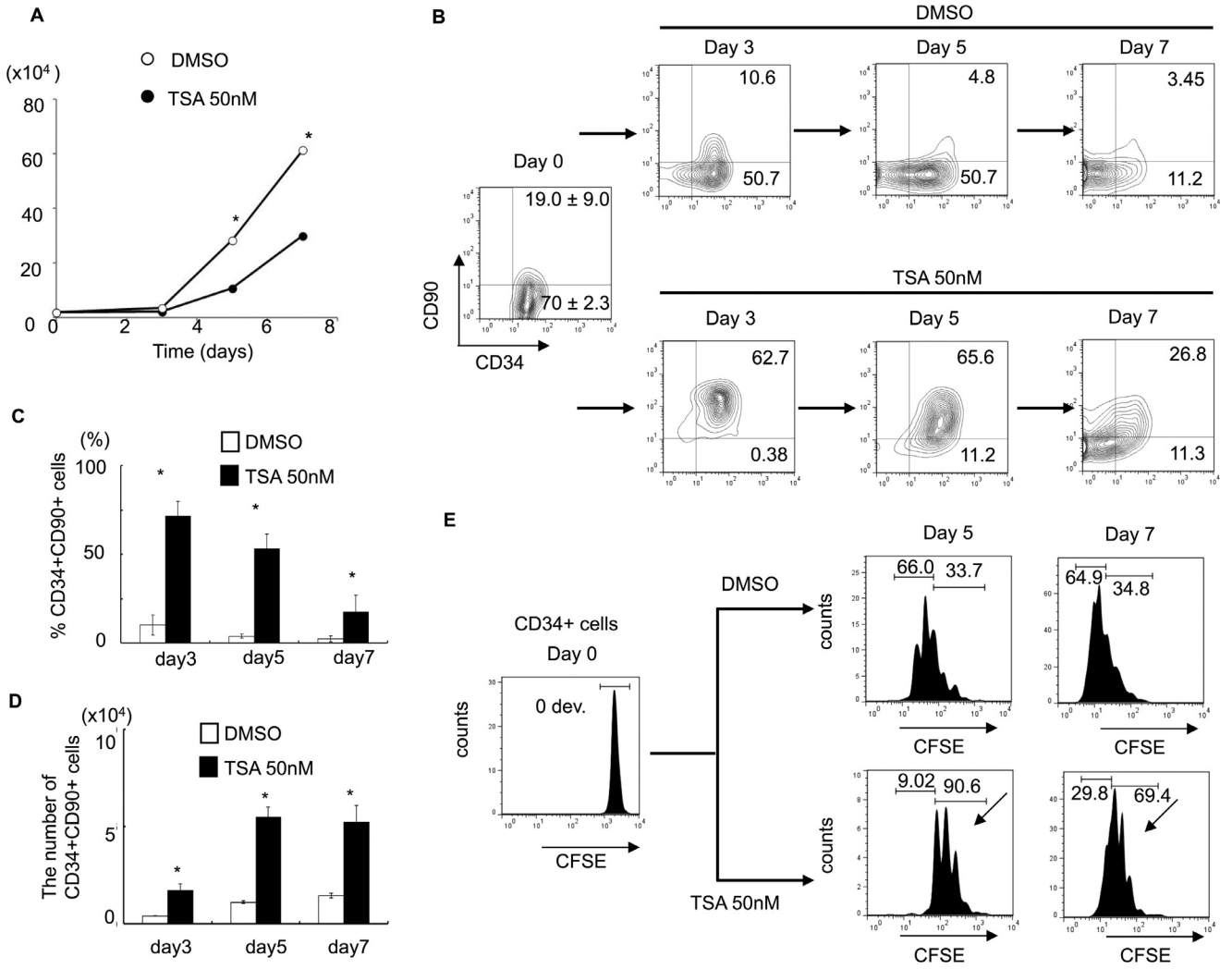

Figure 2. TSA-treated culture leads to preferential expansion of slowly dividing PBSC $CD34^+CD90^+$ PBSCs. **(A)** Cell growth of PBSC $CD34^+$ cells cultured in the presence of cytokines with various concentrations of TSA or with DMSO (control) after 5 days of culture. The data are the means of three independent experiments. Error bars indicate the SD. **(B)** Results of time course experiment from day 0 to 7 days revealing that TSA increased the number of $CD34^+CD90^+$ cells. The data are the means of three independent experiments. **(C)** Percentage of $CD34^+CD90^+$ cells at 3, 5 and 7 days of culture in the presence or the absence of 50 nmol/L TSA. Error bars indicate the SD. *$p < 0.05$. **(D)** Absolute number of $CD34^+CD90^+$ cells at 3, 5, and 7 days of culture in the presence or the absence of 50 nmol/L TSA. Error bars indicate the SD. *$p < 0.05$. **(E)** CSFE-labeled $CD34^+$ cells were cultured in the presence of cytokines with TSA or DMSO treatment for 7 days. Shown here is a representative (one of three experiments) flow cytometric profile of CFSE fluorescence intensity after 5 and 7 days of culture. The *arrow* indicates the fraction of cells that have undergone fewer cell divisions when compared with $CD34^+CD90^-$ cells.

Poisson distribution analysis revealed SRC frequencies of 1 in 247,567 (95% confidence interval [CI]: 1 in 367,071 to 1 in 166,969) in TSA-treated cells and 1 in 1,164,807 (95% CI: 1 in 1,784,517 to 1 in 760,304) in control cells, indicating the effective expansion of SRC number (about 4.7-fold) with TSA treatment (Figure 3A). The SRC frequency of fresh untreated CD34+ cells was 1 in 355,285 (95% CI: 1 in 555,478 to 1 in 227,241) (Figure 3B).

We next asked whether cells treated with TSA have the ability to give rise to mature hematopoietic cells. We observed that TSA-treated cells were capable of differentiating into multiple lineages after transplantation in vivo (Figure 3C). The overall differentiation properties were similar for TSA-treated and control cells (Supplementary Figure E7B). We also assessed their functional capacity in colony-forming cell assays and found that TSA-treated and control cells had similar ability for differentiation (Supplementary Figure E7C). To explore the long-term reconstitution capacity of the transplanted cells, the engraftment rates in the BM were evaluated at 18 weeks post-transplantation (Figure 3D). Mice transplanted with 6×10^5 TSA-treated day 5 progeny exhibited a higher level of human hematopoietic cell engraftment (average engraftment = 13.5%), whereas transplantation of the same number of control cells resulted in a lower level of BM engraftment (average engraftment = 0.68%, $p < 0.05$).

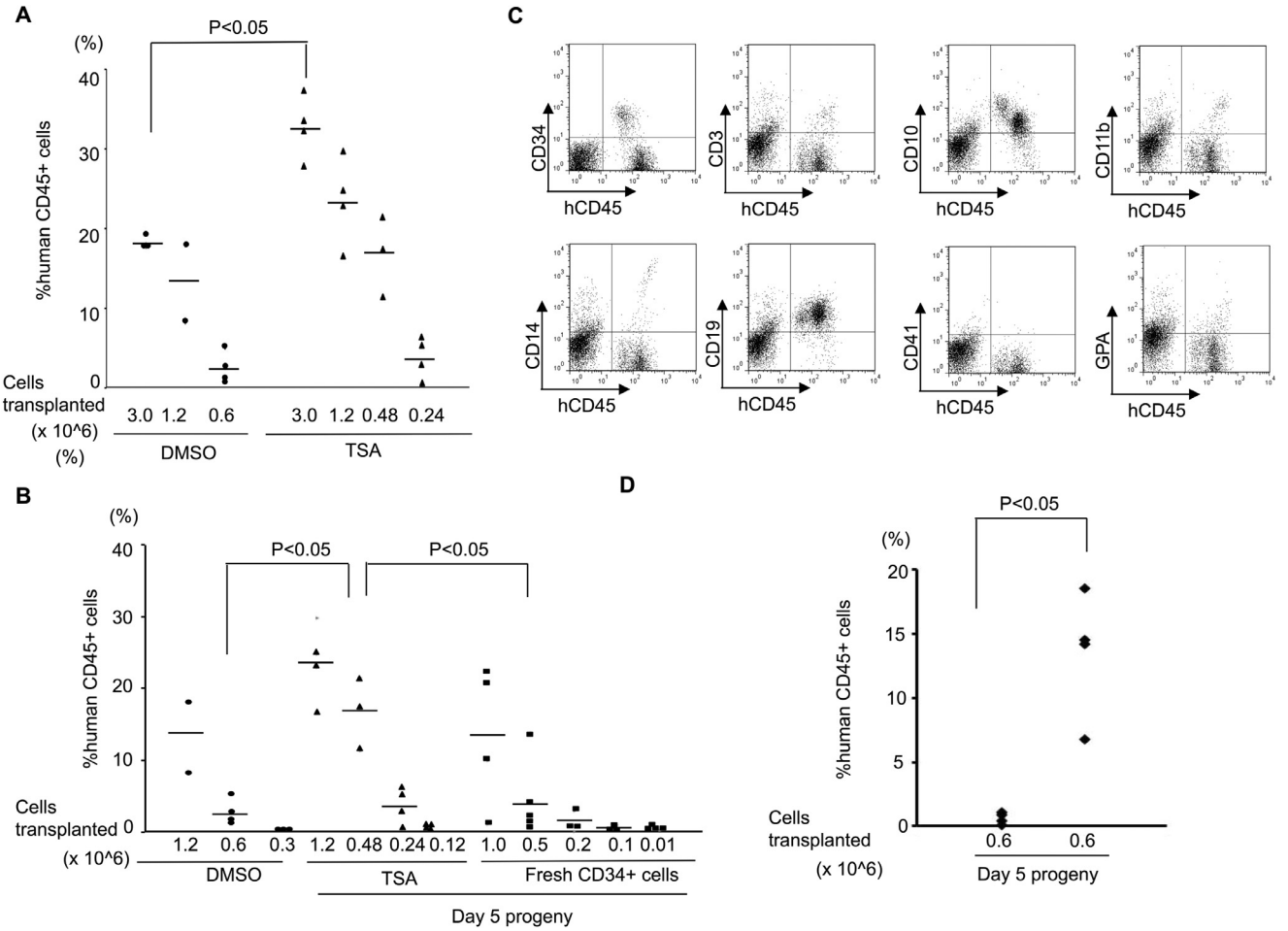

Figure 3. Treatment of PBSCs with TSA enhanced marrow-repopulating potential. **(A)** A scatterplot revealing the levels of human CD45$^+$ cell engraftment in the BM of NSG mice 8 weeks after transplantation with CD34$^+$ cells cultured with TSA or DMSO. **(B)** Limiting dilution analysis comparing the frequency of SRCs in the progeny of TSA-treated and control cultured cells with the frequency in the progeny fresh uncultured CD34$^+$ cells. **(C)** Representative BM flow cytometric analysis of 8-week multilineage hematopoietic differentiation potential of engrafted human hematopoietic cells treated with TSA in NSG mice. **(D)** Scatterplot revealing the levels of human CD45$^+$ cell engraftment in the BM of NSG mice 18 weeks after transplantation with that of their progeny after culture with TSA or DMSO.

Multilineage engraftment in the BM was also observed at 18 weeks posttransplantation with TSA treatment in vivo (Supplementary Figure E7D).

In addition, to evaluate whether TSA-treated cells retain their self-renewal capacity after primary transplantation, BM cells from the primary NSG (NOD-scid gamma null) recipients were harvested at 8 weeks and re-infused into sublethally irradiated (220 rad) secondary NSG recipients (Supplementary Figure E8, Supplementary Table E3, online only, available at www.exphem.org). We observed a trend toward increased engraftment in TSA-treated cells. This result is in agreement with our conclusion from the long-term (18 weeks post-xenotransplantation) and limiting dilution analyses, both of which suggested that TSA treatment can enhance PBSC engraftment.

In summary, these results indicate that our TSA-mediated ex vivo CD34$^+$ cell culture technique can successfully expand SRCs with long-term and multilineage hematopoietic differentiation potential.

SALL4 contributes to TSA-mediated expansion of CD34$^+$CD90$^+$ cells

Next, we investigated the molecular mechanism(s) responsible for the expansion of functional HSPCs after TSA treatment. We hypothesized that cytokine-alone based HSPC culture conditions stimulate CD34$^+$ cells to proliferate and differentiate, along with downregulation of HSPC-related genes. Therefore, we examined the levels of expression of a panel of genes known to be involved in self-renewal or differentiation of HSCs by quantitative real-time polymerase chain reaction (qRT-PCR). When compared with fresh uncultured CD34$^+$ cells, we observed a decrease in the transcript levels of *GATA2, BMI1, HOXB4, SALL4,* and *PTEN* after cytokine-mediated ex vivo culture (Supplementary Figure E9,

online only, available at www.exphem.org). We theorized that by adding TSA to the cytokine-based culture, we could limit the downregulation of these genes. Therefore, we compared the expression profiles of these genes with or without TSA treatment during the ex vivo culture period. We observed higher levels of transcripts for *GATA1, GATA2, HOXB4,* and *SALL4* in cells treated with TSA (Figure 4A; $p < 0.05$). To assess the potential contribution(s) of these four genes to $CD34^+CD90^+$ expansion, TSA-treated $CD34^+CD90^-$ and $CD34^+CD90^+$ cells were sorted and analyzed. The levels of expression of *GATA2* and *SALL4* were increased in $CD34^+CD90^+$ cells when compared with those in $CD34^+CD90^-$ cells (Figure 4B), whereas the levels of expression of *GATA1* and *HOXB4* did not significantly differ between the two cell populations. These data suggest that GATA2 and SALL4 may contribute to TSA-mediated $CD34^+CD90^+$ expansion.

Both GATA2 and SALL4 are known transcription factors involved in HSC function. Enforcing GATA2 expression can increase the quiescence of CB $CD34^+$ cells and reduce proliferation and cell performance in long-term culture-initiating cell and colony-forming cell (CFC) assays [27]. We previously reported that SALL4 is a key regulator in normal human hematopoiesis [28]. Overexpression of SALL4 led to rapid and efficient expansion of $CD34^+$ cells with enhanced engraftment and long-term repopulation capacity in vivo [8].

To investigate whether SALL4 and GATA2 may play a role in TSA-mediated $CD34^+CD90^+$ expansion, we downregulated their expression by shRNA (Figure 5A). Transfection efficiency was evaluated using a vector expressing GFP only (Figure 5B). shRNA-mediated knockdown led to markedly reduced expression of *GATA2* or *SALL4* transcripts (Figure 5C, D). We observed a significant

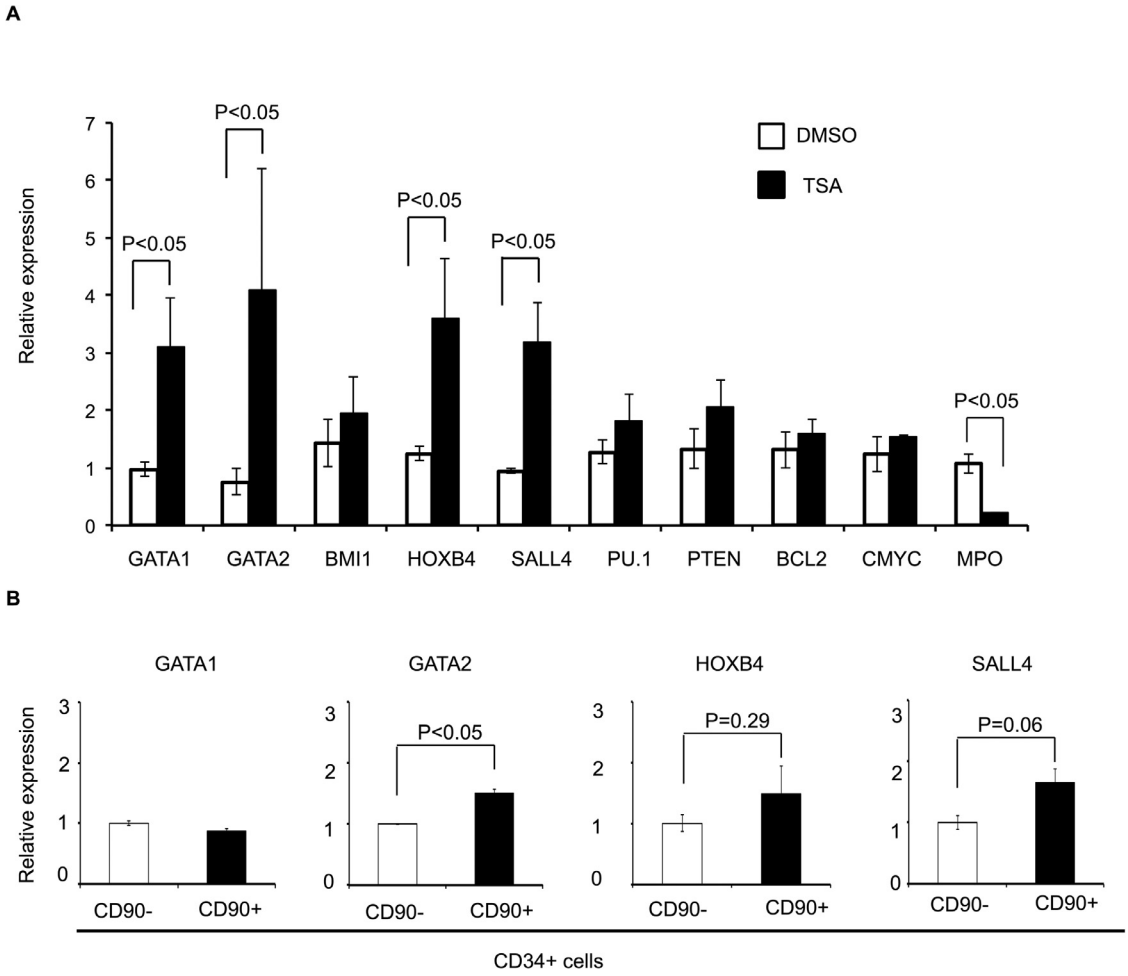

Figure 4. Treatment of $CD34^+$ PBSCs with TSA modulates expression of stem cell-related genes. **(A)** Effects of TSA treatment on the relative transcript levels of genes (*GATA1, GATA2, NOTCH1, BMI1, HOXB4, SALL4, PU.1, PU.1, PTEN, BCL2, c-MYC,* and *MPO*) were evaluated by qRT-PCR. Total RNA was extracted from cells obtained after 3 days of culture in the presence of cytokines with TSA or DMSO. GAPDH was used as internal calibrator (control gene). Measurements were obtained in duplicate using at least two independent samples. **(B)** TSA-treated $CD34^+CD90^+$ cells and $CD34^+CD90^-$ cells were sorted, and the levels of expression of *GATA1, GATA2, HOXB4,* and *SALL4* were analyzed. Measurements were obtained in duplicate using at least two independent samples. Error bars indicate the SE. $^*p < 0.05$.

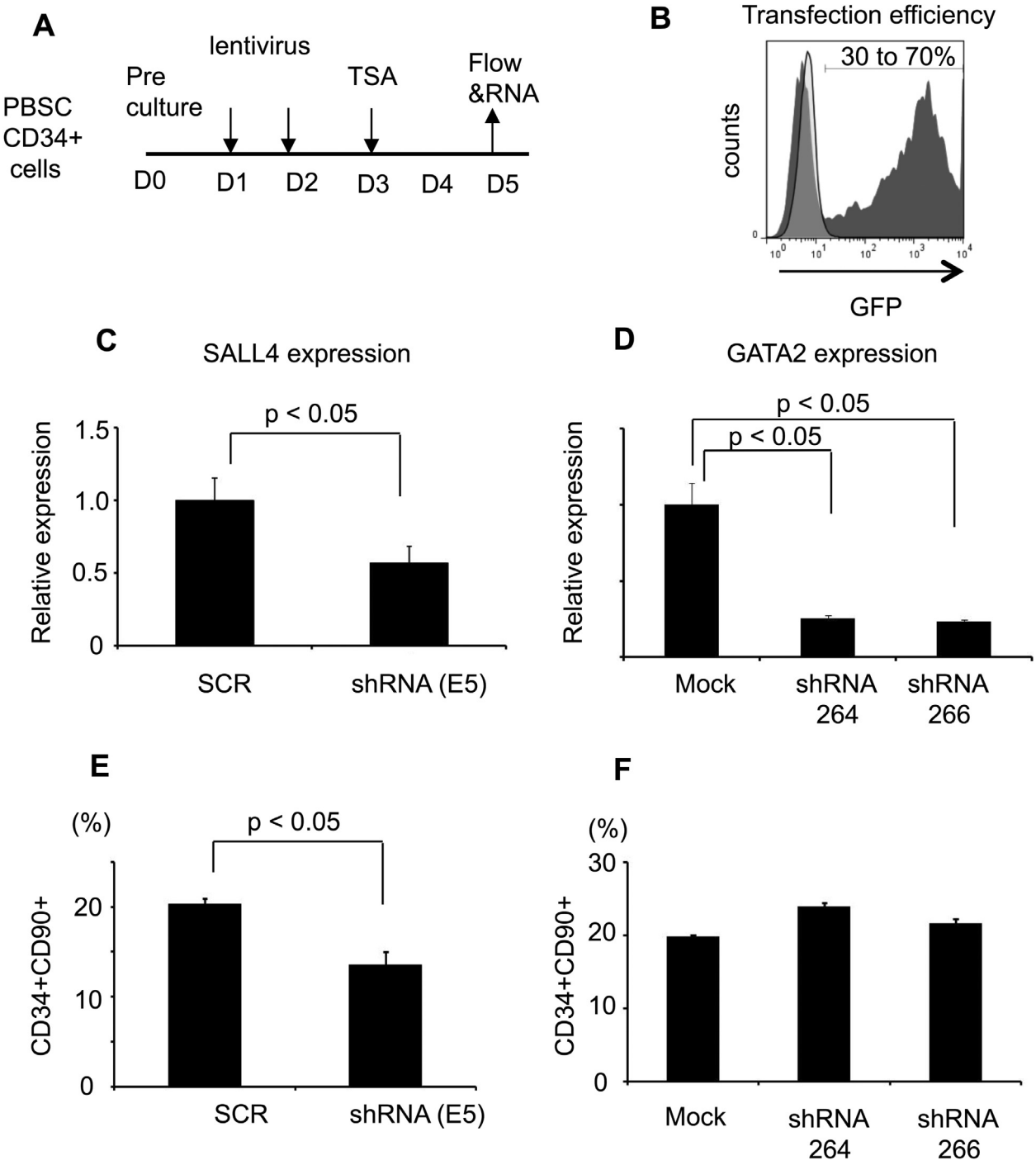

Figure 5. SALL4 silencing decreased TSA-mediated expansion of $CD34^+CD90^+$ cells. **(A)** Strategy of knockdown of SALL4 and GATA2. **(B)** Transfection efficacy was evaluated by using lentiviral vectors pLL3.7 and pLKO.3G expressing GFP. **(C)** SALL4 mRNA expression after shRNA targeting in $CD34^+$ PBSCs. **(D)** GATA2 mRNA expression after shRNA targeting. **(E)** Percentage of TSA-mediated $CD34^+CD90^+$ cells after SALL4 silencing. **(F)** Percentage of TSA-mediated $CD34^+CD90^+$ cells after GATA2 silencing. N = 3. Error bars indicate SD.

reduction in the percentage of $CD34^+CD90^+$ cells after treatment with only SALL4 shRNA (Figure 5E), but not GATA2 (Figure 5F). These data suggest that SALL4 is involved, at least partially, in TSA-mediated expansion of $CD34^+CD90^+$ cells.

Discussion

Compared with cord blood, $CD34^+$ PBSCs have reduced marrow-repopulation potential (Supplementary Figure E4) [24,25], and there is a lack of systematic efforts in the search for a method(s) that can maintain

and expand HSPCs from a PBSC source. However, there is a growing need for expanding PBSCs for transplant-related practices such as gene therapy or genome editing via TALENs or CRISPR/Cas9. Developing a technology that allows for HSPC ex vivo expansion is a key step toward these applications. We have set up a HTS assay to screen for small-molecule compounds for this purpose. Previously, the use of TSA in combination with 5-aza-2′-deoxycytidine (5azaD) or VPA was reported in the expansion of the $CD34^+CD90^+$ population [29–32]. We have further simplified and optimized the TSA-only culture period to 5 days to better fit a clinical transplant setting, in which a shorter culture period is associated with less cost.

The fate of HSPCs is governed by transcription factors, which are downregulated during ex vivo expansion. The combination of cytokines and chromatin modifiers could expand HSPC and favor self-renewal during cell division. This phenomenon can be attributed to, at least in part, the maintained expression of key transcription factors. On the basis of this hypothesis, in further searching for potential mechanism(s) underlying TSA-mediated PBSC expansion, we examined gene expression profiles with a focus on transcription factors. The expression of several key HSPC function-related genes, such as *SALL4* and *GATA2*, in PBSC $CD34^+$ cells is affected by TSA treatment. Both genes are also enriched in the $CD34^+CD90^+$ population

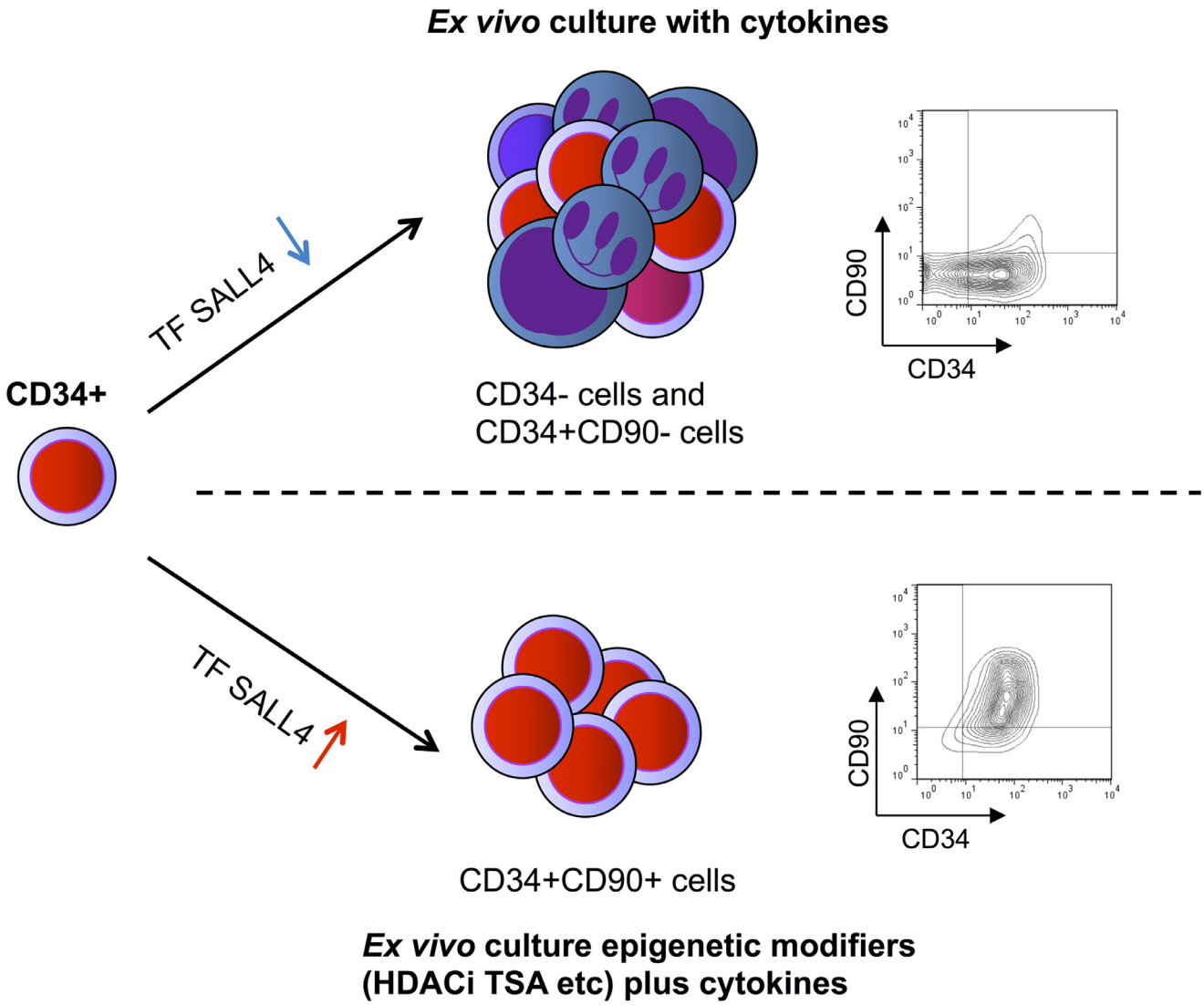

Figure 6. Schematic model of ex vivo $CD34^+CD90^+$ expansion from human PBSC HSPCs. We propose the following model: Under culture conditions with stimulating cytokines, $CD34^+$ HSPCs tend to differentiate and become $CD34^-$ or $CD34^+CD90^-$ cells. Addition of epigenetic modifiers, such as HDACi TSA to the culture medium results in expansion of $CD34^+CD90^+$ cells. TSA-mediated HSPC expansion functions, at least in part, through the transcription factor SALL4 (TF SALL4).

when compared with $CD34^+CD90^-$ cells. To evaluate which of these two genes is important for TSA-mediated expansion of the $CD34^+CD90^+$ population, we performed loss-of-function studies on these two genes. Knocking down SALL4, but not GATA2, can block TSA-mediated expansion of $CD34^+CD90^+$ cells. SALL4 is known to be important in self-renewal and differentiation of HSPCs and embryonic stem cells [8,28,33–38], but it has never been invested in HDACi-mediated $CD34^+$ cell expansion/maintenance.

We propose the following model for our ex vivo PBSC expansion technology (Figure 6). The cytokine-based HSPC culture conditions stimulate $CD34^+$ cells to proliferate ad differentiate; as a result, the majority of the population after culture will be $CD34^-$, $CD34^+CD90^-$, $CD34^+CD90^+$ cells. This could be due to the decreased expression of HSPC-related transcription factors such as SALL4 during this process (Supplementary Figure E9, online only, available at www.exphem.org). With the addition of TSA to the cytokine-based culture, the level of expression of some of these HSPC-related transcription factors such as SALL4 is maintained/increased (Figure 4A), and our TSA-mediated HSPC expansion method can induce the $CD34^+$ cells to divide and result in the expansion of the $CD34^+CD90^+$ population. The mechanism underlying HDACi TSA-mediated expansion of $CD34^+CD90^+$ cells is, at least in part, the maintenance of expression of SALL4 during the ex vivo culture period. This model is further supported by the fact that downregulation of SALL4 can reduce TSA-mediated $CD34^+CD90^+$ cell expansion (Figure 5E).

Conclusions

We report the use of a robust, 5-day TSA-mediated ex vivo culture method to expand the $CD34^+CD90^+$ HSPC population obtained from a PBSC source, which has the potential to be used in PBSC-related transplants such as gene therapy. We further report that for the first time, SALL4, a transcription factor known to be important in self-renewal and differentiation of HSPCs, is important for this TSA-mediated ex vivo $CD34^+CD90^+$ HSPC expansion process.

Author contributions

HT designed and performed research, interpreted the data, and wrote the article. MA prepared $CD34^+$ PBSCs and interpreted data. FW, CG, and SU performed research. XT carried out work on mice. AF, JQ, and JB provided compounds and interpreted data, DGT was responsible for critical reading of the manuscript and important intellectual content. LC was responsible for the study concept, design, and execution of the research, interpretation of data, and writing and revision of the draft paper.

Conflict of interest disclosure

The authors have no competing financial interests.

Acknowledgments

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Supplemental Materials and methods

Flow cytometric analysis

Cells were stained with anti-human CD34 monoclonal antibody conjugated to phycoerythrin (PE), or allophycocyanin (APC), anti-human CD90 conjugated fluorescein isothiocyanate (FITC) or APC, CD38 APC, CD45RA APC, CD49f APC, CD133 APC, CXCR4 APC, CD62L APC, CD 3 tri-color (TC), CD19 TC, CD11b TC, CD41 TC, CD45 FITC, and mouse CD45 APC. The flow cytometry data were collected using FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed by using FLOWJO software. For cell cycle analysis, cells were stained with 10 μg/mL propidium iodide and treated with 200 μg/mL RNase A for 30 min at room temperature. To assess cell division, primary $CD34^+$ cells were labeled for 10 minutes at 37° C with 0.5 uM of carboxyfluorescein diacetate succinimidyl ester (CSFE; Invirtogen, NY, USA) in PBS. After 9 days of culture, cell were labeled with CD34 PE and analyzed for a progressive decline of fluorescence intensity of CSFE using FACSCalibur flow cytometer.

Real-time PCR

We prepared RNA from cells treated with wild-type or scrambled peptide using TRIzol (Invitrogen). We synthesized cDNA using iScript cDNA Synthesis kit (Bio-Rad, CA, USA). To quantitate the level of mRNAs expression, we carried out polymerase chain reaction (PCR) amplification using iScript one-step RT-PCR kit with SYBER Green (Bio-Rad) and the PCR products were detected by use of SYBR green technology (ABI, Foster City, CA, USA). GAPDH was used as an endogenous control. All samples were run in duplicate. Thermal cycler conditions were 50°C for 2 minutes, 95°C for 10 minutes, and 45 cycles of 95°C for 0.30 minutes and 60°C for 1 minute. Results were obtained as threshold cycle (Ct) values and normalized gene expression with GAPDH. Data were analyzed based on 2-ΔΔCT method using CFX Manager software (Bio-Rad). For QPCR primers see Table S2.

Lentivirus production and transduction

For SALL4 knockdown in CD34+ cells, Lentiviral particles were generated by transient co-transfection of 293T cells with the lentiviral vectors pLL3.7, pHR'8.9ΔVPR, and pCMV-VSVG. $CD34^+$ cells were infected in medium containing lentiviral particles and 8 μg/ml protamine. The culture medium was then removed and replaced with fresh media. pLKO.1 puro and pLKO.3G were obtained from Addgene. shRNA pLKO.1 lentivectors for GATA2 or HOXB4 to knockdown in $CD34^+$ cells were obtained from Sigma and used according to the manufacturer's protocol.

Supplemental Table E1. The list of the compounds is shown.

Compounds	Targets
TSA	Pan HDAC inhibitor
DLS3	HDAC1/2 inhibitor
Valproic acid	Pan HDAC inhibitor
SAHA	Pan HADC inhibitor
161	HDAC6 inhibitor
Merk60	HDAC1/2 inhibitor
5-Azacytidine	Demethylating agent
JQ1-S	BET inhibitor
UNC0638	H3K9me2 methyltransferase inhibitor
JMJD3 (histone H3 Lys 27 demethylase) inhibitor	
JQ-EZ-05	EZH2 inhibitor; H3K27me3 methyltranferase inhibitor
Tranylcypromine	Lysine-Specific Demethylase 1 (LSD1) Inhibitor
EPZ5676	DOT1L inhibitor
EPZ4777	DOT1L inhibitor
SR1	Aryl hydrocarbon receptor antagonist
DBZ	Gamma Secretase inhibitor
dmPGE2	Stable prostaglandin E2 (PGE2) derivative
CHIR99021	Inhibitor of GSK3
Forskolin	Adenylyl Cyclase Activator
TGF-beta RI kinase inhibitor II	

Supplemental Table E2. The primers for real time PCR.

Genes	Primers
BCL2	f-agtacctgaaccggcacct r-cagccaggagaaatcaaacag
BMI 1	f-tggctctaatgaagatagagg, r-ttccgatccaatctgttctg
GATA 1	f-acaagatgaatgggcagaac, r-tactgacaatcagcgcttc
GATA 2	f-gataccccacctatccctcctatgtg r-gtggcaccacagttgacacactc
HOXB4	f- tcccactccgcgtgcaaaga r-gccggcgtaattggggttta
MPO	f-accctcatccaacccttc, r-gtcaatgccaccttccag
C-MYC	f-tcctcggattctctgctctc, r-cttgttcctcctcagagtcg
PU.1	f-tgttacaggcgtgcaaaatggaagg r-ctcgtgcgtttggcgttggtataga
SALL4	f-gcgagcttttaccaccaaag r-cacaacagggtccacattca
PTEN	f- gcgtgcagataatgacaagg, r- gctagcctctggatttgacg
GAPDH	f-tggaaggactcatgaccaca r-ttcagctcagggatgacctt

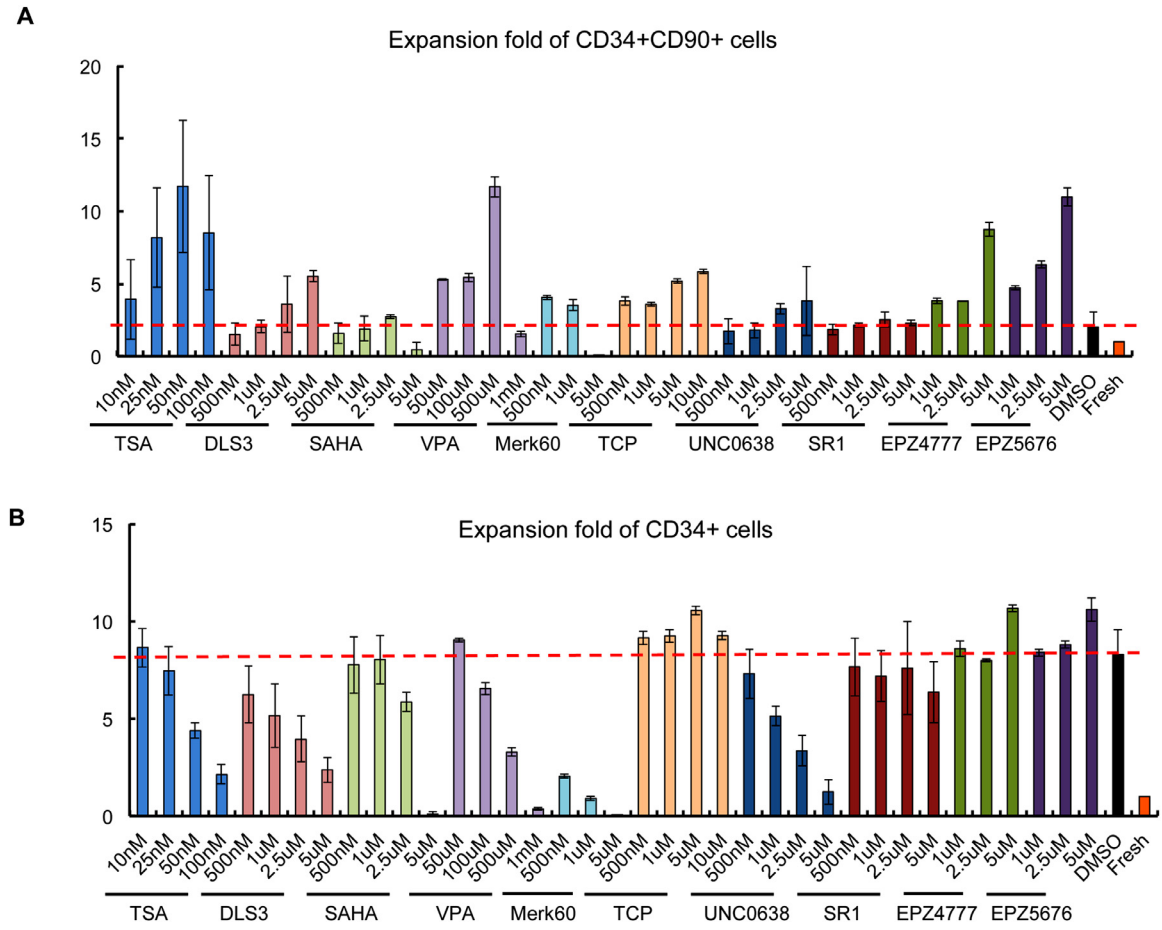

Figure S1. Screening with the compounds, which increased CD34$^+$CD90$^+$ cells. **(A)** Expansion fold of CD34$^+$CD90$^+$cells after 5 days of culture with the compounds. Each value represents mean ± SD of two or three independent experiments. **(B)** Expansion fold of CD34$^+$ cells after 5 days of culture with the compounds. Each value represents mean ± SD of two or three independent experiments.

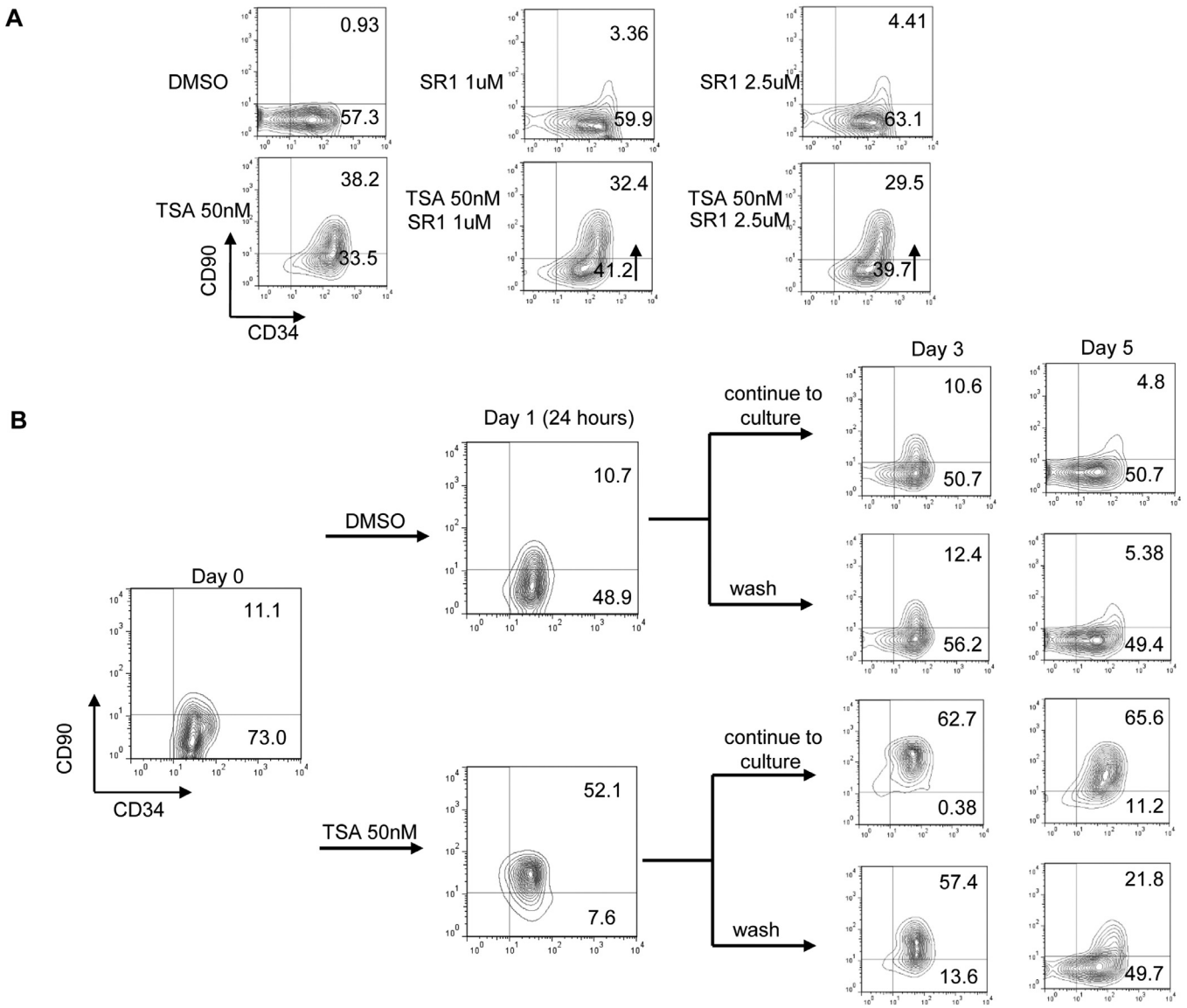

Figure S2. CD34$^+$CD90$^+$ expression adding SR1 or following washing out TSA. (A) CD34$^+$ cells were cultured with SR1 and TSA for 5 days. Representative of two independent experiments flow cytometric analysis were shown. Note the percentage of CD34+CD90+ did not change with combination of SR1 and TSA. **(B)** Washing out the TSA drug after 24 hours' treatment resulted in decreased percentage of CD34$^+$CD90$^+$ cells compared to TSA treatment without washing out step.

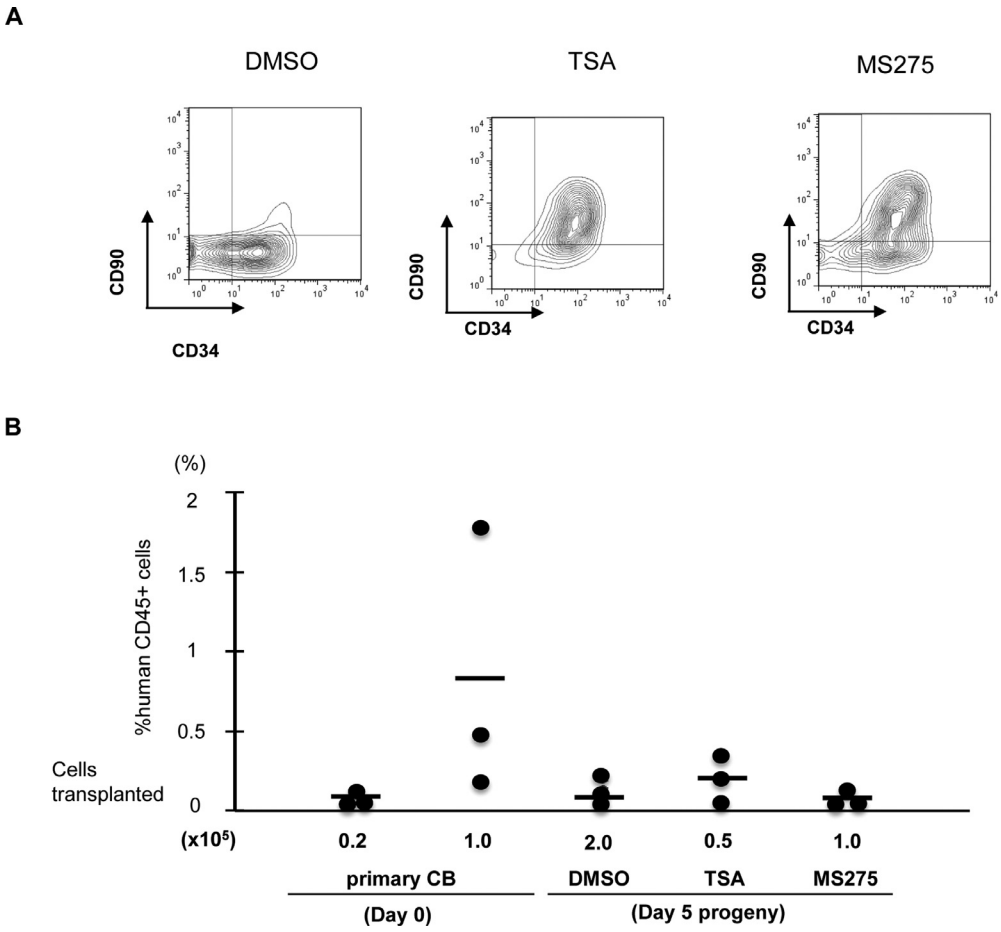

Figure S3. Treatment of CB cells with TSA and MS275 leads to expansion of $CD34^+CD90^+$ Cells (A) Cord blood $CD34^+$ cells were cultured with MS275 and TSA for 5 days. Representative of two independent experiments flow cytometric analysis were shown. Note the percentage of $CD34^+CD90^+$ increased with either TSA or MS275 treatment. **(B)** Scatter plot showing the levels of human $CD45^+$ cell engraftment in the PB of NSG mice 2 weeks after transplantation with the progeny of 2x 10^4 $CD34^+$ cells after culture with DMSO, TSA or MS275.

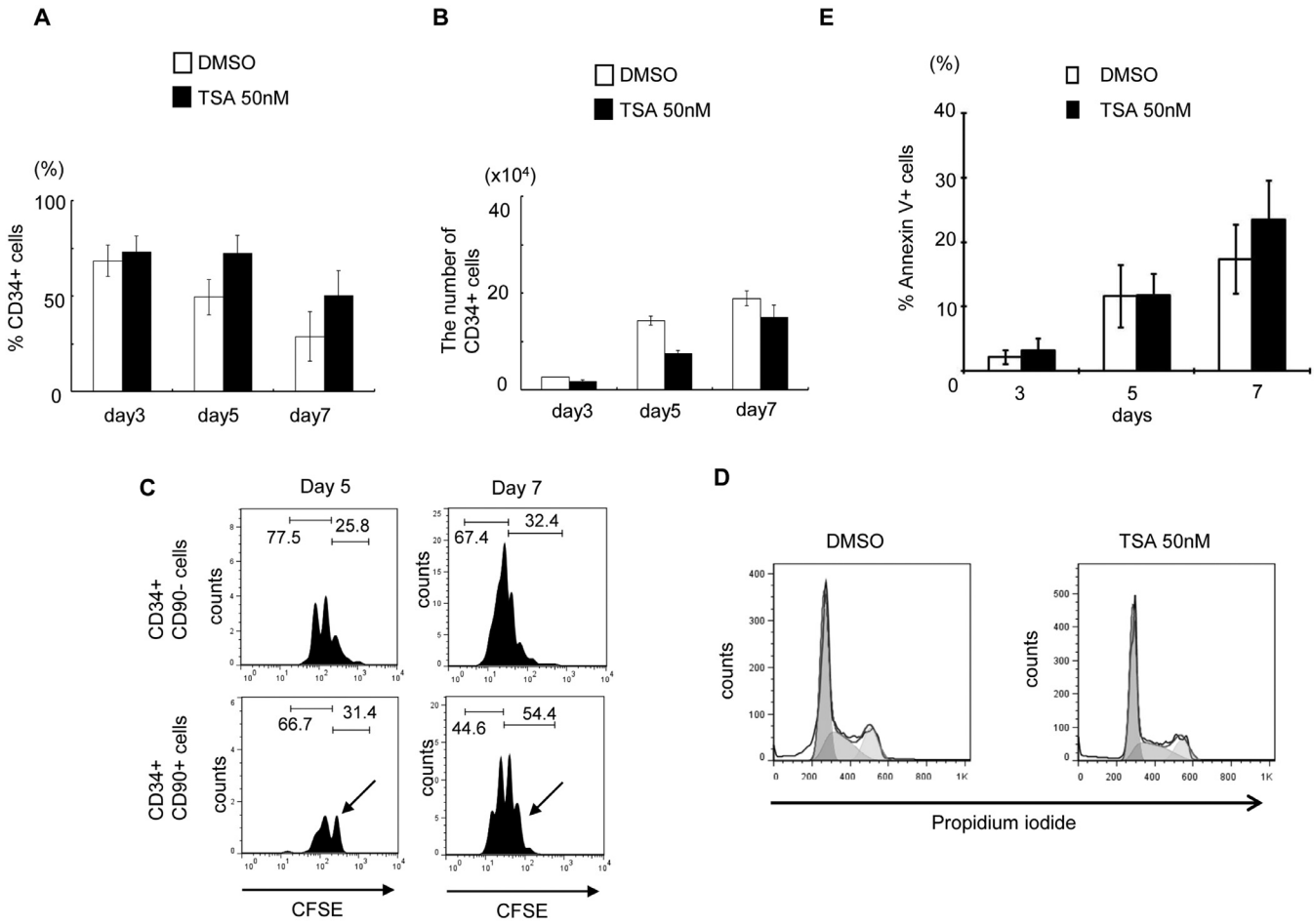

Figure S4. TSA-treated culture leads to preferential expansion of slowly dividing PBSC CD34$^+$CD90$^+$ cells. (A) Percentage of CD34$^+$ cells at 3, 5 and 7 days of culture in the presence or the absence of 50nM TSA. **(B)** Absolute number of CD34$^+$ cells at 3, 5 and 7 days of culture in the presence or the absence of 50nM TSA. **(C)** Cell cycle analysis by PI staining after 5 days of culture. **(D)** Viability was determined using Annexin V expression. The data shown are the mean of three independent experiments. **(E)** The panel shows a representative flow cytometric profile of CFSE fluorescence intensity of CD34$^+$CD90$^+$ and CD34$^+$CD90$^-$ cells after 5 or 7 days of culture.

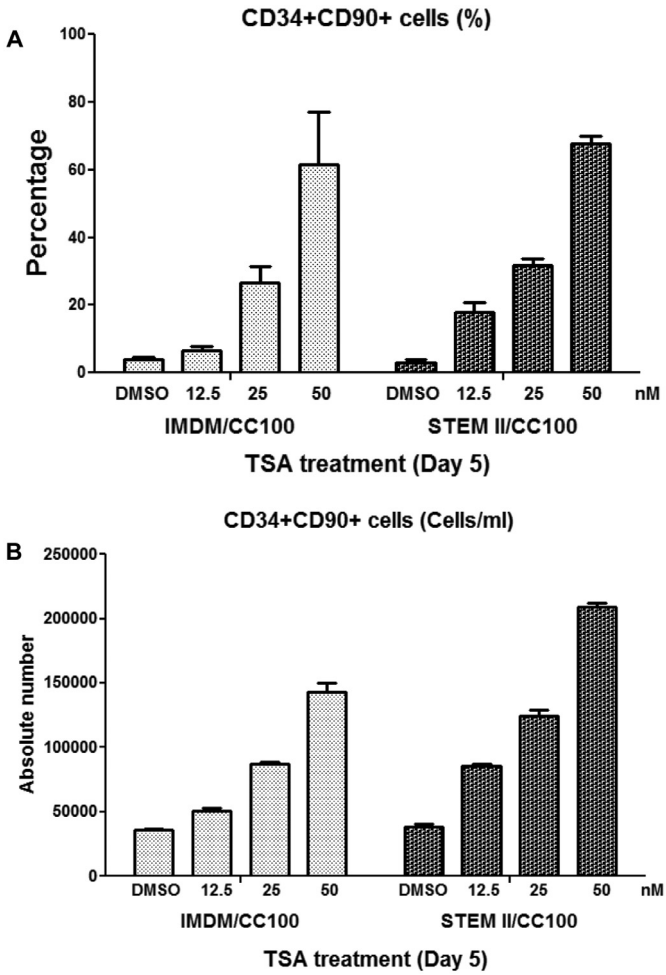

Figure S5. TSA treatment can increase CD34+CD90+ cells in PBMC CD34+ cells cultured in serum free StemSpanTM SFEM II and serum containing IMDM/CC100. **(A)** Effect of TSA on CD34 and CD90 expression following the two culture conditions. Each value represents mean ± standard error of two independent experiments. **(B)** Absolute number of CD34+CD90+cells cultured with the compounds. Each value represents mean ± standard error of two independent experiments. **(C)** Dose effect of TSA on CD34 and CD90 expression following the two culture conditions. **(D)** Time course of TSA on CD34 and CD90 expression following the two culture conditions.

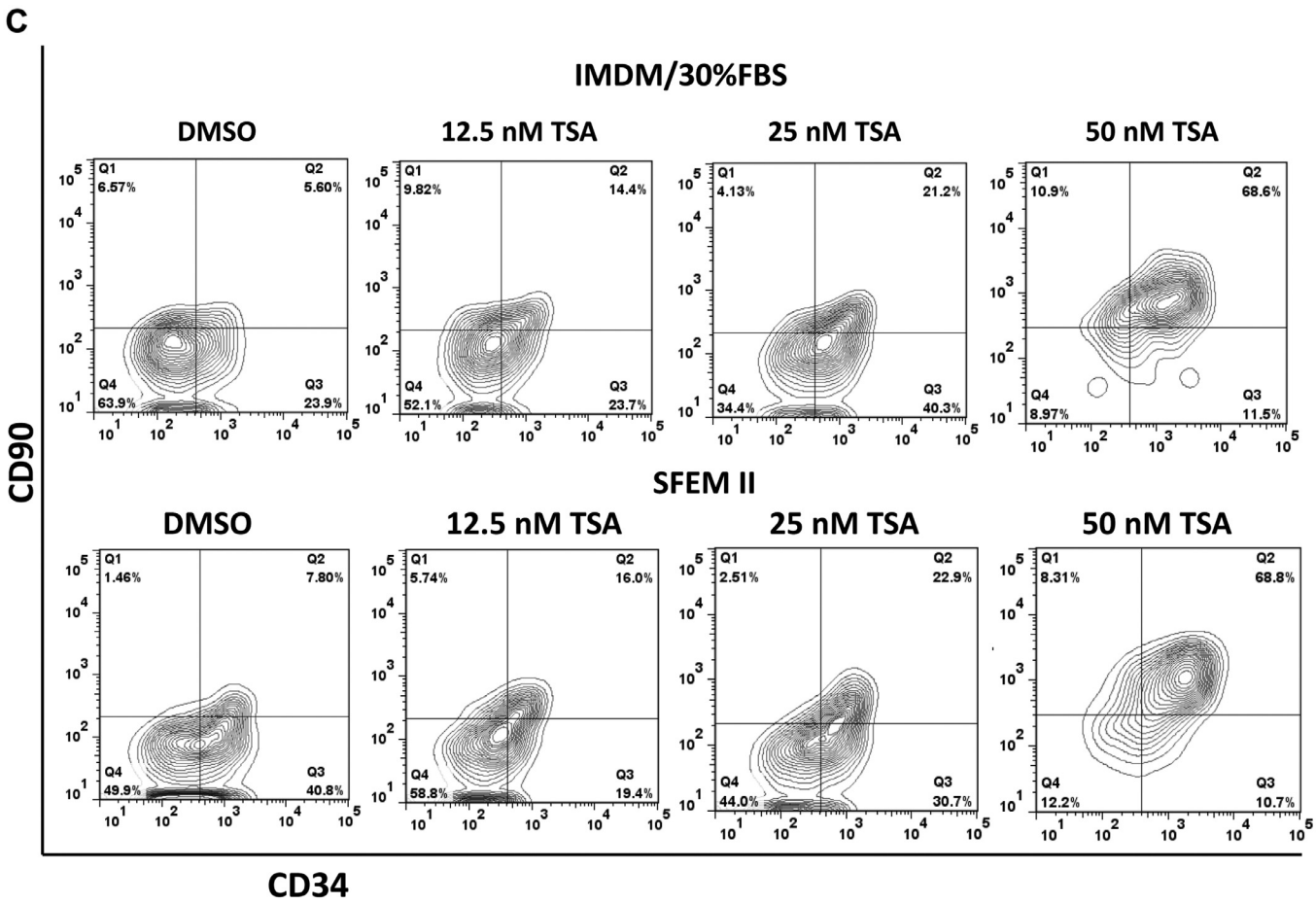

Figure S5. Continued.

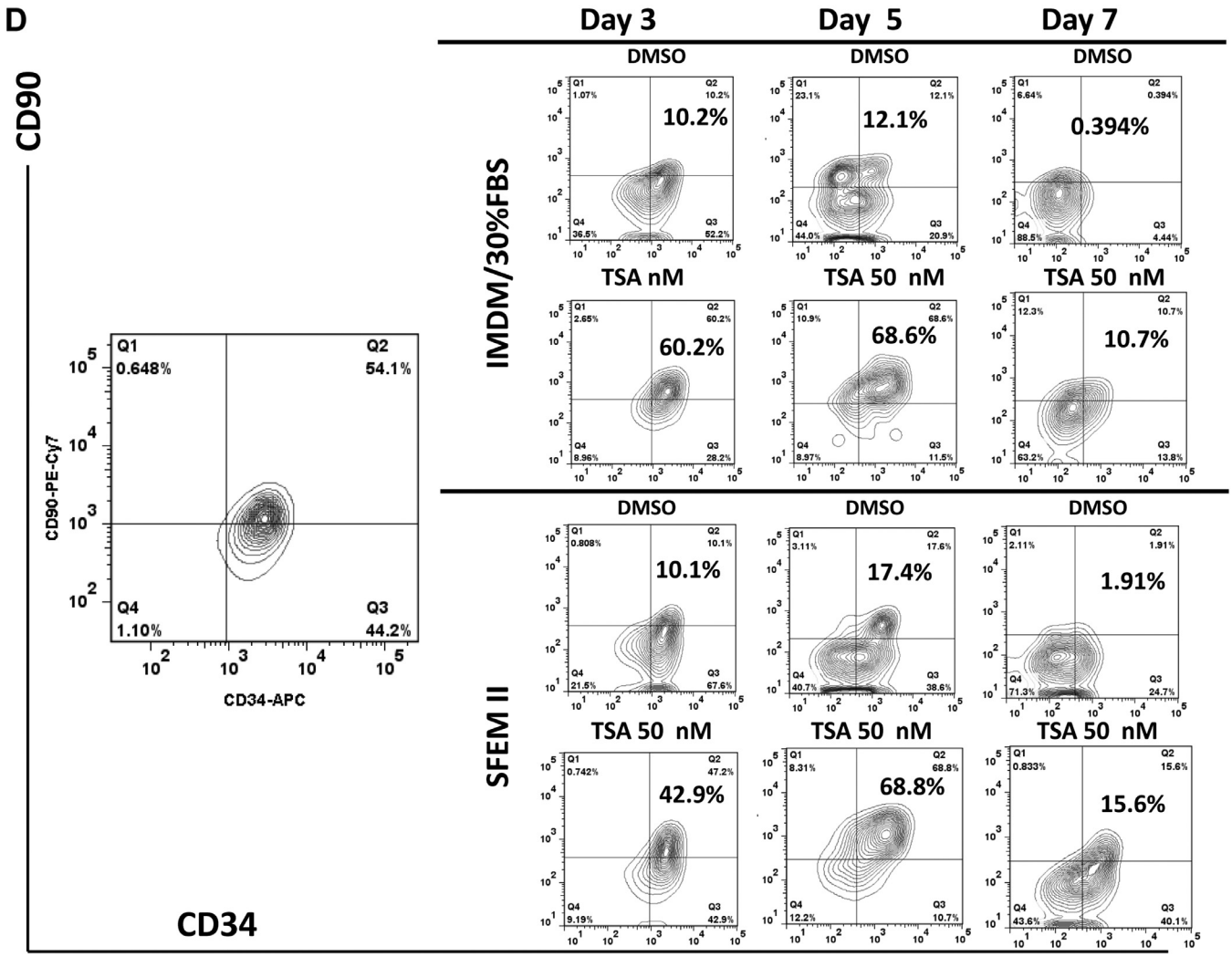

Figure S5. Continued.

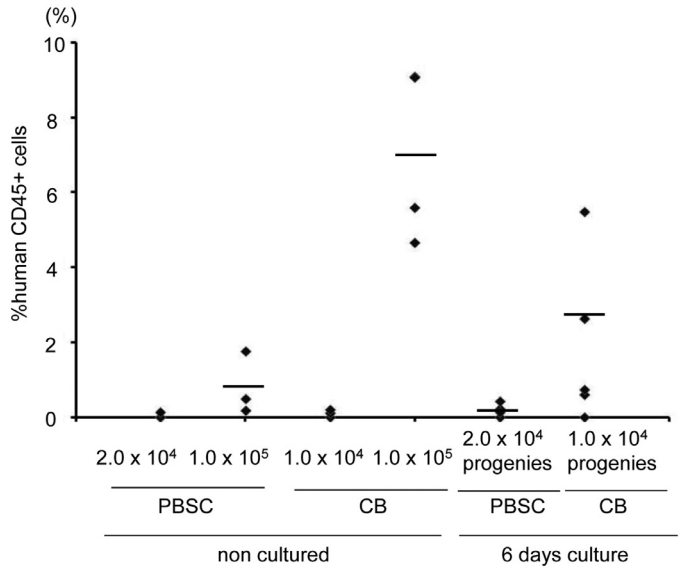

Figure S6. SCID mouse repopulating ability between CB and PBSC. A scatter plot showing the levels of human CD45+ cell engraftment in the BM of NSG mice 8 weeks after transplantation with non-cultured CB or PBSC CD34+ cells or with their progeny after culture with TSA or DMSO.

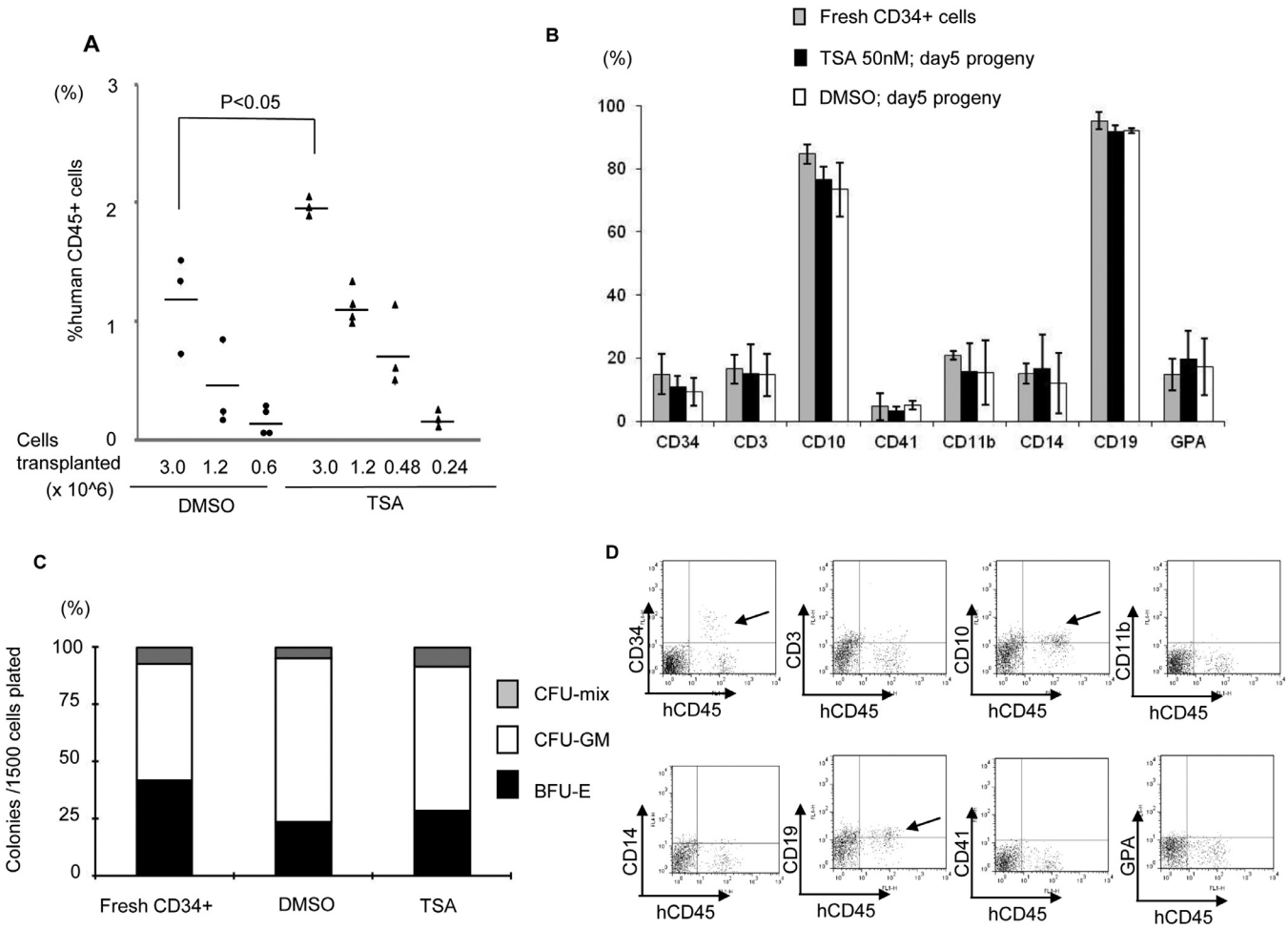

Figure S7. Treatment of PBSCs with TSA enhanced the marrow-repopulating potential. **(A)** Scatter plot showing the levels of human $CD45^+$ cell engraftment in the PB of NSG mice 8 weeks after transplantation with their progeny after culture with TSA or DMSO **(B)** A bar graph shows multilineage differentiation in NSG mice BM at 8 weeks after transplantation with primary $CD34^+$ cells or their progeny after culture with or without TSA. % positive cell = No. human lineage marker such as CD34+ cells/No. human CD45+ cells x 100. **(C)** The effect of TSA treatment on the content of the colony-forming cells (CFC). The CFU content of primary CD34+ cells, cells treated with or without TSA was determined. **(D)** Representative flow cytometric analysis of 18 weeks BM multilineage hematopoietic differentiation potential of engrafted human hematopoietic cells treated with TSA prior to transplantation into NSG mice.

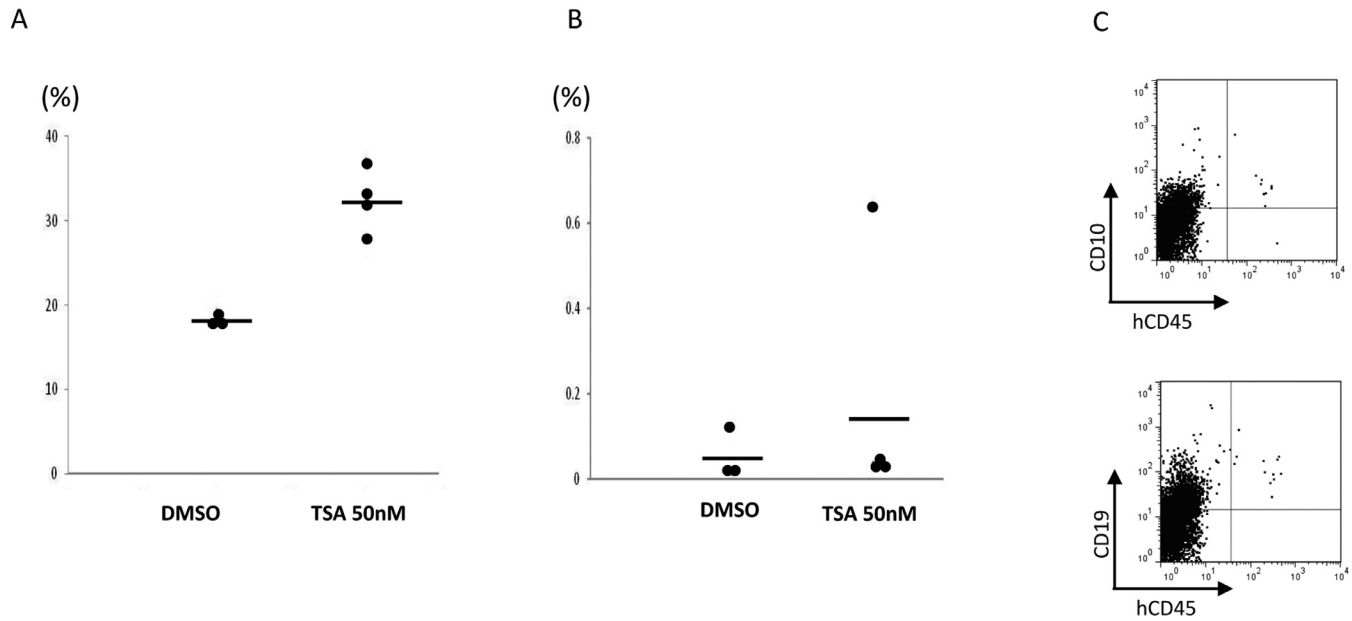

Figure S8. Secondary transplantation (A) Percentage of human CD45 engraftment in primary transplantation, **(B)** Percentage of human CD45 engraftment in secondary transplantation, **(C)** Representative flow cytometric analysis of human CD45, CD10, and CD19 engraftment in secondary transplanted NSG mice.

Supplemental Table E3. Percentage of human CD45 engraftment in primary and secondary transplantation

Recipient Mice	Primary Transplant	Secondary Transplant
Control	17.8	0.014
Control	18	0.014
Control	19.4	0.13
TSA	27.8	0.026
TSA	32.2	0.028
TSA	33.5	0.041
TSA	37.3	0.647

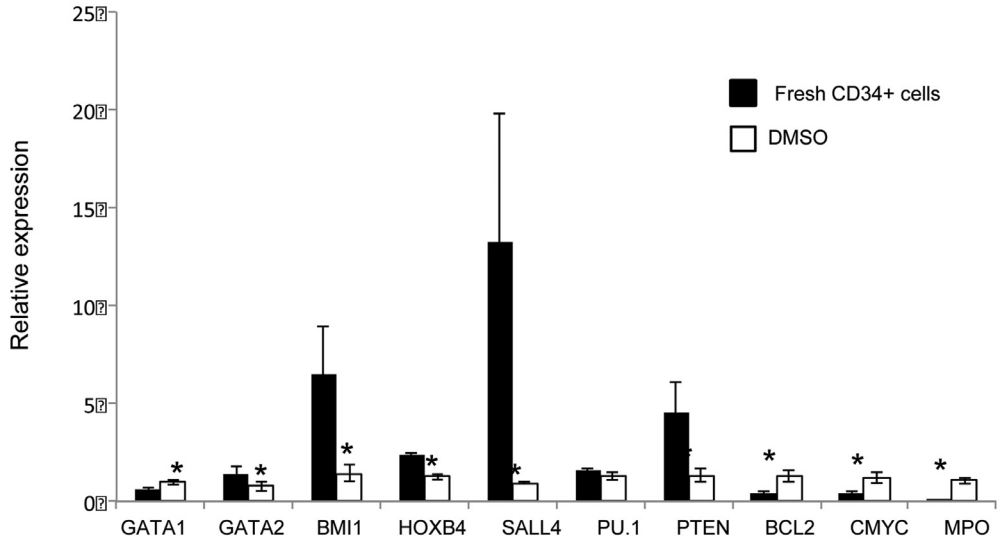

Figure S9. Effects of cytokine treatment on HSCP gene expression. The relative transcript level of genes (*GATA1, GATA2, NOTCH1, BMI1, HOXB4, SALL4, PU.1, PU.1, PTEN, BCL2, c-MYC* and *MPO*) was measured by real-time quantitative PCR. Total RNA was extracted from fresh uncultured $CD34^+$ cells or cells obtained 3 days of culture in the presence of cytokines with DMSO. GAPDH was used as internal calibrator (control gene). Measurements were obtained in duplicate using at least 2 independent samples. Error bars indicate SD; * indicates $p<0.05$.