HHS Public Access

Author manuscript

Exp Hematol. Author manuscript; available in PMC 2020 July 01.

Published in final edited form as:

Exp Hematol. 2019 July ; 75: 53–63.e11. doi:10.1016/j.exphem.2019.06.473.

Maintenance and enhancement of human PBSC engraftment after *ex vivo* culture via an HDACi/SALL4 axis (3465)

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Summary

Currently, there is a growing need for culturing hematopoietic stem/progenitor cells (HSPCs) *in vitro* for various clinical applications including gene therapy. Compared to cord blood (CB) $CD34^+$ HSPCs, it is more challenging to maintain or expand peripheral blood mobilized stem/progenitor cell (PBSC) $CD34^+$ cells *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 (HDACi), could preferentially maintain and expand these cells. Specially, treatment of $CD34^+$ PBSCs with a single dose of HDACi Trichostatin A (TSA) at a concentration of 50nM *ex vivo* yielded the greatest expansion (11.7-fold) of $CD34^+CD90^+$ cells when compared to the control (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 expressions of HSPC-related genes such as *GATA2* and *SALL4*. Furthermore, TSA-mediated

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Author contributions

H.T. designed and performed research, interpreted the data and wrote the paper; M.A prepared PBSC $CD34^+$ cells and interpreted data. F.W, C.G, S.W. performed research; X.T carried out mouse work. A.F, J.Q and J.B provided compounds and interpreted data, D.T. was responsible for critical reading of the manuscript and important intellectual content; and L.C. was responsible for the study concept, design and execution of the research, interpretation of data, and writing and revising the draft paper.

Conflict-of-interest disclosure:

The authors have no competing financial interests.

$CD34^+CD90^+$ expansion was reduced by down-regulation of SALL4 but not GATA2. Overall, we have developed a robust, short-term (5-day), PBSC *ex vivo* maintenance/expansion culture technique and demonstrated that the HDACi-TSA/*SALL4* axis is important for the biological process.

Keywords

hematopoietic stem cell; $CD34^+CD90^+$; self-renewal; expansion; HDACi; SALL4

Introduction

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) (2) (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 event *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 PBSC *in vitro* for transplant-related applications such as gene therapy(12) or 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).

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 in 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 TEPA(21) (22). However, even after several decades of research, the quest for condition(s) that are able to stimulate self-renewal *ex vivo* still continues(23). Compared to cord blood (CB) $CD34^+$ cells, it is more challenging to maintain and expand PBSC $CD34^+$ cells *ex vivo*(24) (25).

In this study, we searched for a robust and short term *ex vivo* culture condition 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 potentials for PBSC expansion. After surveyed 466 compounds, including multiple chromatin modifiers, we found that a single dose of 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*.

Materials and methods

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

PBSC were collected after G-CSF mobilization and enriched by CD34$^+$ immunoselection. Fresh CB collections were obtained from Cell Manipulation Core Facility in Dana-Farber Cancer Institute (DF/HCC; Boston, MA) according to guidelines established by 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). Cells were allotted to 2×10^4 /well and incubated in IMDM containing 30% fetal bovine serum (FBS; GIBCO) supplemented with 1X CC100 cytokine mix (SCF, FL, IL3, and IL6; Stem Cell Technologies) or a serum-free expansion system (StemSpanTM SFEM II, SCF, FL, IL3, and IL6; STEMCELL Technologies) supplemented with 1X 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 has been approved by and done according to the guidelines of the IACUC under protocol 10-10-1832. The CB or PBSC CD34$^+$ cells treated with TSA or DMSO were injected intravenously via the tail vein into sub-lethally irradiated (220 rads) 8 to 16-week-old NSG mice. Transplantation or IV administration was performed within 24 h after irradiation. Peripheral blood (PB) chimerism was monitored at 8 weeks post transplantation. Bone marrow (BM) chimerism was monitored at 8 and 18 weeks post transplantation. These samples were subsequently subjected to flow cytometry analysis utilizing FITC-conjugated anti-human CD45 antibody and APC-conjugated anti-mouse CD45 antibody (eBiosciences, CA, USA). The percentage of human CD45+ cells was calculated as follows: % human CD45+ cells = No. human CD45+ cells/ (No. human CD45+ cells + No. 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 sub-lethally irradiated (220 rads) 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 SRCs was calculated using L-Calc software (StemCell Technologies Inc.)

Statistical analysis

Results are expressed as mean ± Standard Deviation (SD) or Standard Error (SE) when appropriate. Statistical differences were evaluated using the student t test with significance at p of 0.05 or less.

Additional materials and methods are listed supplemental material.

Results

HTS approach to identify small molecule compounds including chromatin modifiers for the maintenance/expansion of PBSC CD34$^+$CD90$^+$ cell *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 severe combined immunodeficiency (SCID)-repopulating cells (SRC). Among the published studies, *ex vivo* cultured CD34$^+$CD90$^+$ cells are most well established to be SRC or have marrow-repopulating potential(26). Therefore, in our study, we use co-expression of CD34 and CD90 to identify compounds with potentials 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 FDA approved compounds and 20 additional small molecule drugs including a panel of chromatin modifiers (Table S1).

Ten compounds were considered to be positive hits based on the increased percentage of CD34$^+$CD90$^+$. Among them, five were histone deacetylase inhibitors, namely trichostatin A (TSA), DLS3, Valproic Acid (VPA), SAHA, and Merk60. The others were H3K9me2 methyltransferase inhibitor UNC0638, Dot1 inhibitors EPZ4777 and EPZ5676 (Figure 1A). A mild increase in the CD34$^+$CD90$^+$ percentage was also observed with treatment of 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 BET inhibitor JQ1-S et al were all negative.

By measuring the absolute CD34$^+$CD90$^+$ cell number, we noticed that while most of the positive hits could expand the CD34$^+$CD90$^+$ population compared to control (DMSO plus cytokines) treated CD34$^+$ cells, there was a range of ability. Among the ten positive hits, TSA, VPA, EPZ4777 and EPZ5676 treatment demonstrated the greatest (11.7-fold, 11.6-fold, 8.7-fold, 10.9-fold) expansion of CD34$^+$CD90$^+$ cells compared to control based on absolute cell number (Figure 1B) and expansion fold (Figure S1A). These compounds did not markedly expand CD34$^+$ and CD34$^+$CD90$^-$ cells in 5 days (Figure S1B). To further enhance expansion, we combined the treatment of SR1 with TSA (Figure S2A). However, this combination treatment only mildly improved the expansion of CD34$^+$CD90$^+$ cells. In addition, we observed that HDACi such as TSA and MS275 can also expand the CD34$^+$CD90$^+$ population from cord blood (Figure S3).

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

TSA-treatment of PBSC CD34$^+$ cells *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 50nM since higher

dosage is toxic to cells (Figure 1, & Figure S1A). 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 day 3, 5, and 7 compared to control treated cells (Figure 2B&2C; $p<0.05$). There was a significant increase in $CD34^+CD90^+$ cells 24 hours post TSA treatment (Figure S2B). To examine whether TSA is required for further expansion of this population during the subsequent culture period, we performed the following experiments. Since the half-life of TSA in culture is 3 days, washing out TSA after 24 hours of treatment was performed, which resulted in decreased $CD34^+CD90^+$ cells (Figure S3B). 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 with TSA treatment was about 2.5 times lower than that with control (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 cell number of $CD34^+$ (Figure S4A&4B), and $CD34^+CD90^+$ subpopulations (Figure 2D). We observed that the absolute number of $CD34^+CD90^+$ cells was significantly increased by TSA treatment especially from day 5 to day 7.

Next, we asked whether the lower total nucleated cell number observed in 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. While 66.0 % of control treated $CD34^+$ cells divided more than 4 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 and control treated cells demonstrated by propidium iodide (PI) staining (Figure S4C). To investigate whether the lower total nucleated cell number observed after TSA treatment was due to increased apoptosis, we performed annexin V and PI staining with TSA- or control-treated cells on day 3, 5 and 7. No difference in apoptosis was observed between the two treatment groups (Figure S4D). We then asked whether TSA treatment could lead to increased $CD34^+CD90^+$ cells through proliferation. Using the CFSE assay, we observed that $CD34^+CD90^+$ cells divided upon TSA treatment. Since the $CD34^+CD90^+$ is the dominant population in TSA treatment, and these cells divide slower than the $CD34^+CD90^-$ cells (Figure S4E), these observations could explain why there are fewer cell divisions in the TSA treated group (Figure 2E).

In addition, we have tested the TSA-mediated protocol in a serum-free StemSpan SFEM II Medium, we observed a similar result in expansion of $CD34^+CD90^+$ population (Figure S5).

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

Treatment of PBSC $CD34^+$ cells with TSA enhances marrow-repopulating potential *in vivo*

Compared to CB, PBSC $CD34^+$ cells have reduced marrow-repopulation potential (Figure S6)(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 culturing PBSC $CD34^+$ cells, we noticed that on day 5, the majority of control-treated cells were

$CD34^+CD90^-$, while a large portion of TSA-treated cells were still $CD34^+CD90^+$ (Figure 2B). To compare the marrow-repopulation potential between control and TSA treatment, we transplanted cells after culture for 5 days with control or TSA into NSG mice as a measurement for HSPC function. Eight weeks after transplantation, the mice were sacrificed and their peripheral blood (PB) and bone marrow (BM) samples analyzed (Figure S7A & Figure 3A). Mice transplanted with 3×10^6 TSA-treated day 5 progeny demonstrated a higher level of human hematopoietic cell engraftment (average engraftment of 32.74%), while transplantation of the same number of control-treated 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 from TSA-treated and control-treated culture conditions. Poisson distribution analysis revealed a SRC frequency of 1 in 247,567 (95% 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-treated cells indicating the effective expansion of SRC number (about 4.7 fold) by 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 capable of differentiating into multiple lineages following transplantation *in vivo* (Figure 3C). The overall differentiation properties were similar between TSA- and control-treated cells (Figure S7B). We also assessed their functional capacity by colony forming cell assays, and found TSA- and control-treated cells had similar differentiation abilities (Figure S7C). 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 demonstrated a higher level of human hematopoietic cell engraftment (average engraftment of 13.5%), while transplantation of the same number of control-treated cells resulted in a lower level of BM engraftment (average engraftment 0.68%; $p<0.05$). Multi-lineage engraftment in the BM was also observed at 18 weeks post-transplant with TSA-treatment *in vivo* (Figure S7D).

In addition, to evaluate whether TSA treated cells still retain their self-renewal capacity after primary transplantation, bone marrow (BM) cells from the primary NSG (NOD-scid gamma null) recipients were harvested at 8 weeks and were reinfused into sub-lethally irradiated (220 rads) secondary NSG recipients (Figure S8 & Table S3). We have 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 analysis, both suggesting that TSA treatment can enhance PBSC engraftment.

In summary, these results indicate that our TSA-mediated $CD34^+$ cell *ex vivo* 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 investigate the molecular mechanism(s) responsible for the expansion of functional HSPCs following TSA treatment. We hypothesized that cytokine-alone based HSPC culture conditions stimulate CD34$^+$ cells to proliferate and differentiate, along with down-regulation of HSPC-related genes. Therefore, we examined the expression levels of a panel of genes known to be involved in self-renewal or differentiation of HSCs by qRT- PCR. When compared to 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 (Figure S9). We theorized that by adding TSA to the cytokine-based culture condition, we could limit the down-regulation of these genes. Therefore, we compared the expression profile 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 the CD34$^+$CD90$^+$ expansion, TSA treated CD34$^+$CD90$^-$ and CD34$^+$CD90$^+$ cells were sorted and analyzed. The expression levels of *GATA2* and *SALL4* were increased in CD34$^+$CD90$^+$ cells when compared to those in CD34$^+$CD90$^-$ cells (Figure 4B), while the expression levels of *GATA1* and *HOXB4* were not significantly different between the two cell populations. These data suggest that GATA2 and SALL4 may contribute to the 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, reduce proliferation and cell performance in long term culture-initiating cell and colony forming cell (CFC) assays (27). We previously demonstrated 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 down-regulated 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 reduction in the percentage of CD34$^+$CD90$^+$ cells after treatment only with 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 to cord blood, PBSC CD34$^+$ cells have reduced marrow-repopulation potential (Supplementary Figure 4)(24) (25), and there is a lack of systematic efforts in searching for method(s) that can maintain and expand HSPC from a PBSC source. However, there is growing need for expanding PBSCs for transplant-related practices such as gene therapy or genome-editing via TALENs or CRISPR/Cas9. Developing a technology, which allows for HSPC *ex vivo* expansion is a key step towards these applications. We have set up a HTS assay to screen for small molecule compounds for this purpose. Previously, TSA, in combination with 5-aza-2'-deoxycytidine (5azaD) or valproic acid (VPA), had been reported

in the expansion of $CD34^+CD90^+$ population (29) (30) (31) (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.

HSPC fate is governed by transcription factors, which are down-regulated 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. Based on this hypothesis, in further searching for potential mechanism(s) of 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 the treatment of TSA. Both genes are also enriched in the $CD34^+CD90^+$ population when compared to the $CD34^+CD90^-$ cells. To evaluate which of these two genes is important for the TSA-mediated expansion of $CD34^+CD90^+$ 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 ES cells (8, 28, 33–38), but it has never been invested in HDACi-mediated $CD34^+$ cell expansion/maintenance.

Altogether, we propose the following model for our *ex vivo* PBSC expansion technology (Figure 6). The cytokine-based HSPC culture condition stimulates $CD34^+$ cells to proliferate ad differentiate; as a result, the majority 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 (Figure S9). With the addition of TSA to the cytokine-based culture condition, the expression level 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 of HDACi TSA-medicated expansion of $CD34^+CD90^+$ cells is, at least in part, by maintaining the expression of SALL4 during the *ex vivo* culture period. This model is further supported by down-regulation of SALL4 can reduce TSA-mediated $CD34^+CD90^+$ cells expansion (Figure 5E).

Conclusion

In summary, we report 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 demonstrate 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

This work was supported in part through NIH grant PO1HL095489, research funds from Leukemia and Lymphoma Society and V foundation (to LC), and by the Singapore Ministry of Health's National Medical Research Council under its Singapore Translational Research (STaR) Investigator Award and the National Research Foundation Singapore and the Singapore Ministry of Education under its Research Centres of Excellence initiative (to DGT). We want to thank Kol Jia Young, Justin Tan, Thalia Chai-Zhang and Alicia Stein for their help in editing the manuscript.

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Highlight

- HTS approach to identify compounds for the maintenance of PBSC $CD34^+CD90^+$ cells
- TSA treated $CD34^+$ cells *ex vivo* leads to preferential expansion of $CD34^+CD90^+$ cells
- Treatment of PBSC $CD34^+$ cells with TSA enhances marrow-repopulating potential *in vivo*
- SALL4 contributes to TSA-mediated expansion of $CD34^+CD90^+$ cells

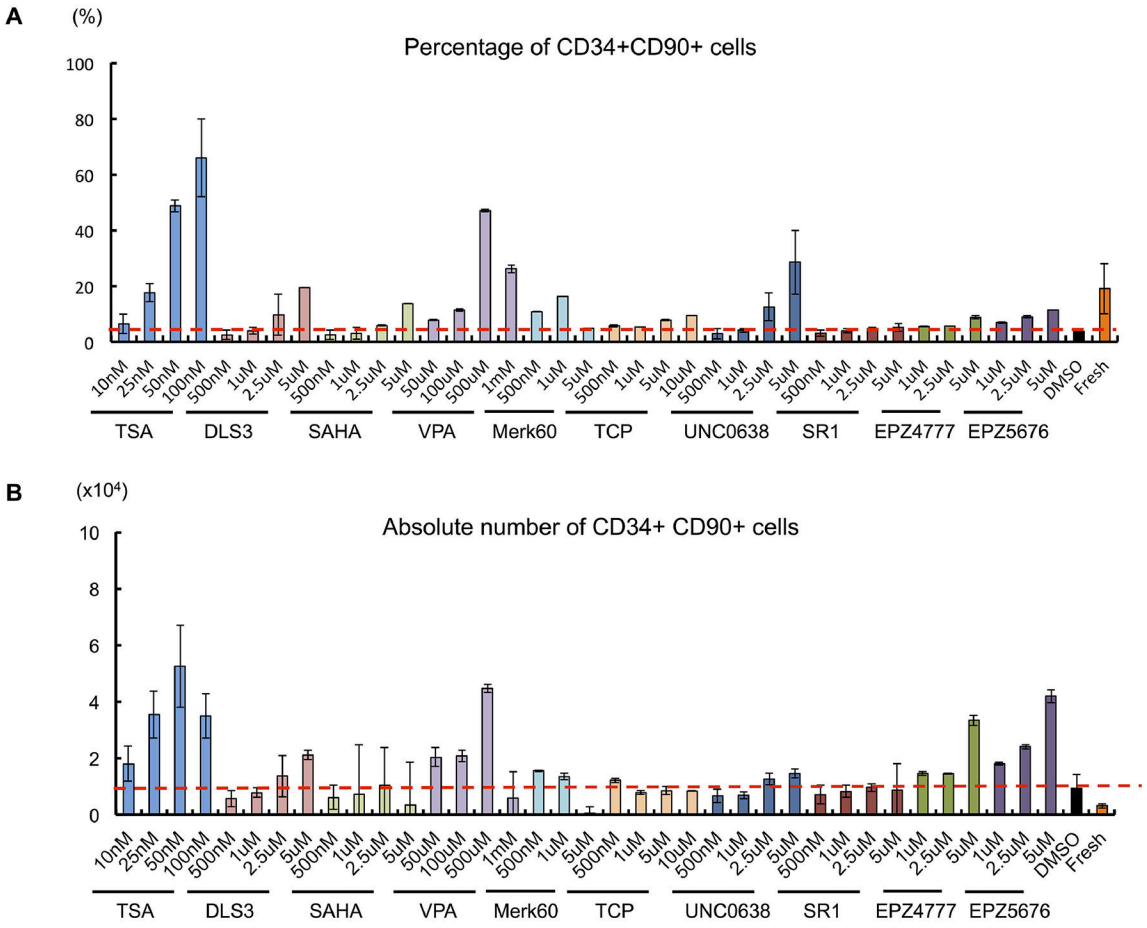

Figure 1. Screening for compounds which increase CD34^{+}CD90^{+} cells from PBSC.

(A) Effect of the compounds on percentage of CD34^{+}CD90^{+} expression following 5 days of culture. **(B)** Absolute number of CD34^{+}CD90^{+}cells after 5 days of culture with the compounds. Each value represents mean ± SE of two or three independent experiments.

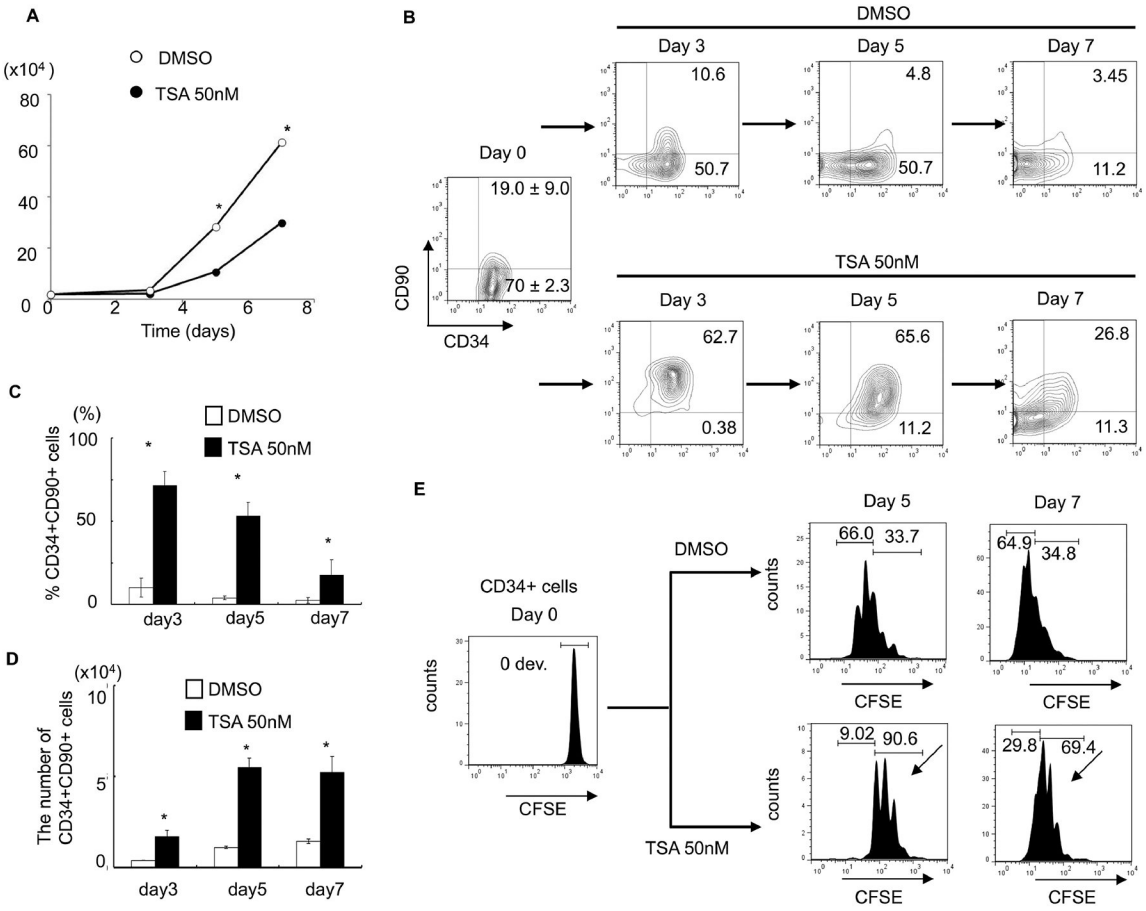

Figure 2. TSA-treated culture leads to preferential expansion of slowly dividing PBSC $CD34^+CD90^+$ cells.

(A) Cell growth of PBSC $CD34^+$ cells cultured in the presence of cytokines with TSA or DMSO at various concentration of TSA after 5 days of culture. The data shown are the mean of three independent experiments. Error bars indicate SD. **(B)** Time course experiment from day 0 to 7 days showing that TSA increased in the number of $CD34^+CD90^+$ cells. The data shown are the mean 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 50nM TSA. Error bars indicate SD; * indicates $p<0.05$. **(D)** Absolute number of $CD34^+CD90^+$ cells at 3, 5 and 7 days of culture in the presence or the absence of 50nM TSA. Error bars indicate SD; * indicates $p<0.05$. **(E)** CSFE-labeled $CD34^+$ cells were cultured in the presence of cytokines with TSA or DMSO treatment for 7 days. The panel shows a representative (1 of 3 experiment) 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 to $CD34^+CD90^-$ cells.

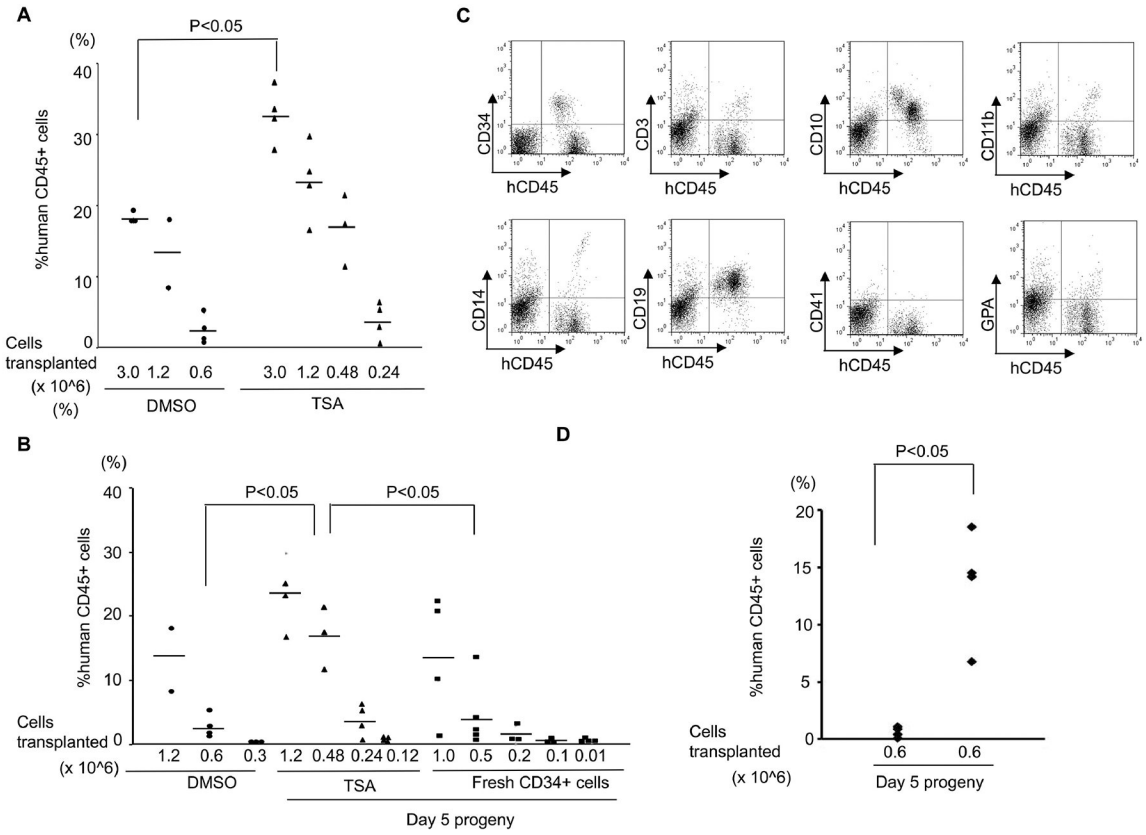

Figure 3. Treatment of PBSCs with TSA enhanced the marrow-repopulating potential.

(A) A scatter plot showing the levels of human $CD45^+$ cell engraftment in the bone marrow (BM) of NSG mice 8 weeks after transplantation with $CD34^+$ cells cultured with TSA or DMSO. **(B)** Limiting dilution analysis to compare the frequency of SRCs in the progeny from TSA-treated, control-treated culture conditions and those from fresh uncultured CD34+ cells. **(C)** Representative BM flow cytometric analysis of 8 weeks multilineage hematopoietic differentiation potential of engrafted human hematopoietic cells treated with TSA in NSG mice. **(D)** A scatter plot showing the levels of human $CD45^+$ cell engraftment in the BM of NSG mice 18 weeks after transplantation with their progeny after culture with TSA or DMSO.

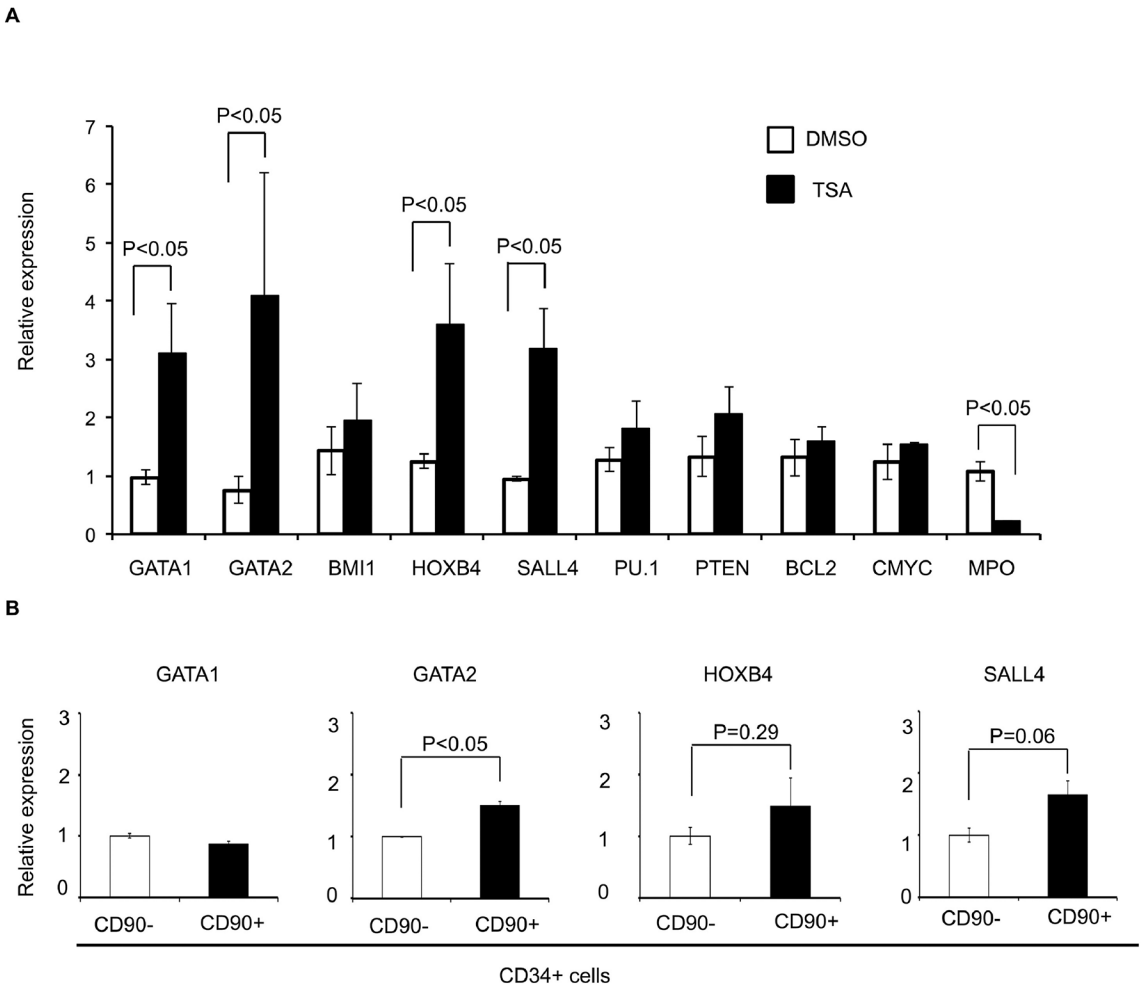

Figure 4. Treatment of PBSC CD34$^+$ cells with TSA modulates expression of stem cell related genes.

(A) Effects of TSA treatment on the relative transcript level of genes (*GATA1, GATA2, NOTCH1, BMI1, HOXB4, SALL4, PU.1, PU.1, PTEN, BCL2, c-MYC*, and *MPO*) were measured by real-time quantitative 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 2 independent samples. **(B)** TSA treated CD34$^+$CD90$^+$ cells and CD34$^+$CD90$^-$ cell were sorted and the expression levels of *GATA1, GATA2, HOXB4, and SALL4* were analyzed. Measurements were obtained in duplicate using at least 2 independent samples. Error bars indicate SE; * indicates $p<0.05$.

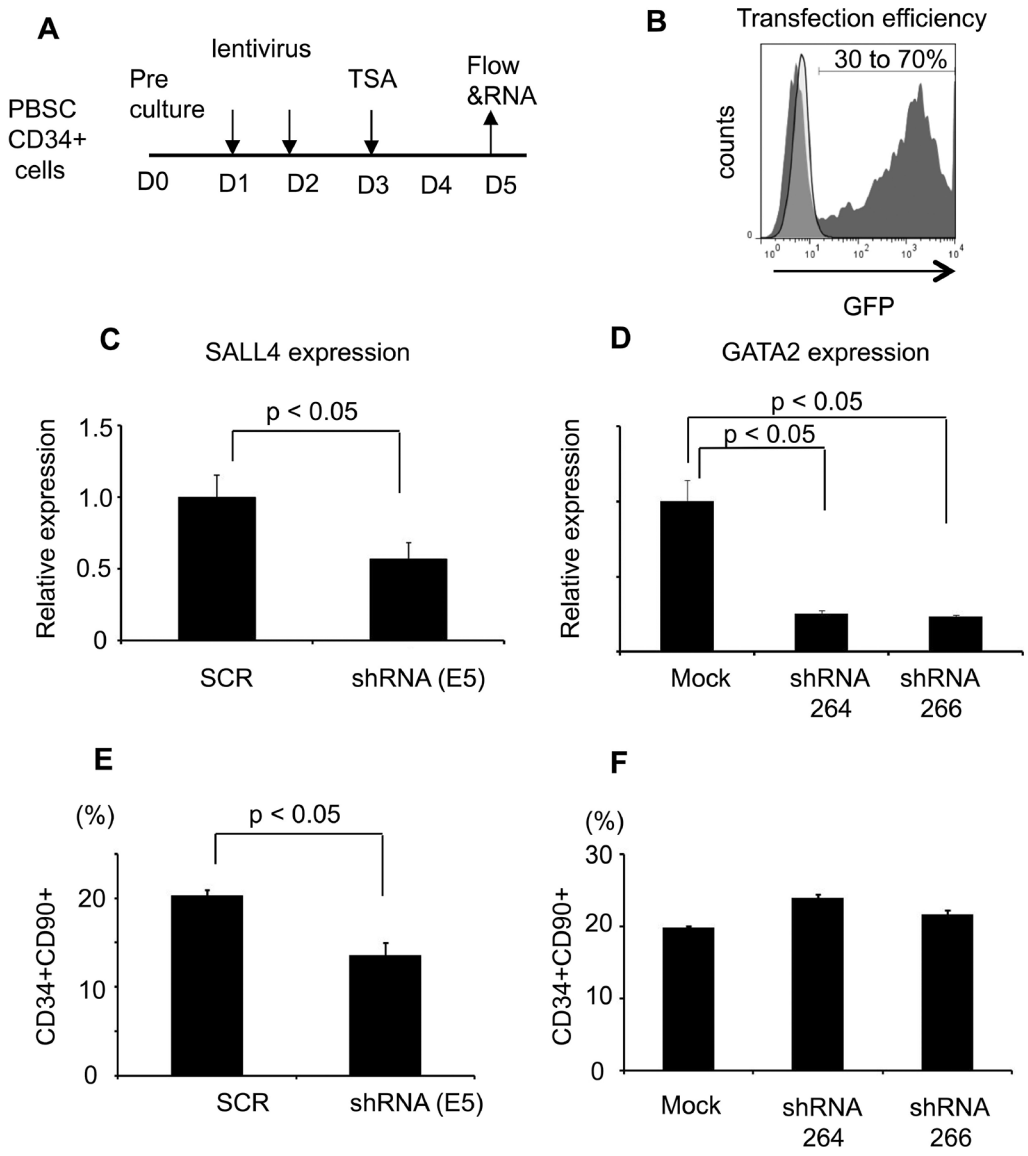

Figure 5. SALL4 silencing decreased TSA-mediated expansion of CD34$^+$CD90$^+$ cells.

(A) The strategy of knockdown of SALL4 and GATA2. **(B)** Transduction efficacy was evaluated by using lentiviral vectors pLL3.7 and pLKO.3G expressing GFP. **(C)** SALL4 mRNA expression after shRNA targeting in PBSC CD34$^+$ cells **(D)** GATA2 mRNA expression after shRNA targeting. **(E)** The percentage of TSA-mediated CD34$^+$CD90$^+$ cells after SALL4 silencing. **(F)** The percentage of TSA-mediated CD34$^+$CD90$^+$ cells after GATA2 silencing. (N=3, Error bars indicate SD)

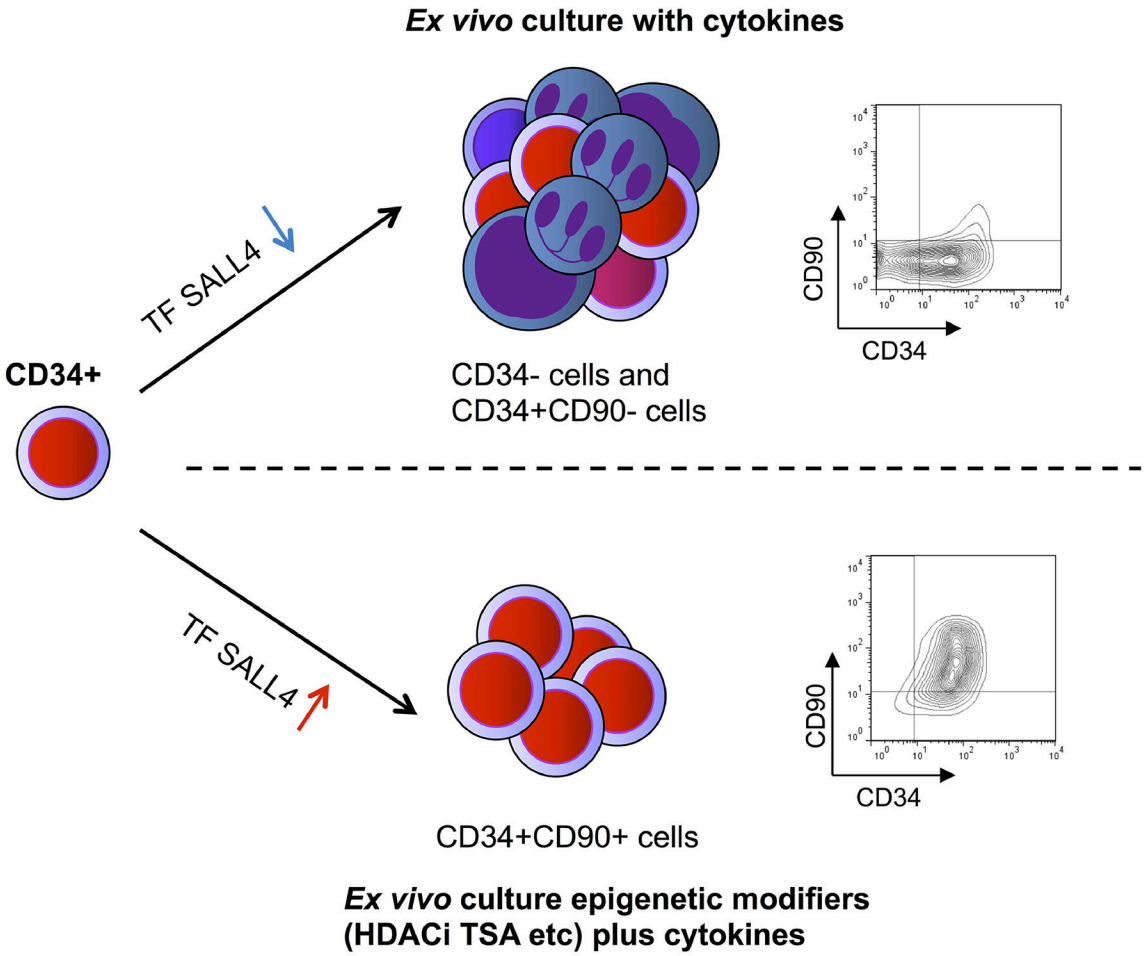

Figure 6. A schematic model of ex vivo CD34^{+}CD90^{+} expansion from human PBSC HSPCs.

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 the HDACi TSA to the culture media, results in expansion of CD34^{+}CD90^{+} cells. The TSA-mediated HSPC expansion functions, at least in part, through transcription factor SALL4 (TF SALL4).