Pharmacological inhibition of AKT activity in human CD34⁺ cells enhances their ability to engraft immunodeficient mice

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Abstract

Although practiced clinically for more than 40 years, the use of hematopoietic stem cell (HSC) transplants remains limited by the ability to expand functional HSCs *ex vivo*. To **determine** the role of PI3K/AKT signaling in human hematopoietic stem and progenitor cell (HSPC) maintenance, **we examined the effect of genetic and pharmacological inhibition of AKT on human cord blood CD34⁺ cells**. We found that knockdown of AKT1 in human cord blood CD34⁺ cells using siRNAs targeting AKT1 **enhances their quiescence and colony formation potential *in vitro***. We treated human cord blood CD34⁺ cells with an AKT-specific inhibitor (AKTi) and performed both *in vitro* and *in vivo* stem and progenitor cell assays. We found that *ex vivo* treatment of human HSPCs maintains CD34 expression and enhances colony formation in serial replating assays. Moreover, pharmacological inhibition of AKT enhances the short-term repopulating potential of human cord blood CD34⁺ cells in immunodeficient mice. Mechanistically, **genetic and pharmacological inhibition of AKT activity promotes human HSPC quiescence**. These pre-clinical results suggest a positive role for AKT inhibitor during *ex vivo* culture of human cord blood HSPCs.

Introduction

Several decades of successful bone marrow transplantations have demonstrated the therapeutic importance of HSCs [1-4]. The use of noninvasively accessible umbilical cord blood (UCB)-derived HSCs provides many advantages over bone marrow cells, including enhanced long-term immune recovery and decreased graft versus host disease [1-4]. However, low cell numbers in single UCB units have limited the suitability of UCB transplantation for adult patients [1-4]. Methods to robustly increase the number of cells that give a rapid and sustained blood count recovery would enable the use of UCB in more patients [1-4].

Culture conditions optimized for HSC expansion (serum-free media supplemented with cytokines, including thrombopoietin, stem cell factor, flt3 ligand, and interleukin-6) result in robust proliferation accompanied by differentiation, leading to loss of HSC activity [5]. Recent advances have allowed *ex vivo* expansion of hematopoietic stem and progenitor cells using cytokine cocktails combined with an array of factors, including aryl hydrocarbon receptor antagonists, Wnt activators, Notch ligands, angiopoietin-like proteins, prostaglandin E2, pleiotrophin or glycogen synthase kinase 3 (GSK-3) inhibitors in combination with insulin [6-12]. These approaches are encouraging, but in all cases have required supplementation with a cocktail of hematopoietic cytokines, which may promote lineage commitment at the cost of long-term HSC maintenance [9-12]. Thus, identification of molecules or factors that expand HSCs during *ex vivo* culture has remained an important goal of the field [6-8].

Deciphering the molecular mechanisms controlling HSC self-renewal is essential for developing clinical strategies that can enhance *ex vivo* HSC expansion [13-14]. HSC self-renewal

requires a complex crosstalk between extrinsic signals from the microenvironment and the cell-intrinsic regulators of HSCs to maintain an undifferentiated state [15-16]. The PI3K/PTEN/AKT signaling has been implicated in regulating mouse HSC self-renewal [17-19]. Although *Pten* deletion, which results in AKT activation, initially leads to a transient expansion of HSCs, the HSC pool is depleted over time [18]. In addition, overexpression of constitutively active AKT also exhausts HSC [19]. Polycomb group protein Bmi1 plays an important role in regulating HSC self-renewal [20] and we found that AKT-mediated phosphorylation of Bmi1 inhibits HSC self-renewal [21], suggesting that activation of PI3K/AKT signaling impairs mouse HSC maintenance.

The role of the PI3K/PTEN/AKT signaling in human hematopoietic stem cells is controversial [22-23]. While transient silencing of PTEN in human CD34⁺ cells enhances their proliferation potential and short-term repopulation capability [22], *ex vivo* rapamycin treatment of human cord blood CD34⁺ cells, which inhibits mTOR activity, enhances their engraftment of immunodeficient mice in serial transplantation assays [23]. Given that rapamycin can induce feedback activation of AKT signaling through an IGF-1R-dependent mechanism [24], there is a critical need to use specific inhibitors to modulate PI3K/AKT signaling in human HSPCs.

There are 3 AKT isoforms in mammalian cells: AKT1, AKT2, and AKT3. AKT1 and AKT2 are ubiquitously expressed and in greater abundance in hematopoietic cells, whereas AKT3 expression is most pronounced in the testes and brain but also can be expressed in lesser amounts in the hematopoietic system [17, 25]. In this study, **we found that knockdown of AKT1 in human cord blood CD34⁺ cells using siRNAs targeting AKT1 enhances their quiescence and**

colony formation potential *in vitro*. Importantly, we discovered that pharmacological inhibition of AKT activity with an AKT-specific inhibitor (AKTi) in human cord blood CD34⁺ cells promotes their quiescence and enhances their engraftment of the immunodeficient mice. Our studies may facilitate the development of innovative clinical strategies that can enhance the engraftment of human cord blood HSPCs.

Materials and methods

Mice

For the repopulation assay, 6 to 8 weeks old NSG (NOD.Cg-Prkdcid IL2rgtm1Wj1/Sz) mice were obtained from an on-site core breeding colony and used. The Institutional Animal Care and Use Committee (IACUC) of the Indiana University School of Medicine approved all experimental procedures.

Western blot analysis

Cells were washed twice with ice-cold phosphate-buffered saline, and lysed on ice in RIPA buffer (Sigma) supplemented with protease inhibitor (Roche) and phosphatase inhibitor (Roche). Cell lysates were sonicated and centrifuged at 13,200 rpm for 10 mins, and boiled with LDS sample buffer (ThermoFisher Scientific), separated by NuPAGE gel (ThermoFisher Scientific), transferred electrophoretically to a PVDF membrane (EMD Millipore), and immunoblotted with Phospho-AKT (Ser473), Phospho-AKT (Thr308), AKT, and GAPDH (Cell signaling) followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz). The blots were developed by the enhanced chemiluminescence technique

using the SuperSignal West Femto Maximum Sensitivity Chemiluminescent Substrate (Fisher Scientific).

Flow cytometry analysis

Human hematopoietic stem and progenitor cells were identified and evaluated by flow cytometry using single cell suspension stained with antibodies against human CD34-FITC, human CD34-PE, human CD3-FITC (BD Biosciences), GlyA (human CD235a)-PE (eBioscience), human CD71-APC, human CD34-PE/CY7, human CD3-APC/CY7, human CD45-APC, human CD19-PerCP-Cy5.5, human CD14-PE/CY7 and human CD33-FITC (BioLegend). Experiments were performed on Accuri C6 and FACSLSR II cytometers (BD Biosciences) and analyzed by using the FlowJo Version 9.3.3 software (TreeStar).

Purification of CD34⁺ human cord blood cells

Normal human CB samples were collected with institutional approval. Mononuclear cells (MNCs) were isolated by Ficoll-Paque™ Plus density gradient centrifugation (1.077g/ml, GE Healthcare Life Sciences). CD34⁺ cells were purified from MNCs by positive selection using human CD34 MicroBead Kit and LS separation columns (Miltenyi Biotec Inc) as manufacturer's instruction.

Cell culture and expansion

40, 000 purified CD34⁺ cells were expanded in StemSpan™ Serum-Free Expansion Medium (SFEM, STEMCELL Technologies) supplemented with rhSCF (100 ng/mL), rhTPO (100 ng/mL)

and rh-Flt-3L (100 ng/mL). To differentiate human HSPCs, purified CD34⁺ cells were cultured in the myeloid-promoting conditions: SCF (100 ng/ml), FLT-3 ligands (10 ng/ml), IL-3 (20 ng/ml), IL-6 (20 ng/ml), GM-CSF (20 ng/ml), and G-CSF (20 ng/ml) and the erythroid-promoting conditions: EPO (6 IU/ml) and SCF (100 ng/ml). Cytokines were purchased from PeproTech. AKT-specific inhibitor AKTi was obtained from Merk [26].

Hoechst 33342/Pyronin Y staining

Purified CD34⁺ cells were stained with 2.5 μ M Hoechst 33342 (Molecular Probes) for 45 minutes at 37 °C. Pyronin Y was added at the final concentration of 1 μ M and cells were further incubated for 45 minutes at 37°C. Thereafter, cells were kept on ice for 30 minutes. Cells were washed 2 times and stained with APC-conjugated anti human CD34 antibody [27].

Colony-Forming Cell (CFC) assay

Hematopoietic clonogenic progenitors were determined in methylcellulose medium (MethoCult H4435, StemCell Technologies) using 1,000 cultured or 250 sorted CD34⁺ cells per well (6-well plate). Colonies were scored 14 days after plating. For secondary CFC assays, cells were harvested and washed with PBS on day 14 after the first plating and 1×10^4 cells were replated as described above. Colonies were scored at Day 14.

NSG mouse transplantation assay

NSG mice were purchased from the Indiana University In vivo therapeutic core facility. Mice at the age of 8 weeks old were irradiated with a single dose of 2.5Gy. 200,000 human CD34⁺ cells were injected into irradiated NSG mice via their tail vein. Peripheral blood of NSG mice was

obtained every 4 weeks following transplantation, stained with anti-human CD45 FITC and anti-mouse CD45 PE antibodies, and then analyzed by flow cytometry. After 12 weeks, bone marrow cells were harvested and analyzed for human CD45 expression, to define the engraftment efficiency.

Transfection of siRNA into human CD34⁺ cells

Accell Non-targeting Pool control siRNA (Control siRNAs) and Accell human AKT1 smart pool siRNA (AKT1 siRNAs) were obtained from GE Dharmacon. 20,000 human CB CD34⁺ cells were suspended in Accell Delivery Medium (Dharmacon) with cytokines and seeded in 96-well plate. siRNAs were added in a final concentration of 1 μ M according to manufacturer's instructions (Dharmacon). Cells were transferred to CD34⁺ basic medium 48hrs after transfection [28].

Senescence analysis of human CD34⁺ cells

Sorted CD34⁺ cells were plated in 8-well chamber slides. Senescent cells were detected by using Senescence β -Galactosidase Staining Kit (Cell Signaling Technology) as manufacturer's instruction [29].

Statistical analysis

Data were analyzed statistically using either the student t test or One-way ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

Results

Transient knockdown of AKT1 increases human hematopoietic stem and progenitor cell quiescence

To determine whether knockdown of AKT1 expression alters the behavior of hematopoietic stem and progenitor cells (HSPCs), we transiently transfected either a Non-targeting Pool control siRNA (Control siRNAs) or a pool of siRNAs directed against AKT1 mRNA (AKT1 siRNAs) into purified CD34⁺ cells. AKT1 mRNA expression was decreased by 50% in AKT1 siRNAs-transfected cells at 48 hours compared to control siRNAs-transfected cells (Figure 1A). To determine how transient knockdown of AKT1 affects the primitiveness of human HSPCs, we examined the proliferative capacity of CD34⁺ cells in early-acting cytokine (rhSCF, rhTPO and rh-Flt-3L) containing liquid cultures following transfection of control siRNAs and AKT1 siRNAs. We quantified the CD34 expression by FACS over time and found that AKT1 deficiency altered neither the frequency nor the absolute number of CD34⁺ cells (Figures 1B and 1C). We then examined the effect of AKT1 deficiency on HSPC quiescence using Pylonin Y and Hoechst 33342 staining and found that transient knockdown of AKT1 is sufficient to increase human hematopoietic stem and progenitor cell quiescence (Figures 1D and 1E). To determine whether genetic inhibition of AKT affects CD34⁺ cell survival and senescence, we assessed the apoptosis as well as senescence of CD34⁺ cells 7 days after transfection. We observed comparable level of apoptotic (Annexin-V⁺/7-AAD⁻) cells in both control siRNAs and AKT1 siRNAs-transfected CD34⁺ cells (Figure 1F). We found that knockdown of AKT1 did not induce senescence in CD34⁺ cells (data not shown). These findings suggest that transient knockdown of AKT1 induces neither apoptosis nor senescence in HSPCs. To examine the effect of knocking down of AKT1 on HSPC function, we performed CFC assays. When we used 250 sorted CD34⁺ cells 7 days after transfection, we observed that AKT1 siRNAs transfected CD34⁺

cells formed more colonies compared to control siRNAs transfected CD34⁺ cells (Figure 1G). Thus, transient knockdown of AKT1 increases human HSPC quiescence and enhances their colony formation potential.

Pharmacological inhibition of AKT enhances colony forming capability of human HSPCs

Previously, we have shown that the AKT specific inhibitor (AKTi) can efficiently inhibit AKT activation in both mouse and human HSPCs [21, 30]. We found that treatment of CD34⁺ cells with AKTi (1 μ M) significantly decreased AKT phosphorylation both at serine 473 and threonine 308 (Figure 2A), demonstrating that AKT activity was inhibited by AKTi treatment. To determine how AKT specific inhibitor (AKTi) affects the primitiveness of human HSPCs, we examined the proliferative capacity of CD34⁺ cells in early-acting cytokine (rhSCF, rhTPO and rh-Flt-3L) containing liquid cultures in the presence of AKTi (1 μ M). We quantified the CD34 expression by FACS over time and found that a significant greater proportion of AKTi treated CD34⁺ cells retained CD34 expression (Figures 2B and 2C). However, AKTi treatment did not increase the absolute number of CD34⁺ cells over time (Figure 2D). We also examined the effect of AKT inhibition on primitive human HSCs (CD34⁺CD38⁻CD45RA⁻CD90⁺) and found that AKTi treatment did not increase the number of primitive HSCs (data not shown). Thus, AKTi promotes the retention of CD34 expression in hematopoietic progenitor cells after *ex vivo* culture. We then performed CFC assays using 1,000 cells from DMSO or AKTi treated cultures at Day 7 and found that AKTi treated cells growing in liquid culture generated more CFUs than DMSO treated cells during both primary and secondary CFC assays (Figures 2E and 2F). When we used 250 sorted CD34⁺ cells 7 days following DMSO or AKTi (0.2 μ M and 0.5 μ M) treatment in the CFC assays, we observed that AKTi treated CD34⁺ cells formed more colonies compared to

DMSO treated CD34⁺ cells (Figure 2G). Thus, pharmacologic inhibition of AKT retains human HSPC primitiveness, leading to a greater capacity to generate CFUs.

Inhibition of AKT activity enhances human HSPC quiescence

Quiescence is an important feature of human hematopoietic stem and progenitor cells [31-34]. Given that knocking down *AKT1* expression in human CD34⁺ cells enhances their quiescence (Figures 1D and 1E), we speculated that pharmacological inhibition of AKT activity promotes human HSPC quiescence. To test this, we cultured CB CD34⁺ cells in the presence or the absence of AKTi (0.1 μ M or 1 μ M) for 7 days and then examined the frequency of quiescent CD34⁺ cells by using Pyronin Y and Hoechst 33342 staining. We found that both low (0.1 μ M) and high (1 μ M) concentration of AKTi treatment significantly increased the frequency of quiescent CD34⁺ cells compared to DMSO treatment (Figures 3A and 3B), indicating that pharmacological inhibition of AKT activity promotes human HSPC quiescence. To determine whether inhibiting AKT activity affects CD34⁺ cell survival, we assessed the apoptosis of CD34⁺ cells 2 days following AKTi treatment and observed comparable level of apoptotic cells (Annexin-V⁺/7-AAD⁻) in both DMSO (0.01%) and AKTi (1 μ M)-treated CD34⁺ cells (Figure 3C). We observed no increase in apoptotic CD34⁺ cells (Annexin-V⁺/7-AAD⁻) 7 days after AKTi (0.1 μ M or 1 μ M) treatment (Figure 3D), suggesting that pharmacological inhibition of AKT activity does not induce apoptosis in HSPCs. To determine the effect of AKTi treatment on HSPC senescence, we performed senescence analysis using SA- β -gal staining [29] and found that neither DMSO nor AKTi treatment induces senescence in human CD34⁺ cells (Figure 3E). We utilized late passage *Bmi1*^{-/-} mouse embryonic fibroblasts (MEFs), which undergo

senescence, as positive controls [35]. Thus, AKT inhibition retains human HSPC primitiveness, at least in part, through promoting quiescence.

Inhibition of AKT activity restricts myeloid and erythroid differentiation of human HSPCs

To investigate whether AKT activity is important for the differentiation of human HSPCs, we first placed purified CD34⁺ cells in myeloid promoting medium along with DMSO (0.01%) or AKTi (1 μ M). The expression of human myeloid marker CD11b was assessed by flow cytometry at Day 9. The percentage of mature myeloid cells (CD11b⁺ cells) was significantly lower in the AKTi treated group compared with DMSO treated cells (Figure 4A), revealing that myeloid differentiation of human CD34⁺ cells may be delayed upon AKT inhibition. We then analyzed the effect of AKT inhibition on erythroid differentiation by culturing purified CD34⁺ cells in erythroid promoting medium along with DMSO (0.01%) or AKTi (1 μ M). The expression of human CD71 and GlyA was assessed by flow cytometry at Day 5. The generation of mature erythroid precursor cells (CD71⁺/GlyA⁺ cells) was significantly decreased following AKT inhibitor treatment compared with DMSO treated cells (Figures 4B and 4C). These findings demonstrated that inhibition of AKT activity delays human HSPCs differentiation toward the myeloid and erythroid lineages.

Pharmacological inhibition of AKT enhances the repopulating capability of human cord blood CD34⁺ cells

Quiescent HSCs exhibit a significantly higher engraftment potential than proliferating HSCs in transplantation assays [32-34]. Therefore, we sought to determine whether the enhanced quiescence seen in AKTi treated CD34⁺ cells would improve their engraftability in

immunodeficient mice. While 1 μ M AKTi treatment enhances the colony formation potential of CD34⁺ cells (Figures 2E, 2F, and 2G), inhibiting AKT activity decreased myeloid and erythroid differentiation (Figure 4). Further, we found that CD34⁺ cells show increased apoptosis following 1 μ M AKTi treatment (data not shown). To avoid the potential negative impact of high concentration of AKTi on transplantation outcome, we decided to utilize low concentration of AKTi (0.1 μ M), which enhanced the colony formation potential as well as the quiescence of CD34⁺ cells (Figures 2F and 3B), in transplantation experiments. Equal number of human CD34⁺ cells were treated with DMSO or AKTi (0.1 μ M) *in vitro*. 7 days later, we harvested cells and transplanted 200,000 cultured cells into sublethally irradiated NOD/SCID IL-2R γ null (NSG) mice. The engraftment of human cells in mice was analyzed every 4 weeks by monitoring human CD45 expression in peripheral blood harvested from the recipient mice. As shown in Figure 5A, we observed significantly increased engraftment in AKTi treated group compared to the control treated group at both 4 weeks and 8-weeks after transplantation. 12 weeks following transplantation, we examined human CD45 expression in the cells isolated from mouse bone marrow and observed a significant enhancement of AKTi treated cell engraftment, compared to DMSO treated CD34⁺ cells (Figure 5B). Next, we analyzed the multilineage reconstitution potential of AKTi treated CD34⁺ cells in the bone marrow of recipient mice. Both control group and AKTi treated group were capable of undergoing myeloid and lymphoid repopulation (Figures 5C, 5D, and 5E). While AKTi treated CD34⁺ cells show enhanced B lineage reconstitution compared to control treated group, inhibition of AKT activity decreased T cell and myeloid differentiation of CD34⁺ cells (Figures 5C, 5D, and 5E), which is consistent with *in vitro* liquid culture experiment shown in Figures 4A and 4B. Thus, AKT appears to restrict the self-renewal capacity of human HSPCs by promoting their cell cycle entry

and terminal differentiation, and pharmacological inhibition of AKT activity leads to enhanced quiescence and better engraftability of human CD34⁺ cells.

Discussion

Clinical hematopoietic transplantation outcomes are strongly correlated with the numbers of cells infused and low cell numbers in single UCB units have limited the suitability of UCB transplantation for adult patients [1-3]. The ability to increase the number of functional human HSCs *in vitro* or *in vivo* could provide improved treatment options for clinical transplantation and gene therapy protocols, however clinically relevant expansion of HSCs has been difficult to achieve [1-4]. In this study, we report that pharmacological inhibition of AKT activity in human cord blood CD34⁺ cells enhances their ability to engraft immunodeficient mice through promoting HSPC quiescence. In addition, our work uncovered an important role of PI3K/AKT signaling in regulating human HSPC maintenance and lineage commitments.

The PI3K/PTEN/AKT signaling regulates murine hematopoietic stem cell function as constitutive activation of AKT depletes HSC pool [17-19]. In addition, we demonstrated that AKT-mediated phosphorylation of Bmi1 inhibits HSC self-renewal [21]. Based upon these observations, we hypothesized that suppressing PI3K/AKT signaling in human HSPCs promotes their quiescence and enhances their self-renewal capability. Utilizing a serum-free culture system containing early acting cytokines, we showed that pharmacological inhibition of AKT activity in CB-derived CD34⁺ cells *ex vivo* improves human HSPC engraftment in NSG mice. Furthermore, inhibition of AKT activity promotes human HSPC quiescence. Transient knockdown of PTEN in

human CD34⁺ cells enhances their proliferation, promoting quiescent HSPCs to enter the cell cycle [22]. Thus, activation of PI3K/AKT signaling promotes human HSPC proliferation. Given that HSC quiescence correlates with its self-renewal capability [32-34], the enhanced engraftability of human HSPCs treated with AKT inhibitor is at least in part due to their enhanced quiescence.

In addition to HSC maintenance, PTEN also regulates terminal differentiation [18]. Loss of PTEN promotes both myeloid and T cell differentiation, but impairs B cell production in mice [18]. We found that pharmacological inhibition of AKT activity in human CD34⁺ cells results in decreased myeloid and T cell differentiation *in vivo*. AKT inhibitor treatment also decreases erythroid differentiation of human HSPCs. Thus, PI3K/AKT signaling pathway promotes differentiation of human HSPCs.

Cancer patients that received chemotherapy normally develop neutropenia, increasing the risk of infections and resulting in treatment delay [36-37]. Neutrophils are essential for the innate immune response and can protect patients from bacterial infections in the initial phase of transplantation, especially for patients undergo myelosuppressive chemotherapy [38-39]. Thus, the capability to generate matured blood cells is also critical for stem cell transplantation [11, 40]. While we observed increased engraftment of CD34⁺ cells treated with AKT inhibitor, the myeloid differentiation capacity of human HSPCs was decreased (Figure 6). To compensate the limitation of AKT inhibitor in human HSPC expansion, other factors, including Wnt activator [38, 41-42], should be used in combination with AKT inhibitor. We expect that the combination

of AKT inhibitor and the activator of canonical Wnt signaling will promote *ex vivo* expansion of functional HSPCs without affecting their differentiation capability (Figure 6).

In summary, our work has identified an important role of PI3K/AKT signaling in regulating human HSPC maintenance. Our data suggest that modulating AKT activity in human HSPCs may be useful to enhance their engraftability. Our findings may have a positive impact on the field of stem cell transplantation and provide novel clinical strategies that can enhance human HSPC transplantation outcome.

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Conflict-of-interest disclosure

The authors declare no competing financial interests.

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Figure legends

Figure 1. Transient knockdown of AKT1 is sufficient to increase human hematopoietic stem and progenitor cell quiescence. (A) AKT1 expression levels were measured by quantitative real-time PCR analysis 48 hours after siRNA transfection and normalized to GAPDH (*** $p < 0.001$, $n=3$). (B) The percentage of human CD34⁺ cells in expanding medium was measured by flow cytometric analysis 7 days after siRNA transfection ($p>0.05$, N.S., $n=4$). (C) The absolute number of human CD34⁺ cells in expanding medium was measured 7 days after siRNA transfection (N.S., $n=4$). (D) Flow cytometry analysis of G0 cells using cellular RNA staining (with Pyronin Y) and DNA staining (with Hoechst 33342). Percentage of CD34⁺ cells in the G0 phase of the cell cycle 7 days after siRNA transfection is shown. (E) Quiescence analysis of CD34⁺ cells 7 days after siRNA transfection (* $p<0.05$, $n=4$). (F) The frequency of apoptotic CD34⁺ cells (Annexin V⁺/7-AAD⁻) was measured 7 days after siRNA transfection ($p>0.05$, $n=4$). (G) Transient knockdown of AKT1 enhances colony formation of human CD34⁺ cells. CFC assays were performed using 250 sorted CD34⁺ cells 7 days after siRNA transfection (*** $p < 0.001$, $n=3$).

Figure 2. Pharmacological inhibition of AKT maintains human HSPCs and enhances colony forming capability. (A) Inhibiting AKT activity in CD34⁺ cells using an AKT-specific inhibitor (AKTi) decreased AKT phosphorylation both at serine 473 and threonine 308. Western blot analysis of AKT phosphorylation in CD34⁺ cells 24 hours after DMSO or AKTi (1 μ M) treatment. (B) Flow cytometric analysis of human CD34 expression 7 days after AKTi (1 μ M) or DMSO treatment. (C) Inhibition of AKT maintains the immature surface marker of human

HSPCs in vitro. The percentage of human CD34⁺ cells in expanding medium supplemented with vehicle (DMSO) or AKTi (1 μ M) is shown (*** p < 0.001, n =3). (D) The absolute number of human CD34⁺ cells in expanding medium was measured 7 days after AKTi (1 μ M) or DMSO treatment (p >0.05, n =3). (E) Inhibition of AKT activity enhances colony formation of human CD34⁺ cells. CFC assays were performed using equal number of cultured cells treated with DMSO (0.01%) or AKTi (0.1 μ M and 1 μ M) for 7 days (* p < 0.05, ** p < 0.01, *** p < 0.001, n =3). (F) 10,000 cells collected from primary colonies were used in the secondary CFC assays (* p < 0.05, *** p < 0.001, n =3). (G) CFC assays were performed using 250 sorted CD34⁺ cells 7 days after DMSO (0.01%) or AKTi (0.2 μ M and 0.5 μ M) treatment.

Figure 3. Inhibition of AKT activity increases human CD34⁺ cell quiescence. (A) Inhibiting AKT activity enhanced HSPC quiescence, as measured by Pyronin Y and Hoechst staining. Flow cytometric analysis of the G0 population of CD34⁺ cells 7 days after AKTi (0.1 μ M and 1 μ M) or DMSO treatment, using cellular RNA staining (with Pyronin Y) and DNA staining (with Hoechst 33342). Percentage of CD34⁺ cells in the G0 phase of the cell cycle following AKTi treatment is shown. (B) Quiescence analysis of CD34⁺ cells 7 days after AKTi (0.1 μ M and 1 μ M) or DMSO treatment (* p <0.05, ** p <0.01, n =3). (C) Flow cytometric analysis of apoptotic CD34⁺ cells 2 days after AKTi (1 μ M) or DMSO treatment, using Annexin-V and 7-AAD staining. The left lower gate, the right lower gate, and the right upper gate represent the live cells, the early apoptotic cell and the late apoptotic cells, respectively. (D) The frequency of apoptotic CD34⁺ cells (Annexin V⁺/7-AAD⁺) was measured 7 days after DMSO or AKTi (0.1 μ M or 1 μ M) treatment (p >0.05, n =3). (E) Senescence analysis of CD34⁺ cells using SA- β -gal

staining following DMSO or AKTi treatment. Senescent *Bmi1*^{-/-} mouse embryonic fibroblasts cells (MEFs) were utilized as positive controls.

Figure 4. Inhibition of AKT activity decreases myeloid and erythroid differentiation of human HSPCs. (A) Inhibition of AKT activity **delays** myeloid differentiation of CD34⁺ cells. Purified CD34⁺ cells were cultured in myeloid-promoting medium containing DMSO (0.01%) or AKT inhibitor (1 μ M). The percentage of myeloid cells (CD11b⁺) was determined at Day 9. (B) Purified CD34⁺ cells were cultured in erythroid-promoting medium containing DMSO (0.01%) or AKT inhibitor (1 μ M). The percentage of mature erythroid cells (CD71⁺/GlyA⁺) was determined at Day 5. (C) Inhibition of AKT activity in CD34⁺ cells decreases the generation of mature erythroid cells (*p<0.05, n=3).

Figure 5. Pharmacological inhibition of AKT enhances the repopulating capability of human cord blood CD34⁺ cells. (A) The percentage of human CD45⁺ cells in the peripheral blood of recipient mice at 4 and 8 weeks following transplantation (*p < 0.05, **p < 0.01, n=5). (B) Frequency of human CD45⁺ cells in the bone marrow of recipient mice 12 weeks following transplantation (**p < 0.01, n=5). (C) Flow cytometric analysis of human myeloid cells (CD33⁺/CD14⁺) in the bone marrow of recipient mice 12 weeks following transplantation. (D) Flow cytometric analysis of human lymphoid cells (CD3⁺ or CD19⁺) in the bone marrow of recipient mice 12 weeks following transplantation. (E) Frequency of donor-derived myeloid cells, T cells and B cells in the bone marrow of recipient mice 12 weeks following transplantation (**p < 0.01, n=5).

Figure 6. Working Model. Pharmacological inhibition of AKT activity in human cord blood CD34⁺ cells enhances their quiescence *in vitro* and promotes their engraftment in immunodeficient mice *in vivo*; however, the myeloid differentiation of human HSPCs was delayed. We predict that the combination of AKT inhibitor and other factors, including the activator of canonical Wnt signaling, will enhance HSPC quiescence and promote *ex vivo* expansion of functional HSPCs without affecting their differentiation capability.

Graphical Abstract

Model

Figure

Figure 1

Figure 2

Figure 3

A

B

C

D

E

A

Myeloid differentiation

B

Erythroid differentiation

C

Figure 5

Figure 6