Effects of signaling pathway inhibitors on hematopoietic stem cells

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Abstract. While there are numerous small molecule inhibitory drugs available for a wide range of signalling pathways, at present, they are generally not used in combination in clinical settings. Previous reports have reported that the effects of glycogen synthase kinase (GSK)3β, p38MAPK, mTOR and histone deacetylase signaling combined together to suppress the stem-like nature of hematopoietic stem cells (HSCs), driving these cells to differentiate, cease proliferating and thereby impairing normal hematopoietic functionality. The present study aimed to determine the effect of HDACs, mTOR, GSK-3β and p38MAPK inhibitor combinations on the efficient expansion of HSCs using flow cytometry. Moreover, it specifically aimed to determine how inhibitors of the GSK3β signaling pathway, in combination with inhibitors of P38MAPK and mTOR signaling or histone deacetylase (HDAC) inhibitors, could affect HSC expansion, with the goal of identifying novel combination strategies useful for the expansion of HSCs. The results indicated that p38MAPK and/or GSK3β inhibitors increased Lin^- cell and $Lin^-Sca\text{-}1^+c\text{-}kit^+$ (LSK) cell numbers *in vitro*. Taken together, these results suggested that a combination of p38MAPK and GSK3β signaling may regulate HSC differentiation *in vitro*. These findings further indicated that the suppression of p38MAPK and/or GSK3β signalling may modulate HSC differentiation and self-renewal to enhance HSC expansion.

Introduction

At present, the treatment of leukemia is dependent upon hematopoietic stem cell (HSC) transplantation (1), as HSCs are multipotent cells which can reconstitute the immune and hematological systems following irradiation (2-4). However, relatively few HSCs can be obtained from donor cord blood or bone marrow samples, and as such, the numbers obtained are insufficient to meet clinical demands (5,6). To date, numerous studies have reported that signaling through the p38MAPK, mTOR, glycogen synthase kinase (GSK)3β and histone deacetylases (HDACs) altered HSCs functionality in ways that were disadvantageous, impairing their ability to proliferate and driving the HSCs to undergo differentiation, thereby impairing the normal hematopoietic restoration of blood cells (7-9).

p38MAPK is an important MAPK, and the activation of p38MAPK signaling has been demonstrated to promote necrosis and differentiation, in addition to impairing the proliferation of HSCs (7,10,11). It was previously reported that the inhibition of p38MAPK rescued the regenerative defects of HSCs caused by reactive oxygen species production, prolonging the life of HSCs and maintaining their self-renewal (12). In addition, it was discovered that the inhibition of p38MAPK induced the homing of HSCs into the bone marrow in response to chemotactic factors (13), thereby improving the proliferation of leukemia cells or lymphocytes (14). Notably, p38MAPK signaling was also observed to be associated with chemotherapy resistance in patients with leukemia (15).

While first studied in the context of glycogen metabolism in the liver, GSK3β has also been identified as an important regulator of HSC homeostasis (16,17). In a previous study, GSK3β activation was reported to drive HSC apoptosis and differentiation through p53 downregulation (18). Moreover, the inhibition of microRNA-126 regulated the PI3K/AKT/GSK3β signaling pathway and promoted the proliferation of HSCs (19). It was also illustrated that GSK3β promoted the migration of hematopoietic stem progenitor cells by regulating cytoskeletal rearrangement (20).

HDACs are able to induce histone lysine deacetylation, thereby modulating the progression of a wide range of diseases and biological processes (21). For example, the HDAC inhibitor valproic acid sodium salt (VPA) was discovered to enhance HSC proliferation, and as such, it is currently used in clinical contexts to treat myelodysplastic syndrome (22-25) and acute myeloid leukemia (AML) (26). Meanwhile, HDAC8 was discovered to aberrantly deacetylate p53 and promote leukemic stem cells(LSC) transformation and maintenance. Conversely, HDAC8 inhibition induced LSC apoptosis and restored p53 activity, suggesting the inhibition of HDAC8 as a promising approach to selectively target LSCs (27). mTOR signaling serves as a mechanism through which cells integrate inputs from numerous different factors, including oxygen,

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nutrients and cytokines, to induce appropriate growth, proliferation or survival responses (28).

At steady-state *in vivo*, the majority of HSCs remain dormant, with only a small fraction of progenitor cells serving to mediate blood cell production (29). However, under conditions of stress or inflammation, these HSCs can become activated and differentiate into additional progenitor cells, thereby enhancing the rate of blood cell replenishment (30). The rate of hematopoietic reconstitution in patients was discovered to be influenced by a wide range of variables, including the source of the donor HSCs, how many were transplanted and whether or not the recipient patients had undergone prior chemotherapy treatment (31). In a highly complex intracellular signaling network, the appropriate regulation of a signaling pathway is strongly dependent on the communication with other signaling molecules, resulting in synergistic or antagonistic relationships and a variety of biological effects (32). Thus, understanding the effects of inhibitors of different signaling pathways on the *in vitro* proliferation of HSCs is a promising strategy to promote the clinical application of HSCs.

Small molecule compounds that hold the potential to expand HSCs are of great promise in the stem cell transplantation field. Notably, current available small molecule compounds primarily affect several important signaling pathways, such as p38MAPK, mTOR, GSK3β and HDAC (31,33-35). Therefore, strategies to regulate these crucial signaling pathways may be of importance for effective HSC expansion *in vitro*. To effectively amplify available HSCs, the bone marrow microenvironment must be effectively mimicked *in vitro* without adversely impacting HSC activity (36). However, such mimicry is complex, as a wide range of mechanical and cytological stimuli work in concert in the bone marrow to modulate signaling pathway activation within these HSCs, thereby governing their ultimate functionality.

At present, research into expanding HSCs has predominantly focused on the following aspects: Promoting self-renewal, inhibiting differentiation, inhibiting apoptosis and promoting homing (13,37-39). HSCs are contained in the LSK cell population; phenotypically, LSK cells express stem cell antigen (Sca)-1 and c-Kit, but lack the lineage (Lin) markers expressed on mature myeloid and lymphoid cells (40). The present study aimed to investigate the efficacy of small molecule inhibitors on the manipulation of HSCs, especially the expansion of HSCs *in vitro*. Different combinations of inhibitors of the p38MAPK, mTOR, GSK3β and HDACs were used to treat HSC cultures at a range of concentrations, in order to explore which signaling pathways could be targeted to expansion HSCs more effectively *in vitro*. SB203580 was the first reported inhibitor of p38 (41,42), which was discovered to permeate cells and inhibit the activation of MAPK activated protein kinase (MAPKAPK)-2 and MAPKAPK-3, which subsequently inhibits the partial signal transduction induced by certain inflammatory factors, such as IL-1β and TNF-α. SB216763 is an effective, selective GSK3β inhibitor (43), which was discovered to activate glycogen synthase, stimulates glycogen synthesis in human hepatocytes, induces the expression of reporter genes regulated by β-catenin and promotes the accumulation of β-catenin, in which β-catenin is an important downstream effector of the Wnt signaling transduction pathway (44). Similarly, CHIR99021 is a GSK3α and GSK3β inhibitor (45), which was illustrated to promote HSC self-renewal, maintain colony morphology and regulate epigenetics by activating the Wnt signaling pathway (46,47). The results of the present study demonstrated that suppressing p38MAPK and/or the GSK3β signaling pathway effectively amplified HSCs *in vitro*.

Materials and methods

Materials. The Cyan ADP flow cytometer, Moflo XDP flow cytometer and cryogenic high-speed centrifuge were all purchased from Beckman Coulter, Inc. The cell counter was obtained from Countstar. In addition, 10X Red Blood Cell Lysis buffer was acquired from eBioscience, 0.4% Trypan blue dye was provided by Sigma-Aldrich; Merck KGaA and FCS, SB203580, CHIR99021, rapamycin, VPA and SB216763 were purchased from Selleck Chemicals. The EasySeo™ Mouse SCA1 Positive Selection kit (cat. no. 18756) was provided by Stemcell Technologies, Inc.

Animal studies. Female C57BL6/J mice (age, 6-8 weeks, weight, 20-25g) were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. All mice were housed under a 12-h light/dark cycle in microisolator cages contained within a laminar flow ventilation system in the animal facility at the experimental Animal Center, Shanghai Normal University under specific-pathogen-free (SPF) conditions and used as donors to collect bone marrow HSCs. The mice were sacrificed by cervical dislocation (n=4 per group). All animal studies were approved by the Institutional Animal Care and Use Committee of Shanghai Normal University.

Cell culture and flow cytometry. Bone marrow cells were isolated from murine femur and tibia bone marrow as previously described (48). Briefly, the bones were dissected and crushed three times with a pestle and, then the cells were collected in 6 ml dissociation solution (PBS with 2% FCS and 145 U/ml type-4 collagenase; Gibco; Thermo Fisher Scientific, Inc.) and incubated at 37˚C for 30 min. Samples were subsequently filtered using a 40-μm Nylon cell strainer to obtain single cell suspensions. The EasySep™ Mouse SCA1 Positive Selection kit was then used to isolate Sca1-PE positive cells.

Subsequently, the pre-prepared primary antibodies mixture (2% FCS-PBS solution) containing anti-CD11b-biotin (clone no. M1/7; eBioscience; cat. no. 13-0112-85; dilution, 1:100), anti-B220-biotin (clone. no. RA3-6B2; eBioscience; cat. no. 13-0452-85; dilution, 1:100), anti-CD3e-biotin (clone no. 145-2C11; eBioscience; cat. no. 13-0031-85; dilution, 1:100), anti-Gr-1-biotin (clone. no. RB6-8C5; dilution, 1:100), anti-Ter119-biotin (clone no. TER-119; eBioscience; cat. no. 13-5921-85; eBioscience; cat. no. 13-5931-85; dilution, 1:100), anti-Sca-1-PE (clone no. D7; eBioscience; cat. no. 12-5981-83; dilution, 1:100) and anti-c-Kit-APC (clone no. 2B8; eBioscience; cat. no. 17-1171-83; dilution, 1:100) was added to the cell suspension (1-2x10^6 cells/100 μl) and incubated at room temperature in the dark for 15 min. After washing the cells with 2% FCS-PBS three times, the cells labelled with biotinylated antibodies were incubated with a streptavidin-FITC secondary antibody (eBioscience; cat. no. 11-4317-87; dilution, 1:100) at room temperature in the dark for 15 min. The cells were subsequently washed three

Table I. Inhibitor combinations and corresponding abbreviations.

Group	Abbreviation
Freshly isolated	A
DMSO	B
VPA	C
SB203580	D
Rapamycin	E
SB216763	F
SB203580 + SB216763	G
SB203580 + VPA	H
SB216763 + VPA	I
Rapamycin + SB216763	J
SB203580 + rapamycin	K
Rapamycin + VPA	L
SB203580 + rapamycin + SB216763	M
SB203580 + rapamycin + VPA	N
SB203580 + VPA + SB216763	O
Rapamycin + SB216763 + VPA	P
VPA, valproic acid	sodium salt

1 μM for the P38MAPK signaling pathway inhibitor SB203580; 15 nM for the mTOR signaling pathway inhibitor Rapamycin; 500 nM for the GSK3β signaling pathway inhibitor CHIR99021; and 1 mM for the HDAC signaling pathway inhibitor VPA. We now combine these inhibitors in different combinations and represent them with corresponding signaling pathway names, and finally we use the letters A-Q for each combination.

times with PBS and resuspended in 2% FCS-PBS, and the LSK hematopoietic progenitor cells were subsequently sorted by flow cytometry on a Moflo XDP cell sorter.

The sorted cells were then cultured in DMEM (Sigma-Aldrich; Merck KGaA), supplemented with 10% FBS (Gibco; Thermo Fisher Scientific), penicillin-streptomycin and gentamicin in humidified incubators with anti-vibration platform at 37˚C with 5% CO_2. The medium also contained 20 ng/ml TPO (cat. no. 315-14-10), 20 ng/ml SCF (cat. no. 250-03-50), 20 ng/ml Flt3-ligand (cat. no. 250-31L-10) (all from PeproTech, Inc.), 10 μg/ml heparin and 0.1 mM β-mercaptoethanol.

For treatment, the cells were treated with combinations of SB203580, CHIR99021, VPA, SB216763 and rapamycin at the indicated concentrations or DMSO (Ctr) at 37˚C with 5% CO_2 for 9 days, followed by flow cytometric analysis.

The optimal inhibitory concentration of these five compounds had been obtained (SB216763, 1 μM; rapamycin, 15 nM; CHIR99021, 500 nM; VPA, 1 mM), cells were treated with different combinations of these inhibitors (Table I) at 37˚C with 5% CO_2, then examined with under a Leica light microscope (magnification, x40).

Statistical analysis. Data are presented as the mean ± SEM of three independent experiments. Statistical differences were determined using a one-way ANOVA, followed by Bonferroni correction, while the statistical differences between the groups presented in Fig. S6 were analyzed using an unpaired Student's t-test with GraphPad prism 6.02 (GraphPad Software, Inc.) software. $P<0.05$ was considered to indicate a statistically significant difference.

Results

SB203580, an inhibitor of the p38MAPK signaling pathway, enhances in vitro HSC functionality. To determine how p38MAPK signaling influences HSC expansion *in vitro*, LSK cells were treated over a 9-day period using a specific inhibitor of this pathway, SB203580. The treatment with this inhibitor increased the number compared with the DMSO control (Fig. 1A and B). Significant changes were observed in the LSK cell counts in a dose-dependent manner. At 5-20 μM, the number of cells began to decline, such that following the treatment with 1 μM SB203580, the number of LSK cells was significantly increased, while the frequency of Lin^- cells at this concentration did not significantly increase, compared with the DMSO control (Fig. 1C-G). In addition, at 1 μM, the absolute number of Lin^- cells (Fig. 1C) and the frequency and absolute of LSK (Fig. 1F and G) significantly increased, compared with DMSO. To improve on the accuracy of these results, whole bone marrow cells should be used for future experiments. These results suggested that inhibiting p38MAPK signaling may alter HSC differentiation and expansion *in vitro*.

Inhibition of GSK3β signaling significantly enhances HSC expansion in vitro. Given the complexities of the *in vivo* bone marrow microenvironment and the role of GSK3β as a regulator of HSC functionality (8), HSCs were treated with SB216763, a specific inhibitor of this pathway. At 2 μM, treatment with SB216763 led to changes in morphology and increased proliferation (Fig. 2A and B). In addition, an increase in the number of total cells (Fig. 2C), number of Lin^- cells (Fig. 2D), LSK cell proportion (Fig. 2E) and LSK cells absolute number (Fig. 2F) was also observed, compared with CHIR99021 treatment (Figs. 2 and S1). Although the increase amplitude of CHIR99021 was higher than that of SB216763 draft at 1 μM, the increase of LSK was not obvious at this concentration (Fig. 2C). By comparison, SB216763 was identified to more effectively enhance HSC proliferation, compared with CHIR99021 (Figs. 2, S2 and S3).

Based on these findings, it was hypothesized that the combined inhibition of p38MAPK and GSK3β signaling pathways may more effectively expand HSCs. Therefore, excluding the cytotoxic effect of DMSO on the cells (Fig. S4), the combination of SB203580 and SB216763 treatment was used to observe the expansion of HSCs; it was identified that the proportion of Lin^- and LSK cells were not significantly different, compared with 1 μM SB203580 treatment alone (Fig. S5). However, compared with the DMSO group, the total number of cells, the frequency and absolute of Lin- cells, the frequency and absolute of LSK cells of G group was significantly increased (Fig. S6 and Table I), suggesting that p38MAPK and GSK3β inhibitors may exert a synergistic effect in promoting HSCs expansion.

HDAC signaling inhibitor VPA alters HSC expansion in vitro. The highest total cell number (Fig. 3B), as well as relative and absolute Lin^- and LSK cell numbers (Fig. 3C, E and G)

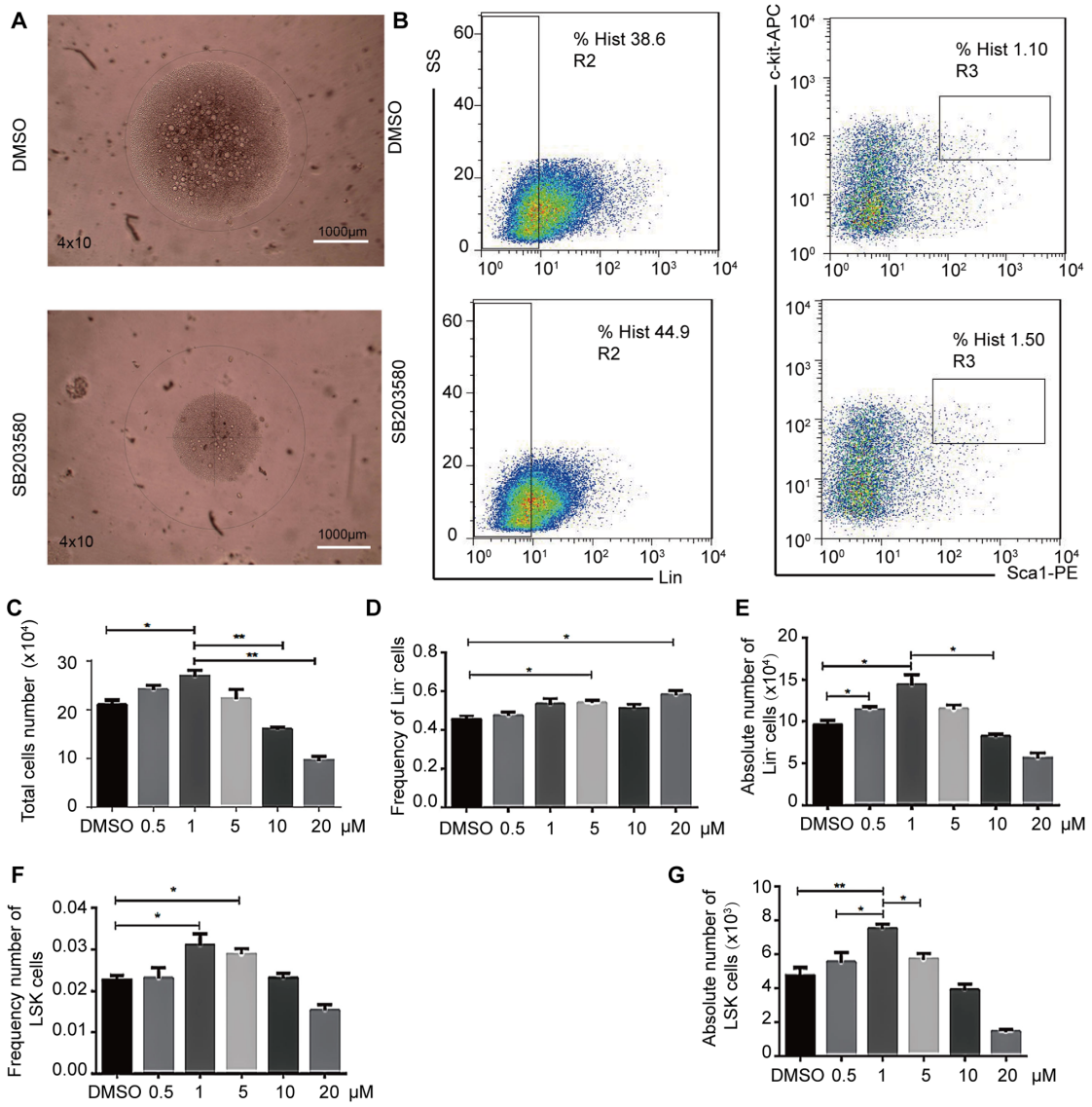

Figure 1. Effects of p38MAPK inhibition on hematopoietic stem cell expansion *in vitro*. LSK cells cultured *in vitro* for 9 days were first microscopically observed and photographed, and then were collected, stained with antibodies against Gr1, CD11b, Ter119, CD3ε, B220, Sca-1, and c-Kit, then analyzed by flow cytometry. (A) LSK cell morphology. n=4. Scale bar, 1,000-μm. (B) Flow cytometric analysis of LSK cells treated with 1 μM SB203580 or equal volume DMSO. n=3. (C) Total cell number. Magnification, x40. Total images were obtained using the confocal Leica DM RXA microscope. (D) Relative and (E) absolute Lin$^-$ cell numbers. (F) Relative and (G) absolute LSK cell numbers. n=4; *P<0.05, **P<0.01. Lin, lineage; APC, allophycocyanin; PE, phycoerythrin; SS, side scatter; Sca-1, stem cell antigen-1.

were achieved at the 0.25 mM dose of VPA (HDAC inhibitor). However, it was found that the frequency of Lin$^-$ and LSK cells increased linearly with increasing VPA concentration (Fig. 3D and F). Meanwhile, when the VPA concentration was 1 mM, the number of cells was significantly reduced and were transparent and uniform, in a very good state under the microscope (Fig. 3A) and maintained the cells in an undifferentiated state. This 1 mM dose was therefore used in the following experiments. At the same time, the number of Lin$^-$ cells was discovered to significantly increase in the H and I groups compared with B, while the combination of VPA and other inhibitors exerted a minimal effect on stem cell expansion, demonstrating population frequencies similar to the DMSO control sample (Fig. S6 and Table I). Several groups of cells were seen to be highly differentiated, especially group B. The number of differentiated cells was much higher than the other experimental groups, while groups C, H, I, N, O and P could maintain an even transparent morphology, but the cells barely grew after all four inhibitors were added, so we did not count this group when we collected the data, i.e., group Q above was invalid (Fig. S6 and Table I).

mTOR signaling inhibition alters in vitro HSC expansion. To determine how mTOR inhibition affects HSC functionality *in vitro*, cells were cultured with (0, 10, 15, 20, 25 or 30 nM) rapamycin for nine days, adding additional rapamycin (0, 10, 15, 20, 25 or 30 nM) every two days during culture to overcome metabolism of rapamycin. Following 9 days of rapamycin treatment, the cells were discovered to be healthy, with very few differentiated cells under the microscope, a lower number of cells overall and uniform cellular morphology (Fig. 4B), suggesting a rapamycin-dependent inhibition of cell growth.

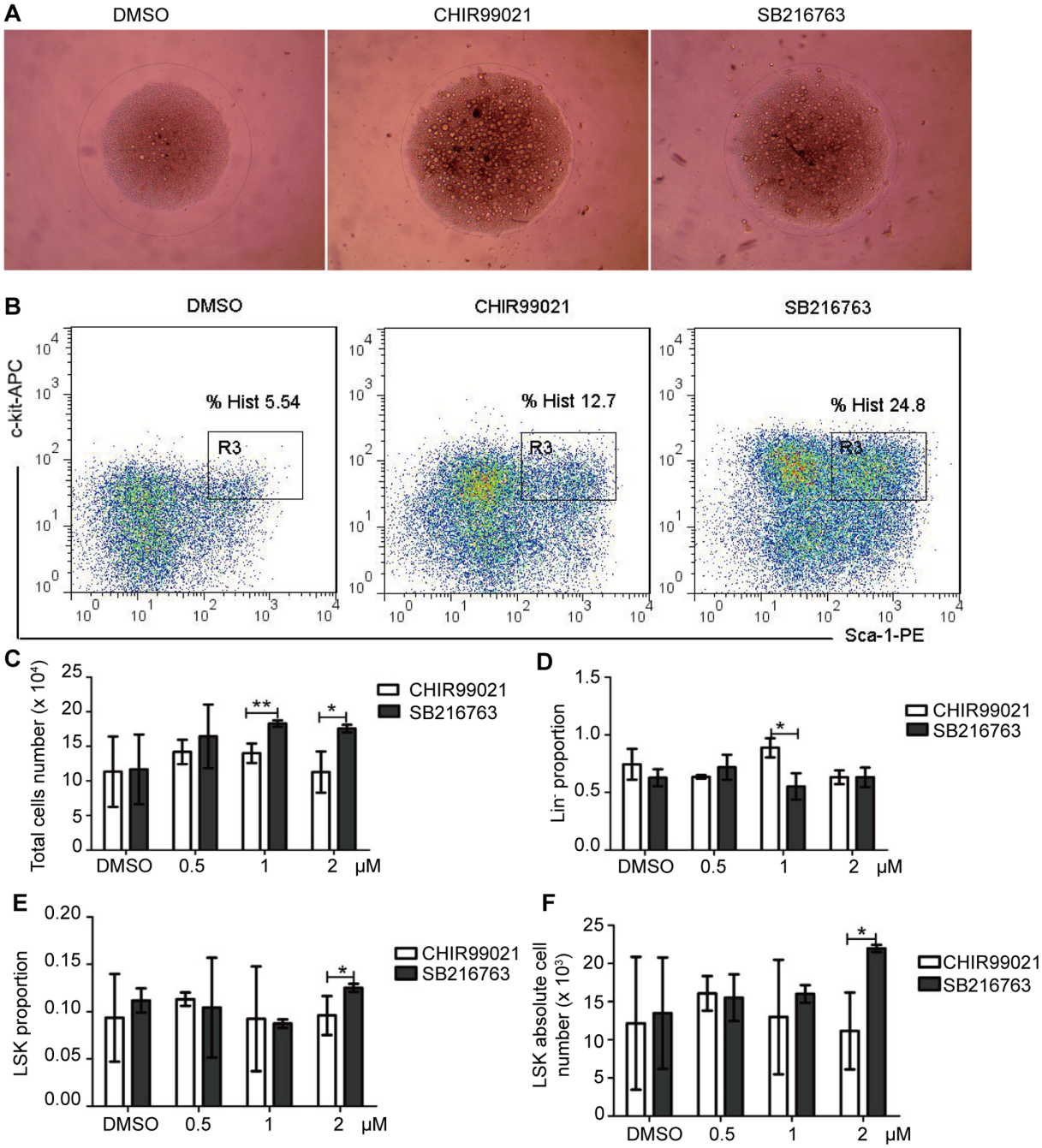

Figure 2. GSK3β inhibition alters hematopoietic stem cell expansion *in vitro*. The GSK3β inhibitors CHIR99021 (0.5, 1, 2 μM) or SB216763 (0.5, 1, 2 μM) were used to treat cells in media supplemented with cytokines for 9 days. (A) Cell morphology. Magnification, x40; scale bar, 1,000 μm. Images were obtained using the confocal Leica DM RXA microscope. (B) After cultured *in vitro* for 9 days, cells were analyzed via flow cytometry to assess the percentage/number of LSK cells. (C) Total number of cell numbers following a 9-day culture. (D) Relative number of Lin$^-$ cells. (E) Relative and (F) absolute LSK cell numbers. n=4; *P<0.05, **P<0.01. APC, allophycocyanin; PE, phycoerythrin; Lin, lineage; GSK3β, glycogen synthase kinase 3β; Sca-1, stem cell antigen-1.

The cells were further analyzed via flow cytometry, which revealed that in response to rapamycin treatment, there was a significant increase in the Lin$^-$ cell ratio (Fig. 4D and E), as well as in the frequency and absolute of LSK cells (Fig. 4F and G), while total cell numbers declined (Fig. 4C) which may be because rapamycin inhibits cell proliferation, thus maintaining cell stemness and inhibiting cell differentiation. Thus, 15 nM was determined as a suitable inhibitory concentration for the mTOR signaling pathway inhibitor rapamycin. Similarly, the proportion of LSK cells in group K and N was increased compared with the other combinations in the random combination of inhibitors (Fig. S6 and Table I). These findings suggested that rapamycin may slow HSC growth, maintaining cells in a relatively undifferentiated and stem-like state.

Discussion

AML is a very common type of cancer; however, patients with AML often still have a poor prognosis (49). The majority of patients currently receive stem cell transplantation therapy as a means of improving patient outcomes and prolonging

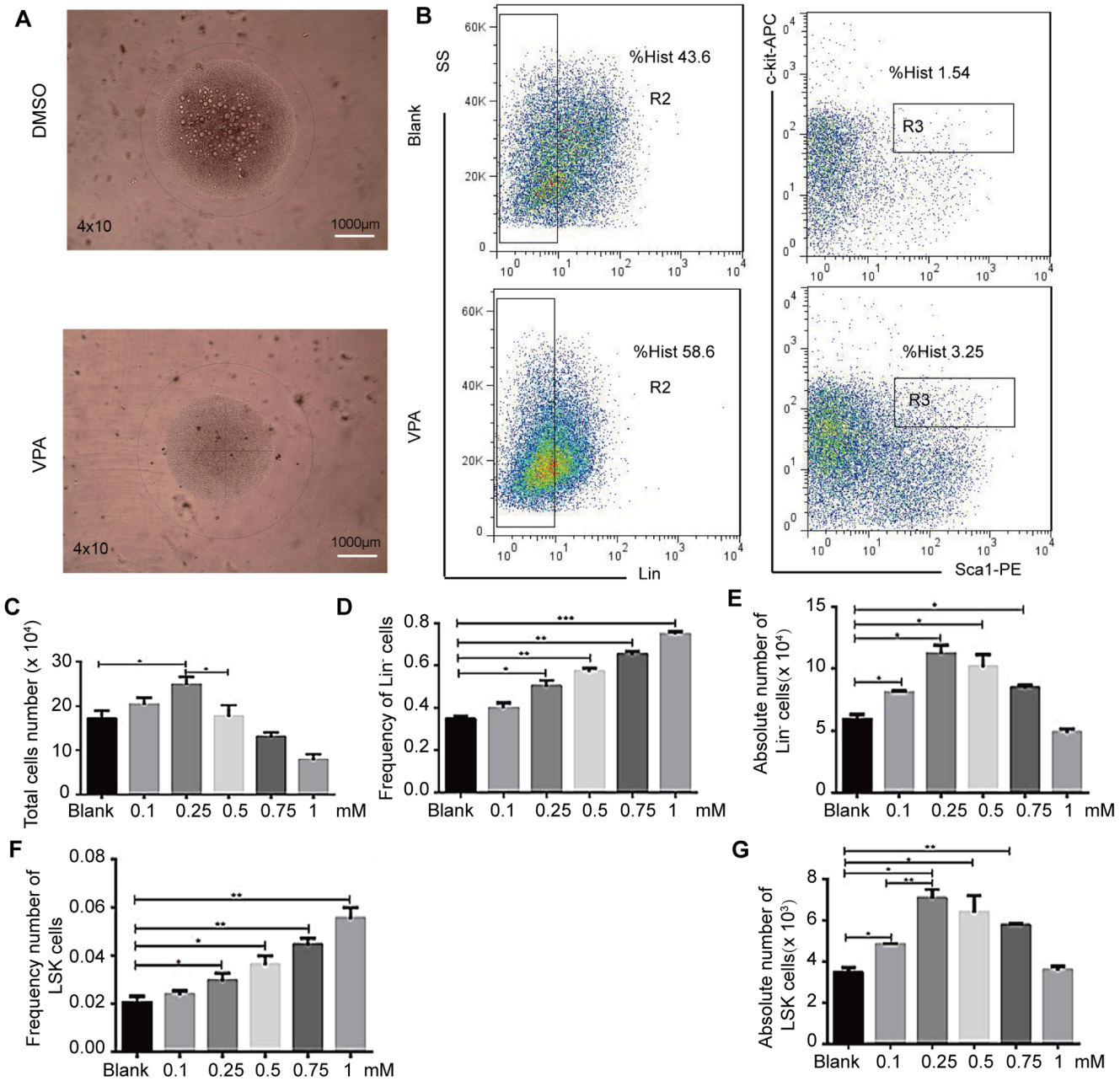

Figure 3. VPA affects hematopoietic stem cell expansion *in vitro*. LSK cells treated with various concentrations of VPA (0.1, 0.25, 0.5, 0.75, 1 mM) were analyzed by flow cytometry after a 9-day culture period. Cells were stained for the expression of Gr1, CD11b, Ter119, CD3ε, B220, Sca-1 and c-Kit. (A) LSK cell morphology was analyzed using a confocal Leica DM RXA microscope. Magnification, x40; scale bar, 1,000 μm. (B) LSK cells were analyzed via flow cytometry. (C) Total cell number. (D) Lin$^-$ cell frequency. (E) Lin$^-$ cell number. (F) LSK cell frequency. (G) LSK cell number. n=4; *P<0.05, **P<0.01, ***P<0.001. APC, allophycocyanin; PE, phycoerythrin; Lin, lineage; SS, side scatter; VPA, valproic acid sodium salt; Sca-1, stem cell antigen-1.

survival (50). For such HSC transplantation (HCT) procedures, donor stem cells are typically derived either from bone marrow, peripheral blood or umbilical cord blood (51). As the number of HSCs available from these donor tissues, and particularly in cord blood samples, can often be very limited, this can lead to very long recovery periods, making it a priority to identify novel means of enhancing HSC expansion *in vitro* (52-54). However, HSC differentiation and homeostatic regulation *in vivo* depends upon a wide array of complex microenvironmental inputs that can be difficult to replicate *in vitro* (55,56).

While there have been numerous efforts made to date to promote *ex vivo* HSC expansion to improve engraftment rates in the clinical setting (57,58), there still remains a significant unmet need in the field of HCT, and as such novel, *ex vivo* expansion strategies are still required. Numerous previous studies revealed that p38MAPK, mTOR, GSK3β and HDAC signaling were all important for regulating the proliferation, differentiation, apoptosis and necrosis of HSCs (7,59-63). Therefore, the present study hypothesized that the simultaneous inhibition of multiple of these pathways may promote the synergistic expansion of HSCs *in vitro*, thereby representing a potentially viable strategy for improving HCT outcomes in patients with leukemia.

In the present study, the expansion of cells using a single molecule inhibitor revealed that the HSC expansion efficiency

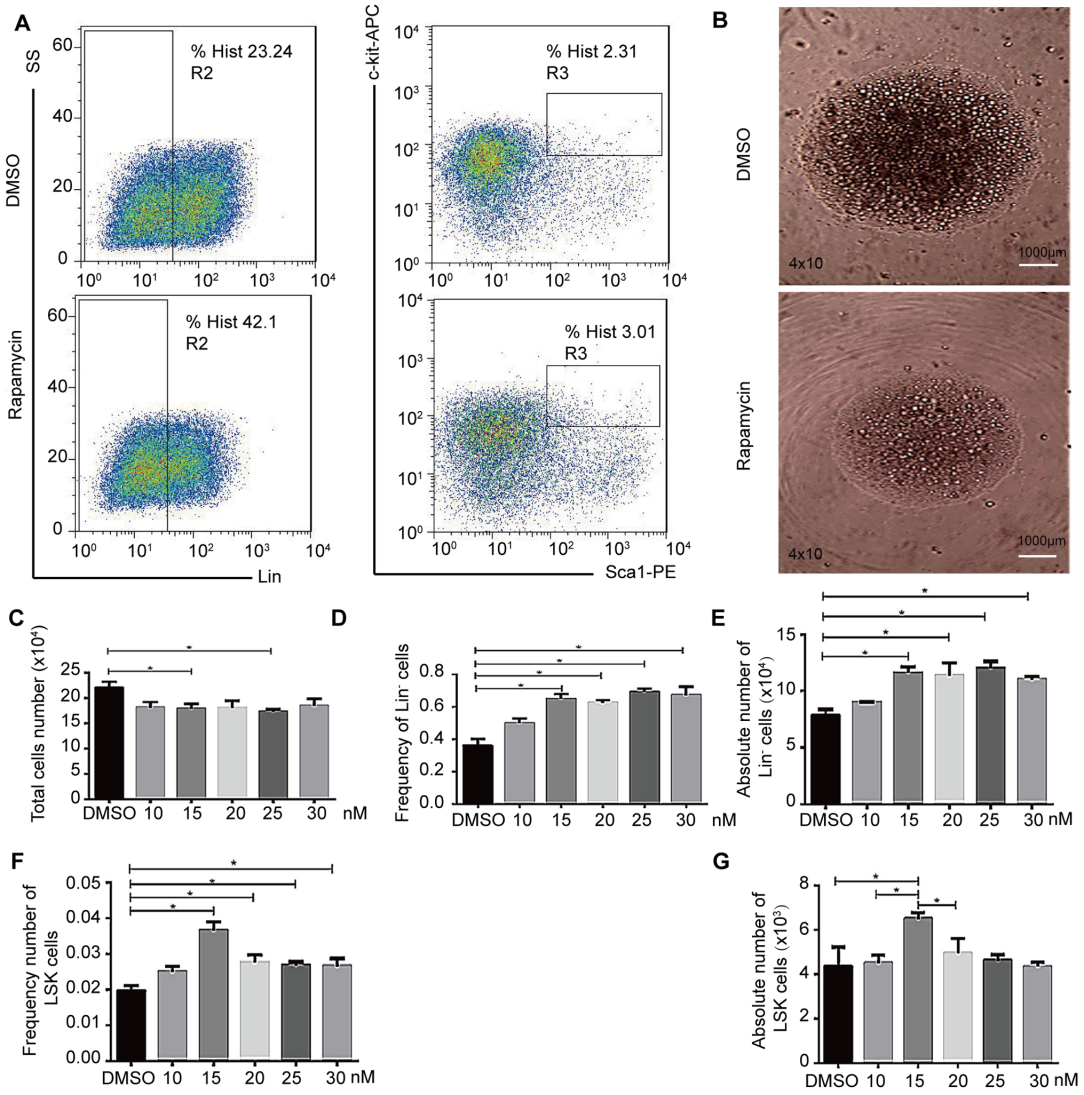

Figure 4. mTOR inhibition affects hematopoietic stem cell expansion *in vitro*. LSK cells were treated with rapamycin (10, 15, 20, 25 and 30 nM) or DMSO. (A) LSK cells were analyzed via flow cytometry. (B) Cell morphology following rapamycin or DMSO treatment. Magnification, x40; scale bar, 1,000-μm. Cells were visualized using the confocal Leica DM RXA microscope. (C) Total cell number following rapamycin (10, 15, 20, 25 and 30 nM) or equal volume DMSO treatment. (D) Lin$^-$ cell frequency following rapamycin or equal volume DMSO treatment. (E) Lin$^-$ cell number following rapamycin or DMSO treatment. (F) LSK cell frequency following rapamycin (10, 15, 20, 25 and 30 nM) or equal volume DMSO treatment. (G) LSK cell number following rapamycin (10, 15, 20, 25 and 30 nM) or equal volume DMSO treatment. n=4. *P<0.05, APC, allophycocyanin; PE, phycoerythrin; Lin, lineage; SS, side scatter; Sca-1, stem cell antigen-1.

was significantly increased when SB203580 and SB216763 were used alone. HDAC signaling was also discovered to be a crucial regulator of HSC homeostasis (64). It was therefore investigated how the suppression of HDAC signaling may alter HSC functionality in an *in vitro* experimental system. High VPA concentrations failed to enhance HSC proliferation, which may have been potentially due to the cytotoxic effects of high concentrations of this inhibitor, which induced apoptotic signaling, and HDAC inhibition was sufficient to maintain HSCs in an undifferentiated state *in vitro*, enhancing their expansion (65,66). Our studies demonstrated that HDAC inhibitors induced HSC expansion, maintained undifferentiated cells *in vitro*. mTOR signaling regulates the growth and metabolism of cells, in addition to regulating HSC homing (62,67,68). mTOR activation has also been illustrated to disrupt HSC quiescence and drive HSC exhaustion, whereas inhibiting this signaling pathway restored HSC self-renewal (61,69). In the present study, inhibiting the mTOR pathway led to decreased cell growth, but also helped maintain the cells in a more stem-like undifferentiated state. These results suggested that the mTOR signaling pathway may serve an important role in regulating the self-renewal of HSCs *in vitro*. Thus, inhibiting the mTOR pathway using rapamycin may represent a novel approach to promote HSC expansion *in vitro* to improve HCT outcomes.

Next, to investigate how combinations of these signaling pathway inhibitors affect HSC expansion and differentiation *in vitro*, cells were treated with combinations of the optimal concentrations of these pathway-specific inhibitors. Consistent with our hypothesis, it was discovered that the combination of SB203580 and SB216763 significantly increased HSC differentiation *in vivo* compared with the other treatment groups. The combined inhibition of p38MAPK and mTOR was also able to amplify HSCs *in vitro*, although not as effectively

as the combination of p38MAPK and GSK-3β inhibition. In contrast, the treatment of cells with HDAC inhibitors was associated with an almost complete loss of differentiation (70), suggesting that HDAC inhibitors may damage cellular responses in this context. Among all the important signaling pathways, using these various combinations, the results indicated that through combining the p38MAPK and GSK3β inhibitors, HSC expansion was significantly enhanced *in vitro*.

In conclusion, the findings of the present study may provide a novel approach to expand HSCs *ex vivo* via inhibiting p38MAPK and GSK3β, either alone or in combination. However, the specific molecular mechanisms through which p38MAPK and GSK3β signaling altered quiescent HSC expansion and proliferation remain unclear and warrant further investigations. Taken together, the aim of future studies is to obtain a series of small-molecule compounds that inhibit p38MAPK and GSK3β signaling pathways that can expand human HSCs *ex vivo*.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YJ performed the experiments, analyzed and extracted the data, generated the figures and wrote the manuscript. ZX, NM and LY analyzed the data; CH conceived the project and reviewed the paper. JL conceived the project, designed and performed the experiments, and analyzed and interpreted results. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal studies were approved by the Institutional Animal Care and Use Committee of Shanghai Normal University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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