

Published in final edited form as:

Transplantation. 2014 January 15; 97(1): . doi:10.1097/TP.0b013e3182a7fcf8.

Rapamycin enhances long-term hematopoietic reconstitution of *ex vivo* expanded mouse hematopoietic stem cells by inhibiting senescence

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Abstract

Background—The mTOR is an important regulator of HSCs self-renewal and its overactivation contributes to HSCs premature exhaustion in part via induction of HSCs senescence. Inhibition of mTOR with rapamycin has the potential to promote long term hematopoiesis of *ex vivo* expanded HSCs to facilitate the clinical application of HSCs transplantation for various hematological diseases.

Methods—A well-established *ex vivo* expansion system for mouse bone marrow HSCs was utilized to investigate whether inhibition of overactivated mTOR with rapamycin can promote long term hematopoiesis of *ex vivo* expanded HSCs and to elucidate the mechanisms of action of rapamycin.

Results—HSCs-enriched mouse bone marrow LSK cells exhibited a time-dependent activation of mTOR after *ex vivo* expansion in a serum-free medium supplemented with SCF, TPO and FL. The overactivation of mTOR was associated with induction of senescence but not apoptosis in LSK cells and a significant reduction in the ability of HSCs to produce long-term hematopoietic reconstitution. Inhibition of overactivated mTOR with rapamycin promoted *ex vivo* expansion and long term hematopoietic reconstitution of HSCs. The increase in long term hematopoiesis of expanded HSCs is likely attributable in part to rapamycin-mediated upregulation of Bmi1 and downregulation of p16, which prevent HSCs from undergoing senescence during *ex vivo* expansion.

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Contribution: LBL and DZ designed the research; YL and LL performed most of the experiments and JW and LS performed some of the experiments. YL and LL collected data. YL, LL, PZ, DZ, and LBL analyzed the data. YL, DZ and LBL wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Conclusions—These findings suggest that mTOR plays an important role in the regulation of HSCs self-renewal *in vitro* and inhibition of mTOR hyperactivation with rapamycin may represent a novel approach to promote *ex vivo* expansion and their long term hematopoietic reconstitution of HSCs.

Keywords

hematopoietic stem cells; *ex vivo* expansion; long term hematopoietic reconstitution; mTOR; rapamycin; senescence

INTRODUCTION

Hematopoietic stem cell (HSC) transplantation is an effective treatment and sometime the only cure for many hematological disorders. Unfortunately, its therapeutic potential has not been fulfilled because of lacking of a suitable donor or insufficient numbers of HSCs for transplantation (1, 2). *Ex vivo* expansion of HSCs could potentially generate ample HSCs to overcome these obstacles. So far, moderate *ex vivo* expansion of HSCs has been achieved by incubation of HSCs with various hematopoietic growth factors, cytokines, Notch ligands, Wnt3a, or angiopoietin-like protein (3–6). Coculture of HSCs with bone marrow stromal cells and endothelial cells also increases expansion of HSCs (7, 8). In addition, ectopic expression of various transcription factors such as HoxB4 by gene transfection can induce robust expansions of HSCs *in vitro* (9). However, these methods have limited utility in clinical practice because of the concerns about the 1) high costs of hematopoietic growth factors, 2) difficulty in standardizing stromal elements to meet FDA regulations, and 3) risks of HSC transformation by gene transfection. In addition, *ex vivo* expansion of HSCs usually occurs at the expense of HSC self-renewal, which leads to a significant reduction in the ability of the expanded HSCs to produce long-term hematopoietic reconstitution after transplantation (10). Therefore, increasing efforts have been devoted to identify small molecules that can help to overcome the shortcomings of these existing methods. Our recent studies showed that *ex vivo* expansion of both mouse bone marrow and human cord blood HSCs activated p38 (10, 11). Activation of p38 was associated with a significant increase in apoptosis and cellular senescence in HSCs and their progeny. Inhibition of p38 with a specific inhibitor can promote HSCs *ex vivo* expansion while preserving HSCs long-term hematopoietic activity. These findings encouraged us to expand our study to uncover other molecular pathways that could be activated to inhibit HSCs self-renewal during *ex vivo* expansion and thus, potentially be targeted by a small molecule inhibitor to promote *ex vivo* expansion and long term hematopoietic reconstitution of HSCs.

The mTOR, a member of the family of PI3K-related kinases, is a central regulator of cellular response to stress and changes in environmental cues, such as changes in nutrients, oxygen tension, and growth factor stimulation (12). It has also emerged as an important regulator for HSCs self-renewal. Activation of mTOR has been found in HSCs during aging or under various pathological conditions such as deletion of the genes encoding PTEN, TSC1 and glycogen synthase kinase 3 (GSK3) (13–15). This activation contributes to premature exhaustion of HSCs in part via induction of apoptosis and senescence, while inhibition of mTOR with rapamycin has been shown to prevent premature exhaustion of HSCs caused by the genetic deletion of *Pten*, *Tsc1* or *Gsk3* in mice and to rejuvenate aging HSCs to extend the lifespan of old mice (13–15).

During *ex vivo* expansion, HSCs are subjected to a variety of stressors, including increases in oxygen tension, fluctuations in various nutrients and growth factor concentrations, and accumulation of toxic metabolites (16). Any of these stressors may cause hyperactivation of mTOR to inhibit HSCs self-renewal and expansion *ex vivo*. Therefore, we hypothesized that

rapamycin has the potential to be used as a small molecule enhancer to promote HSCs *ex vivo* expansion. The present study was designed to test this hypothesis using our well-established *ex vivo* expansion system for mouse bone marrow HSCs.

The results from our study showed that mouse bone marrow LSK cells enriched with HSCs exhibited a time-dependent activation of mTOR after culture in a serum-free medium supplemented with SCF, TPO and FL. The activation of mTOR was associated with a significant reduction in HSCs activity and induction of senescence in LSK cells. Addition of rapamycin to the culture inhibited the activation of mTOR in LSK cells, which led to promotion in *ex vivo* expansion and long term hematopoietic reconstitution of HSCs as shown by the CAFC and competitive repopulation assays. The promotion in HSCs expansion is likely attributable in part to rapamycin-mediated upregulation of Bmi1 and downregulation of p16, which prevent HSCs from undergoing senescence during *ex vivo* expansion. These findings suggest that mTOR plays an important role in the regulation of HSCs self-renewal *in vitro* and inhibition of overactivated mTOR with rapamycin may represent a novel approach to promote hematopoiesis of *ex vivo* expanded HSCs.

RESULTS

Ex vivo expansion of HSCs activates mTOR in a time-dependent manner

S6K1 is a direct substrate of mTOR complex 1 (mTORC1) that consists of mTOR and four other components (12). Its phosphorylation has been widely used as an indicator of mTOR activation. To determine whether *ex vivo* expansion of HSCs activates the mTOR pathway, we analyzed S6K1 phosphorylation in freshly isolated LSK cells and those harvested from HSCs *ex vivo* expansion cultures at various times by the phosphor-flow cytometric method. As shown in Figure 1 A and B, a small percentage ($8.19\% \pm 2.2\%$) of unexpanded LSK cells showed low levels of S6K1 phosphorylation, indicating limited activation of the mTOR pathway. The percentage of LSK cells stained positive for phosphorylated S6K1 (p-S6K1) increased to $24.55\% \pm 4.2\%$ and $67.51\% \pm 8.5\%$ after the cells were cultured *in vitro* for 6 and 9 days with vehicle, respectively. Rapamycin treatment (20 ng/ml) between day 6 and day 9 of the culture resulted in a significant reduction in positive p-S6K1 staining in LSK cells compared to vehicle-treated cells (vehicle: $67.51\% \pm 8.5\%$ vs. rapamycin: $27.85\% \pm 4.8\%$) (Figure 1 A & B). This finding suggests that *ex vivo* expansion of HSCs activates mTOR in LSK cells in a time-dependent manner and the activation can be inhibited by rapamycin. This suggestion is further supported by the observations that the phosphorylation of S6, a substrate of S6K, was also increased in LSK cells after 9 days of culture and the increase was attenuated by rapamycin treatment (Figure 1 C & D). Similar findings were observed in CD48⁻LSK cells (Figure 1 C & E), a primitive hematopoietic cell population that more closely represents HSCs after *ex vivo* expansion by phenotyping (17).

Inhibition of mTOR with rapamycin promotes ex vivo expansion of HSCs

Since activation of mTOR has been shown to impair HSCs self-renewal and inhibition of hyperactivated mTOR with rapamycin can prevent HSCs premature exhaustion caused by the genetic deletion of *Pten*, *Tsc1* and *Gsk3* in mice and rejuvenate aging HSCs (13–15), we investigated whether rapamycin can also promote *ex vivo* expansion of HSCs by inhibiting mTOR overactivation. In a preliminary study, we found that 20 ng/ml rapamycin was the optimal concentration to inhibit mTOR overactivation and promote *ex vivo* expansion of HSCs (data not shown). In addition, we found that rapamycin inhibited cell proliferation if it was added to the *ex vivo* HSC expansion cultures earlier than 6 days after the initiation of the culture (data not shown). Therefore, we added rapamycin (20 ng/ml) or vehicle into HSCs expansion cultures on day 6 when LSK cells exhibited a significant activation of mTOR (Figure 1). On day 10, all cells were harvested from the cultures and counted to

calculate the number of total nucleated cells produced from the expansion. The percentage of LSK cells in the harvested cells was analyzed by flow cytometry after immunostaining in order to calculate LSK cell number for the estimation of HSCs expansion because LSK cells are enriched for HSCs (Figure 2A). As shown in Figure 2B, compared to input cells, the numbers of total nucleated cells increased about 80- and 90-fold after 9 days of culture in the presence of and absence of rapamycin, respectively, indicating that rapamycin treatment slightly reduced the production of nucleated cells in comparison with the cells without rapamycin treatment. However, the cells cultured with rapamycin contained significantly more LSK cells than those without rapamycin (Figure 2C). Compared to the number of input LSK cells, the number of LSK cells increased 9- and 6-fold after 9 days of *ex vivo* expansion with and without rapamycin, respectively. This finding suggests that overactivated mTOR inhibition by rapamycin may promote HSCs *ex vivo* expansion.

LSK cells contain both HSCs and multiple potent progenitors (MPPs). To determine whether rapamycin can increase HSCs expansion *ex vivo*, we quantified the frequencies of CAFCs by CAFC assay on day 35, because the day-35 CAFC assay is considered one of the best *in vitro* assays for measuring HSCs. In addition, day-14 CAFCs were analyzed to quantify HPCs (10). As shown in Figure 2 D & E, cell cultures with rapamycin contained 4.2- and 1.2-fold more day-35 and day-14 CAFCs, respectively, than cultures without rapamycin. More importantly, when the numbers of day-35 and day-14 CAFCs in the cultured cells were expressed as a ratio to those in input, it was found that the majority of HSCs cultured without rapamycin could not self-renew, resulting in a reduction in day-35 CAFCs by about 55% compared to input. This finding agrees with the previous observation showing that HSCs from mouse bone marrow lose radioprotective and long-term engraftment potential after *ex vivo* expansion in the presence of multiple cytokines (10). In contrast, HSCs cultured with rapamycin underwent self-renewing proliferation, leading to a 1.8-fold increase in day-35 CAFCs compared to input, indicating that the inhibition of overactivated mTOR with rapamycin promotes HSCs *ex vivo* expansion.

Inhibition of mTOR with rapamycin increases HSCs long-term engraftment after transplantation

HSCs transplantation is considered the gold standard assay for long-term repopulating HSCs (10). To validate if inhibition of overactivated mTOR with rapamycin can really promote HSCs *ex vivo* expansion, we performed a competitive repopulation assay by transplanting 2000 freshly isolated Lin⁻Sca1⁺ cells or the progeny harvested from a 9-day culture with the same number of input Lin⁻Sca1⁺ cells in the presence or absence of rapamycin into lethally irradiated recipients along with competitors. Peripheral blood from these recipients was analyzed for donor-cell engraftment at 8 and 32 weeks after transplantation. As shown in Figures 3 and 4, a 9-day *ex vivo* expansion of Lin⁻Sca1⁺ cells in the absence of rapamycin resulted in about 31% and 56% decreases in donor cell engraftment at 8 weeks and 32 weeks after transplantation compared to the engraftment produced by the unexpanded input cells, respectively. Rapamycin treatment from day 6 to day 9 of *ex vivo* expansion of Lin⁻Sca1⁺ cells led to 2-fold and 4-fold increases in donor cell engraftment 8 weeks and 32 weeks after transplantation compared to vehicle treatment, respectively. More importantly, we observed a 2-fold increase in donor cell engraftment from rapamycin-expanded cells compared to unexpanded input cells (Figures 3 & 4). In addition, rapamycin treatment also improved the long-term reconstitution of myeloid, B- and T-lymphocyte lineage blood cells in the recipients 8 and 32 weeks after transplantation (Figures 3 & 4). Based on the 32-week long-term engraftment data, we calculated the repopulating unit (RU) for the input and expanded cells with vehicle or rapamycin according to the formulation reported previously (18, 19). It was found that the input cells (e.g. 2000 freshly isolated Lin⁻Sca1⁺ cells) contained 0.56 RU. *Ex vivo* expansion of the cells with vehicle resulted in about 60% reduction of RU

compared to that of the unexpanded input cells (reduced from 0.56 to 0.22 RU). In contrast, the cells expanded with rapamycin exhibited about 250% increases in RU compared to that of the input cells (increased from 0.56 to 1.38 RU). Taken together, these results demonstrate that inhibition of hyper-activated mTOR with rapamycin promotes long term hematopoietic reconstitution of *ex vivo* expanded HSCs.

Inhibition of mTOR with rapamycin promotes HSCs *ex vivo* expansion in part by inhibiting HSCs senescence

Ex vivo expansion of hematopoietic stem cells (HSCs) depends on HSCs self-renewing proliferation, which can be negatively affected by HSCs differentiation, apoptosis, and senescence (3, 10, 11). To elucidate the mechanisms by which mTOR inhibition promotes long term hematopoiesis of expanded HSCs, we analyzed the effects of rapamycin treatment on HSCs differentiation, apoptosis, and senescence by CFC assay, annexin-V staining, and SA- β -gal staining, respectively. As shown in SDC, Supplemental figure 1, rapamycin treatment had no significant effect on the formation of CFU-GM, BFU-E, and CFU-GEMM from the progeny of HSCs, nor did it reduce the percentage of annexin-V positive cells in LSK cell population. However, rapamycin reduced the number of LSK cells that underwent senescence during *ex vivo* expansion, as significantly less LSK cells from rapamycin culture stained positive for SA- β -gal (Figure 5A), a widely used biomarker of senescent cells, than those from vehicle culture. Similar findings were also observed in Figure 5B when we used C12FDG to measure SA- β -gal activity in LSK cells, which can be more sensitively and better quantitatively measured by flow cytometry (20). These findings suggest that inhibition of mTOR with rapamycin promotes *ex vivo* HSCs expansion not by inhibition of HSCs differentiation and apoptosis but probably via suppression of HSCs senescence. This suggestion is in agreement with the finding that rapamycin treatment increased the expression of Bmi1 mRNA while decreasing the expression of p16^{Ink4a} (p16) mRNA in LSK cells (Figure 5C), since both Bmi1 and p16 play an important role in regulation of HSCs senescence but in an opposite direction, e.g. Bmi1 inhibits senescence by repressing the expression of p16 and p19 while p16 is an effector of cellular senescence (21).

DISCUSSION

It has been well-established that overactivation of mTOR impairs HSCs function and causes HSCs depletion *in vivo* under various pathological conditions and during aging (13, 22). To our knowledge, our study is the first to show that *ex vivo* expansion of HSCs also causes overactivation of mTOR in a time-dependent manner in mouse bone marrow LSK cells that are enriched with HSCs. We showed that the activation of mTOR was associated with a significant reduction in the ability of HSCs to produce long-term hematopoietic reconstitution after transplantation of the *ex vivo* expanded cells. Addition of rapamycin after 6 days of *ex vivo* culture when mTOR became significantly activated in LSK cells promoted HSCs expansion, which led to an approximate 2- to 4-fold increase in long-term hematopoietic engraftment by the expanded cells treated with rapamycin compared to unexpanded input cells and the cells expanded without rapamycin treatment, respectively. These findings demonstrate that mTOR plays an important role in HSCs self-renewing proliferation during *ex vivo* expansion and that inhibition of overactivated mTOR with rapamycin has the potential to be employed as a novel strategy to promote hematopoiesis of *ex vivo* expanded HSCs, which can facilitate the clinical application of HSCs transplantation for various hematological diseases.

However, the timing of rapamycin treatment may be important in determining whether inhibition of mTOR can promote hematopoiesis of *ex vivo* expanded HSCs. Rohrabough SL et al. recently showed that the addition of rapamycin at the beginning of *ex vivo* expansion

of human cord blood CD34⁺ cells decreased the expansion of CD34⁺ cells but improved their engraftment in non-obese diabetic, severe combined immunodeficient, and IL2 receptor γ chain null mice (23). In addition, Huang J et al. reported that suppression of mTOR with rapamycin in combination with the activation of the canonical Wnt- β -catenin pathway by a glycogen synthase kinase 3 inhibitor when Lin⁻Sca1⁺ or LSK cells were cultured *ex vivo* in a cytokine-free condition only permitted the maintenance but not expansion of mouse long-term HSCs (24). Therefore, we suspect that a moderate level of mTOR activation at the beginning of HSCs *ex vivo* expansion may be required for HSCs proliferation and HSCs expansion, whereas a high level of mTOR activation at a later stage of HSCs *ex vivo* expansion can impair long term hematopoiesis of expanded HSCs by induction of HSCs senescence. Therefore, to effectively promote *ex vivo* expansion of HSCs, rapamycin should be added to the culture of *ex vivo* expansion of HSCs when mTOR becomes significantly activated in HSCs.

In addition, our studies revealed some of the fundamental mechanisms by which rapamycin promotes HSCs *ex vivo* expansion. Our results showed that inhibition of mTOR with rapamycin reduced HSCs senescence while having no significant effects on HSCs apoptosis and differentiation during *ex vivo* expansion. The inhibition of HSCs senescence may be attributable to rapamycin-mediated upregulation of Bmi1 and downregulation of p16, since Bmi1 can inhibit senescence by repressing the expression of p16 and p19 while p16 is an effector of cellular senescence (21).

Although we showed that inhibition of mTOR by rapamycin can promote *ex vivo* expansion of HSCs, it is possible that the strategies that can inhibit the upstream signaling pathways leading to mTOR activation can also be utilized to increase HSCs expansion either alone or in combination with rapamycin. It has been well-established that mTOR is a central regulator of cellular response to stress and to changes in environmental cues. During *ex vivo* expansion, HSCs are constantly subjected to a variety of stressors, including increases in oxygen tension, fluctuations in various nutrients and growth factor concentrations, and accumulation of toxic metabolites. All of these may cause activation of mTOR via oxidative stress (15), activation of p38 (25) and other unknown mechanisms. Recently, we showed that p38 inhibition can promote *ex vivo* expansion of both mouse bone marrow and human cord blood HSCs (10, 11). It remains to be determined whether the effect of p38 inhibition is mediated in part by inhibition of mTOR and whether simultaneous inhibition of p38 and mTOR can synergistically promote HSCs *ex vivo* expansion. Therefore, further study to elucidate the mechanisms by which *ex vivo* expansion of HSCs causes activation of mTOR in HSCs may lead to the identification of additional molecular targets for intervention to promote HSCs self-renewal and expansion in an *ex vivo* cell culture environment.

DESIGN AND METHODS

Reagents

All the antibodies used to stain various types of hematopoietic cells are presented in Supplemental Table S1. Recombinant mouse SCF, TPO and FL were purchased from Peprotech (Rocky Hill, NJ, USA). StemSpan® SFEM medium was obtained from StemCell Technologies (Vancouver, BC, Canada). Rapamycin was purchased from Cell Signaling Technology (Beverly, MA, USA).

Mice

C57BL/6-Ly-5.1 (Ly5.1) mice were originally obtained from the Jackson Laboratories (Bar Harbor, ME, USA) and were bred in the Laboratory Animal Research Centre (LARC) of Huazhong University of Science and Technology (HUST) in China. C57BL/6-Ly-5.2

(Ly5.2) mice were obtained from LARC and the Jackson Laboratories, respectively, to be used at HUST and at the University of Arkansas for Medical Sciences (UAMS) in the US. Mice received food and water *ad libitum*. All mice were used at 8–12 weeks of age in compliance with the standards for humane care and use of laboratory animals and with the approval of the Institutional Animal Care and Use Committees at HUST and UAMS.

Ex vivo expansion of mouse bone marrow HSCs

Mouse bone marrow Lin⁻Sca1⁺ cells were obtained by a combination of negative selection for Lin⁻ cells and positive selection for Sca1⁺ cells using MicroBeads (Miltenyi Biotec Inc, Auburn, CA, USA) following the protocols provided by the manufacturer. They were seeded into wells of 12-well plates at 3×10^3 cells per well and incubated with 1.5 ml StemSpan® SFEM supplemented with 100 ng/ml SCF, TPO and FL (HSC expansion medium) at 37°C, 5% CO₂ and 100% humidity. The cells were fed with 1 ml fresh HSCs expansion medium every 3 days during the culture. Rapamycin (20 ng/ml in 0.1% dimethyl sulfoxide [DMSO]) was added to the culture on day 6, while DMSO (0.1%) was used as a vehicle control. All progeny harvested from each culture at various times were analyzed as described below.

Flow cytometric analysis of intracellular phosphorylated proteins

Flow cytometric analysis of intracellular phosphorylated proteins were performed as described in supplemental methods.

Colony-forming cell (CFC) and cobblestone-area-forming cell (CAFC) assays

The CFC and CAFC assays were performed as described in supplemental methods.

Competitive repopulation assay

Cells harvested from a 9-day culture of 2000 input Lin⁻Sca1⁺ cells or 2000 freshly isolated Lin⁻Sca1⁺ cells from Ly5.2 mice were mixed with 2×10^5 competitive bone marrow mononuclear cells (BMMNCs) from Ly5.1 mice. The cell mixture was transplanted into lethally irradiated (9.5 Gy TBI) Ly5.1 mice by tail-vein injection. Peripheral blood samples were obtained from the retro-orbital plexus using heparin-coated micropipets (Drummond Scientific, Broomall, PA, USA) at 8 and 32 weeks post transplantation. Following lysis of red blood cells with 0.15 M NH₄Cl, the blood samples were stained with APC-conjugated anti-Ly5.2 antibodies and FITC-conjugated anti-Thy-1.2, anti-CD45R/B220, or anti-Gr-1/Mac-1 antibodies and then analyzed for donor-derived nuclear cells, T-cells, B-cells, and myeloid cells (including granulocytes and monocytes/macrophages), respectively, on a FACS Caliber. We repeated the competitive repopulation assay three times with cells harvested from three separate cultures. Each assay consisted of 7 mice per transplantation group. The donor cell engraftment is presented as mean \pm SE of three independent transplantation experiments. In addition, we calculated the repopulating unit (RU) of donor cells according to the original formula proposed by Dr. Harrison's group (19). Specifically, each RU is the relative repopulating ability of 10^5 fresh marrow cells from the same standard competitor pool. Numbers of RU from each testing donor cell population are calculated from the percentage of testing donor cells where the number of fresh competitor marrow cells used per 10^5 equalled C. $RU = \% (C) / (100 - \%)$ or $RU = \text{Testing cells } \% \times C / \text{Competitors } \%$.

Apoptosis assay

Apoptosis was performed as described in supplemental methods.

Senescence-associated β galactosidase (SA- β -gal) staining

SA- β -gal staining was performed as described in supplemental methods.

Analysis of senescence by C12FDG staining

The analysis of senescence by C12FDG staining was performed as described in supplemental methods.

Quantitative reverse transcriptase PCR (qRT-PCR)

qRT-PCR was performed as described in supplemental methods.

Statistical analysis

The statistical software package SPSS 13.0 was used to analyze data by analysis of variance (ANOVA). In the events that ANOVA justified post hoc comparisons between group means, these were performed using the LSD-*t* test for multiple comparisons. For experiments in which only single experimental and control group were used, group differences were examined by unpaired or paired Student's *t* test. A *P* value of < 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This work was supported by grants from the National Natural Science Foundation of China (No.30871097 and No. 81170524) to Dr. Lingbo Liu and a grant from the National Institutes of Health of the US (AI080421) and a grant from the Edward P. Evan's Foundation to Dr. Daohong Zhou. Dr. Daohong Zhou is also supported by the Winthrop Rockefeller Endowment for Leukemia Research and the Arkansas Research Alliance Scholarship from the Arkansas Science & Technology Authority.

The authors would like to thank Mr. Zhihui Liang and Mrs. Huifen Zhu for the flow cytometric analysis and the Laboratory Animal Research Centre of Huazhong University of Science and Technology for their support in animal care. The Office of Grants and Scientific Publications of the University of Arkansas for Medical Sciences provided editing services.

Abbreviations

BFU-E	burst-forming unit-erythroid
CFU-GEMM	CFU-granulocyte, -erythrocyte, -monocyte, and -megakaryocyte
CAFC	cobblestone area forming cell
CFU-GM	colony-forming unit-granulocyte macrophage
FL	Flt3 ligand
GSK3	glycogen synthase kinase 3
HSCs	hematopoietic stem cells
LSK cells	Lin-Sca1+c-kit+ cells
mTOR	mammalian target of rapamycin
p38	p38 mitogen-activated protein kinase

PTEN	phosphatase and tensin homolog
PI3K	phosphoinositide-3-kinase
SCF	stem cell factor
TPO	thrombopoietin
TSC1	tuberous sclerosis complex 1
Wnt3a	Member 3A of Wingless-type MMTV integration site family

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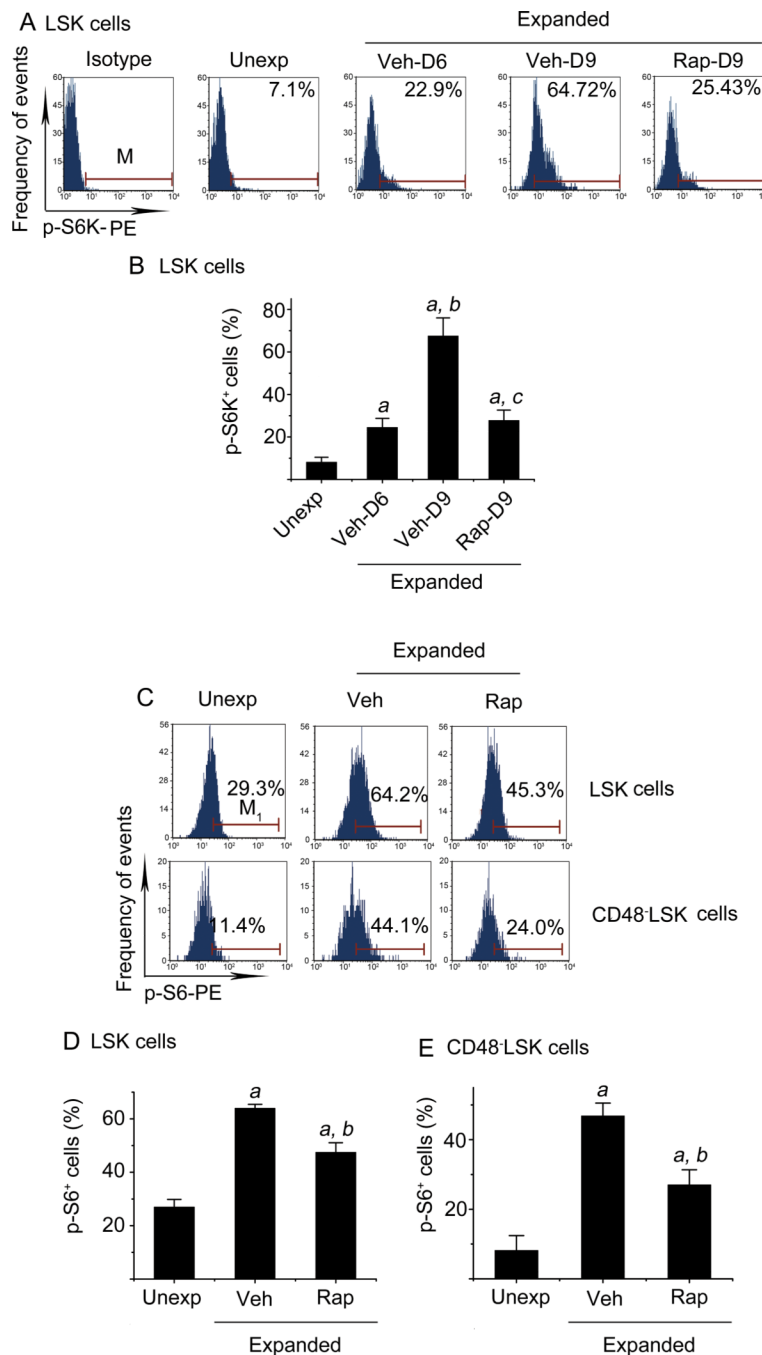


Figure 1. Ex vivo expansion of HSCs activates mTOR in a time-dependent manner

(A) Representative flow cytometric analyses of phosphorylated S6K (p-S6K) in freshly isolated unexpanded Lin⁻Sca1⁺c-kit⁺ cells (LSK cells) (Unexp) and *ex vivo* expanded LSK cells after 6-day (Veh-D6) and 9-day (Veh-D9) culture with vehicle (0.1% DMSO) or 9-day (Rap-D9) culture with rapamycin (20 ng/ml) started on day 6 are shown. Cells stained with an isotype control antibody (Isotype) were included as a control for the staining. (B) Percentages of p-S6K⁺ LSK cells from three independent assays are presented as mean \pm SE. *a*, $p < 0.05$ vs. unexpanded cells; *b*, $p < 0.05$ vs. cells expanded with vehicle for 6 days; *c*, $p < 0.05$ vs. cells expanded with vehicle for 9 days. (C) Representative flow cytometric

analyses of phosphorylated S6 (p-S6) in freshly isolated LSK and CD48⁻LSK cells (Unexp) and *ex vivo* expanded LSK and CD48⁻LSK cells after 9-day culture with vehicle (Veh, 0.1% DMSO) or rapamycin (Rap, 20 ng/ml) started on day 6 are shown. (D) Percentages of p-S6⁺ LSK cells from three independent assays are presented as mean \pm SE. (E) Percentages of p-S6⁺ CD48⁻LSK cells from three independent assays are presented as mean \pm SE. *a*, $p < 0.05$ vs. unexpanded cells; and *b*, $p < 0.05$ vs. cells expanded with vehicle.

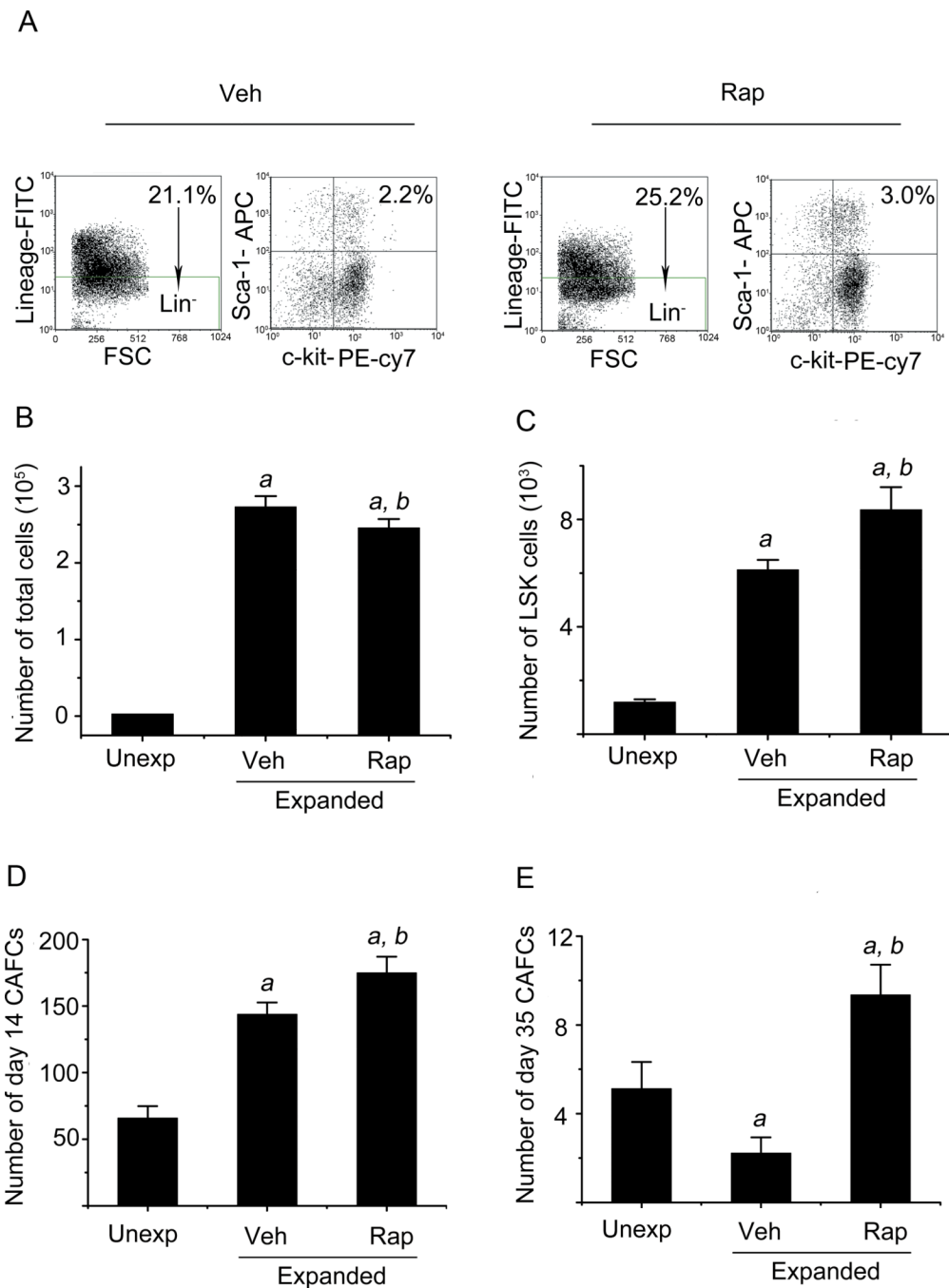


Figure 2. Inhibition of mTOR with rapamycin promotes ex vivo expansion of HSCs

$\text{Lin}^- \text{Sca1}^+$ cells were cultured *in vitro* for 5 days without any treatment and then cultured with either vehicle (Veh, 0.1% DMSO) or rapamycin (Rap, 20 ng/ml) from day 6 to day 9. The cells harvested from the cultures on day 10 were enumerated and analyzed by flow cytometry and CAFC assay. (A) Representative flow cytometric analyses of LSK cells in the cultures with Veh or Rap. The numbers presented in the flow charts are frequencies for each cell population indicated. (B) Number of total nucleated cells. (C) Number of LSK cells. (D) Number of day-14 CAFCs. (E) Number of day-35 CAFCs. The data in B-E are presented as mean \pm SE ($n=3$ independent cultures) recovered from the cultures with 3000 input (Unexp)

Lin⁻Sca1⁺ cells. *a*, $p < 0.05$ vs. unexpanded cells; and *b*, $p < 0.05$ vs. cells expanded with vehicle.

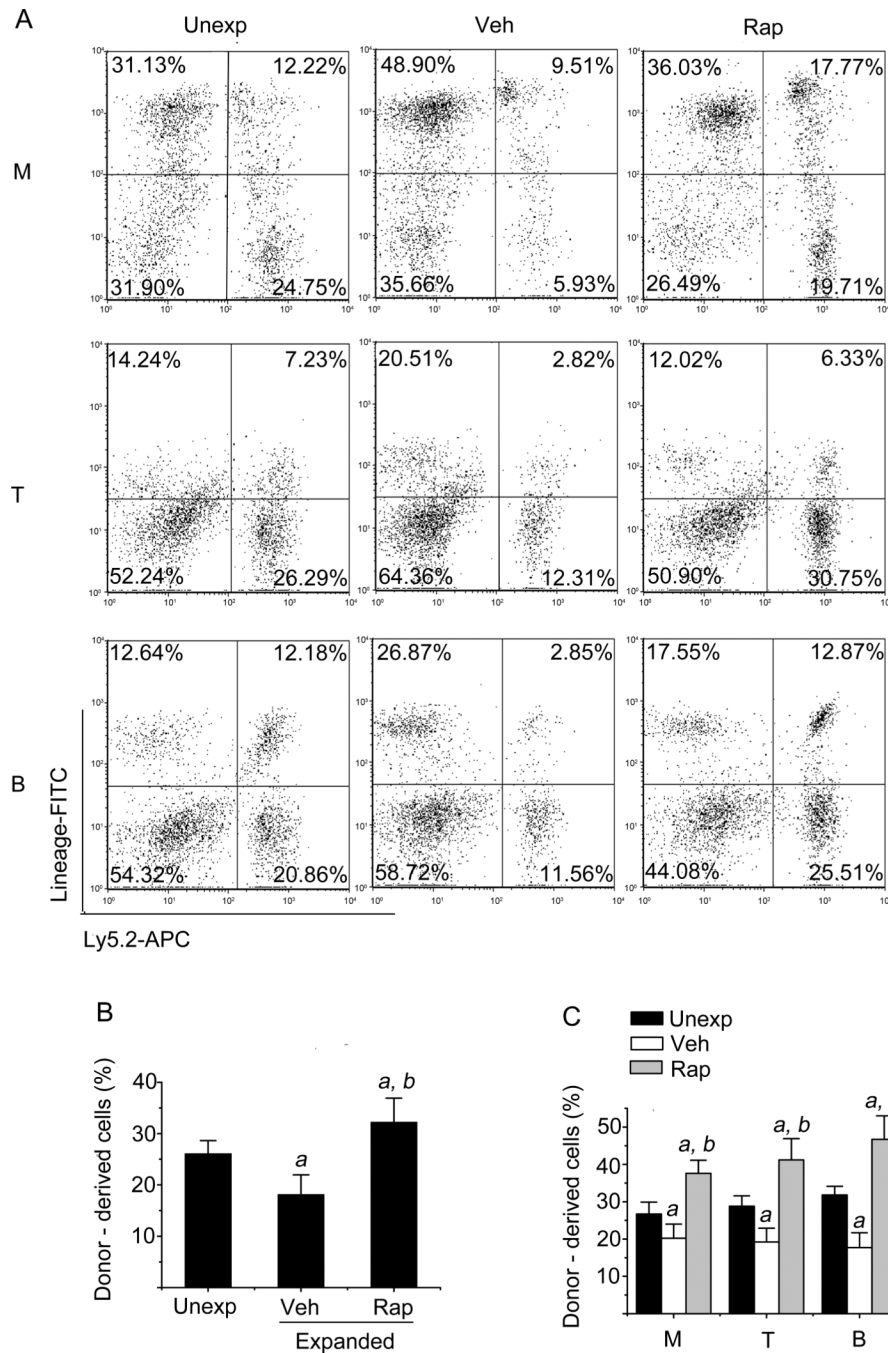


Figure 3. Inhibition of mTOR with rapamycin increases HSC engraftment after transplantation
 $\text{Lin}^{-}\text{Sca1}^{+}$ cells were expanded as described above with vehicle (Veh) or rapamycin (Rap). The progeny of 2×10^3 $\text{Lin}^{-}\text{Sca1}^{+}$ input cells from Ly5.2 mice after 9 days *ex vivo* expansion along with 2×10^5 competitive bone marrow cells from Ly5.1 mice were transplanted into lethally irradiated Ly5.1 mice. Unexpanded 2×10^3 $\text{Lin}^{-}\text{Sca1}^{+}$ cells mixed with the same number of bone marrow cells from Ly5.1 mice were used as controls. Donor cell engraftment in peripheral blood was determined 8 weeks after transplantation by flow cytometry. (A) Representative flow cytometric analyses of donor-derived cells in the peripheral blood of recipients 8 weeks after transplantation. Donor-derived (Ly5.2) myeloid cells (M), T cells (T), and B cells (B) were analyzed by flow cytometry after

immunostaining with APC-conjugated anti-Ly5.2 and FITC-conjugated anti-Gr-1/Mac-1, anti-Thy-1.2, and anti-B220 antibodies, respectively. The numbers presented in the flow charts are percentages of each of these cell populations derived from the donor cells (Ly5.2 positive cells) and competitors (Ly5.1 positive cells). (B) The percentage of total donor-derived cells in the peripheral blood at 8 weeks after transplantation, respectively. (C) The percentage of donor-derived myeloid cells (M), T cells (T), and B cells (B) in the peripheral blood at 8 weeks after transplantation, respectively. The data are presented as mean \pm SE of three independent transplantation experiments with cells from separate cultures and each transplantation consisting of 7 mice per group. *a*, $p < 0.05$ vs. unexpanded cells; and *b*, $p < 0.05$ vs. cells expanded with vehicle.

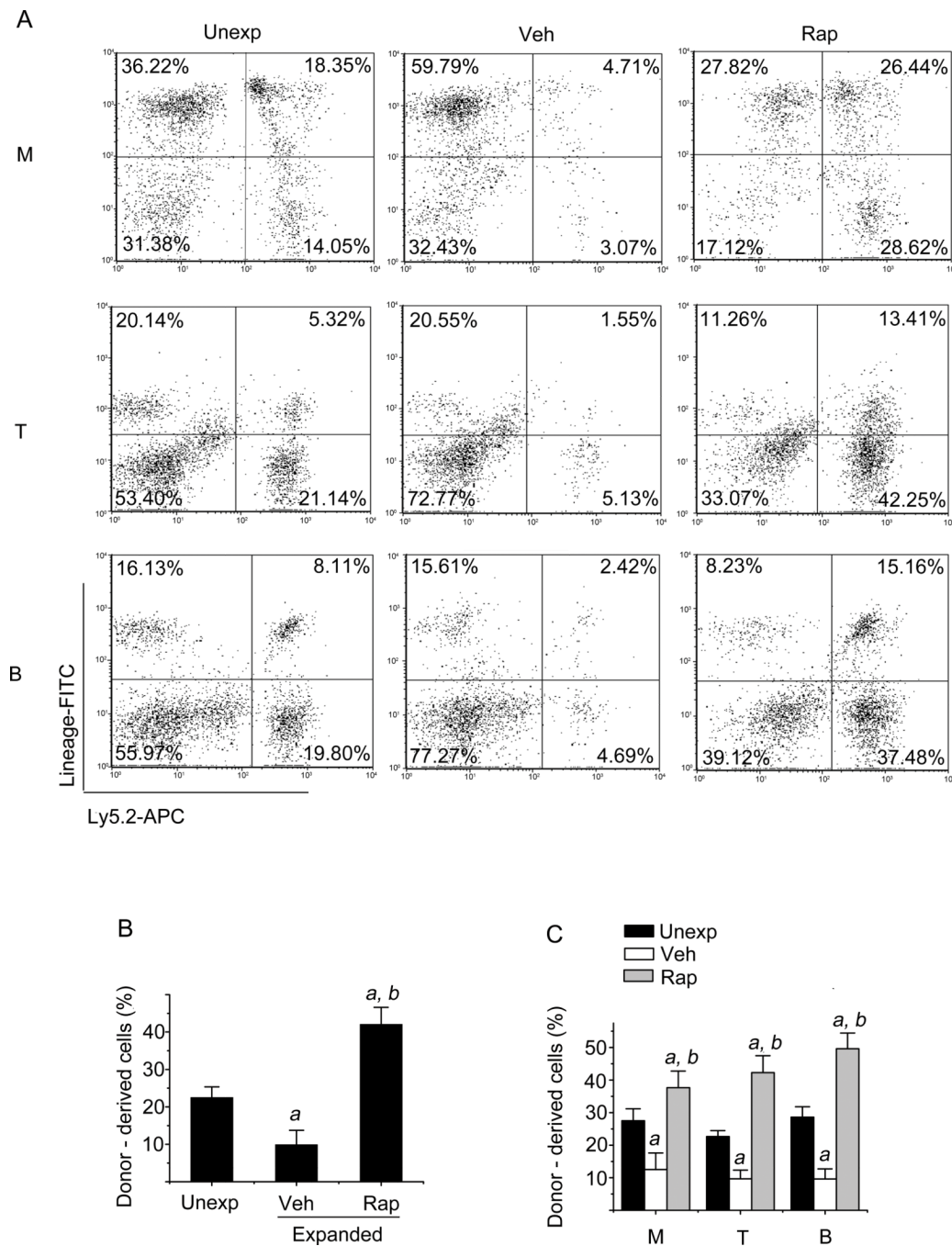


Figure 4. Inhibition of mTOR with rapamycin increases HSC long-term engraftment after transplantation

$\text{Lin}^- \text{Sca1}^+$ cells were expanded with vehicle (Veh) or rapamycin (Rap) and transplanted as described above. Donor cell long-term engraftment in peripheral blood was determined 32 weeks after transplantation by flow cytometry. (A) Representative flow cytometric analyses of donor-derived cells in the peripheral blood of recipients 32 weeks after transplantation. Donor-derived (Ly5.2) myeloid cells (M), T cells (T), and B cells (B) were analyzed by flow cytometry after immunostaining with APC-conjugated anti- Ly5.2 and FITC-conjugated anti-Gr-1/Mac-1, anti-Thy-1.2, and anti-B220 antibodies, respectively. The numbers presented in the flow charts are percentages of each of these cell populations derived from the donor cells

(Ly5.2 positive cells) and competitors (Ly5.1 positive cells). (B) The percentage of total donor-derived cells in the peripheral blood at 32 weeks after transplantation, respectively. (C) The percentage of donor-derived myeloid cells (M), T cells (T), and B cells (B) in the peripheral blood at 32 weeks after transplantation, respectively. The data are presented as mean \pm SE of three independent transplantation experiments with cells from separate cultures and each transplantation consisting of 7 mice per group. *a*, $p < 0.05$ vs. unexpanded cells; and *b*, $p < 0.05$ vs. cells expanded with vehicle.

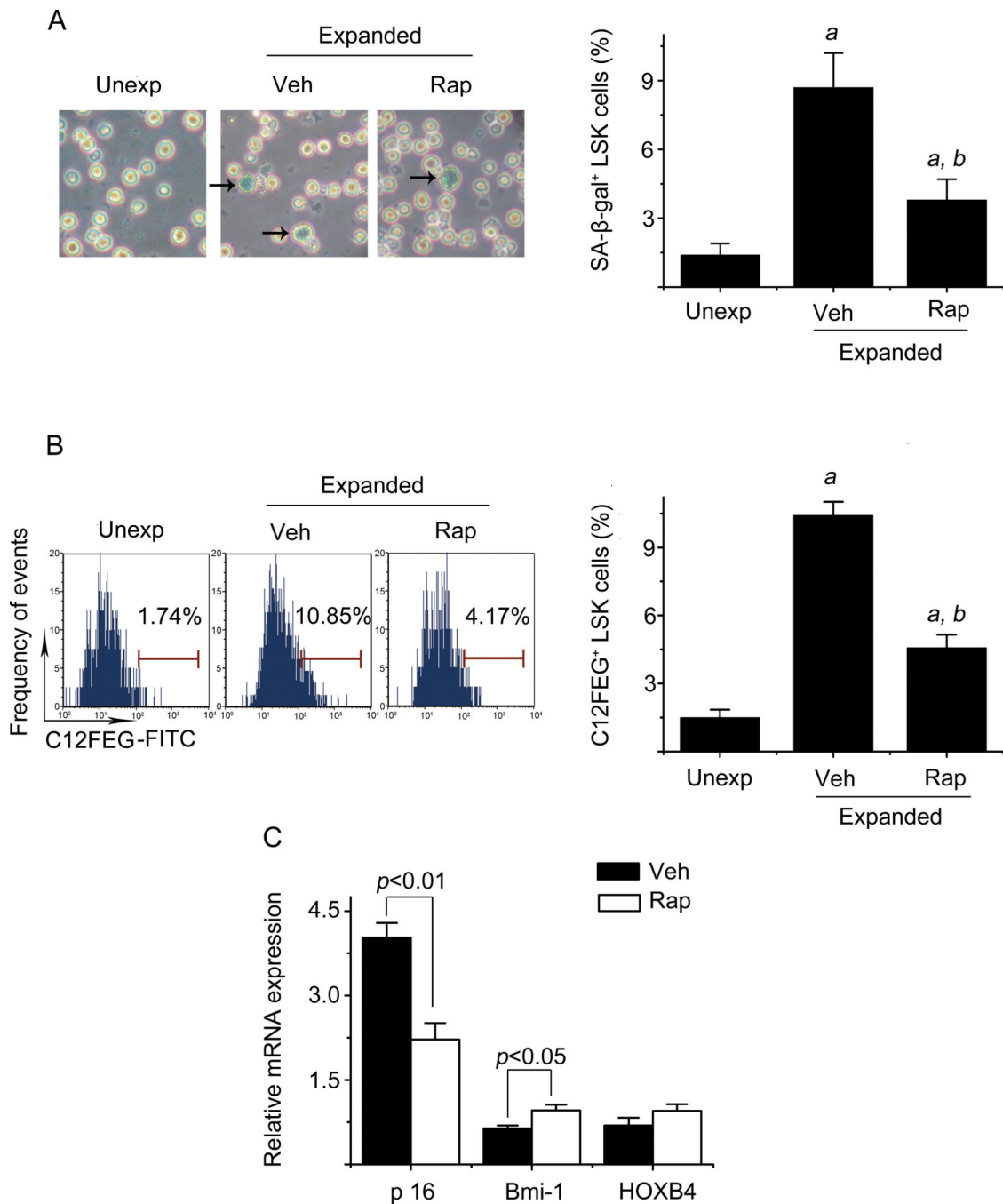


Figure 5. Inhibition of mTOR with rapamycin reduces HSCs senescence after ex vivo expansion
 (A) Analysis of HSCs senescence by SA- β -gal staining. Left panel: Representative photomicrographs of SA- β -gal staining in freshly isolated unexpanded LSK cells (Unexp) and sorted LSK cells after *ex vivo* expansion with vehicle (Veh) or rapamycin (Rap) are shown. The cells pointed to by arrows are SA- β -gal positive cells. Right panel: percentages of SA- β -gal positive LSK cells are presented as mean \pm SE ($n = 3$). (B) Analysis of HSCs senescence by flow cytometry after C12FEG staining. Left panel: Representative flow cytometric analyses of C12FEG staining in freshly isolated unexpanded LSK cells (Unexp) and *ex vivo* expanded LSK cells with vehicle (Veh, 0.1% DMSO) or rapamycin (Rap, 20 ng/ml) started on day 6 are shown. Right panel: percentages of C12FEG positive LSK cells are

presented as mean \pm SE ($n = 3$). (C) Analysis of p16, Bmi1, and HoxB4 mRNA expression in sorted LSK cells after *ex vivo* expansion with vehicle (Veh) or rapamycin (Rap) by real-time RT-PCR. The data are expressed as a ratio of these to freshly isolated LSK cells and presented as mean \pm SE ($n = 3$). *a*, $p < 0.05$ vs. unexpanded cells; and *b*, $p < 0.05$ vs. cells expanded with vehicle.