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Maintenance of Hematopoietic Stem Cells through Regulation of Wnt and mTOR Pathways

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Abstract

Hematopoietic stem cell (HSC) self-renewal and lineage commitment depend on complex interactions with the microenvironment, and the ability to maintain or expand HSCs for clinical applications or for basic research has been significantly limited because these interactions are not well defined. Recent evidence suggests that HSCs reside in a low perfusion, reduced nutrient niche and that nutrient sensing pathways contribute to HSC homeostasis. Here we report that suppression of the mammalian target of rapamycin (mTOR) pathway, an established nutrient sensor, combined with activation of canonical Wnt/β-catenin signaling, allows the *ex vivo* maintenance of human and mouse long-term HSCs under cytokine-free conditions. We also show that combining two clinically approved medications that activate Wnt/β-catenin signaling and inhibit mTOR increases the number (but not the proportion) of long-term HSCs *in vivo*.

Hematopoietic stem cells (HSCs) represent a rare population of cells that self-renew and generate a diversity of mature blood cells. HSCs serve as a major model system for the study of stem cells and are widely used in HSC transplantation to treat human diseases, including hematopoietic malignancies and bone marrow failure[1–3]. However, significant obstacles remain in the use of HSCs both clinically and as a model system for basic stem cell biology, largely because it has not been possible to maintain functional long-term HSCs (LT-HSCs) outside of the complex environment of the hematopoietic niche[4–6].

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Author Contributions

JH designed and performed experiments, interpreted data, and wrote the manuscript. MN performed experiments, interpreted data, and contributed to writing of the manuscript. EOH contributed to design and interpretation of experiments and writing of the manuscript. GADD assisted with design and interpretation of xenograft experiments. PSK oversaw design and interpretation of all experiments and writing of the manuscript.

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HSCs reside within a low-perfusion environment in the bone marrow, with low oxygen tension and reduced nutrient supply[7–10]. Nutrient sensing systems, such as liver kinase B1 (Lkb1) and mammalian target of rapamycin (mTOR), integrate diverse signals, including mitogenic growth factors, hormones, and nutrient levels, and play critical roles in HSC homeostasis. Loss of Lkb1[11–14] or activation of mTOR[15–17], increases proliferation of committed progenitors at the expense of HSCs, suggesting that low nutrient availability is an important feature of the niche that restrains lineage commitment and supports HSC quiescence and/or self-renewal.

Isolation and culture of functional, LT-HSCs has been an elusive goal, as it is not clear what signals provided by the niche are required to maintain quiescence of HSCs[4,18]. 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 E_2, pleiotrophin, and GSK-3 inhibitors plus insulin[19–26]. 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 LT-HSCs[4,18].

Although controversial, Wnt signaling has been repeatedly implicated in HSC self-renewal[27–32]. Wnt signaling is required for normal HSC function, as loss of function of Wnt3a[34] or *β*-catenin[35] or overexpression of the Wnt inhibitor *Dkk1* in osteoblasts[36] impairs HSC self-renewal, whereas Wnt activation enhances renewal under certain conditions[23,32,37,38]. In contrast, deletion of *β-catenin* in adult mice[39–41] does not affect hematopoiesis, and overexpression of an activated form of *β*-catenin impairs HSC repopulating function.

Several nonexclusive explanations could reconcile these differences: Varying strength of Wnt signaling could result in differing hematopoietic phenotypes, as suggested by an elegant study using an allelic series of *Apc* mutants, which result in varying degrees of Wnt/*β*-catenin signaling[33]. In addition, Wnt signaling could activate multiple downstream effectors with distinct and potentially contrasting functions that depend on the mode of activation[25]. For example, inhibition of GSK-3 activates Wnt signaling and increases phenotypic HSCs and progenitor cells (HPCs), but these effects are not sustained, most likely because downstream pathways apart from *β*-catenin signaling are also activated[25,32]. We have found that the increase in phenotypic HSCs associated with GSK-3 inhibition requires Wnt/*β*-catenin signaling and that the subsequent loss of LT-HSCs occurs because inhibition of GSK-3 also activates mTOR.

Thus we hypothesized that both low nutrient availability and activation of Wnt signaling contribute to HSC maintenance and self-renewal. Here we show that human and mouse LT-HSCs can be maintained *ex vivo* in the absence of cytokines, serum, or support cells by inhibiting the mTOR-dependent nutrient sensing pathway and at the same time activating canonical Wnt signaling. Furthermore, clinically well-established inhibitors of GSK-3 (lithium) and mTOR (rapamycin) increases the number (but not the proportion) of functional LT-HSCs in mice. These observations support a role for Wnt signaling and nutrient sensing

in HSC maintenance and identify an approach for culture of HSCs in the absence of exogenous cytokines.

Results

Cytokine-free culture of hematopoietic stem cells

Maintenance of LT-HSCs outside of the hematopoietic niche remains a significant challenge, presumably because conventional culture conditions include a complex mixture of hematopoietic cytokines that promote lineage commitment and/or because of a lack of critical factors normally supplied by the niche. A number of protein and small molecule factors have been described that enhance culture of HSCs and HPCs, but in all cases these are used in conjunction with multiple cytokines. Our published data suggest that GSK-3 regulates both self-renewal and lineage commitment of HSCs; inhibition of GSK-3 activates Wnt/β-catenin signaling, which promotes self-renewal, but GSK-3 inhibition also activates mTOR[43], which promotes lineage commitment and HSC depletion[15–17]. Thus, we found that adding an mTOR inhibitor in the setting of *Gsk3* knockdown prevents HSC depletion and maintains Wnt-dependent HSC expansion *in vivo*.

As these experiments were performed *in vivo*, it was not clear whether additional factors within the hematopoietic niche contribute to this response. To explore the mechanism further, we tested whether inhibition of GSK-3 and mTOR would be sufficient for the preservation of HSCs *ex vivo* under defined culture conditions. We cultured mouse c-Kit$^+$ or Lin$^-$Sca1$^+$cKit$^+$ (LSK) cells, which are enriched for HSCs and HPCs, in serum-free, cytokine-free medium in the presence of the GSK-3 inhibitor CHIR99021 and the mTOR inhibitor rapamycin (CR) for 7 d (Fig. 1a, Supplementary Fig. 1), and then assessed hematopoietic potential by serial passage in stromal coculture (Fig. 1b, Supplementary Fig. 2a–c) and functional HSCs by competitive repopulation and serial transplantation in lethally irradiated mice (Fig. 2).

Coculture on OP9 stromal cells has been used extensively as a surrogate to test the hematopoietic potential of HSCs and HPCs[44–46]. Thus, mouse c-Kit$^+$ cells were cultured for 7 d in cytokine-free medium in the presence of CR or DMSO, but without stromal cells, and then hematopoietic potential was assessed using the OP9 system. Cultured cells were transferred at three different concentrations into either OP9 or OP9-DL1 stromal cocultures (in triplicate). After two passages and 21 d, myeloid and lymphoid differentiation was assessed by flow cytometry (FCM) and wells were scored as positive if more than 1% of cells expressed mature lineage markers. All wells (9/9) in the CR-treated group gave rise to mature myeloid cells (>54% myeloid cells per well), whereas only 1 well (1/9) of control (DMSO-treated) cells was weakly positive (~4% myeloid) for myeloid lineage, and this was at the highest cell density (2,500 cells) (Supplementary Fig. 2b). Both myeloid and lymphoid lineages developed from CR treated c-Kit$^+$ cells when cultured on OP9 (myeloid cells) or OP9-DL1 (T, B cells) stromal cells (Supplementary Fig. 2c).

These experiments were repeated with a more purified population of cells. The LSK population is highly enriched for HSCs and HPCs. LSK cells were sorted and cultured in cytokine-free, serum-free medium with either CR or DMSO-control. After 7 d, cultured

LSK cells were transferred to stromal cocultures for 21 d and assessed by FCM as above. Both myeloid and lymphoid lineages developed from the CR treated group but not from the control group when cultured on OP9 or OP9-DL1 stromal cells, respectively (Fig. 1b).

These data demonstrate that hematopoietic cells capable of multilineage hematopoiesis can be maintained in cytokine-free culture, and raise the possibility that these treated cells contain true HSCs, which to our knowledge have not previously been successfully cultured in cytokine-free conditions. However, stromal coculture systems may allow nonphysiologic lineage commitment of hematopoietic precursors, and are not a definitive test of HSC function[47]. Bone marrow transplantation remains the gold standard to assess HSC function. As a rigorous test of LT-HSC function after 7 d in cytokine-free culture, we performed competitive repopulation assays. c-Kit$^+$ cells cultured in CR for 1 week were injected with competitors into lethally irradiated 1° recipients. CR cultured cells were CD45.1$^+$ to distinguish them from competitors (CD45.2$^+$). As lithium is a well-established GSK-3 inhibitor that is widely used clinically for bipolar disorder, we also tested whether LiCl could replace CHIR99021 in cytokine-free culture conditions as a structurally distinct GSK-3 inhibitor. Thus c-Kit$^+$ cells were cultured in LiCl $^+$ rapamycin (LR) and tested in competitive repopulation assays in parallel with the CR treated group. Bone marrow was harvested at 4 months and chimerism within the LSK,Flk2$^-$CD150$^+$CD48$^-$ population was assessed by FCM. The degree of chimerism in CR (48.4%) and LR (20.8%) treated groups was significantly higher than DMSO, lithium, or rapamycin alone; the number of positive chimeric mice, defined by convention as > 5% donor-derived (CD45.1) cells in either bone marrow or peripheral blood, was also significantly higher in CR (8/9 mice; $p < 0.01$, one-way ANOVA) and LR (7/9; $p < 0.05$) treated mice than in the DMSO-control group (3/9) (Fig. 2a). CR treated cells also achieved significantly higher chimerism than cells cultured with CHIR99021 alone (5/9; $p < 0.01$). Trilineage reconstitution was also detected after 8 and 16 weeks in both LR and CR groups, as a substantial percentage of peripheral blood cells were donor-derived, including T cells (CD4$^+$ and CD8$^+$), B cells (B220$^+$), myeloid cells (Gr1$^+$ and Mac1$^+$[CD11b$^+$]), and erythroid cells (TER119$^+$) (Supplementary Fig. 3). In contrast, there was minimal chimerism in peripheral blood of DMSO control recipients. CR treated cells also achieved significantly higher chimerism in secondary recipients than cells cultured in control (DMSO) medium (data not shown).

Mouse c-Kit$^+$ cells represent a heterogeneous population. To confirm the effect of the drugs was directly on HSCs, sorted LSK cells were cultured with DMSO or CR for 7 d, then transplanted into lethally irradiated hosts. As with cultured c-Kit$^+$ cells, significant chimerism was observed in recipients receiving CR-treated cells but not vehicle-control cells (Fig. 2b). As a further test of the long-term self-renewal capability of HSCs cultured in cytokine-free CR medium, we performed serial transplantation to secondary and tertiary lethally irradiated hosts. CR-treated donors achieved high chimerism in 2° (mean = 40.5%) and 3° (mean = 30.3%) recipients, whereas DMSO-treated cells demonstrated low chimerism (mean = 3.5% and 0.6% respectively; $p < 0.01$, Fig. 2c,d), indicating that HSCs cultured with CR maintain their potential for long term reconstitution.

To measure the number of HSCs present after culture, we performed a limiting dilution analysis with c-Kit$^+$ cells cultured in CR or vehicle control. We also used freshly isolated c-

Kit$^+$ cells (not cultured) as a positive control. Donor cells were mixed at several dilutions (from 1×10^3 to 1×10^4 cells) with a constant number (2×10^5) of recipient cells and transplanted into lethally irradiated recipient mice. After four months, marrow was harvested, donor chimerism was measured, and frequency of HSCs was calculated (Fig. 2e). HSC frequency in CR-treated cultures (1/5,700; CI: 4,203–7,715) were similar to uncultured c-Kit$^+$ control cells (1/3,540; CI: 2,640–4,751; $p = 0.1433$), indicating that inhibition of GSK-3 and mTOR indeed preserves HSCs in cytokine-free culture.

Human HSCs are maintained *ex vivo* by GSK-3 and mTOR inhibition

Human umbilical cord blood (UCB) holds tremendous promise for clinical HSC transplantation (UCBT). The major obstacle to wider application of UCBT is the limited number of cells available in single UCB grafts. Attempts to expand HSCs in UCB using cytokine-based cocktails have so far not met with clinical success. Several newer approaches to expand HSCs from UCB have been described recently[19–24,26], but have either not been introduced into clinical practice or require multiple UCB grafts, and all of these approaches use cocktails of multiple cytokines, which may promote lineage commitment at the cost of LT-HSCs. To address whether human HSCs can be maintained in culture without cytokines, we cultured human UCB CD34$^+$ cells in the presence of CR without serum or cytokines. CR treatment increased the total cell number up to 7-fold by day 3 (Supplementary Fig. 4).

To determine functional HSC frequency of CR-cultured human cells, we performed xenografts into NOD.Cg-*Prkdc*^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice. At 12 weeks, all mice receiving cells treated with standard cytokines (SCF, Flt3-L, and Tpo) alone or with CR alone engrafted, in contrast to GSK-3 inhibitor alone or vehicle control (Fig. 3a,b). The CR-cultured human CD34$^+$ cells gave rise to all major lineages (Fig. 3c). We also performed secondary transplantation to assess LT-HSC function. CR-cultured HSCs achieved efficient long-term engraftment, comparable to uncultured CD34$^+$ cells; in contrast, no secondary recipients of HSCs cultured in a standard cytokine cocktail engrafted (Fig. 3d). We also directly compared CR medium to a serum-free medium containing SCF, Tpo, IGF-2, and FGF-1 (STIF medium), as this cytokine cocktail was shown to expand mouse and human LT-HSCs in *ex vivo* culture[19,20,26]. Human UCB CD34$^+$ cells were cultured in serum-free medium for 7 d in the presence of CR or STIF medium, and then competitive repopulation assays were performed. HSCs cultured in either CR or STIF medium engrafted in all primary receipients and in the majority of secondary recipients (data not shown). These data indicate that CR treatment preserves LT-HSCs in human UCB in *ex vivo*, cytokine-free conditions. Furthermore, a limiting dilution analysis revealed that the frequency of SCID repopulating cells in UCB CD34$^+$ cells cultured in CR (1/28,187) was comparable to UCB CD34$^+$ cells that were directly transplanted without culture (1/21,901; $p = 0.7088$), indicating that inhibition of GSK-3 and mTOR preserves human HSCs in cytokine-free culture (Table 1).

Wnt/β-catenin signaling is required for *ex vivo* HSC maintenance

Gsk3 loss of function or inhibition leads to stabilization of β-catenin and constitutive activation of Wnt signaling, but the requirement for β-catenin in the hematopoietic response to GSK-3 inhibitors has not been tested. We confirmed that GSK-3 inhibition activates

canonical Wnt signaling using Batgal mice, which express a LacZ reporter for canonical Wnt signaling[48]. C-Kit$^+$ cells were isolated from Batgal mice and cultured in vehicle, CHIR99021, or CR for 3 d, and then assayed for lacZ activity by FCM. CR (and CHIR99021 alone) activated the reporter in LSK,Flk2$^-$ phenotypic HSCs and also activated the endogenous Wnt target gene *Axin2* approximately 4-fold in wild-type c-Kit$^+$ cells (Supplementary Fig. 5), similar to previous reports using Wnts or GSK-3 inhibitors[32,33,38].

To test whether β-catenin is required for HSC maintenance in our *ex vivo* conditions, we used a conditional *β-catenin* loss of function allele (*β-cat$^{fl/fl}$*) crossed to tamoxifen inducible cre mice (*cre-ERT2*). LSK cells from *β-cat$^{fl/fl}$*; *cre-ERT2$^+$* or *β-cat$^{fl/fl}$*; *cre-ERT2$^-$* mice were treated with 4-hydroxytamoxifen for 2 d to induce deletion of β-catenin (Supplementary Fig. 5c) and were cultured with CR or vehicle for 7 d. Cultured cells were plated on OP9 cells and FCM was performed after 3 weeks. CR-treated wild-type but not β-catenin deficient cells gave rise to mature myeloid cells (Fig. 4a). To test the requirement for β-catenin in long-term HSC assays *in vivo*, we used mx-cre to delete *β-catenin* in hematopoietic cells. Cre was induced in *β-cat$^{fl/fl}$*; *mx-cre^{tg}* mice with poly-I/poly-C (Supplementary Fig. 5d) and c-Kit$^+$ cells were harvested and treated with CR or vehicle. After 7 d in culture, cells were mixed with 3×10^5 competitors and transplanted into lethally irradiated hosts. After 4 months, marrow from these 1° recipients was harvested and analyzed by FCM. CR maintains HSCs derived from wild-type hosts, and loss of *β-catenin* blocked this effect in 1° recipients (Fig. 4b), showing that *β-catenin* is required for the effect of CR on HSCs. These data, taken together with previously published observations[25,32], support a positive role for the Wnt/β-catenin pathway in HSC maintenance in the context of reduced GSK-3 activity.

GSK-3 and mTOR inhibition changes the HSC cell cycle profile

To explore the mechanism by which GSK-3 and mTOR inhibition preserves HSCs and HPCs, we examined the cell cycle and survival status of cultured c-Kit$^+$ cells. Mouse c-Kit$^+$ cells were cultured with CR for 4 d and stained with Ki-67 antibody and DAPI followed by FCM to assess cell cycle in the LSK population. Compared to controls, >2-fold more CR-treated LSK cells were in G_0, indicating an increased percentage of quiescent cells (Fig. 4c). There was also a small increase in the percent of cells in S/G2/M phases.

Cell death was also analyzed by FCM using 7AAD and annexin V. Control and CR treated cells showed mostly similar patterns, except for an increase in early apoptotic cells (annexin V$^+$ 7ADD$^-$) in the CR treated group (Fig. 4d), suggesting that the maintenance of LSK cells is not due to reduced cell death. These data indicate that the combination treatment results in maintenance or enhancement of quiescence within the LSK cell population as well as modestly accelerated cell cycle progression.

GSK-3 and mTOR inhibition increases the number of LT-HSCs *in vivo*

Both lithium and rapamycin are used extensively in clinical settings, and combining lithium and rapamycin to enhance hematopoietic function could have therapeutic implications. Thus, we performed serial, noncompetitive transplantation with c-Kit$^+$ cells cultured in cytokine-free medium with lithium and rapamycin (LR). c-Kit$^+$ cells were cultured in LR or control medium for 7 d and then transplanted to lethally irradiated recipients. 60% of mice

receiving LR-cultured cells (n = 10 mice) survived 4 months or longer while 100% of control mice (receiving cells cultured in control medium, lithium alone, or rapamycin alone) died within 14 d (Fig. 5a). All surviving recipients of LR-treated cells showed long-term multilineage reconstitution with >90% of donor derived cells contributing to multiple lineages in bone marrow (Fig. 5b) and peripheral blood (data not shown).

We then treated mice with LR for 2 weeks and compared them to mice receiving vehicle, LiCl alone, or rapamycin alone. The overall bone marrow cellularity increased in both LiCl (as observed previously) and LR treated groups compared to vehicle control (Fig. 5c). The absolute number of immunophenotypic LT-HSCs (LSK,CD34$^-$Flk2$^-$CD150$^+$CD48$^-$) also increased in the LR group (Fig. 5d). In a competitive repopulation assay, the absolute number of competitive rescue units was increased 2-fold in bone marrow from mice treated with lithium and rapamycin (Fig. 5e) ($p < 0.01$). Furthermore, β-catenin protein increased in whole bone marrow of the LiCl-treated and the LR-treated groups, consistent with activated Wnt signaling under GSK-3 inhibition. An increase in the phosphorylation of S6 and 4EBP, which are downstream of mTOR, was also observed in the LiCl-treated group (Fig. 5f), and this increase was blocked by rapamycin. These observations demonstrate mTOR activation by LiCl, consistent with the known inhibition of mTOR by GSK-3[43]. Taken together, our data suggest that the combination of two clinically approved drugs increases the number of LT-HSCs, as well as the entire bone marrow compartment, *in vivo*.

Discussion

The data presented here identify activated Wnt and reduced mTOR signaling as critical pathways that support the maintenance of LT-HSCs in defined, cytokine-free conditions. These findings also support an essential role for GSK-3 in HSC homeostasis as a suppressor of self-renewal (Wnt) and nutrient sensing (mTOR) pathways. Wnt/β-catenin signaling promotes stem cell renewal[28] whereas mTOR activation promotes lineage commitment and stem cell depletion[15–17,50]. Inhibition of GSK-3 activates both Wnt/β-catenin and mTOR pathways in HSCs, and concurrent inhibition of mTOR prevents HSC depletion, allowing *ex vivo* maintenance (in the absence of growth factors) and an *in vivo* increase in the number of LT-HSCs. The ability to maintain LT-HSCs in the absence of exogenous hematopoietic cytokines and to increase the number (but not the proportion) of LT-HSCs *in vivo* using two clinically well-tolerated medications may have a significant impact on stem cell transplantation in hematopoietic malignancies and bone marrow failure.

Culture of HSCs has been a major roadblock in both the basic research of HSCs and in improving human HSC transplantation, as it is difficult to reconstitute the endogenous conditions required to maintain or expand HSCs. The use of multiple hematopoietic cytokines, apparently essential for the survival of HPCs *ex vivo*, may also contribute to these difficulties, as these factors likely promote lineage specification at the cost of LT-HSC function[4,18]. Thus an important remaining goal is to achieve HSC expansion while limiting or eliminating exposure to lineage promoting cytokines.

Wnt/β-catenin has been implicated in stem cell self-renewal in multiple tissues[28], but its role in HSC self-renewal has been controversial. The conflicting reports concerning the role of

Wnt/β-catenin signaling in adult HSCs may in part be explained by differential responses to varying levels of Wnt signaling, as proposed recently[33]. In addition, Wnt signaling, which inhibits GSK-3, can activate both β-catenin and mTOR[43], which could lead to transient expansion of HSCs that is counteracted by lineage commitment and HSC depletion; in this context, the relative contributions of β-catenin and mTOR activation to HSC fate could also depend on the level of Wnt signaling. The opposing effects of Wnt/β-catenin and mTOR could also explain the limited response of HSCs to GSK-3 inhibitors when given alone. The effects we observe with CR treatment clearly involve both β-catenin and mTOR, as conditional knockout of *β-catenin* blocks the response and inhibition of mTOR is required for the response. However, additional GSK-3 regulated pathways could also play a role in the response.

The use of GSK-3 inhibitors to improve hematopoietic function began with lithium, before it was known to be a GSK-3 inhibitor[51]. Lithium increases circulating $CD34^+$ cells[52] and reliably increases production of granulocytes and platelets in most patients taking lithium. However, trials with lithium met with limited success, possibly because the effects of Wnt activation are mitigated by concurrent activation of mTOR signaling, leading to expansion of progenitor cells and lineage commitment. Consistent with this, in mice, treatment of transplant recipients with CHIR99021 accelerates recovery of mature myeloid cells and expands HPCs without increasing LT-HSCs[32]. Similarly, GSK-3 inhibitors enhance the progenitor population and hematopoietic reconstitution in mouse primary transplant recipients[23], as well as in control mice, and preserve long-term culture initiating progenitor cells when human $CD34^+$ cells are co-cultured with stromal cells and cytokines[49]. Furthermore, inhibition of GSK-3 combined with insulin expands LT-HSCs in culture in the presence of hematopoietic cytokines[25]. Although the targets downstream of insulin/PI3K signaling were not addressed, this exciting work suggests that inhibition of GSK-3 combined with additional signals may allow expansion of HSCs.

Recent work has shown that HSCs and other somatic stem cells reside in low perfusion niches[7–10] and that activation of nutrient sensing pathways enhances lineage commitment at the expense of HSCs. Thus, Lkb1, a multifunctional nutrient sensing kinase, is required to maintain HSC quiescence under reduced nutrient conditions and loss of Lkb1 promotes lineage commitment and HSC exhaustion in an mTOR-independent manner[11–14]. However, mutations that activate mTOR, mimicking nutrient replete states, also promote lineage commitment and HSC depletion[15–17]. These observations, together with the findings reported here, suggest that reduced nutrient availability, as in a low perfusion niche, or pharmacological inhibition of nutrient sensing pathways, may contribute to the maintenance of LT-HSCs. These findings are also consistent with observations that activation of Wnt signaling combined with inhibition of mTOR maintains long-term epidermal stem cells[50], suggesting that the interplay between Wnt and mTOR might be a general mechanism to regulate the self-renewal and differentiation of stem cells.

In summary, we show for the first time that LT-HSCs can be maintained *ex vivo*, in the absence of growth factors, under conditions of reduced nutrient sensing (inhibition of mTOR) combined with activation of Wnt signaling, establishing these signals as critical factors in the maintenance of LT-HSCs. We also show that a novel combination of clinically

tolerated medications increases the number of LT-HSCs as well as overall bone marrow cellularity *in vivo*. These studies lay the preclinical groundwork for testing whether outcomes of clinical UCBT can be enhanced through *in vivo* or *ex vivo* exposure to GSK-3 inhibitors and rapamycin. The clinical application of GSK-3 inhibitors should be approached with caution, as activation of Wnt signaling has been implicated in malignancies, including colorectal cancers and acute leukemias[53,54]; however, lithium has been used to treat bipolar disorder for over 50 years and is not associated with increased risk of malignancies[55]; furthermore, the approaches described here involve transient *ex vivo* or *in vivo* exposure to GSK-3 inhibitors, rather than the prolonged activation associated with colorectal cancers. Recent studies also suggest that GSK-3 and mTOR regulate T cell memory, and that inhibition of GSK-3 and/or mTOR may enhance the number and functionality of $CD8^+$ memory T cells[56,57]. Thus this approach, using agents with already well defined safety profiles, hold promise for improved hematopoietic recovery and immune reconstitution in UCBT.

Methods

Mice

C57 B/6 WT ($CD45.2^+$), $CD45.1^+$ congenic (Jackson lab), and *mx-cre*, *β-catenin*$^{fl/fl}$ mice were bred in-house in a pathogen-free mouse facility of University of Pennsylvania. Transplant recipients were 8–10 week old females. Animal experiments were in accordance with guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania.

Cytokine-free HSC culture

HSC culture medium consists of X-VIVO 15 (BioWhittaker) supplemented with 1% penicillin and streptomycin (Sigma). CHIR99021 (3μM) (Stemgent) and rapamycin (5nM) (Calbiochem) were added where indicated. BM cells were harvested from C57Bl/6 ($CD45.2^+$) or SJL ($CD45.1^+$) mice and distributed into a single cell suspension by gently drawing through a 22-gauge needle three to five times. The red blood cells were lysed in ACK (ammonium chloride-potassium) buffer. Mouse c-Kit^+ cells were purified with Miltenyi MACS cell separation kit. 5×10^4 mononucleated cells were plated in 1 ml of culture medium per well in a 24-well U-bottom plate (Becton Dickinson Company). For sorted cultures, LSK cells were sorted into 96-well U-bottom plates at 1,000 cells per well with 200 μl medium. One-half volume of medium was replaced every other day. After 7 d, the total culture product was harvested and cells were washed and transplanted into lethally irradiated mice.

Flow Cytometry (FCM) and Isolation of HSCs

BM cells were flushed from the long bones (tibias and femurs) of mice with Hank's buffered salt solution without calcium or magnesium. For detection of lineage$^-$Sca-1^+c-Kit^+ (LSK) cells, whole BM cells were incubated with biotin-conjugated monoclonal antibodies to lineage markers including B220 (6B2), CD4 (GK1.5), CD8 (53-6.7), Gr-1 (8C5), Mac-1 (M1/70), Ter119, IL-7R (A7R34), PerCP Cy5.5-conjugated antibody to Sca-1 (Ly6A/E; D7), and APC-Alexa Fluor® 750-conjugated antibody to c-Kit (ACK2). Flk2, CD34,

CD150, and CD48 were measured with the following antibodies: PE-conjugated antibody to Flk2 (Ly-72/A2F10), Alexa Fluor® 647-conjugated antibody to CD34, PE Cy5-conjugated antibody to CD48, PE Cy7-conjugated antibody to CD150. Biotin-conjugated lineage markers were detected using streptavidin-conjugated PE-Texas Red. Mouse antibodies were used at a dilution of 1:100. Nonviable cells were excluded using the viability dye DAPI (1 g ml^{-1}). Cells were sorted with a FACSAria (Becton Dickinson) automated cell sorter. Analysis was performed on LSR II, FACSCanto or FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star).

Wnt reporter assay by flow cytometry—Mouse c-Kit^+ cells were harvested from the Batgal Wnt reporter mouse line. The FluoReporter® *lac*Z Flow Cytometry Kit (Invitrogen) was used for quantitating β-galactosidase activity with fluorescein di-V-galactoside in single cells using flow cytometry.

OP9 Cultures

OP9-MigR1 (OP9) and OP9-DL1 cells were used as described[58]. Mouse c-Kit^+ or LSK progenitors were seeded into 12-well plates containing a confluent stromal monolayer of OP9 or OP9-DL1 cells which were irradiated at 30 Gys. The cytokines mFlt3L (5 ng ml^{-1}) and mIL-7 (5–10 ng ml^{-1}) (Peprotech) were added to the coculture. The cells were passed every 7 d to freshly irradiated OP9 or OP9-DL1 stromal cells.

Long-Term Repopulation Assays

For long-term non-competitive repopulation, adult recipient mice were irradiated with a Cs-137 Irradiator in two equal doses of 500 rads separated by at least 2 hrs. Cells were injected into the retro-orbital venous sinus of anesthetized recipients. Beginning 4 weeks after transplantation and continuing for at least 16 weeks, blood was collected from the tail veins of recipient mice and analyzed by FCM for the lineage markers B220 (6B2), Mac-1 (M1/70), CD4 (L3T4) and CD8 (Ly-3), and Gr-1 (8C5) to monitor engraftment by FCM. For long-term competitive repopulation, CD45.1^+ c-Kit^+ cells from CR or DMSO treated cultures were transplanted into lethally irradiated B6 recipients together with 3×10^5 competitor B6 BM cells (CD45.2^+). In limiting dilution analyses, uncultured c-Kit^+ cells or cells cultured in CR or control medium for one week were injected together with 3×10^5 competitors into lethally irradiated 1° recipients. Beginning 4 weeks after transplantation and up to 16 weeks, peripheral blood was collected and analyzed by FCM as above to monitor engraftment. BM was harvested and analyzed 16 weeks after transplantation. In serial transplantations, the BM was harvested from primary recipients after 4 months and 20×10^6 BM cells were transplanted into each lethally irradiated secondary recipient.

Xenotransplant assays

Gender balanced NOD-SCID-IL2Rgc^{null} (NSG) mice were used in this study. Adult recipients were irradiated with a Cs-137 Irradiator in two equal doses of 400 rads separated by at least 2 h. Cells were injected into the retro-orbital venous sinus of anesthetized recipients. In serial transplantation experiments, BM was harvested from primary recipients after 4 months and 90% of total BM cells (4 tibias, 4 femurs and 2 hips) were transplanted into each sub-lethally irradiated secondary recipient mouse (NSG).

Administration of pIpC and rapamycin

Polyinosine-polycytidine (pIpC; Sigma) was resuspended in Dulbecco's-PBS at 2 mg ml^{-1}. Mice received 25 μg of pIpC per gram every other day for 2 weeks. Rapamycin (LC Laboratories) was dissolved in absolute ethanol at 10 mg ml^{-1} and diluted in 5% Tween-80 (Sigma) and 5% PEG-400 (Hampton Research) before injection and was administered by intraperitoneal injection at 4 mg kg^{-1} rapamycin in 200 μl total volume/injection every other day for 2–3 weeks.

Statistical Methods

Pairwise comparisons were analyzed using a two-tailed Students's t-test and results were considered significant when $p < 0.05$. Data involving multiple samples (as in Fig. 2a) were analyzed with a one-way ANOVA followed by a Dunn's post hoc analysis when a significant difference was found among the groups.

Supplementary Material

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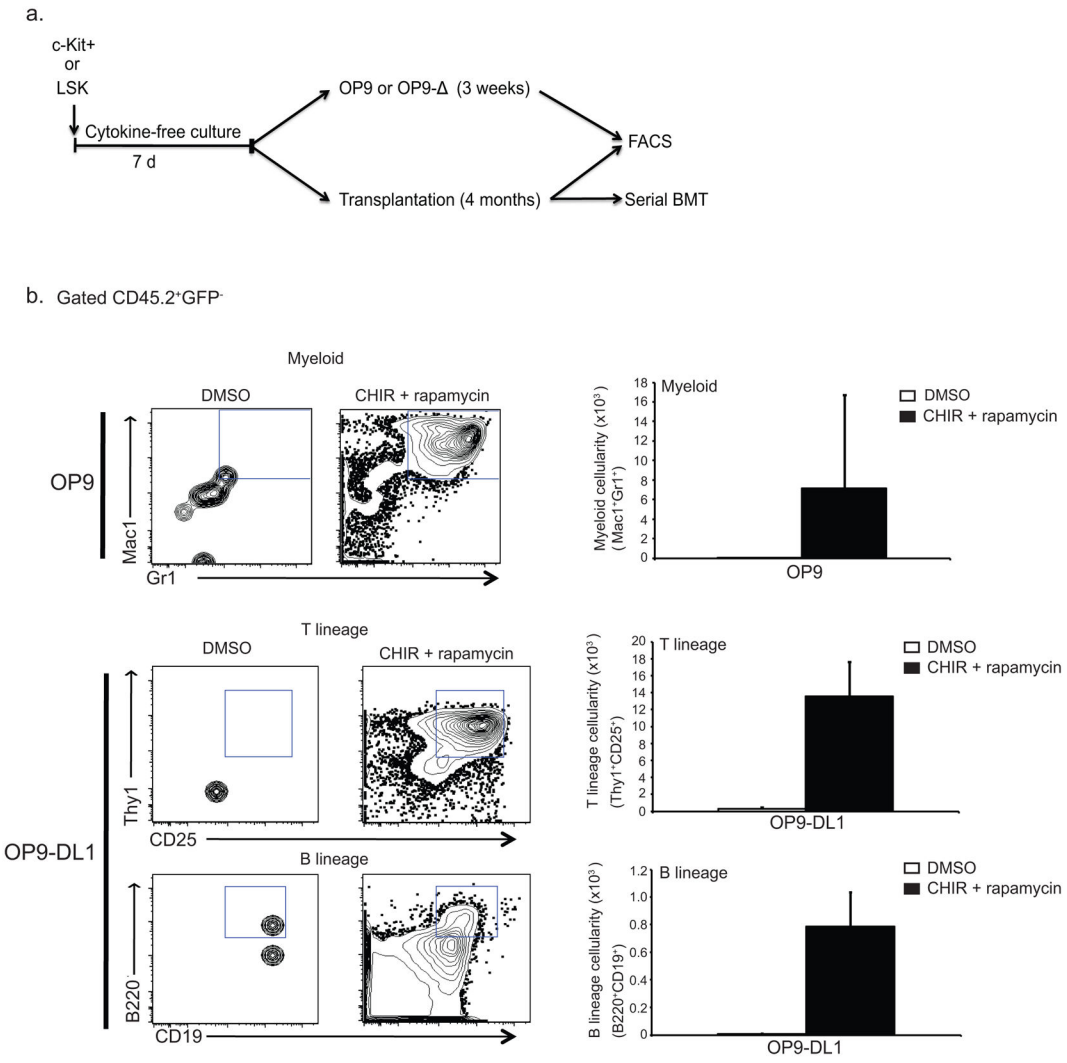

Figure 1. Inhibition of GSK-3 and mTOR preserves HSPCs

(a) Experiment design: Mouse c-Kit^{+} or LSK cells were cultured in cytokine-free medium with inhibitors (CR) or vehicle for 7 d, then either plated on OP9 stromal cells or transplanted into lethally irradiated mice. FCM was performed after 3 weeks (OP9) or 4 months (transplantation, Figure 2). **(b)** Sorted LSK cells were cultured for 7 d, then plated on OP9 cells. Markers for myeloid (OP9), T and B lineages (OP9-DL1) (left) were measured at 3 weeks by FCM. Representative FCM data on left, quantification of myeloid, T and B lineages on right.

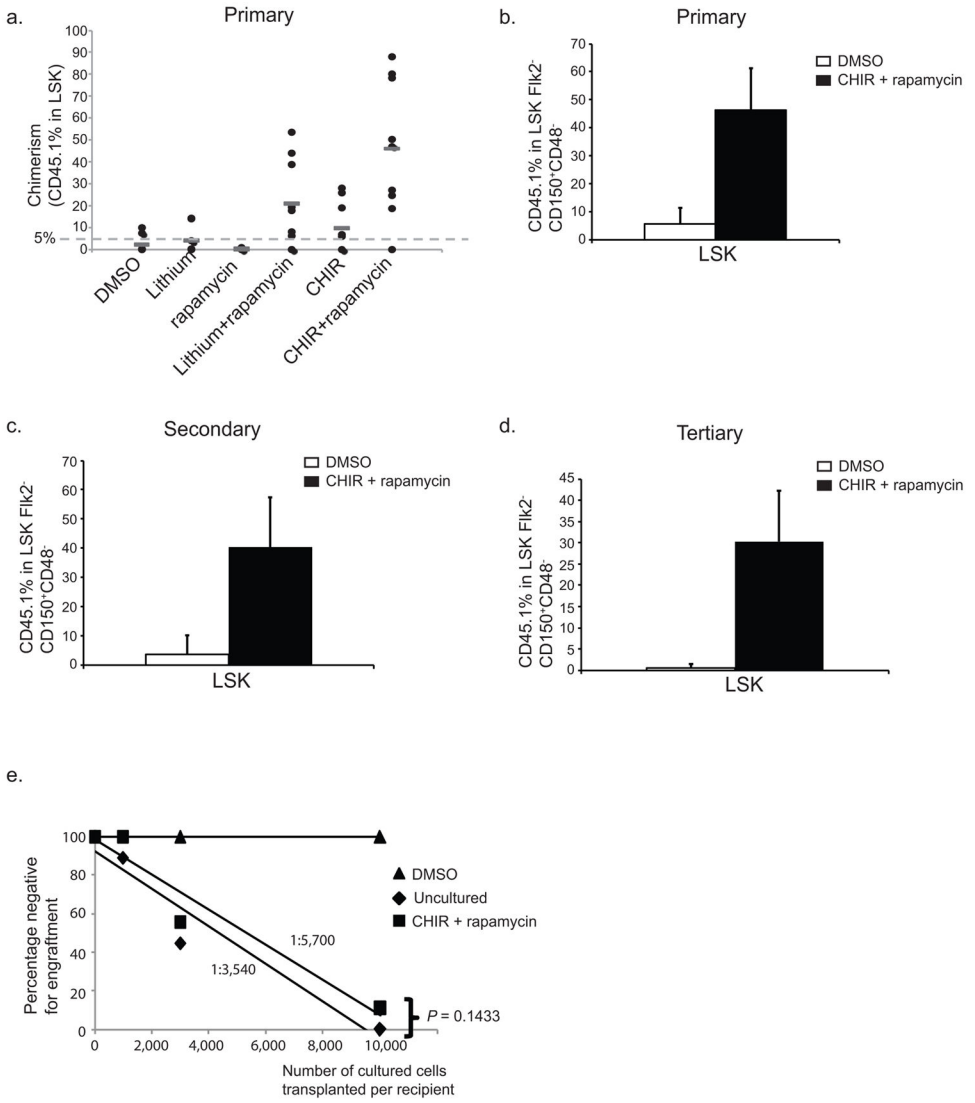

Figure 2. Maintenance of long-term HSCs *ex vivo* by inhibition of GSK-3 and mTOR

(a) Mouse c-Kit$^+$ cells were cultured in cytokine-free medium for 7 d with the inhibitors as indicated, then competitive serial transplants were performed. Chimerism in bone marrow (BM) was measured by FCM after 4 months. The data are from 3 independent experiments. **(b–d)** LSK cells were cultured in cytokine-free medium for 7 d, then competitive serial transplants were performed in primary **(b),** secondary **(c),** and tertiary recipients **(d).** Bone marrow was harvested after 4 months for analysis of chimerism and for transplant to subsequent recipients. **(e)** Limiting dilution experiments were performed with three doses of cultured c-Kit$^+$ test marrow (CD45.1$^+$) from control or CR treated cells combined with 2×10^5 competing cells (CD45.2$^+$) transplanted into groups of at least nine recipients per dose. Chimerism at 4 months for each dose is represented as the percentage of mice negative for engraftment in BM for vehicle control (green triangle), CR treated cells (red square), and uncultured cells (blue diamond).

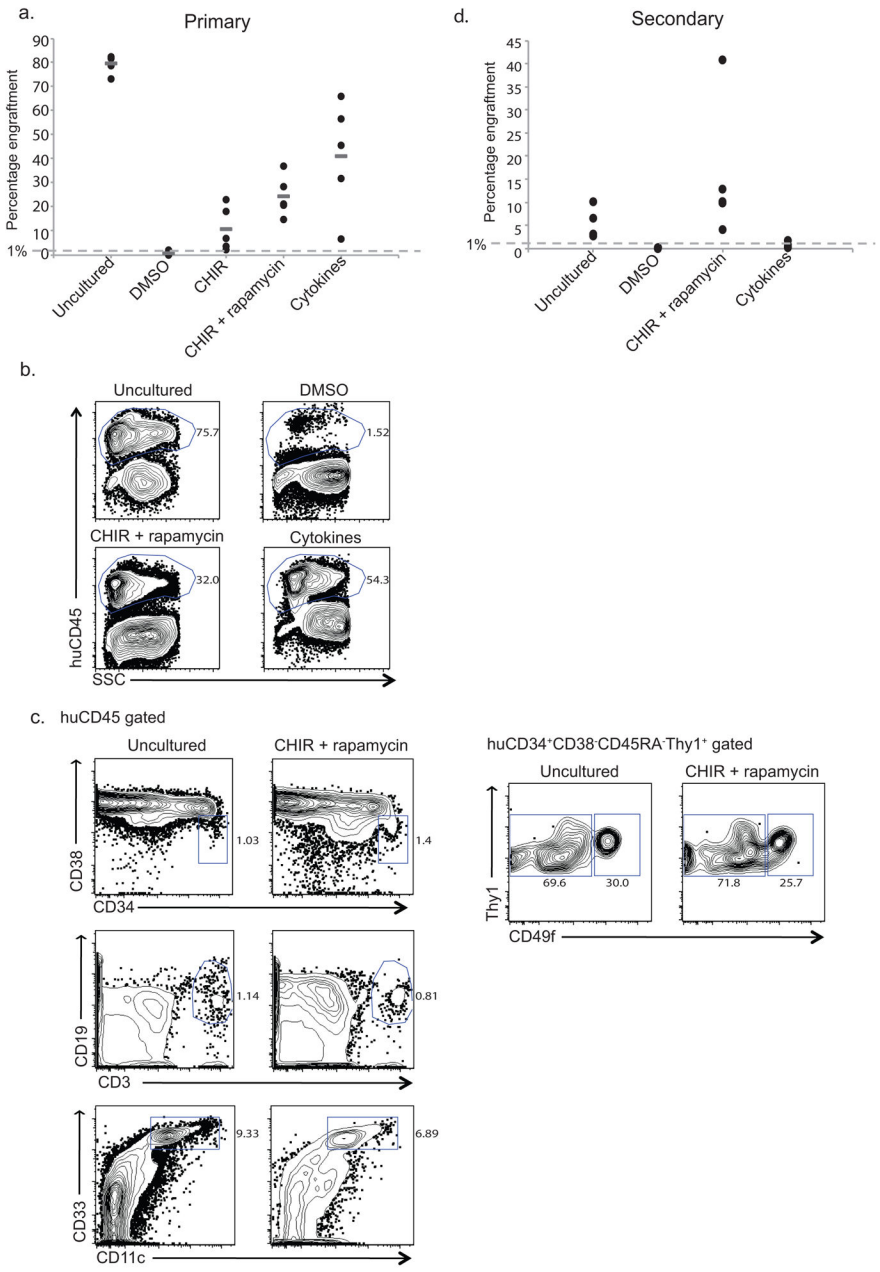

Figure 3. Human long-term HSCs maintained in cytokine-free culture

(a) Human umbilical cord blood (UCB) $CD34^+$ cells were cultured in cytokine-free medium with or without $Chiron^+$rapamycin (CR) for 7 d, then competitive serial transplants were performed by transplanting all the cells into sub-lethally irradiated NSG recipients. Engraftment was assessed as percent of human $CD45^+$ cells in BM after 4 months. **(b)** Representative FCM data ($CD45^+$) are shown for BM from primary recipients of human $CD34^+$ cells that were directly transplanted (uncultured) or cultured in vehicle (DMSO), CR, or conventional cytokines. **(c)** Progenitors, lymphoid (T-cell and B-cell), and myeloid lineages derived from human $CD34^+$ cells that were directly transplanted (uncultured) or cultured in CR for 7 d before transplant. Similar numbers of each lineage were detected by

FCM. **(d)** Four months after primary transplantation, BM was transplanted into 2° NOD-SCID recipients and chimerism was assessed after 4 months as in A.

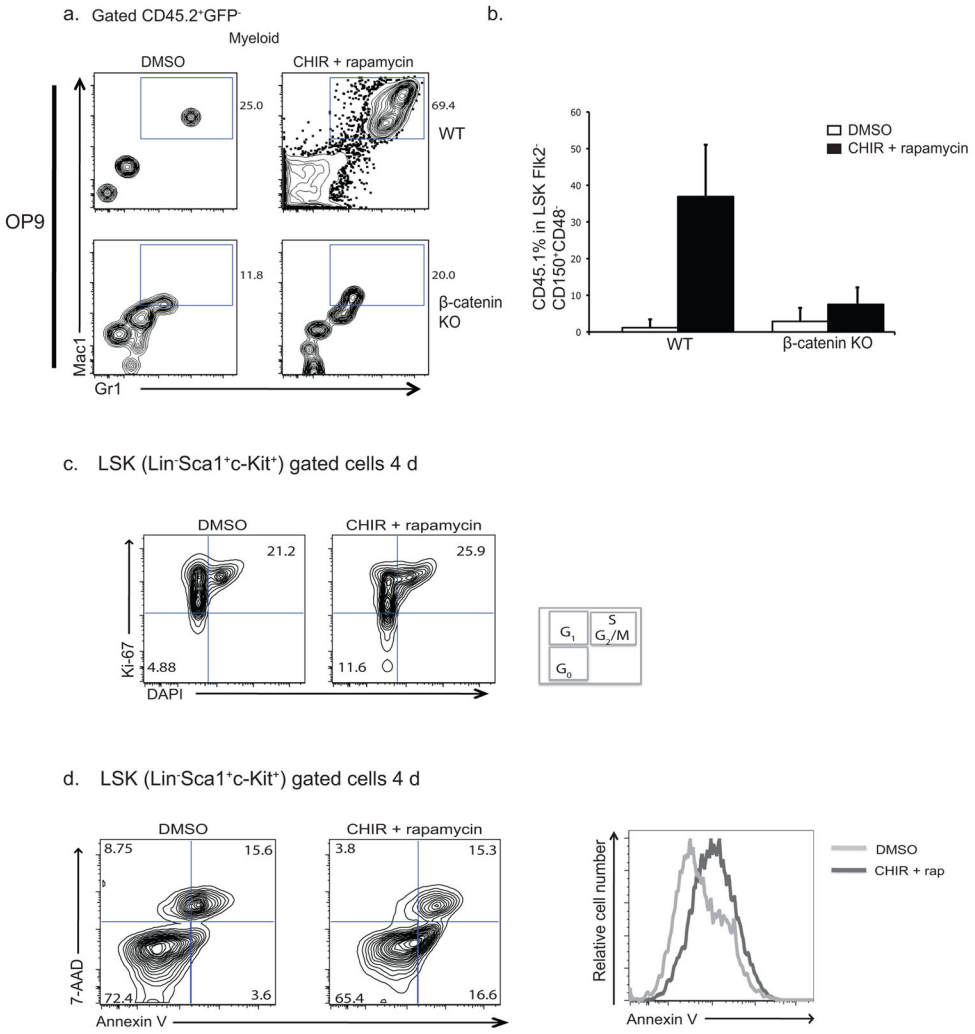

Figure 4. Maintenance of long-term HSCs in culture requires *β*-catenin

(a) BM was harvested from mice with a conditional deletion of *β-catenin* (using cre-ERT2) or cre- controls, and LSK cells were cultured in cytokine-free medium with or without inhibitors (CR) for 7 d. Cultured cells were then plated on OP9 cells and assessed by FCM after 3 weeks as in Fig. 1. (b) C-Kit$^+$ cells were harvested from mice with a conditional deletion of *β*-catenin (using mx-cre) or cre- controls and cultured in cytokine-free medium with or without inhibitors (CR) for 7 d, then competitive transplants were performed by transplanting all the cells in each group mixed with 3X10^5 fresh BM cells into lethally irradiated recipients. Chimerism in BM was measured by FCM for CD45.1$^+$LSK,CD150$^+$CD48$^-$Flk2$^-$ cells after 4 months. For panels (a) and (b), depletion of *β-catenin* was confirmed by western blot (Supplementary Fig. 5c,d). **(c)** To assess cell cycle status, c-Kit$^+$ cells were cultured for 4 d and analyzed by FCM for LSK markers, DAPI staining, and Ki67. Representative FACS data are shown for control (DMSO) vs. CR cultured cells. **(d)** To assess apoptosis, c-Kit$^+$ cells were cultured as in A and analyzed by FCM for LSK, 7AAD, and Annexin V.

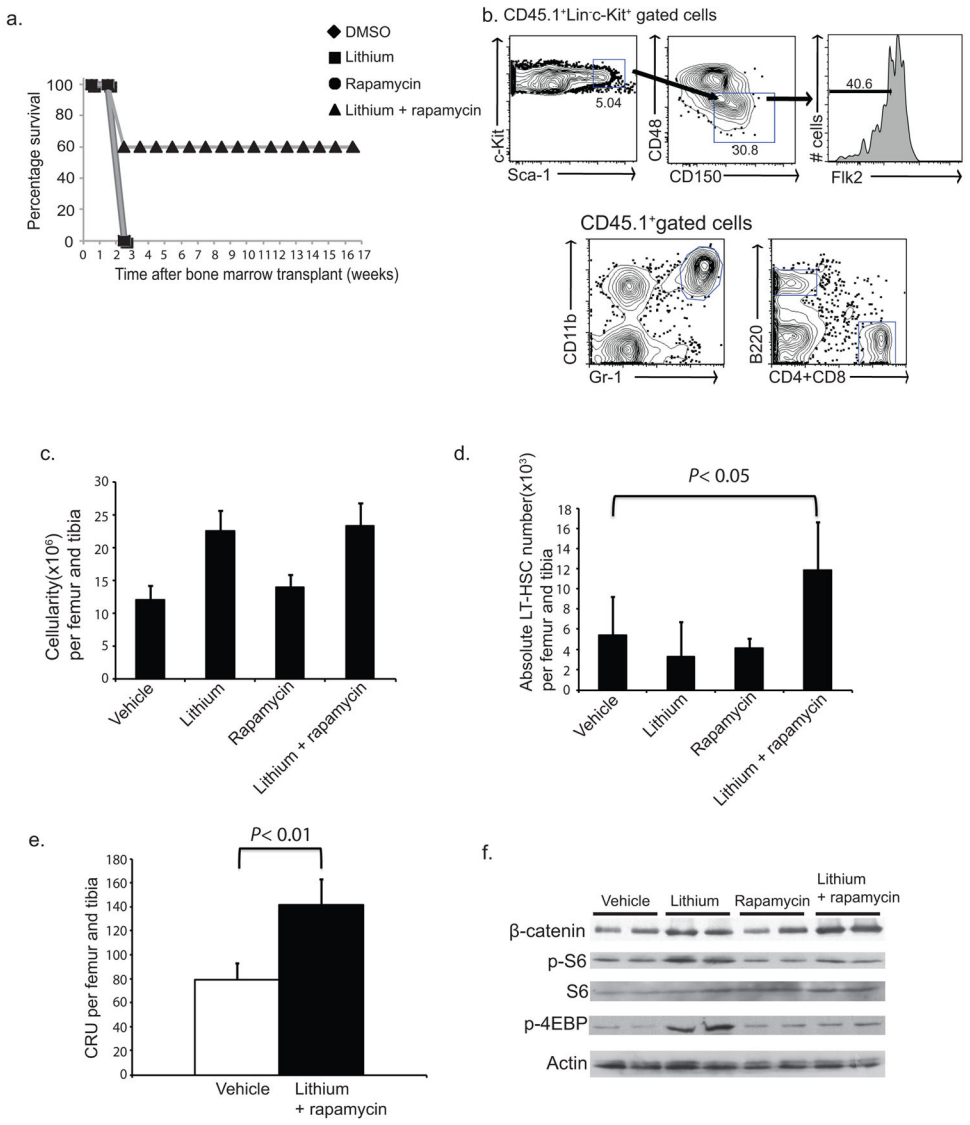

Figure 5. Lithium and rapamycin increase the number of long-term HSCs *in vivo*

(a) Mouse c-Kit$^+$ cells were cultured for 7 d in cytokine-free medium with or without lithium and rapamycin (LR) and transplanted into lethally irradiated recipients. Survival of recipients receiving cells cultured with vehicle (blue diamond), lithium (red square), rapamycin (purple circle), or LR (green triangle) is shown as a Kaplan-Meier plot. **(b)** Representative FCM of donor-derived phenotypic HSCs in BM (upper panels) and myeloid and lymphoid lineages in peripheral blood (lower panels) for recipients of LR cultured cells. **(c)** Mice received lithium and rapamycin for 3 weeks. Cellularity of BM in vehicle control, lithium, rapamycin, and LR treated mice was measured as the number of nucleated cells/ mouse recovered from one femur and tibia. **(d)** Absolute number of phenotypic HSCs (LSK,CD34$^-$Flk2$^-$CD150$^+$CD48$^-$) in BM in control, lithium, rapamycin, and LR treated mice. **(e)** Mice were treated with lithium and rapamycin for 3 weeks. BM was harvested, limiting dilution assay was performed, and competitive rescue units for control and LR are shown. **(f)** Western blot analysis for β-catenin, phosphorylated-S6, S6,

phosphorylated-4EBP in BM cells harvested from control, lithium, rapamycin, and LR treated mice.

Table 1

Effect of CR on CB-derived CD34+ cells after 7 days in culture

Human $CD34^+$ cells from UCB were transplanted directly (uncultured) or cultured for 7 d (CR or vehicle) before transplantation. A serial dilution of uncultured or CR-cultured cells (based on the number of cells present before culture) was injected into NOD-SCID hosts. The percentage of human $CD45^+$ cells in peripheral blood at 12 weeks was measured and >1% was considered positive for engraftment. The frequency of SCID repopulating cells (SRCs) calculated with L-Calc software was not significantly different between uncultured and cells cultured in CR ($p = 0.7088$).

Conditions	#Cells transplanted (or equivalent starting dose for cultured cells)	# reconstituted mice/# primary recipients	SRC frequency (confidence interval)
	1,000	1/4	
Uncultured	10,000	2/4	1 in 21,901 (13,273–36,138)
	50,000	3/4	
	1,000	2/5	
CR	10,000	2/5	1 in 28,187 (17,914–44,352)
	50,000	3/5	
DMSO	150,000	0/3	