Maintenance of hematopoietic stem cells through regulation of Wnt and mTOR pathways

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Hematopoietic stem cell (HSC) self renewal and lineage commitment depend on complex interactions with the microenvironment. The ability to maintain or expand HSCs for clinical applications or basic research has been substantially 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 mTOR pathway, an established nutrient sensor, combined with activation of canonical Wnt–β-catenin signaling, allows for the ex vivo maintenance of human and mouse long-term HSCs under cytokine-free conditions. We also show that the combination of two clinically approved medications that together activate Wnt–β-catenin and inhibit mTOR signaling increases the number (but not the proportion) of long-term HSCs in vivo.

HSCs represent a rare population of cells that self renew and generate a diversity of mature blood cell types. 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 failure1–3. However, major 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 niche4–6.

HSCs reside within a low-perfusion environment in the bone marrow with low oxygen tension and reduced nutrient supply7–10. Nutrient-sensing systems, such as Lkb1 and mTOR, integrate diverse signals, including mitogenic growth factors, hormones and nutrient availability, and have crucial roles in HSC homeostasis. Loss of Lkb1 (refs. 11–14) or activation of mTOR15–17 increases the proliferation of committed progenitors at the expense of HSC maintenance, suggesting that low nutrient availability is an important feature of the niche that restrains lineage commitment and supports HSC quiescence, self renewal or both.

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 HSC quiescence4,18. 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 E2, pleiotrophin or glycogen synthase kinase 3 (GSK-3) inhibitors in combination with insulin19–26. 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-HSC maintenance4,18.

Although the issue is still under debate, Wnt signaling has repeatedly been implicated in HSC self renewal27–35. Wnt signaling is required for normal HSC function, as loss of Wnt3a34 or β-catenin35 function or overexpression of the Wnt inhibitor Dkk1 in osteoblasts36 impairs HSC self renewal, whereas Wnt activation enhances renewal under certain conditions33,32,37,38. In contrast, β-catenin deficiency in adult mice39–41 does not affect hematopoiesis, and overexpression of an activated form of β-catenin impairs HSC repopulating function42,43. 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 signaling33. In addition, Wnt signaling could activate multiple downstream effectors with distinct and potentially contrasting functions that depend on the mode of activation25. For example, inhibition of GSK-3 activates Wnt signaling and increases the number of immunophenotypic HSCs and hematopoietic progenitor cells (HPCs), but these effects are not sustained, probably because downstream pathways apart from β-catenin signaling are also activated25,32. We have previously found that the increase in the number of immunophenotypic 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 mTOR44.

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, the combination of clinically well-established inhibitors of GSK-3 (lithium) and mTOR (rapamycin) increase the number (but not the

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Figure 1 Inhibition of GSK-3 and mTOR preserves hematopoietic stem and progenitor cells. (a) Experimental design. Mouse c-Kit+ or LSK cells were cultured in cytokine-free medium with inhibitors (C+R) or vehicle for 7 d and then either plated on OP9 or OP9-DL1 stromal cells or transplanted into lethally irradiated mice. FCM was performed after 3 weeks of coculture with stromal cells or 4 months after transplantation; the transplantation results are shown in Fig. 2. (b) Representative FCM data (left) and quantification of myeloid, T and B lineages (right) of sorted LSK cells cultured for 7 d, plated on OP9 or OP9-DL1 cells and assessed for myeloid (OP9), T and B (OP9-DL1) lineage markers. Cultured LSK cells were plated on OP9 cells in triplicate; histograms on right represent the mean value from three wells (error bars, s.d.). (The overall experiment was performed twice). Hematopoietic cells were distinguished from OP9 cells (which are GFP+) by gating on the CD45.2+GFP− population.

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 culturing 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 substantial challenge, presumably because conventional culture conditions include a complex mixture of hematopoietic cytokines that promote lineage commitment, lack crucial factors normally supplied by the niche, or both. A number of protein and small-molecule factors have been described that enhance culture of HSCs and HPCs, but these are always used in conjunction with multiple cytokines. Our previously published data suggest that GSK-3 regulates both the self renewal and lineage commitment of HSCs and mTOR inhibition of GSK-3 activates Wnt-β-catenin signaling, which promotes self renewal, but GSK-3 inhibition also activates mTOR, which promotes lineage commitment and HSC depletion. Thus, the addition of an mTOR inhibitor in the setting of GSK3 knockdown prevents HSC depletion and maintains Wnt-dependent HSC expansion in vivo.

As our previous work was in the in vivo setting, it was not clear whether additional factors within the hematopoietic niche contributed to HSC maintenance in the context of GSK-3 and mTOR inhibition. To explore the mechanism further, we tested whether inhibition of GSK-3 and mTOR would be sufficient for the preservation of HSCs under defined culture conditions. We cultured mouse c-Kit+ or Lin−Sca1+c-Kit+ (LSK) cells, which are enriched for HSCs and HPCs, in serum-free, cytokine-free medium in the presence of the GSK3 inhibitor CHIR99021 plus the mTOR inhibitor rapamycin (C+R) for 7 d (Fig. 1a and Supplementary Fig. 1). We then assessed hematopoietic potential by serial passage in stromal coculture (Fig. 1b and 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. Thus, we cultured mouse c-Kit+ cells for 7 d in cytokine-free medium in the presence of C+R or DMSO but without stromal cells and then assessed their hematopoietic potential using the OP9 system. We transferred the cultured cells at three different concentrations into either OP9 or OP9-DL1 stromal cocultures (in triplicate). After culturing for 21 d (with two passages), we assessed myeloid and lymphoid differentiation by flow cytometry (FCM) and scored wells as positive if more than 1% of the cells expressed mature lineage markers. All wells (9/9) in the C+R-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 for myeloid lineage cells (~4% myeloid cells), and this result was seen only at the highest cell density (2,500 cells). Myeloid and lymphoid lineage cells were generated from C+R-treated c-Kit+ cells when cultured on OP9 or OP9-DL1 stromal cells, respectively (Supplementary Fig. 2b).

We next repeated these experiments with a more purified population of cells. The LSK population is highly enriched for HSCs and HPCs. We sorted LSK cells and cultured them in cytokine-free, serum-free medium with either C+R or DMSO (control). After 7 d, we transferred the cultured LSK cells to stromal cocultures for 21 d and assessed them by FCM as described above. Myeloid and lymphoid lineage cells were generated from the C+R-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 that are 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 nonphysiological lineage commitment of hematopoietic precursors and are not a definitive test of HSC function. 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. We cultured c-Kit+ cells in C+R for 1 week and then injected them with competitor cells into lethally irradiated primary recipients. C+R-cultured cells were CD45.1+ to distinguish them from competitors (CD45.2+). As lithium is a well-established GSK-3 inhibitor that is clinically widely used for bipolar disorder, we also tested whether lithium chloride...
could replace CHIR99021 in cytokine-free culture conditions as a structurally distinct GSK-3 inhibitor. Thus, we cultured c-Kit+ cells in lithium chloride plus rapamycin (L+R) and tested them in competitive repopulation assays in parallel with the C+R-treated cells. We then harvested bone marrow from the transplant recipient mice after 4 months and assessed chimerism within the LSK Flk2-CD150-CD48- population by FCM. The degree of chimerism for the C+R (48.4%) and L+R (20.8%) treatment groups was substantially higher than for the DMSO, lithium or rapamycin-alone treatment groups.

The number of chimeric-positive mice, defined by convention as >5% donor-derived (CD45.1) cells in either the bone marrow or peripheral blood, was also significantly higher for the C+R (8/9 mice; P < 0.01, one-way analysis of variance) and L+R (7/9; P < 0.05) treatment groups compared to the DMSO control treatment group (3/9) (Fig. 2a). C+R-treated cells also achieved significantly higher chimerism than did cells cultured with CHIR99021 alone (5/9; P < 0.01). Both the L+R and C+R treatment groups showed tri-lineage reconstitution after 8 and 16 weeks; in these groups we detected donor-derived peripheral blood 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 the peripheral blood of DMSO-treated control recipients. We also harvested bone marrow at 4 months from primary recipients of either C+R-treated or DMSO-treated donor cells and performed transplants directly into secondary recipients. After 4 months, the C+R treatment group achieved substantially higher chimerism in secondary recipients than did the DMSO control treatment group (data not shown).

Mouse c-Kit+ cells represent a heterogeneous population. To confirm that the effect of the drugs was directly on HSCs, we cultured sorted LSK cells with DMSO or C+R for 7 d and then transplanted them into lethally irradiated recipients. As with the cultured c-Kit+ cells, we found substantial chimerism in recipients receiving C+R-treated cells but not in those receiving DMSO-treated control cells (Fig. 2b). As a further test of the long-term self-renewal capability of HSCs cultured in cytokine-free C+R medium, we performed serial transplantation to secondary and tertiary lethally irradiated hosts. Bone marrow from primary recipients that had been transplanted with C+R-treated donor cells achieved high chimerism in secondary (mean, 40.5%) and tertiary (mean, 30.3%) recipients, whereas serial transplants from primary recipients of DMSO-treated donor cells had low chimerism in secondary and tertiary recipients (mean, 3.5% and 0.6%, respectively; P < 0.01; Fig. 2c,d). These results indicate that HSCs cultured with C+R 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 C+R or vehicle control. We also used freshly isolated c-Kit+ cells (not cultured) as a positive control. We mixed donor cells at several dilutions (from 1 × 10^5 to 1 × 10^6 cells) with a constant number (2 × 10^5) of recipient cells and transplanted them into lethally irradiated recipient mice. After 4 months, we harvested bone marrow, measured the donor chimerism and calculated the frequency of HSCs (Fig. 2e). The frequency of HSCs in C+R-treated cultures (1/5,700; 95% CI 1/3,140–1/10,326) was similar to that in uncultured c-Kit+ control cells (1/3,540; 95% CI 1/1,991–1/6,300; P = 0.1433), indicating that combined inhibition of GSK-3 and mTOR indeed preserves HSCs in cytokine-free culture.

Inhibition of GSK-3 and mTOR maintains human HSCs ex vivo

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 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 and reduce LT-HSC maintenance. To address whether human HSCs can be maintained in culture without cytokines, we cultured human UCB CD34+ cells in the presence of C+R without serum or cytokines. C+R treatment increased the total cell number up to sevenfold by day 3 (Supplementary Fig. 4).

To determine the functional HSC frequency of C+R-cultured human cells, we performed xenografts into nonobese diabetic severe combined immunodeficient IL-2Rγnull (NSG) mice. At 12 weeks, all mice receiving cells treated with standard cytokines (SCF, Flt3 ligand and Tpo) alone or with C+R alone were engrafted, in contrast to mice receiving cells treated with GSK-3 inhibitor alone or vehicle control, which were not successfully engrafted (Fig. 3a,b). C+R-cultured human CD34+ cells gave rise to all major lineages (Fig. 3c).

We also performed secondary transplantation to assess LT-HSC function. C+R-cultured HSCs achieved efficient long-term engraftment...
Figure 3  Maintenance of human long-term HSCs in cytokine-free culture. (a) Human UCB CD34+ cells were cultured in cytokine-free medium with the indicated inhibitors or with conventional cytokines for 7 d. Alternatively, UCB CD34+ cells were used without culturing. Competitive serial transplants were performed using sublethally irradiated recipient NSG mice. Percentage engraftment is shown in primary recipients, assessed as the percentage of human CD45+ (huCD45+) cells in the bone marrow 4 months after transplantation. (b) Representative FCM data (CD45+) for bone marrow from primary recipients of human CD34+ cells that were directly transplanted (uncultured) or cultured with vehicle (DMSO), C+R or conventional cytokines. (c) Bone marrow was harvested 4 months after transplantation and analyzed by FCM to detect progenitors (CD34+ CD19+), T and B lymphocytes (CD3 and CD19), myeloid cells (CD11c and CD33) and immunophenotypic HSCs (huCD34+CD38−CD45RA−Thy1+CD49f+) derived from human CD34+ cells that were directly transplanted (uncultured) or cultured in C+R for 7 d before transplant. In b and c, the numbers next to the small rectangles indicate the percentage of cells in the indicated region relative to total cells in the histogram. (d) Four months after primary transplantation, bone marrow was transferred into secondary NSG recipients and chimerism was assessed after 4 months, as in a.

Table 1 Effect of CR on UCB-derived CD34+ cells after 7 d in culture

| Conditions          | Number of cells transplanted (or equivalent starting dose for cultured cells) | Number of reconstituted mice/number of primary recipients | SRC frequency (95% CI) |
|---------------------|--------------------------------------------------------------------------------|----------------------------------------------------------|------------------------|
| Uncultured         | 1,000                                                                           | 1/4                                                      | 1 in 21,901 (8,207–58,447) |
|                     | 10,000                                                                          | 2/4                                                      |                         |
|                     | 50,000                                                                          | 3/4                                                      |                         |
| C+R                 | 1,000                                                                           | 2/5                                                      | 1 in 28,187 (11,593–68,532) |
|                     | 10,000                                                                          | 2/5                                                      |                         |
|                     | 50,000                                                                          | 3/5                                                      |                         |
| DMSO                | 150,000                                                                         | 0/3                                                      |                         |

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

**Figure 3**

**Wnt signaling is required for ex vivo HSC maintenance**

Deficiency or inhibition of Gsk3 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 previously been tested. We first confirmed that GSK-3 inhibition activates canonical Wnt signaling in c-Kit+ cells using BAT-gal mice, which carry a lacZ reporter transgene for monitoring canonical Wnt signaling. We isolated c-Kit+ cells from the bone marrow of these mice and cultured them in vehicle, CHIR99021 or C+R for 3 d and then assayed the treated cells for β-galactosidase activity by FCM. Either C+R or CHIR99021 alone activated the reporter in LSK Flk2+ immunophenotypic HSCs and increased expression of the endogenous Wnt target gene Axin2 approximately fourfold in c-Kit+ cells (Supplementary Fig. 5). These results are similar to previous reports using Wnt or GSK-3 inhibitors.

To test whether β-catenin (encoded by Ctnnb1) is required for HSC maintenance in our ex vivo conditions, we crossed mice carrying a conditional Ctnnb1 loss-of-function allele (Ctnnb1flox/flox) to mice expressing a tamoxifen-inducible Cre allele (cre-ERT2). We treated LSK cells from Ctnnb1flox/flox, cre-ERT2+ or Ctnnb1flox/flox (control) mice with 4-hydroxytamoxifen for 2 d to induce deletion of Ctnnb1 (Supplementary Fig. 5c) and cultured them with C+R or DMSO control for 7 d. We plated the cultured cells on OP9 cells and performed FCM after 3 weeks. C+R-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, we used mice carrying the Mx-cre allele to delete Ctnnb1 in hematopoietic cells. We induced Cre in Ctnnb1flox/flox, Mx-cre mice with polyinosine-polycytidine (pIpC) (Supplementary Fig. 5d), harvested c-Kit+ cells from bone marrow, and treated them with C+R or vehicle.
After 7 d in culture, we mixed the cells with 3 × 10^5 competitor cells and transplanted them into lethally irradiated recipients. After 4 months, we harvested marrow from these primary recipients and analyzed it by FCM. C+R maintained HSCs in the c-Kit+ cell cultures, and loss of Ctnnb1 blocked this effect (Fig. 4b). These results show that Ctnnb1 is required for the ability of C+R treatment to maintain HSCs, and, taken together with previously published observations\(^5\),\(^3\),\(^2\), support a positive role for the Wnt–β-catenin pathway in HSC maintenance when GSK-3 is inhibited.

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

To explore the mechanism by which inhibition of GSK-3 and mTOR preserves HSCs and HPCs, we examined the cell cycle and survival status of cultured c-Kit+ cells. We cultured mouse c-Kit+ cells with C+R for 4 d, stained them with antibody to Ki-67 and DAPI and then performed FCM to assess cell cycle status in the LSK cell population. Compared to DMSO-treated control cells, more that twice as many C+R-treated LSK cells were in the G0 phase, indicating an increased percentage of quiescent cells (Fig. 4c). C+R treatment also led to a small increase in the percentage of cells in the S/G2/M phases, indicating a modestly accelerated cell-cycle progression.

We also analyzed cell death by assessing 7-aminoactinomycin D (7AAD) and annexin V staining by FCM. DMSO- and C+R-treated cells showed similar staining for the most part, except for an increase in early apoptotic cells (annexin V positive and 7AAD negative) in the C+R-treated group (Fig. 4d). These results suggest that the maintenance of LSK cells by C+R treatment is not the result of reduced cell death.
GSK-3 and mTOR inhibition increases 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 using c-Kit+ cells cultured in cytokine-free medium with lithium and rapamycin (L+R). We cultured c-Kit+ cells in L+R or control medium for 7 d and then transplanted them into lethally irradiated recipients. Sixty percent of mice receiving L+R-cultured cells (n = 10 mice) survived 4 months or longer, whereas 100% of control mice (receiving cells cultured in DMSO control medium, lithium alone or rapamycin alone) died within 14 d of transplant (Fig. 5a). All surviving recipients of L+R-treated cells showed long-term multilineage reconstitution, with >90% of donor-derived cells contributing to multiple lineages in the bone marrow (Fig. 5b) and peripheral blood (data not shown).

In the next experiment, we treated mice with L+R for 2 weeks and compared them to mice receiving vehicle, lithium chloride alone or rapamycin alone. The overall bone marrow cellularity was higher in both the lithium chloride (as observed previously44) and L+R treatment groups compared to vehicle control treatment or the rapamycin treatment group (Fig. 5c). The absolute number of immunophenotypic LT-HSCs (LSK CD34+ Flk2+ CD150− CD48−) also increased in the L+R treatment group compared to the control group (Fig. 5d). In a competitive repopulation assay, the absolute number of competitive rescue units was increased twofold in bone marrow from mice previously treated with L+R compared to mice receiving vehicle control (5% Tween-80 and 5% PEG-400) (P < 0.01; Fig. 5e). Furthermore, the amount of β-catenin protein was increased in whole bone marrow of lithium chloride– and L+R-treated mice, consistent with activated Wnt signaling under conditions of GSK-3 inhibition. The amounts of phosphorylated S6 and 4EBP, which are downstream of mTOR, were increased in lithium chloride–treated mice (Fig. 5f), and this increase was blocked by rapamycin. These observations show that lithium chloride treatment leads to mTOR activation, consistent with the known ability of GSK-3 to inhibit mTOR44,45. Taken together, our data indicate that the combination of two clinically approved drugs increases the number of LT-HSCs, as well as the overall number of cells in the bone marrow in vivo.

DISCUSSION

The data presented here show that the combination of activated Wnt and reduced mTOR signaling supports the maintenance of LT-HSCs defined, cytokine-free conditions. These findings also indicate that GSK-3 has an essential role in HSC homeostasis as a suppressor of self-renewal (Wnt) and nutrient-sensing (mTOR) pathways. Wnt–β-catenin signaling promotes stem-cell renewal28, whereas mTOR activation promotes lineage commitment and stem-cell depletion15–17. Inhibition of GSK-3 activates both the Wnt–β-catenin and mTOR pathways in HSCs; 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 increase the number (but not the proportion) of LT-HSCs in vivo using two clinically well-tolerated medications may have a major impact on stem-cell transplantation in hematopoietic malignancies and bone marrow failure.

Difficulty in culturing HSCs has been a major roadblock for both basic research involving HSCs and improving human HSC transplantation outcomes, as it is difficult to reconstitute the in vivo conditions that maintain or expand HSCs. The use of multiple hematopoietic cytokines, which seem to be essential for the survival of HPCs ex vivo, may also contribute to these difficulties, as these factors probably promote lineage specification at the cost of LT-HSC function4,18. Thus, an important goal is HSC expansion while limiting or eliminating exposure to lineage-promoting cytokines.

Wnt–β-catenin signaling has been implicated in stem-cell self renewal in multiple tissues58, but its role in HSC self renewal is not clear. The conflicting reports regarding the role of Wnt–β-catenin signaling in adult HSCs may be explained in part by differential responses to varying levels of Wnt signaling, as has been recently proposed53. In addition, Wnt signaling, which inhibits GSK-3, can activate both β-catenin and mTOR52, 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. Opposing effects of Wnt–β-catenin and mTOR signaling could also explain the limited response of HSCs to GSK-3 inhibitors when given alone52. The ability of C+R treatment to maintain HSCs clearly involves effects on both β-catenin and mTOR, as either β-catenin deficiency or mTOR activity blocks this response. However, additional GSK-3–regulated pathways could also have a role.

The use of GSK-3 inhibitors to improve hematopoietic function began with lithium before it was known to be a GSK-3 inhibitor52. Lithium treatment increases the amount of circulating CD34+ cells and increases the production of granulocytes and platelets in most patients53. However, clinical trials of 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 concept, treatment of bone marrow–transplanted mice with CHIR99021 accelerates the recovery of mature myeloid cells and expands HPCs without increasing the number of LT-HSCs32. Similarly, GSK-3 inhibitors enhance the progenitor population and hematopoietic reconstitution in mouse primary transplant recipients53. GSK-3 inhibitors enhance the progenitor population and hematopoietic reconstitution in mouse primary transplant recipients53, and preserve long-term–culture initiating progenitor cells ex vivo when human CD34+ cells are cocultured with stromal cells and cytokines54. Furthermore, inhibition of GSK-3 combined with insulin expands LT-HSCs in culture in the presence of hematopoietic cytokines55, suggesting that inhibition of GSK-3 combined with additional signals may allow the expansion of HSCs, although the targets downstream of insulin/PI-3 kinase signaling were not addressed.

Recent work has shown that HSCs and other somatic stem cells reside in low-perfusion niches7–10 and that activation of nutrient sensing pathways enhances lineage commitment at the expense of HSC maintenance. 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 manner11–14. However, mutations in genes that inhibit mTOR lead to its activation, mimicking the nutrient-replete state; these mutations also promote lineage commitment and HSC depletion15–17. 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 cells51, 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 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 pathways as crucial for the maintenance of LT-HSCs. We also show that a combination of clinically tolerated medications increases the number of LT-HSCs and 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; however, lithium has been used to treat bipolar disorder for over 50 years and is not associated with an increased risk of malignancies. Furthermore, the approaches described here involve transient ex vivo or in vivo exposure to GSK-3 inhibitors rather than the prolonged activation that is associated with colorectal cancers. Recent studies have also suggested that GSK-3 and mTOR regulate T cell memory and that inhibition of GSK-3, mTOR or both may enhance the number and functionality of CD8+ memory T cells. Thus, this approach, using agents with already well-defined safety profiles, holds promise for improved hematopoietic recovery and immune reconstitution in UCBT.

METHODS

Methods and any associated references are available in the online version of the paper. The authors declare no competing financial interests.

1. Weissman, I.L. Stem cells: units of development, regions of renewal, and units in evolution. Cell 100, 157–168 (2000).
2. Purton, L.E. & Scadden, D.T. Limiting factors in murine hematopoietic stem cell assays. Cell Stem Cell 1, 263–270 (2007).
3. Orkin, S.H. & Zon, L.I. Hematopoiesis: an evolving paradigm for stem cell biology. Cell 132, 631–644 (2008).
4. Chou, S., Chu, P., Hwang, W. & Lodish, H. Expansion of human cord blood hematopoietic stem cells for transplantation. Cell Stem Cell 7, 427–428 (2010).
5. Lamperti, S., Ferraro, F. & Scadden, D.T. The HSC niche concept has turned 31. Has our knowledge matured? Ann. NY Acad. Sci. 1192, 12–18 (2010).
6. Bowman, T.V. & Zon, L.I. Lessons from the niche for generation and expansion of hematopoietic stem cells. Drug Discov. Today Ther. Strateg. 6, 135–140 (2009).
7. Potten, C.S., Mauch, P., Verfaillie, C.M. & Dons, J.D. Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. Proc. Natl. Acad. Sci. USA 104, 5431–5436 (2007).
8. Simsek, T. et al. The distinct metabolic profile of hematopoietic stem cells reflects their distinct location in a hypoxic niche. Cell Stem Cell 7, 380–390 (2010).
9. Mihara,da, K. et al. Cripto regulates hematopoietic stem cells as a hypoxic-niche-related factor through cell surface receptor GRP78. Cell Stem Cell 9, 330–344 (2011).
10. Takubo, K. et al. Regulation of the HIF-1α level is essential for hematopoietic stem cell biology. Cell Stem Cell 7, 391–402 (2010).
11. Krock, B., skull, N. & Simon, M.C. The tumor suppressor LKB1 emerges as a critical factor in hematopoietic stem cell biology. Cell Metab. 13, 8–10 (2011).
12. Gan, B. et al. Lkb1 regulates quiescence and metabolic homeostasis of hematopoietic stem cells. Nature 468, 659–663 (2010).
13. Gurumurthy, S. et al. The Lkb1 metabolic sensor maintains hematopoietic stem cell survival. Nature 468, 3431–3423 (2010).
14. Nakada, D., Saunders, T.L. & Morrison, S.J. Lkb1 regulates cell cycle and energy metabolism in hematopoietic stem cells. Nat. Genet. 41, 528–541 (2009).
15. Himnanz, O.H. et al. Pten dependence distinguishes hematopoietic stem cells from leukaemia-initiating cells. Nature 441, 475–482 (2006).
16. Zhang, J. et al. Pten maintains hematopoietic stem cells and acts in lineage choice and leukaemia prevention. Nature 441, 518–522 (2006).
17. Chen, C. et al. Tsc-mtor maintains quiescence and function of hematopoietic stem cells by repressing mitochondrial biogenesis and reactive oxygen species. J. Exp. Med. 205, 2397–2408 (2008).
18. Hofmeyr, C.C., Zhang, J., Knight, K.L., Le, P. & Stiff, P.J. Ex vivo expansion of umbilical cord blood stem cells for transplantation: growing knowledge from the hematopoietic niche. Bone Marrow Transplant. 39, 11–23 (2007).
19. Zhang, C.C., Kaba, M., Iizuka, S., Huyhn, H. & Lodish, H.F. Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID-T cell chimaerism. Blood 111, 3431–3423 (2008).
20. Chong, C.C. et al. Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. Nat. Med. 12, 240–245 (2006).
21. Himburg, H.A. et al. Pleiotrophin regulates the expansion and regeneration of hematopoietic stem cells. Nat. Med. 16, 475–482 (2010).
22. Delaney, C. et al. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. Nat. Med. 16, 232–236 (2010).
23. Geisslinger, W. et al. Genetic interaction of PGE2 and Wnt signaling regulates developmental specification of stem cells and regeneration. Cell 136, 1136–1147 (2009).
24. Boitano, A.E. et al. Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. Science 329, 1334–1338 (2010).
25. Perry, J.M. et al. Cooperation between both Wnt/b-catenin and Pten/Pi3k/Akt signaling promotes primitive hematopoietic stem cell self-renewal and expansion. Genes Dev. 25, 1928–1942 (2011).
26. Zhang, C.C. & Lodish, H.F. Murine hematopoietic stem cells change their surface phenotype during ex vivo expansion. Blood 105, 4314–4320 (2005).
27. Staal, F.J., Burgering, B.M., van de Wetering, M. & Clevers, H.C. Tcf-1–mediated transcription in T lymphocytes: differential role for glycogen synthase kinase-3 in fibroblasts and T cells. Int. Immunol. 11, 317–323 (1999).
28. Reya, T. & Clevers, H. Wnt signalling in stem cells and cancer. Nature 434, 843–850 (2005).
29. Staal, F.J. & Sen, J.M. The canonical Wnt signaling pathway plays an important role in lymphopoiesis and hematopoiesis. Eur. J. Immunol. 38, 1788–1794 (2008).
30. Malhotra, S. & Kincade, P.W. Wnt-related molecules and signaling pathway equilibrium in hematopoiesis. Cell Stem Cell 4, 27–36 (2009).
31. Satoh, N., Meijer, L., Skalskova, L., Greigengard, P. & Bryan, J. A.H. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK3-3-specific inhibitor. Nat. Med. 10, 55–63 (2004).
32. Tombrigue, J.J., Xerostomas, A., Moon, R.T. & Bhattacharya, M. Glycogen synthase kinase-3 is an in vivo regulator of hematopoietic stem cell repopulation. Nat. Med. 12, 89–98 (2006).
33. Lui, T.C. et al. Canonical Wnt signaling regulates hematopoiesis with a dosage-dependent fashion. Cell Stem Cell 9, 345–356 (2011).
34. Lui, T.C. et al. Wnt3a deficiency irreversibly impairs hematopoietic stem cell self-renewal and leads to defects in progenitor cell differentiation. Blood 113, 546–554 (2009).
35. Zhao, C. et al. Loss of β-catenin impairs the renewal of normal and CML stem cells in vivo. Cancer Cell 12, 528–541 (2007).
36. Fleming, H.E. et al. Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo. Cell Stem Cell 2, 272–283 (2008).
37. Willert, K. et al. Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature 423, 448–452 (2003).
38. Reya, T. et al. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature 423, 409–414 (2003).
39. Cobas, M. β-catenin is dispensable for hematopoiesis and lymphopoiesis. J. Exp. Med. 199, 221–229 (2004).
40. Jeannet, G. et al. Long-term, multilineage hematopoiesis occurs in the combined absence of β-catenin and γ-catenin. Blood 111, 160–164 (2008).
41. Koch, U. et al. Simultaneous loss of β- and γ-catenin does not perturb hematopoiesis or lymphopoiesis. Blood 111, 160–164 (2008).
42. Kirstetter, P., Anderson, K., Porse, B.T., Jacobsen, S.E. & Nerlov, C. Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. Nat. Immunol. 7, 1048–1056 (2006).
43. Scheller, M. et al. Hematopoietic stem cell and multilineage defects generated by constitutive β-catenin activation. Nat. Immunol. 7, 1037–1047 (2006).
44. Huang, J. et al. Pivotal role for Gsk3 in hematopoietic stem cell homeostasis in mice. J. Clin. Invest. 119, 3519–3529 (2009).
45. Inoki, K. et al. TSC2 integrates Wnt/β-catenin signaling during mouse development and in colorectal tumors. Proc. Natl. Acad. Sci. USA 100, 3299–3304 (2003).
46. Holmes, R. & Zúñiga-Pflücker, J.C. The OP9–DL1 system: generation of T-lymphocytes from embryonic or hematopoietic stem cells in vitro. Cold Spring Harb. Protoc. published online, doi:10.1101/pdb.prot5156 (February 2009).
47. Kodama, H., Nose, M., Niida, S., Nishikawa, S. & Nishikawa, S. Involvement of the c-Kit receptor in the adhesion of hematopoietic stem cells to stromal cells. Exp. Hematol. 22, 979–984 (1994).
48. Zediak, V.P., Maillard, I. & Bhandoola, A. Multiple prethymic defects underlie age-related loss of T progenitor competence. Blood 110, 1161–1167 (2007).
49. Richie Ehrlich, L.I., Serwold, T. & Weissman, I.L. In vitro assays misrepresent in vivo lineage potentials of murine lymphoid progenitors. Blood 117, 2618–2624 (2011).
50. Maretto, S. et al. Mapping Wnt/β-catenin signaling during mouse development and in colorectal tumors. Proc. Natl. Acad. Sci. USA 100, 3299–3304 (2003).
51. Castilho, R.M., Squarize, C.H., Chodosh, L.A., Williams, B.O. & Gutkind, J.S. mTOR mediates Wnt-induced epidermal stem cell exhaustion and aging. Cell Stem Cell 5, 279–289 (2009).
52. Boggs, D.R. & Joyce, R.A. The hematopoietic effects of lithium. Semin. Hematol. 20. 129–138 (1983).
53. Balin, A., Lehman, D., Sirola, P., Litvinjuk, U. & Meytes, D. Increased number of peripheral blood CD34+ cells in lithium-treated patients. Br. J. Haematol. 100, 219–221 (1998).
54. Holmes, T. et al. Glycogen synthase kinase-3β inhibition preserves hematopoietic stem cell activity and inhibits leukemic cell growth. Stem Cells 26, 1288–1297 (2008).
55. Polakis, P. Wnt signaling and cancer. Genes Dev. 14, 1837–1851 (2000).
56. Wang, Y. et al. The Wntβ-catenin pathway is required for the development of leukemia stem cells in AML. Science 327, 1650–1653 (2010).
57. Cohen, Y., Chetrit, A., Sirola, P. & Mosdan, B. Cancer morbidity in psychiatric patients: influence of lithium carbonate treatment. Med. Oncol. 15, 32–36 (1998).
58. Gattinoni, L. et al. A human memory T cell subset with stem cell-like properties. Nat. Med. 17, 1290–1297 (2011).
59. Gattinoni, L., Klebanoff, C.A. & Restifo, N.P. Pharmacologic induction of CD8+ T cell memory: better living through chemistry. Sci. Transl. Med. 1, 11ps12 (2009).
Cytokine-free HSC culture. HSC culture medium used for serum-free culture of c-Kit+ or LSK cells isolated from mouse bone marrow or CD34+ cells from human UCB consists of X-VIVO 15 (BioWhittaker) supplemented with 1% penicillin and streptomycin (Sigma). CHIR99021 (3 μM) (Stemgent) and rapamycin (5 nM) (Calbiochem) were added and were diluted 1:275. Antibody to CD34 was diluted 1:35. Nonviable cells were excluded using the viability dye DAPI (1 g ml−1). Cells were sorted with a FACSAria using the single-cell suspension by gently drawing through a 22-gauge needle three to five times. Red blood cells were lysed in ammonium chloride–potassium (ACK) buffer. Mouse c-Kit+ cells were purified with the Miltenyi MACS cell separation kit. After purification, 50,000 c-Kit+ cells were plated in 1 ml of culture medium per well in a 24-well U-bottom plate (Becton Dickinson Company). For experiments using sorted LSK cells, sorted cells were distributed 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.

FCM and isolation of HSCs. Bone marrow cells were flushed from the long bones (tibias and femurs) of mice with HBSS without calcium or magnesium. For detection of LSK cells, whole bone marrow cells were incubated with biotin-conjugated monoclonal antibodies to lineage markers, including B220 (6B2), CD4 (GK1.5), CD8 (53-6.7), Gr1 (8C5), Mac1 (M1/70), Ter119 (Ter119) and interleukin-7 receptor (IL-7R) (A7R34), PerCP Cy5.5-conjugated antibody to c-Kit (ACK2). Flk2, CD34, CD150 and CD48 were measured with the following antibodies: phycoerythrin (PE)-conjugated antibody to Flk2 (Ly-72/clone #A2F10), Alexa Fluor 647–conjugated antibody to CD34 (RAM34), PE Cy5-conjugated antibody to CD48 (HM48-1) and PE Cy7-conjugated antibody to CD150 (TC15-12F 12.2). All antibodies were purchased from eBioscience except for antibodies to CD150 and CD48, which were purchased from Biogeneg. Biotin-conjugated lineage markers were detected using streptavidin–conjugated PE–Texas Red. Antibodies to B220, CD4, CD8, Gr1, Mac1 and interleukin-7 receptor (IL-7R) were diluted 1:550. Antibodies to Sc1, c-Kit, CD150, CD48 and Flk2 were diluted 1:80. Antibodies to Ter119 and Gr1 were diluted 1:275. Antibody to CD34 was diluted 1:35. Nonviable cells were excluded using the viability dye DAPI (1 g ml−1). Cells were sorted with a FACSArta (Becton Dickinson) automated cell sorter. Analyses were performed on LSR II, FACSCanTo or FACSCalibur flow cytometers (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star).

Wnt reporter assay by flow cytometry. Mouse c-Kit+ cells were harvested from the BAT-gal Wnt reporter mouse line (provided by S. Piccolo, University of Padova). The FluoroReporter lacZ Flow Cytometry Kit (Invitrogen) was used for quantifying β-galactosidase activity by assessing fluorescein di-V-galactoside staining in single cells using flow cytometry.

OP9 cell cultures. OP9-MigR1 (OP9) and OP9-DL1 cells (provided by A. Bhandoola, University of Pennsylvania School of Medicine) were used as described40. Mouse c-Kit+ or LSK progenitors were seeded into 12-well plates containing a confluent stromal monolayer of OP9 or OP9-DL1 cells, which had been γ-irradiated (137Cs) at a dose of 30 Gy. The cytokines mouse Flt3 ligand (5 ng ml−1) and mouse IL-7 (5–10 ng ml−1) (Peprotech) were added to the coculture. The cells were passaged every 7 d to freshly irradiated OP9 or OP9-DL1 stromal cells.

Long-term repopulation assays. For long-term noncompetitive repopulation, adult recipient mice were irradiated with a cesium-137 irradiator in two equal doses of 500 rads separated by at least 2 h. 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), Mac1 (M1/70), CD4 (L3T4), CD8 (Ly-3) and Gr1 (8C5) to monitor engraftment by FCM. For long-term competitive repopulation, CD45.1-c-Kit+ cells from C57 or DMSO-treated cultures were transplanted into lethally irradiated C57BL/6 recipients together with 3 × 105 competitor B6 bone marrow cells (CD45.2+). In limiting dilution analyses, uncultured c-Kit+ cells or cells cultured in C+R or DMSO control medium for 1 week were injected together with 3 × 105 competitor cells into lethally irradiated primary recipients. Beginning 4 weeks after transplantation and continuing for up to 16 weeks, peripheral blood was collected and analyzed by FCM as described above to monitor engraftment. Bone marrow was harvested and analyzed 16 weeks after transplantation.

In serial transplantation assays, the bone marrow was harvested from primary recipients after 4 months, and 20 × 106 bone marrow cells were transplanted into each lethally irradiated secondary recipient.

Xenotransplant assays. Gender balanced severe combined immunodeficient IL-2Rγnull (NSG) mice (provided by the Stem Cell and Xenograft Core Facility at the University of Pennsylvania School of Medicine) were used in this study. Adult recipients were irradiated with a cesium-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, bone marrow was harvested from primary recipients after 4 months, and 90% of the total bone marrow cells (from four tibias, four femurs and two hips) was transplanted into each sublethally irradiated secondary recipient mouse (NSG).

Administration of plpC, rapamycin and lithium chloride. plpC (Sigma) was suspended in Dulbecco’s PBS at 2 mg ml−1. Mice received 25 mg of plpC per kg body weight 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 per kg body weight rapamycin in 200 μl total volume per injection every other day for 2–3 weeks. For lithium treatment, mice were fed 0.2% (w/w) lithium chloride (LiCl) mouse chow (Harlan Teklad) for 3 d followed by 0.4% (w/w) LiCl mouse chow for the duration of the experiment. All mice had access to supplemental 450 mM sodium chloride drinking solution.

Statistical methods. Pairwise comparisons were analyzed using a two-tailed Student’s t-test, and results were considered significant when P < 0.05. Data involving multiple samples (as in Fig. 2a) were analyzed with one-way analysis of variance followed by Dunn’s post hoc analysis when a significant difference was found among the groups.