Supplemental Results

Lithium increases hematopoietic stem/progenitor cells

Lithium increases circulating CD34+ stem cells (1) in humans, increases neutrophil count in a high percentage of treated patients, and may also stimulate other lineages (2-9). In rodents, lithium increases peripheral blood counts and enhances stem and progenitor cell numbers in ex vivo and in vivo assays. These studies led to the hypothesis that the effects of lithium are mediated at the level of the HSC and/or HPC (6, 10, 11). As sophisticated immunophenotypic markers to detect diverse hematopoietic cell types have become available since those early studies were performed, we have re-examined the effect of lithium on hematopoiesis in C57/B6 mice using FCM.

Mice received dietary LiCl (or NaCl as control) at a dose that achieves a serum lithium concentration of 1.0 mEq/L (12), similar to therapeutic concentrations in bipolar disorder patients (there was no change in the overall well being of the animals after two-three weeks on lithium). After 2 weeks, bone marrow was isolated and cells were analyzed by FCM. Lithium caused a significant increase in the number of LSK cells compared with NaCl treated animals (Supplemental Figure 1A), consistent with an increase in HSCs and HPCs, and a doubling of the overall marrow cellularity (Supplemental Figure 1B). To confirm this, we also examined expression of the SLAM family receptors CD150, CD48, and CD244 (13) and observed an approximately 2.3-fold increase in the number of CD150+CD48−CD244− cells, an immunophenotypic population highly enriched for HSCs (data not shown). Histological analysis of bone marrow morphology showed no significant differences in maturity or cellular morphology in lithium-treated versus control marrows, as reported previously (not shown). Consistent with published observations in humans and rodents, the percentage of neutrophils in peripheral blood also increased 30-40% in lithium-treated mice (data not shown). Inclusion of CD34 and
Flk2 in FCM analysis shows that lithium primarily increases the CD34⁺ Flk2⁻ population, consistent with an increase in ST-HSCs (Supplemental Figure 1A).

**GSK-3 as the target of lithium in HSC/HPCs**

Inhibition of GSK-3 provides a compelling explanation for many of the known effects of lithium (14), but lithium also inhibits inositol monophosphatase and structurally related phosphomonoesterases, some of which are highly sensitive to lithium (15). To test further whether inhibition of GSK-3 explains the hematopoietic effects of lithium, we used alternative, selective GSK-3 inhibitors that are unlikely to have off-target effects that overlap with lithium (16-18). The selective GSK-3 inhibitor, 6-bromo-indirubin 3’-oxime (6BIO) has an IC₅₀ for GSK-3 in the nanomolar range (17). 6BIO caused a pronounced increase in the number of LSK cells after a 2-week treatment (Supplemental Figure 1C). 6BIO also increased marrow cellularity, similar to lithium. These observations are consistent with Trowbridge et al, who observed an approximately 50% increase in LSK cells in mice treated with the GSK-3 inhibitor CHIR-911 (19). Goessling et al also reported that 6BIO increases HSCs in mice as measured by long-term competitive repopulation assay (20), consistent with our observation of increased LSK cells after 6BIO treatment. Taken together, these pharmacological data support the hypothesis that lithium increases HSCs through inhibition of GSK-3.

To test whether progenitor cells are increased by lithium treatment, we performed colony formation assays. Previous work in the 1980’s with lithium-treated rodents demonstrated an increase in colony forming units (CFU) in ex vivo assays and by CFU-S formation in short-term transplants (6, 21). To confirm these studies, bone marrow cells were isolated from lithium-treated mice and cultured in methylcellulose with hematopoietic cytokines. Marrow from lithium-treated animals showed a two-fold increase in total colony initiating cells compared to control, similar to earlier reports (data not shown) and consistent with more recent observations with
small molecule GSK-3 inhibitors, which increase CFUs ex vivo and CFU-S approximately 1.5 to 2-fold (19, 20).

To test in a side-by-side comparison whether structurally diverse GSK-3 inhibitors expand HPCs similar to lithium, c-Kit^+ cells were purified from control mice and treated for three days with GSK-3 inhibitors including lithium, 6BIO (17, 20), AR-A014418 (22), and the organometallic GSK-3 inhibitor DW21 (18). (The number of cells after three days was similar in each group (Supplemental Figure 1E). Treated cells were washed and an equal number from each sample was then added to methylcellulose with hematopoietic cytokines and cultured for 10-14 days. Each of the GSK-3 inhibitors induced a marked increase in hematopoietic colony number (Supplemental Figure 1D), strongly supporting the hypothesis that lithium expands the HPC population by inhibiting GSK-3 within hematopoietic cells.

**Supplemental References**


Supplemental Figure Legends

Supplemental figure 1. Lithium and other GSK-3 inhibitors expand HSC/HPCs: (A) Absolute number of Lineage-Sca-1^+cKit^+(LSK) fraction, which is enriched for HSCs, and immunophenotypic LT-HSC (LSK; CD34^-Flk2^-) and ST-HSC (LSK CD34^+Flk-2^-) fractions in bone marrow harvested from mice treated with control or lithium diet for 2 weeks. (B) Cellularity of bone marrow, thymus and spleen in control and lithium treated mice, shown as the number of nucleated cells/mouse recovered from both femurs and tibias, thymus, and spleen. (C) Percentage (left) and absolute number (right) of HSC/HPCs (as LSK) in mice treated with 6BIO vs control for two weeks. (D) Colony formation assay: Purified total c-Kit^+ cells were treated with lithium, 6BIO, AR-A014418, or Ru (1-OH) (also known as DW12) for 3 days and plated in methylcellulose with hematopoietic cytokines for 12 days. Colonies of >30 viable cells were counted and the mean colony number/50,000 plated cells for each of three separate experiments is shown. (E) The total numbers of c-Kit^+ cells in each drug treatment after 3 days primary culture were shown.

Supplemental figure 2. Annexin V staining and cellularity of bone marrow harvested from transplant recipients: (A) Flow cytometric detection of Annexin V using bone marrow cells harvested from 1° recipients after 4 month bone marrow transplantation from both control and Gsk3-mai. Annexin V was measured in the GFP^-LSK gated population. (B) Total number of bone marrow cells and number of GFP^- cells recovered in bone marrow from 1° recipients after 4 month transplant. (C) Total number and number of GFP^- cells recovered in bone marrow from 2° recipients after 4 months. (D) Total number and number of GFP^- cells recovered in bone marrow from 3° recipients after 4 months.
Supplemental Figure 3. Increased colony formation induced by Gsk3-rnai requires β-catenin: Colony formation assay was performed (as in figure 1) using sorted GFP+ cells harvested from 1º recipients (A) or 2º recipients (B) of wild-type (left) and β-catenin KO (right) marrow transduced with control (open boxes) or Gsk3-rnai (filled boxes) lentivirus. Data represent mean number of colonies per well for five mice per construct repeated in three separate experiments.

Supplemental Figure 4. Effect of β-catenin conditional knockout on colony formation in Gsk3-depleted bone marrow: Colony formation assay was performed (as in figure 1) using sorted GFP+ cells from each group in 1º (A) and 2º (B) recipients. Data represent mean number of colonies per well for five mice per construct repeated in three separate experiments.
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