Thioredoxin-Interacting Protein Regulates Hematopoietic Stem Cell Quiescence and Mobilization under Stress Conditions

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J Immunol 2009; 183:2495-2505; Prepublished online 22 July 2009;
doi: 10.4049/jimmunol.0804221
http://www.jimmunol.org/content/183/4/2495

Supplementary Material

http://www.jimmunol.org/content/suppl/2009/07/23/jimmunol.0804221.DC1

References

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Hematopoietic stem cells (HSCs) are distinct cells that are predominantly located in the bone marrow (BM) microenvironment, which is referred to as the niche (1). These cells can self-renew, differentiate into various types of cells of the blood lineage, mobilize from the BM into secondary hematopoietic organs through the circulation, or undergo apoptosis. In the niche, the most primitive HSCs are in a quiescent state to continue to produce all types of blood cells throughout a prolonged life span without depleting the regenerative cell pool. Disruption of HSC quiescence prematurely exhausts the stem cell pool and causes hematological failure under various stresses, such as oxidative stress, cell cycling, and aging (2, 3). In response to stress or stimulation, the HSCs can move from the osteoblastic niche to the vascular niche to undergo cell division and migrate into secondary organs, such as the spleen and liver. Following this, the mobilized HSCs may return to the BM niche and regain their quiescent state (4).

HSC quiescence, activation, and mobilization are coordinately regulated in their niche by intrinsic and extrinsic mechanisms (5). Several genes, including those that encode transcriptional repressors, cell cycle regulatory proteins, tumor suppressors, and proto-oncogenes, intrinsically regulate the balance between HSC quiescence and activation. A deficiency in specific tumor suppressors or an increased activity of specific proto-oncogenes can activate HSCs and increase cell cycle entry (6, 7). A number of intrinsic factors, including c-Myc, p21, Mef/Eif4, Foxo3a, Gfi1, Pbx1, and Fbw7, have been implicated in the regulation of HSC self-renewal (6–12). In addition, there are extrinsic-specialized microenvironment factors, such as supportive cells that express adhesion molecules and secrete soluble factors (e.g., N-cadherin, Tie2/Ang-1, osteopontin (Opn)/integrins, Notch/Jagged1, and Dkk-1/Lrp5), which regulate HSC maintenance and migration (12–16). Whereas numerous molecular factors that contribute to quiescence exist in the HSCs and the BM niche, the mechanism that coordinates the cell cycle regulation of HSC and the interactions between HSCs and the niche remains unclear.

Thioredoxin-interacting protein (TXNIP) is a 397-aa residue, 50-kDa protein that belongs to the arrestin family. TXNIP was originally identified as a gene that was induced following the treatment of leukemia with 1,25-dihydroxyvitamin D3 (17). TXNIP acts as a mediator of oxidative stress, either by inhibiting thioredoxin activity or by limiting its bioavailability. TXNIP is also a transcriptional repressor that may act as a bridge-molecule between transcription factors and corepressor complexes; its overexpression induces G1/G0 cell cycle arrest. TXNIP interacts with JAB1 and restores JAB1-induced suppression of p27 stability, blocking the JAB1-mediated translocation of p27 from the nucleus to the cytoplasm (18). TXNIP functions as a tumor and metastasis suppressor. TXNIP expression has been shown to be reduced in many types of tumors, and TXNIP overexpression inhibits tumor growth by blocking cell cycle progression (19). TXNIP interacts with the von Hippel-Lindau protein (pVHL)/HIF1α complex to accelerate the nuclear export of pVHL/HIF1α via the chromosome region maintenance 1-dependent pathway, and then increases HIF1α degradation in the cytoplasm. Blocking the translocation of TXNIP, either by mutations in the nuclear export signal or by leptomycin B, inhibited the nuclear export of pVHL/HIF1α and relieved the destabilization of HIF1α. In addition, TXNIP suppressed cell invasiveness and tumor metastasis, which were also.
recovered by blocking the nuclear export of TXNIP (20). TXNIP is a critical regulator of a broad range of cellular functions, including maturation of NK cells, immune regulation, glucose and lipid metabolism, and regulation of the life span, aging, renal function, homeostasis, and hematopoiesis (21, 22). In this study, we examined the role of TXNIP in the regulation of HSC function. We hypothesized that TXNIP plays a pivotal role in controlling HSC self-renewal and the interaction between HSCs and the BM niche.

Materials and Methods

Mice and 5-fluorouracil (5-FU) administration

Txnip−/− mice were generated, as previously described (21). CD45.1+ C57BL/6 mice were purchased from The Jackson Laboratory. The i.v. administration of 5-FU (Sigma-Aldrich) was performed at a dose of 150 mg/kg. On days 2, 4, or 6 post-5-FU treatment, BM, spleen, and peripheral blood (PB) cells were analyzed by flow cytometry. Animal care and use were in accordance with institutional guidelines.

BM cell and osteoblast (OB) isolation

Total BM cells were isolated from mouse femurs, tibias, hiphones, and shoulder bones by grinding tissues in RPMI 1640 medium plus 2% FBS. RBCs were lysed using ACK buffer (0.15 M NH4Cl, 1.0 mM KHCO3, 0.1 mM EDTA (pH 7.4)), and the cells were filtered through a strainer. OBs were isolated from marrow-depleted bones by grinding and enzymatic digestion in 0.1% collagenase plus 0.05% dispase for 1 h. The resulting cell suspension was centrifuged for 5 min at 400 × g to pellet the OBs. CD45-FITC, Ter119-Cy7 (BD Biosciences), and rat anti-OPN (Millipore) were used for OB isolation using FACSaria.

Flow cytometry and sorting

BM-HSCs were isolated and analyzed based on the expression pattern of surface markers by flow cytometry. Lineage staining was performed with a mixture of biotinylated anti-mouse Abs to Mac-1 (CD11b), Gr-1 (Ly-6G and 6C), Ter119 (Ly-76), NK1.1, CD2, and B220 (CD45R) (BD Biosciences). For detection, we used streptavidin-conjugated allopheyocyanin/ Cy7 (BD Biosciences) and multiple directly conjugated Abs were used, as follows: PE-Cy7-conjugated anti-c-Kit (CD117), PerCP-Cy5.5-conjugated anti-Sca1, FITC-conjugated anti-CD34, allopheyocyanin-conjugated anti-CD16/32, PE-Cy5-conjugated anti-IL-7Ra, and PE-conjugated anti-Fik2 (CD135) (BD Biosciences). For congenic strain discrimination, a FITC-conjugated anti-CD45.1 Ab (BD Biosciences) was used in combination with PE-conjugated anti-CD11b, anti-Gr-1, anti-B220, anti-NK1.1, and anti-CD3. Following treatment with 5-FU, staining for the primitive BM populations was done with PE-conjugated anti-CD150 and FITC-conjugated anti-CD48 and lineage Abs and then stained with PE-conjugated anti-c-Kit, PerCP-Cy5.5-conjugated anti-Sca1, FITC-conjugated anti-CD34, allopheyocyanin-conjugated anti-CD16/32, PE-Cy5-conjugated anti-IL-7Ra, and PE-conjugated anti-Fik2 (CD135) (BD Biosciences). For lineage staining, a mixture of biotinylated anti-mouse Abs to Mac-1 (CD11b), Gr-1 (Ly-6G and 6C), Ter119 (Ly-76), PE-conjugated anti-CD45 (BD Biosciences), and rat anti-OPN (Millipore), or hamster anti-integrin β1 (BD Biosciences). For detection, PE-conjugated streptavidin, FITC-conjugated anti-rat, and PE-conjugated anti-hamster were used. The data were acquired using a FACSCalibur (BD Biosciences) and were analyzed with the CellQuestpro and WinMDI software. Fluorescence-activated cell sorting was performed using a FACSaria cell sorter (BD Biosciences).

In vitro long-term culture

Lineage− ckit+ Sca1− (LKS) cells (1 × 106) were isolated by FACS Aria (BD Biosciences) and cultured in myelocult medium (ST05350; StemCell Technologies) supplemented with 30 ng/ml Flt3 ligand, 30 ng/ml stem cell factor, 20 ng/ml IL-6, and 20 ng/ml thrombopoietin (PeproTech). One-half of the medium was removed every 3–4 days and was replaced with fresh medium.

 Colony-forming assays

The in vitro colony-forming cell activity was assessed using a methylcellulose colony assay (Methocult GF M3434; StemCell Technologies). BM or spleen cells were seeded into methylcellulose medium. The cultures were plated in triplicate and incubated for 7 days with 5% CO2 at 37°C. On day 7, colonies containing at least 50 cells were scored.

Reconstitution assay

Lethally irradiated (800 rad) C57BL/6-C57DL.1 congenic mice were reconstituted with 2 × 106 BM cells from Txnip−/− or wild-type (WT) mice. The reconstitution of donor-derived cells was monitored by staining BM cells with mAbs against CD45.1, CD45.2, c-Kit, Sca1, and lineage markers. Reconstitution was determined by flow cytometry of BM from the recipient mice.

In vivo mobilization assay

Recipient Txnip−/− and control WT mice received a daily s.c. injection of 200 μg/kg G-CSF (PeproTech) for 5 days, and PB was collected and analyzed by flow cytometry.

In vivo homing assay

Donor cell suspensions were prepared from CD45.1 congenic BM. Recipient Txnip−/− and control WT mice were injected with 5-FU 48 h before transplantation. The cells (2 × 106) were injected via the tail vain into 5-FU-treated recipients. After 24 h, recipient BM cells were collected and analyzed by flow cytometry.

ELISA

The BM supernatant was assayed for cytokines by grading cleaned bones with 700 μl of precooled PBS. The PBS was then centrifuged at 1500 rpm for 5 min. The supernatant was removed to a new tube and stored at −20°C until analysis. The stored supernatants were assayed for the wnt10b and CXCL12 level using an anti-wnt10b Ab (Santa Cruz Biotechnology) or a CXCL12 Quantimke Immunoassay (R&D Systems).

In vivo BrdU assay

The BrdU incorporation into LKS cells in vivo was analyzed using a FITC-BrdU Flow Kit (BD Biosciences), according to the manufacturer's instructions. Mice were treated with 100 μg/ml BrdU by i.p. injection. After 4 h, BM cells were stained with anti-BrdU, anti-CD34, anti-Sca1, anti-c-Kit, and lineage markers, as described above.

Cell cycle analysis

To analyze the cell cycle status, freshly isolated BM cells were stained with Hoechst 33342 and pyronin Y. Costaining with the RNA dye, pyronin Y, allows for the discrimination of the G0 and G1 cell populations. BM cells were first stained with FITC-conjugated anti-CD34, PE-Cy7-conjugated anti-c-Kit, PerCP-Cy5.5-conjugated anti-Sca1, FITC-conjugated anti-CD34, allopheyocyanin-conjugated anti-CD16/32, PE-Cy5-conjugated anti-IL-7Ra, and PE-conjugated anti-Fik2 (CD135) (BD Biosciences).

Quantitative real-time PCR

RNA was isolated from sorted long-term HSC (LTHSC), short-term HSC (ST-HSC), multipotent progenitor cells (MPP), and LKS subpopulations using TRIzol and RNeasy microRNA isolation kit (Qiagen). Quantitative PCR was performed using SYBR Premix Ex Taq (Takara Bio) and a Thermal Cycler Dice Real-Time System TP800 instrument (Takara Bio). PCR amplification began with a 10 s denaturation step at 95°C, 40 cycles of denaturation at 95°C for 5 s, and annealing and extension at 60°C for 20 s. The samples were analyzed in triplicate, and the expression of Dkk-1, Lrp5, p21, and Lef1 Tcf4 was normalized to the expression of GAPDH. The primer sequences are provided in Table S1.

Confocal imaging

Total BM cells were first stained with surface markers and then fixed and permeabilized with the Cytofix/Perm Solution (BD Biosciences). The cells were then incubated with an anti-OPN (Chemicon International) or anti-active β-catenin Ab (Millipore) for 1 h, washed with PBS for 30 min, and then incubated with a secondary PE-conjugated anti-mouse IgG Ab (BD Biosciences). The images were captured using a LSM510 confocal microscope (Carl Zeiss).

Luciferase reporter assay

The TOPflash (500 ng) and p21-Luc plasmids were transfected into 293T, HCT116, or SW620 cells using lipofectamine (Life Technologies). In addition, the cells were cotransfected with a control Renilla luciferase-expression vector (Promega) to monitor the transfection efficiency. The firefly and Renilla luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega). Briefly, the cells were washed with PBS and lysed with the passive lysis buffer. The cell lysates were mixed with the Luciferase Assay Reagent II, and the firefly luminescence was measured using a luminometer (Turner Designs). Next, the samples were measured using a luminometer (Turner Designs).
mixed with the stop reagent, and the Renilla luciferase activity was measured as an internal control. The relative luciferase activity was calculated as the ratio of firefly luciferase activity to Renilla luciferase activity.

Western blot

Cells were transfected using lipofectamine (Life Technologies), according to the manufacturer’s protocol. The transfected cells were lysed in lysis buffer (20 mM HEPES (pH 7.9), 100 mM KCl, 300 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.5 mM PMSF, and protease inhibitors mixture (Roche)) for 20 min on ice, and then centrifuged at 13,000 × g for 20 min. The supernatants were heat denatured in the presence of 2-ME and SDS, and separated electrophoretically in a 10% SDS-polyacrylamide gel under denaturing conditions. The proteins were then transferred electrophoretically onto a polyvinylidene difluoride membrane (Millipore), and the membrane was incubated sequentially with a primary Ab and a HRP-conjugated anti-mouse or anti-rabbit IgG secondary Ab (Amersham Biosciences). The protein bands were visualized using ECL. Western blot detection reagents (Amersham Biosciences) on x-ray film. The primary Abs were purchased from Sigma-Aldrich for anti-β-catenin Ab and from MBL International for the Txnip Ab. For the β-catenin ubiquitination assay, 293T cells were transfected with ubiquitin, β-catenin, and TXNIP expression plasmids, as indicated. After 24 h, the cells were incubated for 4 h with 10 μM MG-132. The cell lysates were analyzed by Western blot using an anti-β-catenin mAb (Sigma-Aldrich).

Results

Decreased TXNIP expression during HSC activation

To investigate the impact of TXNIP deficiency on HSC fate decisions, we examined TXNIP expression by quantitative real-time RT-PCR in populations of LT-HSC (Lin− c-Kit+ Sca1− CD34− Flk2−), ST-HSC (Lin− c-Kit+ Sca1− CD34+ Flk2−), and MPP (Lin− c-Kit+ Sca1+ CD34+ Flk2+) isolated from 2-mo-old mouse BM. Although Txnip was detected in all of the HSCs, TXNIP expression was the highest in the most quiescent LT-HSCs. TXNIP expression was substantially reduced as the HSCs differentiated into ST-HSC and MPP (Fig. 1A), a process that is accompanied by the loss of potential for self-renewal and an increased rate of proliferation (23, 24). These results imply that TXNIP has the potential to control HSC fate.

Reduced LT-HSC populations in aged Txnip−/− mice

To understand the role of TXNIP in HSC maintenance and regulation, we used Txnip−/− mice, which were generated by disrupting exons one to eight with lacZ and a neo cassette (21). Previously, we have shown that TXNIP deficiency disrupts the differentiation of NK cells, but not other hematopoietic lineages. In this study, we analyzed the immunophenotype and HSC pool size in 2- and 12-mo-old Txnip−/− mice by flow cytometry. HSCs and HPCs are comprised of a heterogeneous mixture of LT-HSCs, ST-HSCs, MPPs, common myeloid progenitors, granulocyte/monocyte progenitors, megakaryocyte/erythroid progenitors, and common myeloid progenitors. Multiple fluorescently labeled Abs were used to analyze all of the HSC and HPC populations (Fig. S1). We found that various lineages of the HSC populations were not significantly altered in young animals under steady-state conditions (Fig. 1B). These data indicate that the frequency of all hematopoietic cells is normal in the Txnip−/− mice. Next, we investigated the capacity for proliferation and differentiation of Txnip−/− BM cells using a colony-forming assay. Freshly isolated BM cells were cultured for 1 wk in methylcellulose medium. The number of colonies from Txnip−/− BM cells was comparable to that of wild-type
FIGURE 2. TXNIP deficiency results in defective HSC long-term repopulating ability. A, Lethally irradiated (800 rad) CD45.1 congenic mice were reconstituted with $2 \times 10^7$ BM cells from Txnip$^{-/-}$ or WT mice (both CD45.2). The BM of recipient mice was analyzed by CD45 staining to examine the contribution of donor-derived cells after 12 and 16 wk of transplantation. The data shown are a representative dot plot, and the bar graph shows the percentage of the donor-derived cells ± SD (n = 5). B, CD45.2-positive donor-derived cells were further analyzed for the expression of lineage markers, c-Kit, and Sca1 to examine the contribution of donor-derived LKS cells 16 wk after transplantation. The data shown are representative dot plots from the BM and the mean percentage ± SD of LKS cells (n = 5). C, CD45.2-positive, donor-derived LKS cells were analyzed for CD34 expression. The data shown are in a representative histogram and the mean percentage ± SD (n = 5). D, Decreased number of LT-HSCs after long-term culture. After 4 wk of culture, the total number of CD34$^+$ LKS cells was analyzed by flow cytometry. The data shown are representative dot plots and the mean percentage ± SD of cultured LKS cells (n = 3).

(WT) BM cells (Fig. S2A). Moreover, the colony morphologies demonstrated that Txnip$^{-/-}$ BM cells have the capacity to differentiate into the full range of myeloid and lymphoid lineages (Fig. S2B). Next, we compared the level of TXNIP expression in the HSCs of 2-mo-old (young) and 13-mo-old (aged) mice. We found that HSCs isolated from aged mice, which have a reduced capacity for self-renewal and an increased mobilization, had a lower level of TXNIP expression than the cells isolated from young HSCs (Fig. 1C) (25). This suggests that aging has an effect on the level of TXNIP in the HSC populations, and that a reduced expression of TXNIP in the HSCs from aged mice causes a defect in HSC self-renewal and mobilization. Therefore, we further analyzed the HSC pool in aged mice. BM cellularity of old TXNIP$^{-/-}$ mice slightly reduced, but not statistically significant (Fig. S3). As shown in Fig. 1D, there was a decrease in the number of LT-HSC (LKSCD34$^+$ Flk2$^+$) and LKSCD150$^+$CD48$^-$ cells, but the number of ST-HSCs was increased in the BM of Txnip$^{-/-}$ mice compared with WT mice. Taken together, these results demonstrate that Txnip$^{-/-}$ plays a pivotal role in preserving HSC maintenance during hematopoietic stress derived from aging.

Defective HSC self-renewal ability in Txnip$^{-/-}$ mice

To further assess TXNIP function in vivo, we next performed a reconstitution assay in which $2 \times 10^6$ BM cells from Txnip$^{-/-}$ or WT mice (CD45.2) were transplanted into lethally irradiated recipient mice (CD45.1). After transplantation, the recipient BM cells were then analyzed by flow cytometry (Fig. 2A). The Txnip$^{-/-}$ and WT BM cells were equally capable of hematopoietic reconstitution. However, we found a reduced number of LT-HSCs 16 wk following BM transplantation (Fig. 2, B and C). To determine whether TXNIP was required for the maintenance of functional LT-HSCs, we conducted a long-term in vitro culture of LKS derived from Txnip$^{-/-}$ BM was significantly decreased when compared with the number derived from WT BM (Fig. 2D). In addition, we checked the viability of LKS cells using annexin V and propidium iodide staining. There was no difference in the proportion of apoptotic cells between the Txnip$^{-/-}$ and WT LKS cells (Fig. S4A). Therefore, these results suggest that the self-renewal of the LT-HSCs and repopulating capacity in the BM are profoundly affected by TXNIP deficiency.

Reduced quiescence of Txnip$^{-/-}$ HSCs under stress

To determine whether TXNIP is required for the function of primitive HSCs under stress, we treated mice with 5-FU, a nucleotide analog that is used to deplete the pool of actively cycling cells. After 5-FU treatment, whole blood cells generated from spared, more quiescent HSC pools. The time to recovery correlated with
the relative preservation of the HSC pool (16). After 5-FU treatment, there was a significant time delay in the recovery of hematopoietic cells in the BM of 5-FU-treated \textit{Txnip}^{-/-} mice. The total BM cellularity was significantly decreased in the \textit{Txnip}^{-/-} mice (Fig. 3A), and these results were not because of cell death caused by the toxicity of 5-FU (Fig. S4B). The acquisition of CD34 expression on the cell surface of HSCs is one of the earliest events during the transition from the most primitive LT-HSC state to the activated ST-HSC state, and actively expanding HSCs, such as G-CSF-mobilized adult HSCs, are all CD34+ (26, 27). As shown in Fig. 3B, a significantly higher proportion of \textit{Txnip}^{-/-} LT-HSCs acquired CD34 expression and escaped quiescence compared with WT LT-HSCs.

The number of LT-HSCs dramatically decreased, and the number of ST-HSCs was increased (Fig. 3C; Fig. S5A, B). Similar results have been observed with other primitive HSC markers, such as the signaling lymphocyte activation molecule family markers, CD150 and CD48. After being exposed to 5-FU, \textit{Txnip}^{-/-} BM contained fewer primitive cells (lin^-CD150^+CD48^-; Fig. 3D), whereas the recovery of the other hematopoietic lineages was also delayed. The high forward light scatter, medium to high side light scatter, and Gr-1^- population (the granulocyte population) recovered slowly in \textit{Txnip}^{-/-} BM (Fig. 3, E and F). The frequency of NK cells and CD11b^-Gr-1^- cells also showed decreased frequencies, whereas the T and B cell populations remained normal (Fig. 3G). These
findings again suggest that following 5-FU treatment of Tnip−/− mice, there is a reduced quiescent LT-HSC pool in the BM and a delayed recovery from the hematopoietic stress of 5-FU. It is possible that the defect in HSC quiescence could lead to the premature exhaustion of LT-HSCs and shorten the LT-HSC life span in vivo; these effects would become evident as the mice aged, as we have already shown in Fig. 1D (8). Taken together, our data demonstrate that TXNIP plays a pivotal role in maintaining HSC quiescence during hematopoietic stress derived from aging or chemical treatment.

**Increased mobilization of Tnip−/− HSCs under stress conditions**

As already mentioned in Fig. 3A, the BM of Tnip−/− showed a reduced cellularity following 5-FU treatment, whereas another histological analysis also showed a significant reduction in the BM cellularity after 5-FU treatment and a mild reduction in aged Tnip−/− (Fig. 4A). Two-month-old (young) and 12-mo-old (aged) mice were treated with 5-FU, and 4 days after treatment, femur sections were stained with H&E. In contrast to the BM, there was a significant increase in the cellularity of the spleen and peripheral blood in the aged and 5-FU-treated Tnip−/− mice. The size of the spleen was larger in the aged and 2-mo-old 5-FU-treated Tnip−/− mice than in the WT control (Fig. 4, B and C). In addition, the cellularity of the PB was also dramatically increased in the aged and 5-FU-treated Tnip−/− mice (Fig. 4D; Table S2). The frequency of LKS cells in the spleen and peripheral blood was also significantly higher (Fig. 4E). These results suggest that more Tnip−/− HSCs were mobilized to the secondary hematopoietic organs. To determine whether the Tnip−/− BM niche had an impaired capacity for the retention of HSCs, we next performed an in vivo mobilization assay using G-CSF. After G-CSF treatment, the number of HSCs in the PB was more elevated in Tnip−/− mice (Fig. 5, A and B). We also performed an in vitro migration assay using a Transwell system. The Transwell migration efficiency of the CD45.1 LKS cells toward Tnip−/− BM was lower than toward the WT BM (Fig. S6A). We also did this Transwell mobilization assay using BM supernatants instead of the BM cells and found that fewer HSCs migrated toward the Tnip−/− BM supernatant (Fig. S6B). We also checked the motility features of the Tnip−/− HSC themselves in an in vitro Transwell assay (Fig. S6C); there was no difference between the WT and Tnip−/− in their migratory capability. For the in vivo homing assay, WT and Tnip−/− mice were treated with 5-FU for 2 days, and CD45.1 donor BM cells were transplanted. The donor-derived cells in the BM were then analyzed by flow cytometry after 24 h. In the BM of Tnip−/− mice, there were fewer donor-derived LKS cells (Fig. 5, C and D). In addition, we checked the motility features of the Tnip−/− HSC themselves using an in vivo homing assay (Fig. S6D). There was no difference between homing capacity of the WT and Tnip−/− cells. Together, these results suggest that TXNIP regulates HSC mobilization by modulating the expression of extrinsic components in the BM niche.

**Reduced niche interactions cause HSC mobilization**

HSC mobilization appears to depend on both direct cell-to-cell contact and the cellular and extracellular matrix components that support and regulate the HSCs that form the niches. There is accumulating evidence suggesting that CXCL12, through its interaction with CXCR4, plays an important role in regulating HSC mobilization (28). Studies of CXCL12- or CXCR4-deficient mice
have established that these genes are necessary for the normal migration of HSC from the fetal liver to the BM and in the efficient retention of myeloid precursors in the adult BM (29). Elevation of CXCL12 levels in the blood is associated with a significant mobilization of HSC into the blood. To understand the molecular events involved in TXNIP-regulated HSC mobilization, we examined the expression of CXCL12 in the BM niche. We checked the CXCL12 level in the BM supernatant of 5-FU treated mice. After 5-FU treatment, the CXCL12 level was down-regulated in the Txnip\(^{-/-}\) BM (Fig. 5E), and the CXCR4 expression level in the Txnip\(^{-/-}\) HSCs was also down-regulated 6 days after 5-FU treatment (Fig. 5F), but not without treatment (Fig. S7A).

Recent studies have shown that osteoblastic suppression can lead to HSC mobilization (30) and that OPN-positive OBs modulate HSC proliferation and mobilization (31). We detected OBs based on the absence of the hematopoietic markers CD45 and Ter119 and the expression of OPN (31). The frequencies of OBs were not different between WT and Txnip\(^{-/-}\) mice BM (Fig. S7B). The OPN expression level in CD45\(^{-/-}\) Ter119\(^{-/-}\) OBs was reduced after 5-FU treatment in Txnip\(^{-/-}\) mice (Fig. 5G; Fig. S7C). These data suggest that, when exposed to 5-FU, the BM niche undergoes dramatic changes in the Txnip\(^{-/-}\) mice. These results also highlight the important role of CXCL12 and OPN in the communication between the HSCs and the BM niche. This communication can be mediated by both direct OPN\(^{+}\) OB-HSC contact and soluble signals secreted by the BM niche. The down-regulation of the CXCL12- and OPN-mediated interactions impaired the ability to retain and mobilize the HSC in the Txnip\(^{-/-}\) BM niche. Therefore, these changes may contribute to the increased cycling and mobilization of Txnip\(^{-/-}\) HSCs within the BM niche.

**Txnip\(^{-/-}\) HSCs promote active cell cycling**

Next, we tested whether Txnip\(^{-/-}\) HSCs demonstrate accelerated cell cycling or hyperproliferation. We performed in vivo BrdU labeling in mice to determine the proliferative status of HSCs in the BM. Three days after 5-FU treatment, WT and Txnip\(^{-/-}\) mice were exposed to BrdU for 4 h, and the percentage of primitive BM cells that incorporated BrdU was measured by flow cytometry. The percentage of CD34\(^{-}\) LKS cells that incorporated BrdU was elevated in the Txnip\(^{-/-}\) mice compared with the WT mice (Fig. 6A).
To evaluate HSC quiescence directly, flow cytometry was performed following staining with pyronin Y and Hoechst 33342 to monitor RNA and DNA content, respectively, and to distinguish between cells in G0 and G1. Quiescent primitive G0 populations are identified by low pyronin Y staining and a 2N DNA content (10). In the steady state, the number of pyronin Y-positive LT-HSCs was not different in the WT and Txnip/H11002/H11002 mice. However, after 5-FU treatment, TXNIP deficiency resulted in a significant loss in the number of G0-phase LT-HSC cells (43 vs 10%), and an increase in the number of cells in the G1/S/G2/M phase (57 vs 90%), compared with the WT controls (Fig. 6B). To investigate the molecular mechanism underlying the increased cell cycle of Txnip/-/- HSCs, we examined the mRNA expression of several genes that had previously been implicated as candidate molecules for HSC cell cycle regulation by quantitative RT-PCR. BM cells were isolated from mice 6 days after 5-FU treatment and were lineage depleted using MACS. LKS cells were then sorted using a FACSaria. The expression levels were normalized GAPDH expression. The results are presented as the mean ± SD.

**FIGURE 6.** TXNIP deficiency activates the cell cycle in HSCs. A, Increased incorporation of BrdU by Txnip/-/- LT-HSCs. Six days after 5-FU treatment, mice were administered 100 μg/g BrdU through an i.p. injection and analyzed 4 h later. Total BM cells were stained with anti-BrdU, anti-Scal, anti-c-Kit, anti-CD34, and lineage markers. The data shown are representative dot plots from five mice over three independent experiments. B, HO/PY staining in HSCs. Six days after 5-FU treatment, total BM cells stained with nucleic acid dyes HO and PY, anti-Scal, anti-c-Kit, anti-CD34, and lineage markers were analyzed by flow cytometry. The data shown are representative dot plots from five mice over three independent experiments. C, The mRNA expression of c-myc, Dkk-1, Lrp5, p21, Lef1, and Tcf4 was measured by real-time PCR. BM cells were isolated from mice 6 days after 5-FU treatment and were lineage depleted using MACS. LKS cells were then sorted using a FACSaria. The expression levels were normalized GAPDH expression. The results are presented as the mean ± SD.

Wnt activation in Txnip/-/- HSCs

We analyzed the Wnt signaling pathway under stress conditions to explain the loss of quiescence and impaired function of the Txnip/-/- HSCs under stress conditions. Wnt signaling was acutely activated under stress conditions, such as 5-FU treatment, but reverted to the baseline activation quickly. We also investigated the WNT10B level in the BM supernatant. The level of WNT10B was much higher in HSCs 6 days after 5-FU treatment (Fig. 7A), and the activated β-catenin level was much higher in the Txnip/-/- CD34/- LKS cells (Fig. 7B).
FIGURE 7. TXNIP activates Wnt signaling. A, Wnt10b expression levels in the BM niche. The BM supernatant was assayed for Wnt10b 6 days after 5-FU treatment. B, The expression of activated β-catenin in CD34+ LKS cells was analyzed by confocal microscopy 6 days after 5-FU treatment. C, The TOPflash plasmid was transfected into 293T cells, and the luciferase activity was measured. D, The p21-Luc plasmid was transfected into 293T cells, and the expression of luciferase was measured. E, 293T cells were transfected with β-catenin plasmid, TXNIP plasmid, and GFP plasmid. After 24-h transfection, cell lysates were immunoblotted with anti-β-catenin mAb. F, 293T cells were transfected with hemagglutinin-ubiquitin plasmid, β-catenin plasmid, and TXNIP plasmid. After 24-h transfection, cell lysates were immunoblotted with anti-β-catenin mAb.

The direct effect of TXNIP on Wnt signaling was measured in a 293T cell line using the TOPflash reporter system, which contains T cell-specific factor binding sites. TXNIP overexpression significantly reduced the TOPflash reporter activity in a dose-dependent manner (Fig. 7C) and increased the expression of a reporter under the control of the p21 promoter (Fig. 7D). Similar results were obtained in two other cell lines, HCT116 and SW620 (Fig. 8A, B). In addition, when TXNIP was overexpressed in the 293T cell line, the degradation of β-catenin was increased in a dose-dependent manner (Fig. 7E and F). We previously reported that TXNIP functions as a nuclear export mediator important in the degradation of HIF1α (20). TXNIP interacts with the β-domain of pVHL and enhances the interaction between pVHL and HIF1α to promote the nuclear export and degradation of HIF1α. It has been recently reported that β-catenin is also subject to negative regulation by pVHL (33). From these results, we propose a model in which the impaired degradation of β-catenin, mediated by the TXNIP/pVHL complex, results in overactive and sustained Wnt signaling in HSC. Overall, the increased Wnt signaling and decreased p21 expression in Txnip−/− HSCs could influence HSC cycling and cause the loss of quiescence.

Discussion

In this study, we demonstrate that TXNIP is a regulator of HSC quiescence and migration under stress conditions. Our results suggest that different mechanisms regulate HSCs under steady-state and stress conditions, such as aging, anti-cancer treatment, or following transplantation. Txnip−/− mice have a similar frequency of HSC pools in the BM compared with WT mice, indicating that TXNIP partially controls the steady-state HSC number in young mice. In contrast, Txnip−/− HSCs have a markedly lower ability to recover following 5-FU treatment and repeated transplantation, indicating that TXNIP is a key regulator that maintains HSC quiescence under stress conditions.

We also demonstrate that TXNIP regulates Wnt signaling. Wnt signaling has been implicated as a key signal for self-renewal (34). Constitutive Wnt activation following the expression of a constitutively active form of β-catenin blocks the differentiation of HSCs, thereby leading to the loss of stem cell activity (35, 36). Conversely, Fleming et al. (16) has demonstrated that blocking Wnt signaling in the mouse BM microenvironment with the overexpression of DKK-1, a soluble secreted protein, antagonized Wnt signaling and altered the HSC pools. The inhibition of Wnt signaling by DKK-1 in OBs reduces the nuclear function of β-catenin in HSCs and increases HSC cell cycling; this inhibition also markedly reduces the long-term repopulation activity of the hematopoietic system. These data suggest that both excessive activation and inhibition of Wnt signaling can disrupt HSC homeostasis.

Wnt activation plays an important role in hematopoietic regeneration repair. The Wnt signal in the microenvironment, and in the
ROLE OF TXNIP IN HSC QUIESCENCE AND MOBILIZATION

HSC itself, acutely and finely acts to drive efficient hematopoietic repair. However, TXNIP deficiency results in overactivated and sustained Wnt signaling in both the BM niche and the HSCs, and therefore, exhausts the *Txnip*+/− HSCs. Our *Txnip*+/− mice did not show any abnormalities in the number or function of HSCs in the absence of exogenous stress; however, the abnormal phenotype arose during aging and after stress. It is therefore possible that short-term activation of Wnt is needed for the homeostasis of HSCs, but that chronic activation of Wnt will exhaust the HSCs (37). Thus, the finely tuned regulation of Wnt/β-catenin signaling is required to properly control HSC quiescence (34). This is most likely due to the complexity of Wnt signaling and the existence of compensatory and redundant pathways. Moreover, it is possible that the regulation is different under steady-state and stress conditions. However, based on the results of this study, we can conclude that TXNIP plays an important role in regulating Wnt signaling under stress conditions.

The HSC niche has an interesting, complex structural and molecular network. It is likely that many cell types contribute to the HSC niche, including OBs, osteoclasts, endothelial cells, adipocytes, and the HSCs themselves. N-cadherin has been proposed to be an important factor in the niche for the localization and adhesion of quiescent HSC. Recently, however, it has been reported that N-cadherin is not expressed in long-term repopulating HSCs and that N-cadherin(high) cells are primarily mature cells that are incapable of reconstituting the HSC population (38, 39). These results illustrate the uncertainty surrounding HSC interactions in their niche (40), and suggest that other unrecognized molecules and chemokines may also participate in this interaction.

Recent studies have suggested that under steady-state conditions, the HSCs are associated with OBs (12) and localized near CXCL12-abundant reticular cells (41). HSC proliferation and mobilization appear to depend on both direct cell-to-cell contact and the cellular and extracellular matrix components that support and regulate the HSCs that form the niche. There is accumulating evidence that suggests that CXCL12, through its interaction with CXCR4, plays an important role in regulating HSC mobilization (28). Additionally, HSCs bind to OPN, which regulates the trans-marrow migration of transplanted HSC via β1 integrins on the HSC surface. OPN forms a complex with fibronectin and collagen within the extracellular matrix and is involved in anchoring the HSCs to the endosteal niche (38, 42). It remains unclear, however, how much of the OPN binds to the extracellular matrix in the endosteum and how much exists as a free cytokine; these forms of OPN could have differential effects on HSCs. Our data demonstrate that the reduced expression of CXCL12 and OPN in the BM niche may be important factors responsible for the increased mobilization of *Txnip*+/− HSCs.

A recent study has shown that HSCs reversibly switch from dormancy to self-renewal under conditions of hematopoietic stress (43). Dormant HSCs can be robustly activated to proliferate and mobilize in response to injury signals, such as S-FU, and they can reversibly return to a dormant state. A BM endosteal niche may keep the HSCs dormant during homeostasis to preserve their self-renewal capacity and prevent stem cell exhaustion. HSC dormancy is an important strategy for the adult organism to prevent stem cell exhaustion. Serial HSC transplantation is only possible for approximately six rounds, demonstrating that the capacity of HSCs to self-renew is limited. It can be speculated that an impaired BM niche in the TXNIP-deficient mice induces the HSCs to proliferate and mobilize in response to hematopoietic stress, but prevents return of HSCs to dormancy. This could be another explanation for the paradoxical phenotype of *Txnip*+/− HSC between active cycling and reduced HSC number.

In conclusion, our data demonstrate that TXNIP is a key component of the HSC niche under stress conditions. TXNIP plays a dual role in maintaining LT-HSC quiescence and migration by decreasing Wnt signaling and enhancing the niche interaction. Further studies examining the mechanism underlying the TXNIP-regulated maintenance of HSC quiescence and niche interactions will help to better define the molecular mechanisms that control HSC function after anticancer treatment or BM transplantation. These studies will aid in the development of strategies to effectively stimulate the ex vivo expansion of HSCs for clinical use and to limit the overproliferation of HSCs that causes leukemia.

Acknowledgments

We thank Dr. Chul-Ho Lee and his team at Korea Research Institute of Bioscience and Biotechnology, especially Dong-Hee Choi, for experimental animal care.

Disclosures

The authors have no financial conflict of interest.

References


Supplementary Figure legends

Figure S1. Multiparametric analysis of HSC Populations.

A. FSC and SSC gating of total BM cells. B. Gating strategy used to identify the Lin’ cell population. BM cells were stained with a cocktail of biotin-conjugated antibodies specific for different lineage markers (CD11b, Gr1, B220, CD2, NK1.1, and TER119), followed by incubation with fluorochrome-conjugated streptavidin. C. BM cells were stained for surface markers (c-Kit and Sca1) and gated. D. The LT-HSC, ST-HSC, and MPP populations were identified based on CD34 and Flk2 expression. E. The GMP, CMP, and MEP cell populations were determined based on CD34 and CD16/32 expression. F. CLP cells were identified based on CD127 expression.

Figure S2. BM colony-forming assay

A. BM cells (1×10^5) were plated in methylcellulose medium with growth factors for 7 days. All colonies with more than 50 cells were counted from duplicated samples. The data are presented as the mean number of colonies from five mice in two independent experiments. B. Morphology of colonies in the colony-forming assay.

Figure S3. Total BM cellularity. The total BM cellularity was counted from two
femurs, tibias, hipbones, and shoulder bones. The data are expressed as the absolute number of BM cells from four mice tested over two independent experiments.

**Figure S4. Analysis of apoptosis.**

A. Apoptotic LKS cells were measured by flow cytometry following staining with Annexin V and propidium iodide. The data shown are a representative dot plot. B. Representative flow cytometric analyses of apoptotic BM cells 24 hours after 5-FU injection.

**Figure S5. Reduced LT-HSC and Increased ST-HSC.**

A. Absolute number of LT-HSC after 5-FU treatment. B. Absolute number of ST-HSCs after 5-FU treatment. The data described and presented in Fig. 3C is converted to absolute cell number.

**Figure S6. Migration and homing assays.**

A. LKS cells were collected from CD45.1 mice, and 1×10^6 cells were added to the upper chamber of a transwell. Total BM cells (1×10^6) collected from WT and *Txnip^-/-* mice were added to the lower chamber of a transwell (5 μm pore size; Corning). After a
4 h incubation, the upper chamber was carefully removed, and the number of LKS cells that had migrated into each lower chamber was determined by flow cytometry using a FITC-conjugated CD45.1-specific antibody (BD Bioscience). The data shown in a representative dot plot and the mean percentage ± SD. B. Total BM cells from WT *Txnip*<sup>-/-</sup> were cultured for 24 hr and the culture supernatant was placed in the lower chamber. LKS cells were isolated from CD45.1 mice and added to the upper chamber. After a 4 h incubation, the number of CD45.1 LKS cells that had migrated into each lower chamber was counted. C. LKS cells were isolated from WT and *Txnip*<sup>-/-</sup> mice and added to the upper chamber of a transwell (1x10<sup>6</sup> cells per well), and total BM cells isolated from CD45.1 mice were added to the lower chamber. After a 4 h incubation each lower chamber was determined by flow cytometry using a FITC-conjugated CD45.1-specific and an APC-conjugated CD45.2-specific antibody (BD Bioscience, USA). D. LKS cells were isolated from 6 month old *Txnip*<sup>-/-</sup> or WT mice and injected into lethally irradiated CD45.1 mice (2x10<sup>6</sup> cells per mouse). After 24 h, BM cells were isolated from the recipient mice, and the donor-derived CD45.2 cells were analyzed by flow cytometry.

**Figure S7. CXCL2-OPN-mediated mobilization**

A. CXCR4 expression on LKS cells from WT *Txnip*<sup>-/-</sup> control group (without 5FU
treament). The data are presented as a histogram from five mice over two independent experiments. B. CD45^TER119^-Osteoblast frequencies in the collagenase treated bone cell (6 days after 5-FU treatment). The data shown are representative dot plots from five mice over three independent experiments. C. The expression of OPN in the CD45^-TER119^- osteoblasts was analyzed by flow cytometry 6 days after 5-FU treatment. Freshly isolated osteoblasts were stained with CD45-, TER119-, and OPN-specific antibodies. The CD45^-TER119^- cells were analyzed and the *Opn* expression was assayed. The cells were gated on the CD45^-Ter119^- population. The data are presented as a histogram showing the OPN expression on osteoblasts.

**Figure S8. TOPflash reporter assay.** The TOPflash plasmid was transfected into the HCT116 and SW620 cell lines, and the luciferase activity was measured. A. HCT116 cells, B. SW620 cells.
Figure S6

A

WT

KO

27 ± 2.3

18 ± 2.0

CD45.1

B

Relative cell number (%)

WT sup

KO sup

* 

C

D

CD45.1

CD45.2

12.3

13.1

WT

KO

6.48

6.9

CD45.2

CD45.1

6.9

6.9

KO
Figure S7

A

WT  KO

378 ± 10.6  398 ± 13.6

CXCR4

B

WT  KO

5 ± 1  4.6 ± 0.7

CD45

Ter119

C

CD45-TER119+ gated

*  

MFI

WT  KO

240  0

QPN

Isotype  WT  KO
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Table S2. Blood cell counts

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Values shown are the mean ± SD from 5 mice of three independent experiments (*p < 0.05).