Latexin Inactivation Enhances Survival and Long-Term Engraftment of Hematopoietic Stem Cells and Expands the Entire Hematopoietic System in Mice

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Repository Citation

Liu, Yi; Zhang, Cuiping; Li, Zhenyu; Wang, Chi; Jia, Jianhang; Gao, Tianyan; Hildebrandt, Gerhard C.; Zhou, Daohong; Bondada, Subbarao; Ji, Peng; St. Clair, Daret K.; Liu, Jinze; Zhan, Chang-Guo; Geiger, Hartmut; Wang, Shuxia; and Liang, Ying, "Latexin Inactivation Enhances Survival and Long-Term Engraftment of Hematopoietic Stem Cells and Expands the Entire Hematopoietic System in Mice" (2017). Toxicology and Cancer Biology Faculty Publications. 67.  
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Notes/Citation Information
Published in Stem Cell Reports, v. 8, issue 4, p. 991-1004.

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Digital Object Identifier (DOI)
https://doi.org/10.1016/j.stemcr.2017.02.009

This article is available at UKnowledge: https://uknowledge.uky.edu/toxicology_facpub/67
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http://dx.doi.org/10.1016/j.stemcr.2017.02.009

SUMMARY

Natural genetic diversity offers an important yet largely untapped resource to decipher the molecular mechanisms regulating hematopoietic stem cell (HSC) function. Latexin (Lxn) is a negative stem cell regulatory gene identified on the basis of genetic diversity. By using an Lxn knockout mouse model, we found that Lxn inactivation in vivo led to the physiological expansion of the entire hematopoietic hierarchy. Loss of Lxn enhanced the competitive repopulation capacity and survival of HSCs in a cell-intrinsic manner. Gene profiling of Lxn-null HSCs showed altered expression of genes enriched in cell-matrix and cell-cell interactions. Thrombospondin 1 (Thbs1) was a potential downstream target with a dramatic downregulation in Lxn-null HSCs. Enforced expression of Thbs1 restored the Lxn inactivation-mediated HSC phenotypes. This study reveals that Lxn plays an important role in the maintenance of homeostatic hematopoiesis, and it may lead to development of safe and effective approaches to manipulate HSCs for clinical benefit.

INTRODUCTION

Hematopoietic stem cells (HSCs) persist throughout life to produce hematopoietic progenitor cells (HPCs) and all types of blood cells. In the adult, HSCs reside in bone marrow (BM), are rare but have the unique capability for self-renewal and multilineage differentiation (Eaves, 2015). The maintenance of a steady-state HSC pool adept at dynamic change in response to stress depends on the balance of self-renewal, differentiation, survival, and proliferation (Goodell et al., 2015). Loss of this balance could lead to an overexpansion or exhaustion of HSC population, and result in an increased risk for cancer or tissue degeneration. HSCs have been used therapeutically in the clinic for several decades in life-saving treatment of malignant diseases and hematological disorders through BM transplantation protocols (Doulatov et al., 2012). However, insufficient stem cell numbers significantly limit the efficacy and success of these regimes. Expansion of HSCs while maintaining their self-renewal capability has been one of the most desired, yet elusive, goals in experimental hematology and transplantation medicine (Walasek et al., 2012). HSC fate decisions require strict control. Multiple signaling pathways regulate HSC functions through cell-intrinsic and cell-extrinsic mechanisms (Rossi et al., 2012; Gottgens, 2015). Genetic manipulation of transcription factors and signal transduction pathways enhances HSC expansion ex vivo (Andrade et al., 2010, 2011); these include the homeobox gene family (Antonchuk et al., 2002), immobilized Notch ligand (Delaney et al., 2010), Wnt-associated prostaglandin E2 (Goessling et al., 2011), the soluble growth factors angiopoietin-like 5 (Zheng et al., 2012), pleiotrophin (Himburg et al., 2010; Himburg et al., 2012), and miR-126 (Lechman et al., 2012), and the aryl hydrocarbon receptor inhibitor (Boitano et al., 2010). Moreover, employing an induced pluripotent stem cell population and targeting the HSC microenvironment hold promise for HSC expansion (Blanpain et al., 2012;
Kunisaki and Frenette, 2012; Huang et al., 2013; Chen et al., 2014). However, all these attempts have had limited success clinically, due to a failure to expand sustainable and self-renewable stem cells (Walasek et al., 2012).

Our understanding of the molecular pathways for HSC fate decision is insufficient to allow safe manipulation of HSCs for clinical benefit. Large natural variations in HSC number and function exist in humans (Nathan and Orkin, 2009; Sankaran and Orkin, 2013) as well as in different mouse strains (Jordan and Van Zant, 1998; Geiger et al., 2001; Henckaerts et al., 2002; Abiola et al., 2003; Hsu et al., 2007; Cahan et al., 2009; Avagyan et al., 2011). Such natural genetic diversity is an important yet largely unused tool for unraveling the genes and signaling networks associated with stem cell regulation (Van Zant and Liang, 2009). Using this genetic diversity tool, we previously identified latexin (Lxn) as a stem cell regulatory gene with expression that negatively correlates with HSC number variation in different mouse strains (de Haan, 2007; Liang et al., 2007). Lxn is a negative regulator of HSC function and works as a “brake” to constrain the HSC pool to a physiological range. In addition, the canonical function of Lxn protein is its inhibitory effect of carboxypeptidase A (Liu et al., 2000; Uratani et al., 2000; Pallares et al., 2005; Mouradov et al., 2006). Studies have also shown that LNX has high structural similarity with cystatin and tumor suppressor TIG1 gene, suggesting its potential role in inflammation and transformation (Aagaard et al., 2005). However, the in vivo function of Lxn in hematopoiesis and the underlying regulatory cellular and molecular mechanisms remain largely elusive. Particularly, when drawing upon genetic diversity to identify genes (usually multiple ones) associated with a complex trait (HSC number), all contributing genes are important (Van Zant and Liang, 2009). Therefore, it warrants to know to what extent Lxn contributes to the natural variation of the size of HSC population and how it specifically regulates HSC function and hematopoiesis.

In this study, we showed that Lxn deletion in vivo leads to increased numbers of HSCs, HPCs, and all blood cell lineages. Loss of Lxn enhanced long-term repopulating capacity and survival of HSCs. Mechanistically, gene array analysis showed that genes involved in cell-cell and cell-matrix interaction were dysregulated in Lxn−/− HSCs, and thrombospondin 1 (Thbs1) was identified as a potential downstream target of Lxn. Thbs1 mRNA and protein levels were significantly decreased in Lxn−/− HSCs, and ectopic expression of Thbs1 rescued the Lxn−/− HSC phenotype. Our study reveals the cellular and molecular regulatory mechanisms of Lxn in HSC homeostasis, and highlights the importance of Lxn-mediated intrinsic and extrinsic cellular signaling in the control of HSC function.

RESULTS

Lxn−/− Mice Have Increased Blood Cell Numbers and Balanced Lineage Differentiation

The constitutive Lxn knockout (Lxn−/−) mice were generated on the C57BL/6 background. Because Lxn gene lies within the mitochondrial elongation factor G (Gfm1) gene, only exons 2 to 4 were targeted for deletion to minimize any potential effect on Gfm1 (Figure 1A). Western blot showed complete deletion of the LNX protein in BM, spleen, liver, and brain without affecting GFM1 protein expression (Figure 1B). Peripheral blood (PB) analysis of Lxn−/− mice showed a significant increase in the counts of complete white blood cells, neutrophils, monocytes, lymphocytes, and platelets compared with those of wild-type (WT) mice (Figure 1C). The percentages of macrophages, granulocytes (Mac-1/Gr-1+), and B lymphocytes (B220+) in Lxn−/− mice showed a slight but significant increase (Figure 1D), whereas the T lineage was not affected (data not shown). These data suggest that Lxn deletion increases the number of mature blood cells of both myeloid and lymphoid lineages without skewing differentiation.

Lxn Inactivation Leads to Expansion of the HSC and HPC Populations

Analysis of the BM compartment of Lxn−/− mice showed a significant increase in cellularity compared with WT mice (Figure 2A), despite similar body weights (data not shown). Using flow cytometry and immunostaining of HSCs and HPCs (Figure 2B), we found that the absolute numbers of HPCs, including common myeloid progenitor (CMP), granulocyte/monocyte progenitor (GMP), and common lymphoid progenitor (CLP) cells, were significantly increased in Lxn−/− mice (Figure 2C). Moreover, Lxn−/− mice consistently presented a 56% increase in the absolute number of HSC/HPC-enriched LSK cells (Figure 3D). Among the LSK population, the absolute numbers of long-term (LT)-HSCs, short-term (ST)-HSCs, and multipotent progenitor cells (MPPs) were all significantly increased in Lxn−/− mice compared with WT mice (Figures 2E–2G). These data suggest that Lxn inactivation leads to an expansion of the hematopoietic stem and progenitor pool, which contributes to the increased blood cell counts.

Lxn Inactivation Increases HSC Clonogenic and Repopulating Capacity in a Cell-Intrinsic Manner

To define the effect of Lxn inactivation on the function of HSCs and HPCs, we used the in vitro culture system, including cobblestone area-forming cell (CAFC) and colony-forming cell (CFC) assays, to assess the clonogenic potential of HSCs and HPCs, respectively. Figure 3A shows that the number of primitive HSCs (CAFC day 35) was...
3-fold higher in Lxn−/− BM than WT BM. Moreover, the total number of CFCs generated by Lxn−/− BM cells was increased by nearly 2-fold compared with WT cells (Figure 3B). We next evaluated the capability of Lxn−/− HSCs to reconstitute the hematopoiesis of myeloablated mice by a competitive repopulation assay (Figure 3C). The results showed that Lxn−/− HSCs made a greater contribution to the repopulation of PB leukocytes at both 8 weeks and 16 weeks post transplantation compared with WT cells (Figure 3D). Analysis of PB lineage chimerism at 16 weeks showed that the engrafted Lxn−/− HSCs retained multilineage differentiation potential and contributed to more circulating myeloid and B cells than the WT cells (Figure 3E). This recapitulates the intrinsic changes of Lxn−/− cells in physiological condition (Figure 1D). Analysis of BM chimerism showed that mice transplanted with Lxn−/− cells presented with 66.4% (±9.9%) donor-derived cells, whereas in mice receiving WT cells, the contribution was dramatically lower at 30.9% (±16.9%) (Figure 3F). In the BM LSK compartment, the percentage and absolute number of Lxn−/−-derived LSK cells was nearly 3-fold higher than that of WT-derived cells (Figures 3G and 3H, respectively). Altogether, these results reinforce findings of increased numbers of functional HSC and HPC, as measured by their
clonogenic activity in Lxn<sup>−/−</sup> mice. The data suggest that Lxn inactivation significantly enhances HSC long-term reconstitution capacity and maintains multilineage differentiation potential in a cell-intrinsic manner. Moreover, the extent of expansion of Lxn<sup>−/−</sup>-derived LSK population in this transplantation setting (3-fold) is higher than that in situ condition (1-fold, Figure 2D), indicating the likelihood of increased self-renewal of Lxn<sup>−/−</sup> HSCs. Future studies are needed to address this by the serial transplantability of Lxn<sup>−/−</sup> HSCs.
Figure 3. Lxn Inactivation Increases the Number of Functional HSCs and HPCs, and Enhances the Competitive Repopulation Capacity of HSCs

(A) Absolute number of clones, defined by the cobblestone area-forming cell (CAFC) assay, at d35 of culture for HSC cells from Lxn−/− and WT mice (***p = 0.0002).

(B) Absolute number of clones, defined by colony-forming cell (CFC) assay, at day 14 of culture for HPC cells from Lxn−/− and WT mice (*p = 0.01). Presented data are the average ±SD pooled from three independent experiments with three mice per experiment for both strains (n = 9 each strain).

(C) Experimental scheme for competitive repopulation assay.

(D–H) Frequencies of Lxn−/− or WT donor (CD45.2)-derived leukocytes at 4, 8, and 16 (***p < 0.001) weeks after transplantation in the PB of recipient mice (CD45.1). At 16 weeks after transplantation in the BM of recipient mice, frequencies of Lxn−/− or WT donor...
Lxn Inactivation Enhances HSC Survival without Affecting Cell Cycling

The HSC pool size is maintained by the balance of apoptosis and proliferation. Apoptosis analysis with Annexin V showed that the percentage of Annexin V+ propidium iodide-negative (PI–) apoptotic cells (Figure 4A) was significantly decreased by nearly 50% in Lxn/C0/C0 LSK cells, and subsets of HSCs (LT-HSCs, ST-HSCs, and MPPs) compared with the WT cells (Figure 4B). Increased survival was further confirmed by flow cytometric analysis of LSK cells positive for active caspase-3 (Figure 4C). The percentage of positive cells was significantly lower in Lxn/C0/C0 mice than that in WT mice (Figure 4D). Moreover, expression of active caspase-3, as measured by the mean fluorescence intensity, was lower in Lxn/C0/C0 LSK cells than in WT cells (Figure 4E). These data suggest that Lxn inactivation can intrinsically enhance HSC survival. We further followed the response of WT and Lxn/C0/C0 HSCs to 5-fluorouracil (5-FU)-induced hematopoietic stress, and found that LSK cells in Lxn/C0/C0 BM recovered faster than that in WT mice (Figure 4F). Lxn/C0/C0 mice had a faster recovery in LSK cells after 5-FU-induced hematopoietic stress at days 1, 3, 6, 10, and 14. (G) Less Lxn/C0/C0 LSK cells underwent apoptosis at 24 hr after 5-FU injection. Data were the average of five mice at each time point in each group. ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05.

Figure 4. Lxn/C0/C0 HSCs have Increased Survival

(A) Representative FACS plots of Annexin V+ and 7-AAD apoptotic LT-HSCs (left plots). Thymocytes were used as control to determine the Annexin V+ population, as we published previously (Liang et al., 2007).

(B) Percentage of apoptotic cells in populations of LSK cells, MPPs, ST-HSCs, and LT-HSCs of Lxn/C0/C0 and WT mice. Presented data are the average ± SD of five mice/group.

(C) Representative flow cytometry profile of active caspase-3 immunofluorescence signal in LSK cells.

(D) Frequency of LSK cells positive for active caspase-3 (*p = 0.04).

(E) The level of the active caspase-3 protein in LSK cells of WT and Lxn/C0/C0 mice. Shown is the histogram of active caspase-3 protein in flow cytometry analysis. Data were the average of three independent experiments with three mice in each one (n = 9).

(F) Lxn/C0/C0 mice had a faster recovery in LSK cells after 5-FU-induced hematopoietic stress at days 1, 3, 6, 10, and 14.

(G) Less Lxn/C0/C0 LSK cells underwent apoptosis at 24 hr after 5-FU injection. Data were the average of five mice at each time point in each group. ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05.

(CD45.2)-derived (E) myeloid cells (M/G; *p = 0.02), T and B lymphocytes (***p < 0.001), (F) BM nucleated (**p < 0.001), and (G) LSK cells (***p < 0.001), as well as (H) absolute numbers of LSK cells in one femur (***p < 0.001), were measured.

Presented data in (D–H) are the average ± SD pooled from two independent experiments with five recipients receiving either Lxn/C0/C0 or WT BM cells per experiment (n = 10 per donor group).
and less Lxn−/− LSK cells underwent apoptosis (Figure 4G). These results indicate that Lxn deletion protects hematopoietic stem/progenitor cells from 5FU-induced myelosuppression by enhancing their survival. We next examined the cell-cycle status with Ki-67/PI staining and bromodeoxyuridine (BrdU) incorporation, and did not observe any significant difference in cell-cycle distribution and cycling dynamics between Lxn−/− and WT LT-HSCs (Figures S1A and S1B, respectively). This result suggests that Lxn did not affect stem cell cycling under physiological conditions.

**Lxn−/− Mice Do Not Develop Hematological Malignancy during Aging**

We and others have shown that Lxn is downregulated in leukemia, lymphoma, and several other cancers (Li et al., 2011; Liu et al., 2012; Mitsunaga et al., 2012; Abd Elmageed et al., 2013; Muthusamy et al., 2013; Ni et al., 2014). Since young Lxn−/− mice did not show any obvious sign of hematological malignancy, we asked whether aging could promote tumor development in Lxn−/− mice. Cohorts of Lxn−/− and WT mice were physiologically aged for 28 months and the hematopoietic profile in PB and BM was analyzed. No significant difference in either complete or differentiated blood cell counts was identified between aged-matched Lxn−/− and WT mice (Figure 5A). Lineage differentiation in old Lxn−/− mice showed a slight but significant increase in myeloid lineage and decrease in B lymphocytes (Figure 5B), although both strains demonstrated age-associated myeloid skewing and immunodeficiency compared with their young counterparts (Figure 1D). No significant difference was found in BM cellularity (Figure 5C) and the number of CFCs (Figure 5D). Aged Lxn−/− mice had nearly 2-fold more LT-HSCs in both frequency and absolute numbers than age-matched WT counterparts, but no significant difference was observed in other HSC sub-populations (Figures 5E and 5F). Importantly, the overall health of old Lxn−/− mice was comparable with the controls, and no apparent pathological changes were detected in blood, BM, spleen, and liver of Lxn−/− mice (data not shown). These data suggest that Lxn−/− mice are not inherently prone to hematological malignancy.

**Figure 5. Aged Lxn−/− Mice Do Not Spontaneously Develop Hematologic Malignancy**

In age-matched old Lxn−/− and WT mice, the following hematopoietic phenotypes were measured.

(A) Differential blood cell counts.

(B) Lineage differentiation analysis of myeloid cells (M/G; *p = 0.02) and T and B lymphocytes (*p = 0.03).

(C) Total BM cell counts (cellularity).

(D) Absolute number of CFC progenitor cell clones on day 14 of culture.

(E) Frequency of LSK cells, LT-HSCs (*p = 0.02), ST-HSCs, and MPPs in one femur.

(F) Absolute number of LSK cells, LT-HSCs (*p = 0.02), ST-HSCs, and MPPs in one femur. Presented data in (A–F) are the average ± SD pooled from Lxn−/− (n = 15) and WT (n = 19) mice.
Thbs1 Is a Downstream Target of Lxn
We performed gene array analysis in phenotypically defined LT-HSCs and MPPs, respectively, to determine Lxn inactivation-induced molecular changes. A total of 3,561 genes are differentially expressed, among them one-third (1,235) are upregulated and two-thirds (2,326) are downregulated. Gene set enrichment analysis (GSEA) showed alteration in six signaling pathways enriched in Lxn/C0/C0 cells, and three of them involved cell-cell and cell-extracellular matrix interaction (Figure S2 A). Among the top ten up- and downregulated genes (Figure S2B), Thbs1 is one of the most interesting candidates. The rationale is that Thbs1 is a multidomain matrix glycoprotein that interacts with numerous adhesion receptors and proteases, and mediates cell-cell and cell-matrix interactions (Adams and Lawler, 2011). Thbs1 has been shown to enhance cell survival and regulate hematopoietic progenitor recovery under stress conditions (Isenberg et al., 2008). These functions are consistent with the functional effects of Lxn (You et al., 2014), which prompted us to further investigate whether Thbs1 is the downstream target of Lxn. Our microarray data showed a significant decrease in Thbs1 expression in Lxn/C0/C0 HSCs. Quantitative real-time PCR validated the downregulation of Thbs1, a reduction in mRNA expression of at least 2-fold was identified in different subsets of Lxn/C0/C0 HSCs (Figure 6A). It is noteworthy that the expression level of Thbs1 increased with the content of primitive hematopoietic cells, reaching the highest in the most primitive HSC population. Such expression pattern is similar to that of Lxn, as we reported previously (Liang et al., 2007), further corroborating the association between Thbs1 and Lxn. THBS1 protein, measured by western blot, also decreased in Lxn/C0/C0 BM/HPC-enriched Lin– cKi+ cells (Figure 6 B, left). THBS1 is a secreted protein (Adams and Lawler, 2011). We therefore measured its level in BM fluid and found that Thbs1 content in the Lxn/C0/C0 BM environment was also reduced (Figure 6B, right). Immunofluorescence staining of THBS1 protein level in BM sections of Lxn/C0/C0 mice confirmed reduced protein content of THBS1 (Figure 6 C). Furthermore, Figure 6D shows that the THBS1 protein content was reduced in individual Lxn/C0/C0 LSK cells compared with WT ones (left), and the percentage were flow cytometrically analyzed for the LSK population. Data shown are the mean ± 1 SD. **p = 0.01.

Figure 6. Thbs1 Is the Downstream Target of Lxn
(A) Real-time PCR data demonstrating Thbs1 mRNA levels in LSK, MPP, and HSCs. **p < 0.01 and *p < 0.05. HSCs are the combination of both LT-HSC and ST-HSCs (Lin– Sca1+ c-Kit+ Flt3 cells). Presented data are the average ± 1 SD of 12 measurements derived from three separate samples.
(B) Western blot analysis of Thbs1 protein in BM LIN-C-KIT+ (LK) cells and BM fluid. Actin (ACT) was normalization control. Blots are representative of three independent experiments.
(C) Immunofluorescence staining of the Thbs1 protein (red) in BM sections from Lxn/C0/C0 and WT mice. Scale bars, 50 µm.
(D) Immunofluorescence staining of Thbs1 protein (green) in individual Lxn/C0/C0 or WT LSK cells (left panel) and quantification of LSK cells positive for Thbs1 staining (right panel). All cells (50–100) in the field were captured and analyzed in each sample. Scale bars, 5 µM. ***p = 0.0003.
(E) Thbs1 heterozygous knockout mice (Thbs1+/−) had reduced THBS1 levels. Western blot was performed on LSK cells and shown is one representative blot of two replicates.
(F) Increased number of LSK cells in Thbs1+/− BM. BM cells from Thbs1+/− mice were flow cytometrically analyzed for the LSK population. Data shown are the mean ± 1 SD. **p = 0.01.
(G) Increased number of long-term colony-forming cells (CAFC day 35) in Thbs1+/− BM. Data shown are the mean ± 1 SD. ***p = 0.003.
of Thbs1+ LSK cells was significantly decreased in Lxn−/− BM cells compared with that in WT cells (right).

To further confirm the positive correlation between Lxn and Thbs1 expression, we overexpressed Lxn in WT LSK cells, and found that Thbs1 mRNA level was significantly increased (Figure S3). Moreover, we analyzed BM HSCs in Thbs1+/− mice in which Thbs1 protein was decreased (Figure 6E), and found that the number of LSK cells and CAFC day 35 cells was significantly higher in Thbs1+/− BM than those in WT BM cells (Figures 6F and 6G), a phenotype similar to Lxn−/− mice (Figures 2D and 3A). These data strongly suggest that Thbs1 is a downstream target of Lxn.

Thbs1 is a natural inhibitor of angiogenesis, megakaryopoiesis, and thrombopoiesis (Adams and Lawler, 2011), all of which have been shown as critical niche components implicated in HSC regulation (Kfoury and Scadden, 2015; Schepers et al., 2015; Bruns et al., 2014; Zhao et al., 2014). We asked whether the reduced Thbs1 levels in Lxn−/− HSCs and surrounding environment could affect these niche components. There was no significant difference in the number of megakaryocytes and megakaryocyte progenitor cells in Lxn−/− mice (Figures S4A and S4B, respectively) compared with WT mice. No difference in the expression of von Willebrand factor and CD41, two megakaryocyte markers, was shown in the microarray of Lxn−/− cells (data not shown). Moreover, the number of endothelial cells and the function of platelets also remain unchanged in Lxn−/− mice (Figures S4C and S4D, respectively). Altogether, these data suggest that Thbs1 is a specific downstream target of Lxn, and the decreased level of Thbs1 might affect Lxn+/− HSCs in a cell-intrinsic manner.

Enforced Expression of Thbs1 Reverts the Lxn−/− HSC Phenotype

To directly address the involvement of Thbs1 in the HSC phenotype of Lxn−/− mice, we overexpressed Thbs1 in WT and Lxn−/− LSK cells (Figures 7A and S5). Quantitative real-time PCR and western blot analysis confirmed increased expression of Thbs1 at both transcript and protein levels in Lxn−/− cells transduced with a Thbs1 virus compared with cells transduced with empty vector (Figures 7B and 7C, respectively). Figure 7D showed that Thbs1 overexpression dramatically reduced the number of Lxn−/− HSCs assessed by CAFC at day 35. Moreover, when Thbs1-overexpressing Lxn−/− stem cells were transplanted into myeloablated recipients, they showed a significant lower...
capacity to reconstitute recipients’ PB than control Lxn−/− cells at 16 weeks post transplantation (Figure 7E). Consistently, Thbs1 overexpression reduced the BM LSK cell reconstitution by nearly 2-fold (Figure 7F), which reverted the increased repopulation capacity seen in Lxn−/− HSCs (Figures 3D and 3G). However, we did not see any significant change in CAFC day 35 number in Thbs1-overexpressing WT cells, suggesting Thbs1 as a specific downstream target of Lxn (Figure S5). Overall, the data indicate that enforced Thbs1 expression can revert the Lxn−/− phenotype, suggesting that the functional effect of Lxn may act, in part, through Thbs1.

DISCUSSION

Lxn was originally identified by the natural variation of HSC numbers between C57BL/6 and DBA/2 mice, in which the C57BL/6 strain had a lower HSC number than the DBA/2 strain (Liang et al., 2007). Lxn expression negatively correlates with this variation, that is, its expression is higher in C57BL/6 HSCs than DBA/2 stem cells. However, the specific role of Lxn in hematopoiesis remains largely unknown.

In this study, we determined the in vivo function of Lxn in regulating HSC function and maintaining homeostatic hematopoiesis, and uncovered the cellular and molecular mechanisms that control these processes. Using the Lxn knockout mouse model generated on the C57BL/6 background, we found that loss of Lxn in vivo led to an expansion of the entire hematopoietic hierarchy, from the stem cell pool to the PB cells. Very interestingly, the increased number of HSCs in Lxn−/− mice is comparable with that of the DBA/2 strain (Liang et al., 2007), suggesting that Lxn, as the natural regulator, has the unique characteristic of maintaining the physiologic range of the HSC pool size. Therefore, reduction or even deletion of Lxn activity results in a controlled expansion of HSCs without impairment of stem cell repopulating and multilineage differentiation capacity. Indeed, the transplantation studies show that Lxn−/− HSCs have a much better long-term repopulation capacity with a balanced output of blood cells. Moreover, controlled expansion reduces the risk of malignant hyperplasia, supported by the observation that Lxn−/− mice did not spontaneously develop hematologic malignancy even during aging. Altogether, antagonism of Lxn function may have a therapeutic potential through a controlled expansion of HSCs without exhaustion or transformation.

The molecular mechanisms by which Lxn regulates HSC function remain largely unknown. Here, we provide strong evidence to support Thbs1 as a downstream target that mediates control of HSC by Lxn: (1) loss of Lxn dramatically decreased Thbs1 mRNA and protein levels; (2) ectopic expression of Lxn significantly increased Thbs1 transcript level; (3) Thbs1−/− mice phenocopied Lxn−/− mice in their BM HSC population by showing an enlarged HSC pool; and (4) ectopic expression of Thbs1 reverted the Lxn−/− HSC phenotype. Thbs1 is a multidomain adhesive glycoprotein that mediates cell-cell and cell-matrix interactions (Adams and Lawler, 2011). It interacts with a wide range of cell adhesion receptors and numerous proteases, and is involved in various cellular processes, such as inhibition of angiogenesis, megakaryocytopoiesis, and platelet function (Yang et al., 2003; Kopp and Rafii, 2007; Isenberg et al., 2009). However, our results indicate that decreased expression and secretion of Thbs1 in Lxn−/− HSCs and surrounding environment does not alter these niche cellular components; Since Thbs1 is a matrix protein, one of the regulatory mechanisms of Lxn may involve cell-cell and/or cell-niche interactions by altering the abundance of matrix proteins (Figure 7G). This discovery is consistent with a previous report from the Furukawa group, although they identified different adhesion molecules: N-cadherin, Tie2, and Roundabout 4 (Mitsunaga et al., 2012). This discrepancy may be due to the reasons that they used a less primitive cell population (SCA-1+) for proteomic analysis and/or they deleted the entire Lxn gene in the knockout mouse model. Nevertheless, additional investigations on the role of Lxn in stem cell trafficking, including mobilization and homing would be valuable.

Thbs1 can induce apoptosis via upregulation of active caspase-3 (Li et al., 2003). Consistently, Lxn−/− HSCs demonstrated decreased apoptosis and reduced levels of active caspase-3, suggesting that downregulation of Thbs1 might also intrinsically enhance Lxn−/− stem cell survival (Figure 7G). An increased survival could boost stem cell tolerance to genotoxic or cytotoxic stress. Indeed, absence of Thbs1 confers near complete resistance to injury from high-dose radiation, partly through mitigation of radiation-induced BM cell apoptosis (Isenberg et al., 2008; Soto-Pantoja et al., 2013). Moreover, Thbs1 knockout mice demonstrated an accelerated hematopoietic recovery following 5-FU-induced myelosuppression (Kopp et al., 2006). Interestingly, our published work has shown that Lxn is involved in radiation response and it increases the radiation sensitivity of myeloid progenitor cells (You et al., 2014). Therefore, we speculate that loss of Lxn may protect HSCs from radiation- and/or chemotherapy-induced myelosuppression and long-term damages. We indeed showed that Lxn−/− mice had a much faster recovery in hematopoietic stem/progenitor cells from 5-FU-induced stress.

HSCs have served as a fundamental model for the study of stem cell biology and as a vital therapeutic modality for the treatment of hematopoietic malignancies and BM failure syndromes (Daley, 2012; Doulatov et al., 2012). Our results show that inhibition of Lxn activity leads to
the controlled expansion of HSC population without impairment of self-renewal capacity and induction of malignant hyperplasia. Therefore, Lxn might be a promising genetic target for HSC expansion. More importantly, since Lxn was identified by the genetic diversity underlying HSC natural variations, it is highly likely that Lxn may function similarly in humans and manipulation of Lxn activity could provide translational impact (Van Zant and Liang, 2009; Doulatov et al., 2012).

**EXPERIMENTAL PROCEDURES**

**Animals**

C57BL/6 mice and B6.SJL/Boj (CD45.1) recipient mice were purchased from The Jackson Laboratory. Latexitn constitutive knockout mice (Lxn−/−) were generated by TaconicArtemis. Thbs1 heterozygous knockout mice (Thbs1+/-) were kindly provided by Dr. Shuxia Wang (Li et al., 2016; Maimaitiyiming et al., 2016). All mice used were 8–12 weeks old. Mice were housed in the University of Kentucky animal facilities following NIH-mandated guidelines for animal welfare and with IACUC approval. Complete blood count was performed on a Hemavet 950 (Drew Scientific). 5-FU was intraperitoneally injected at a concentration of 100 mg/kg body weight.

**Immunostaining and Flow Cytometry**

HSCs, HPCs, and stromal cells: BM cells were stained with lineage antibodies, including CDS (clone S3-7.3), CD8a (clone S3-6.7), CD4SR/B220 (clone RA3-6B2), CD11b/MAC-1 (clone M1/70), LY-6G/GR-1 (clone RB6-8C5), and TER119/Ly-76 (clone TER-119) antibodies, including CD5 (clone 53–7.3), CD8a (clone 53–6.7), CD45R/B220 (clone RA3-6B2), CD11b/MAC-1 (clone M1/70), LY-6G/GR-1 (clone RB6-8C5), and TER119/Ly-76 (clone TER-119), APC-Cy7, and stem cell markers, C-KIT-APC, SCA-1-PE-Cy5.5, CD34-FTTC, and FLT3-PE. HPCs were stained with FcyR and IL7R. HSC/HPC-enriched LSK cells, LT-HCSs and ST-HSCs, MPPs, and HPCs, including CMP, GMP, megakaryocyte/erythroid progenitor (MEP) and CLP cells were immunophenotypically defined as different markers. BM megakaryocytes and endothelial cells were stained with CD41 (clone MWR/REG30) and CD31 (clone MEC13.3), respectively. Dead cells were excluded by PI selection. PB lineage chimera staining was stained with CD45.2 and CD45.1 markers and MAC-1/GR1, THY1, and B220 markers. Cell-cycle analysis was performed using Ki-67 and 7-aminoactinomycin D (7-AAD) staining. In vivo BrdU incorporation was used to study cell-cycle dynamics. BrdU was administered in drinking water (0.5 mg/mL) for 3 days and the percentage of BrdU+ LT-HSCs was analyzed daily. Apoptosis was performed with Annexin V and 7-AAD. Antibodies were purchased from eBioscience, BioLegend, or BD Pharmingen. Negative controls for fluorescence-activated cell sorting (FACS) gating was set by cells without any antibody staining. Flow cytometry was performed on a FACSAria II (Becton Dickinson) and the data were analyzed using FlowJo software (TreeStar).

**In Vitro Colony Assays**

(1) CFC assay was performed as described previously in complete MethoCult medium (STEMCELL Technologies), Colony formation was observed on days 7 and 14 (Liang et al., 2005). (2) CAFC assay was performed as described previously (Liang et al., 2007). The most primitive HSCs showed cobblestones at day 35, whereas HPCs show colonies at early dates, such as CAFC day 7. CAFC frequencies were calculated by using L-Calc limiting dilution analysis software (STEMCELL Technologies).

**BM Transplantation**

In competitive repopulation assays, 1×10⁶ donor cells from Lxn−/− or WT BM (CD45.2) mice were mixed with an equal number of competitor BM cells (B6.SJL/Boj) and retro-orbitally injected into lethally irradiated (9 Gy) recipient mice. For transplantation of Thbs1-overexpressing Lxn−/− cells, sorted 1×10⁵ GFP+ cells that were either Thbs-1 overexpression or empty vector control Lxn−/− BM cells were mixed with 2×10⁵ competitor cells, and injected into B6.SJL/Boj recipient mice. Percentages of donor (CD45.2 or GFP+)-derived PB and BM cells were determined at 16 weeks post transplantation (Liang et al., 2007).

**Microarray Analysis**

Microarray was performed on LT-HSCs and MPPs in the Microarray Facility Center at the University of Kentucky. In brief, nearly 100,000 LT-HSCs and MPPs were sorted from pooled marrows of 10–20 WT or Lxn−/− mice. Total RNA was extracted using RNeasy Kit (Qiagen) and processed onto the Affymetrix Gene Chip MoGene-1_0-st-v1 Arrays. Three independent samples were obtained from each strain. Microarray data were normalized by the robust multiarray averaging method. The limma package (Smyth, 2004) in R/Bioconductor was used to determine the differentially expressed genes. In GSEA (Subramanian et al., 2005) (www.broadinstitute.org/gsea/index.jsp), genes were preranked based on the absolute values of their limma test statistics. Pathways in the Kyoto Encyclopedia of Genes and Genomes database were investigated. Enriched pathways were identified with false discovery rate less than 0.2.

**mRNA and Protein Quantification**

Quantitative real-time PCR was performed as described previously (Liang et al., 2007) with a commercially available primer/probe mix for Thbs1 in ABI PRISM 7700 (Applied Biosystems). Western blot was performed as described previously (Liang et al., 2007). Antibodies were anti-GFM-1 (Abcam), anti-THBS-1 (Cell Signaling Technology), anti-LNX, and anti-Actin (Sigma).

**Overexpression of Thbs-1 in BM Cells**

Flow cytometry sorted LSK cells from C57/BL6 or Lxn−/− mice were stimulated with cytokines including 100 ng/mL FMS-like tyrosine kinase-3 ligand, 50 ng/mL mouse stem cell factor, 10 ng/mL interleukin-3 (IL-3), and 10 ng/mL IL-6 in StemSpan SFEM (STEMCELL Technologies). After 24 hr, the cells were transduced with lentiviral particles encoding either mThbs1 (catalog no. EX-Mm05712-Lv165), mLxn (catalog no. EX-Mm03695-Lv165), or its related empty vector (catalog no. EX-NEG-Lv165), at an MOI of 100 for 6 hr at 37°C. All lentiviral particles were premade and purchased from GeneCopoeia. After 48 hr, the GFP-positive cells were sorted for real-time PCR, western blotting, CAFC assay, and transplantation assays. In transplantation assay, 1×10⁴ GFP+ cells plus 2×10⁵ competitor B6.SJL/Boj BM cells were injected into
B6.SJL/BoyJ mice, and GFP+ chimerism in PB and BM was measured at 16 weeks post transplantation. The CAFC assay of GFP+ cells was as described above.

**Statistical Analysis**

Data were examined for homogeneity of variances (F test), then analyzed by Welch’s t test or one-way ANOVA with post hoc analysis by Tukey’s test. Differences were considered significant at p < 0.05. All statistical analyses were conducted using SPSS 16.0 for Windows.

**ACCESSION NUMBERS**

The accession number for all microarray data reported in this paper is GEO: GSE94665.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.02.009.

**AUTHOR CONTRIBUTIONS**


**ACKNOWLEDGMENTS**

Research reported in this publication was supported by the National Heart, Lung, and Blood Institute of the NIH under Award Number RO1HL124015 (Y. Liang), the Edward P. Evans Foundation (S.B., D.S.C., D.Z., H.G., and Y. Liang), and the Biostatistics and Bioinformatics Shared Resource(s), Flow Cytometry Core of the University of Kentucky Markey Cancer Center (P30CA177558). We thank Carol Swiderski, Yanan You, and Rong Wen for technical assistance, and Garretson Epperly in the Imaging Core of the University of Kentucky Markey Cancer Center (P30CA177558). We thank Carol Swiderski, Yanan You, and Rong Wen for technical assistance, and Garretson Epperly in the Imaging Core of the University of Kentucky Markey Cancer Center for confocal microscope imaging assistance, and the Markey Cancer Center’s Research Communications Office for editing and graphics support.

Received: June 14, 2016
Revised: February 9, 2017
Accepted: February 10, 2017
Published: March 16, 2017

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