

# CLEC-2 in megakaryocytes is critical for maintenance of hematopoietic stem cells in the bone marrow

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**Hematopoietic stem cells (HSCs) depend on the bone marrow (BM) niche for their maintenance, proliferation, and differentiation. The BM niche is composed of nonhematopoietic and mature hematopoietic cells, including megakaryocytes (Mks). Thrombopoietin (Thpo) is a crucial cytokine produced by BM niche cells. However, the cellular source of Thpo, upon which HSCs primarily depend, is unclear. Moreover, no specific molecular pathway for the regulation of Thpo production in the BM has been identified. Here, we demonstrate that the membrane protein C-type lectin-like receptor-2 (CLEC-2) mediates the production of Thpo and other factors in Mks. Mice conditionally deleted for CLEC-2 in Mks (*Clec2<sup>MkΔ/Δ</sup>*) produced lower levels of Thpo in Mks. CLEC-2-deficient Mks showed down-regulation of CLEC-2-related signaling molecules *Syk*, *Lcp2*, and *Plcg2*. Knockdown of these molecules in cultured Mks decreased expression of *Thpo*. *Clec2<sup>MkΔ/Δ</sup>* mice exhibited reduced BM HSC quiescence and repopulation potential, along with extramedullary hematopoiesis. The low level of Thpo production may account for the decline in HSC potential in *Clec2<sup>MkΔ/Δ</sup>* mice, as administration of recombinant Thpo to *Clec2<sup>MkΔ/Δ</sup>* mice restored stem cell potential. Our study identifies CLEC-2 signaling as a novel molecular mechanism mediating the production of Thpo and other factors for the maintenance of HSCs.**

Maintenance of hematopoietic stem cells (HSCs) within the adult BM is crucial for the healthy production of hematopoietic cells (Orkin and Zon, 2008). HSCs reside in a specialized microenvironment in the BM called the niche (Schofield, 1978). Along with cell-intrinsic programs, the niche influences the cell fate of HSCs, which in turn govern the homeostasis of the hematopoietic system (Nakamura-Ishizu et al., 2014a). The HSC niche is chiefly composed of nonhematopoietic cells, including immature osteoblasts (OBLs; Arai and Suda, 2007), endothelial cells (ECs; Butler et al., 2010; Ding et al., 2012), perivascular cells (Sugiyama et al., 2006; Ding et al., 2012), mesenchymal stem cells (MSCs; Méndez-Ferrer et al., 2010), sympathetic nervous cells (Katayama et al., 2006), adipocytes (Naveiras et al., 2009), and nonmyelinating Schwann cells (Yamazaki et al., 2011). Nonetheless, mature hematopoietic cells such as macrophages/monocytes (Chow et al., 2011), osteoclasts (Kollet et al., 2006), and regulatory T cells (Fujisaki et al., 2011) also regulate HSCs, albeit mainly in an indirect manner, through the

modulation of nonhematopoietic niche cells. Recently, mature megakaryocytes (Mks) were described as hematopoietic progeny that directly regulate HSC quiescence (Heazlewood et al., 2013; Bruns et al., 2014; Zhao et al., 2014; Nakamura-Ishizu et al., 2014b); one of the mechanisms underlying Mk niche function is the production of the cytokine thrombopoietin (Thpo) by Mks themselves (Nakamura-Ishizu et al., 2014b). However, among the Mk-related niche factors reported to date, no molecular mechanism that is specific to Mks has been identified.

Thpo is a crucial cytokine for both the maturation of Mks and the maintenance of quiescent HSCs (Zucker-Franklin and Kaushansky, 1996; Qian et al., 2007; Yoshihara et al., 2007). Thpo is produced in multiple organs, including the liver, kidney, spleen, and muscle (Nomura et al., 1997). Baseline production of serum Thpo is thought to be maintained by the liver and regulated in response to inflammatory stress or changes in glycosylation of aged platelets (Kaser et al., 2001; Stone et al., 2012; Grozovsky et al., 2015). Serum Thpo levels also fluctuate according to circulating platelet number: platelets sequester Thpo via the myeloproliferative leukemia virus oncogene (c-Mpl), the receptor for Thpo (Kuter and Rosenberg,

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Abbreviations used: BMT, BM transplantation; EC, endothelial cell; HSC, hematopoietic stem cell; HSPC, hematopoietic stem and progenitor cell; IHC, immunohistochemistry; LT-HSC, long-term HSC; Mk, megakaryocyte; MNC, mononuclear cell; MSC, mesenchymal stem cell; OBL, osteoblast; PB, peripheral blood; Thpo, thrombopoietin.

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1995; de Graaf et al., 2010), thereby lowering *Thpo* levels. Thus, platelet number is not as tightly regulated by *Thpo* production as erythrocyte number is by erythropoietin production (Fandrey and Bunn, 1993). It is likely that BM HSCs depend on *Thpo*, which is produced in the BM by niche cells. Depletion of circulating platelets by neuraminidase does not affect HSCs (Bruns et al., 2014), indicating that serum *Thpo* up-regulation through thrombocytopenia does not affect HSC maintenance. Moreover, HSCs reside near bone-lining OBLs and mature Mk, which both support HSCs by producing *Thpo* (Yoshihara et al., 2007; Nakamura-Ishizu et al., 2014b). However, the main cellular source of *Thpo*, upon which BM HSCs depend, and the molecular signaling pathway that mediates BM *Thpo* production remain elusive.

Recent studies showed that signals mediated through C-type lectin-like domain-containing receptors (CLEC-4H1 and CLEC-4H2; also known as Ashwell–Morell receptor) stimulate *Thpo* production in hepatocytes through recognition of desialylated platelets (Grozovsky et al., 2015). Platelets and Mk express CLEC-2 (Suzuki-Inoue et al., 2006, 2007), which is among the top 25 genes specifically expressed on Mk (Senis et al., 2007). Activation of platelet CLEC-2 through binding to sialylated podoplanin is essential for the segregation of lymphatic and blood vessels during development (Bertozzi et al., 2010; Suzuki-Inoue et al., 2010). CLEC-2–podoplanin signaling also functions in maintenance of lymphocyte- and dendritic cell–related responses in the stroma of lymph nodes (Acton et al., 2012, 2014; Herzog et al., 2013).

The significance of CLEC-2 expression on Mk in BM hematopoiesis, and whether it is involved in *Thpo* production in Mk, has not been previously explored. Here, we demonstrate that Mk-specific deficiency of CLEC-2 disrupts HSC quiescence and alters HSC potential as a result of defective Mk niche function. Moreover, we demonstrate that CLEC-2 signaling is involved in various molecular pathways for production of niche factors, including *Thpo* in Mk. Through the identification of CLEC-2, a novel Mk-specific factor, our data elucidate the organ-dependent production and function of *Thpo* and reinforce the idea that Mk contribute to a niche that regulates HSC quiescence.

## RESULTS

### CLEC-2 is highly expressed on BM Mk

CLEC-2 expression was detected in platelets, dendritic cells, and liver sinusoidal endothelia (Suzuki-Inoue et al., 2011); however, the expression of CLEC-2 in BM has not been previously investigated. Using immunohistochemistry (IHC), we observed expression of CLEC-2 protein on the surface of Mk and ECs in the BM (Fig. 1 A). Hematopoietic stem and progenitor cell (HSPC) fractions also expressed *Clec2* transcripts (Fig. 1 B). Flow cytometry analysis confirmed surface CLEC-2 expression on HSPCs (Fig. 1 C). CLEC-2 protein expression was also compared in various hematopoietic (Fig. 1 D) and niche cells (Fig. 1 E). Among the hematopoietic and niche cells, CLEC-2 expression on CD41<sup>+</sup> Mk and ECs was significantly high.

We investigated mice deficient in CLEC-2 specifically in the Mk lineage (*PF4-Cre:Clec2<sup>lox/lox</sup>* mice [*Clec2<sup>MkΔ/Δ</sup>*]), which are born and survive through adulthood (Osada et al., 2012). PCR analysis confirmed that *Clec2* deletion was specific to Mk lineages: deletion was detected in genomic DNA from CD41<sup>+</sup> (glycoprotein IIb<sup>+</sup>) cells, but not LSK (Lineage negative, Sca-1<sup>+</sup>, c-Kit<sup>+</sup>) cells, in the BM of *Clec2<sup>MkΔ/Δ</sup>* mice (Fig. 1 F). CLEC-2 protein level was significantly reduced in platelets from *Clec2<sup>MkΔ/Δ</sup>* mice but retained in their long-term HSCs (LT-HSCs; Fig. 1 G). Deletion of CLEC-2 from Mk was also confirmed with IHC (Fig. 1 H). *Clec2<sup>MkΔ/Δ</sup>* mice exhibited mild thrombocytopenia along with anemia (Fig. 1 I).

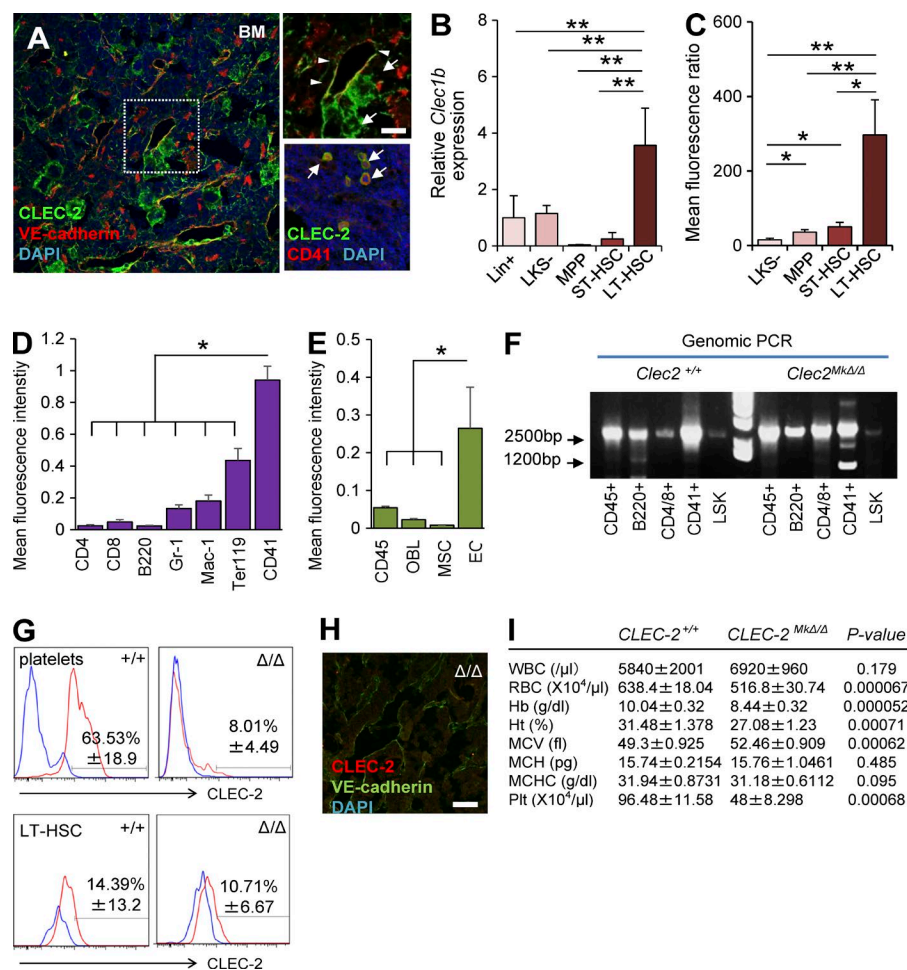
### CLEC-2-deficient mice have impaired Mk cell population

Because CLEC-2 is highly expressed in Mk lineages, we asked whether CLEC-2 deficiency altered the frequency and BM functions of Mk lineage cells. The frequencies and numbers of mature Mk and Mk progenitors (MkP and Pre MegE cells) were unaltered in *Clec2<sup>MkΔ/Δ</sup>* mice (Fig. 2, A–D), and total CD41<sup>+</sup> Mk cell number was unaltered in *Clec2<sup>MkΔ/Δ</sup>* mice (Fig. 2 E). However, ploidy analysis revealed a reduced frequency of mature polyploid Mk with CLEC-2 deficiency (Fig. 2, F and G). IHC revealed a significant reduction in the number of Mk in the metaphyseal region of the BM in *Clec2<sup>MkΔ/Δ</sup>* mice (Fig. 2 H). These observations indicate that CLEC-2 deficiency modestly affects the maturation of Mk but drastically alters the distribution of Mk within the BM.

### CLEC-2-deficient Mk exhibit impaired *Thpo* production and niche function

Mk can support HSC expansion in vitro, chiefly through the production of *Thpo* (Nakamura-Ishizu et al., 2014b). Hence, we asked whether CLEC-2 deficiency would alter the capacity of Mk to support HSCs in vitro. Addition of Mk to HSC cultures resulted in a *Thpo*-dependent increase in HSC number (Nakamura-Ishizu et al., 2014b). Accordingly, addition of *Clec2<sup>+/+</sup>* Mk to cultured HSCs promoted proliferation, as indicated by expansion of LSK cells and LT-HSCs (Fig. 3, A and B). *Clec2*-deficient Mk exhibited a reduced capacity to stimulate HSPC proliferation and could not maintain LT-HSC populations (Fig. 3 B).

Next, we assessed whether *Clec2*-deficient Mk exhibited changes in the production of various niche factors. *Clec2*-deficient Mk exhibited a broad range of decrease in the gene expression of various niche factors, including *Angpt1*, *Cxcl12*, *IL-7*, *Spp1*, *Tnfrsf1*, and *Pf4* (Fig. 3 C). Characteristically, *Clec2*-deficient Mk secreted less *Thpo* and altered Mk niche function. *Clec2*-deficient Mk exhibited significantly lower *Thpo* expression than Mk from *Clec2<sup>+/+</sup>* mice (Fig. 3 C). Furthermore, wild-type Mk treated with CLEC-2–stimulating antibodies expressed significantly higher levels of *Thpo* transcripts (Fig. 3 D). The decrease of *Thpo* protein expression in CLEC-2–deficient Mk was confirmed by IHC (Fig. 3 E).



**Figure 1. CLEC-2 is expressed in Mks and is specifically deleted in Mks of *Clec2<sup>MkΔ/Δ</sup>* mice.** (A) IHC of BM of 10-wk-old wild-type mice. CLEC-2 stains for VE-cadherin-positive ECs (arrowheads) and VE-cadherin-negative Mks (arrows). Enlargement of the dotted box is shown in the top right panel. CLEC-2 stains for CD41-positive Mks (bottom right). (B) Relative expression of CLEC-2 (*Clec2b*) transcripts in various hematopoietic cells. (C) Calculation of mean fluorescence ratios of CLEC-2 in various HSPC populations as measured by flow cytometry. (D and E) Mean fluorescence intensity of various hematopoietic (D) and niche (E) cells. (B–E) Means ± SD. *n* = 4; two independent experiments. \*, *P* < 0.05; and \*\*, *P* < 0.01 by Tukey's test. (F) Detection of *Clec2* deletion by genomic PCR. An intact exon yields a 2,500-bp product, whereas removal of floxed exon 1 results in a 1,200-bp product. (G) Expression of CLEC-2 in platelets and LT-HSCs from *Clec2<sup>MkΔ/Δ</sup>* (Δ/Δ) and *Clec2<sup>+/+</sup>* (+/+) mice. Red, CLEC-2 antibody-stained; blue, isotype control. Means ± SD. *n* = 5; two independent experiments. *P* = 0.0078 for platelets and *P* = 0.69 for LT-HSCs by Student's *t* test. (H) IHC of BM staining CLEC-2 and VE-cadherin in *Clec2<sup>MkΔ/Δ</sup>* (Δ/Δ) mice. Bars: (A and H) 100 μm; (A, top right) 25 μm. (I) Measurement of PB parameters in *Clec2<sup>MkΔ/Δ</sup>* (Δ/Δ) and *Clec2<sup>+/+</sup>* (+/+) mice. Means ± SD. *n* = 5; three independent experiments. *P*-values as indicated by Student's *t* test.

Transcript expression of CLEC-2 signaling pathway genes were decreased in CLEC-2-deficient Mks (Fig. 3 F). To confirm that CLEC-2 signaling affected Thpo production in Mks, we used inducible shRNAs to knock down three downstream molecules involved in CLEC-2 signaling: *Syk*, *Lcp2*, and *Plcg2* (Suzuki-Inoue et al., 2011). Cultured LT-HSCs were transduced with lentiviral clones that inducibly express each shRNA and then cultured to obtain CD41<sup>+</sup>Ter119<sup>+</sup> Mks. Knockdown of *Syk*, *Lcp2*, and *Plcg2* was highly efficient: transcripts of the targeted genes were nearly absent in sorted Mks (not depicted). Knockdown of *Syk*, *Lcp2*, or *Plcg2* significantly decreased the expression of Thpo in the sorted Mks (Fig. 3 G). These data confirmed that CLEC-2 signaling is indeed critical for Thpo production in Mks.

### CLEC-2 deficiency in Mks directly affects HSC cycle quiescence

Because CLEC-2-deficient Mks exhibited diminished Thpo production and could not support cultured HSCs, we next assessed whether HSCs in *Clec2<sup>MkΔ/Δ</sup>* mice were affected in vivo. Lineage and HSPC differentiation were unchanged in the BM of *Clec2<sup>MkΔ/Δ</sup>* mice (Fig. 4, A–C). Functionally, HSPCs from *Clec2<sup>MkΔ/Δ</sup>* mice displayed a significant eleva-

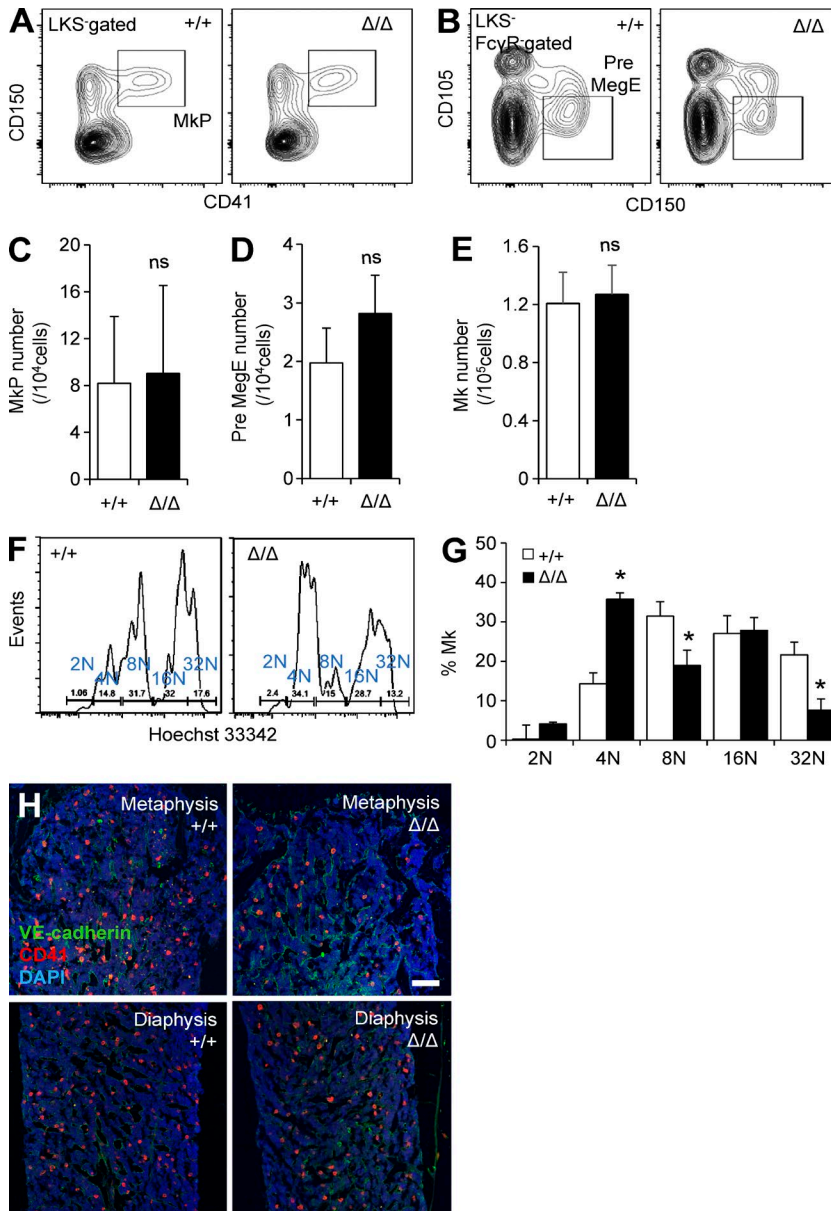
tion in colony-forming capacity, indicating an increase in the proliferation capacity of HSPCs (Fig. 4, D and E). Cell cycle analysis of HSCs, as assessed by Pyronin Y staining, indicated loss of HSC quiescence (Pyronin Y-negative CD34<sup>+</sup> LSK cells) in *Clec2<sup>MkΔ/Δ</sup>* mice (Fig. 4, F and G). c-Mpl expression was significantly up-regulated in LT-HSCs from *Clec2<sup>MkΔ/Δ</sup>* mice, suggestive of deficient Thpo signaling (Fig. 4 H). Loss of nonhematopoietic niche cells (ECs, MSCs, and OBLs) was not observed in BM of *Clec2<sup>MkΔ/Δ</sup>* mice (Fig. 4, I and J).

Although CLEC-2 is also expressed on ECs, EC-specific CLEC-2 deletion did not alter hematopoiesis: *VE-cadherin-Cre:Clec2<sup>flxed/flxed</sup>* mice exhibited no change in peripheral blood (PB) parameters (not depicted), HSPC numbers, HSC quiescence, or repopulation potential (not depicted). These observations indicate that Mk-specific *Clec2* deficiency stimulates HSCs to exit the G<sub>0</sub> phase of the cell cycle.

### HSCs from *Clec2<sup>MkΔ/Δ</sup>* mice exhibit reduced stem cell potential

To further investigate the stem cell potential of HSCs from *Clec2<sup>MkΔ/Δ</sup>* mice, we performed a competitive repopulation assay, using BM transplantation (BMT) of LT-HSCs from *Clec2<sup>+/+</sup>* or *Clec2<sup>MkΔ/Δ</sup>* mice (Ly5.2) into C57BL/6-Ly5.1



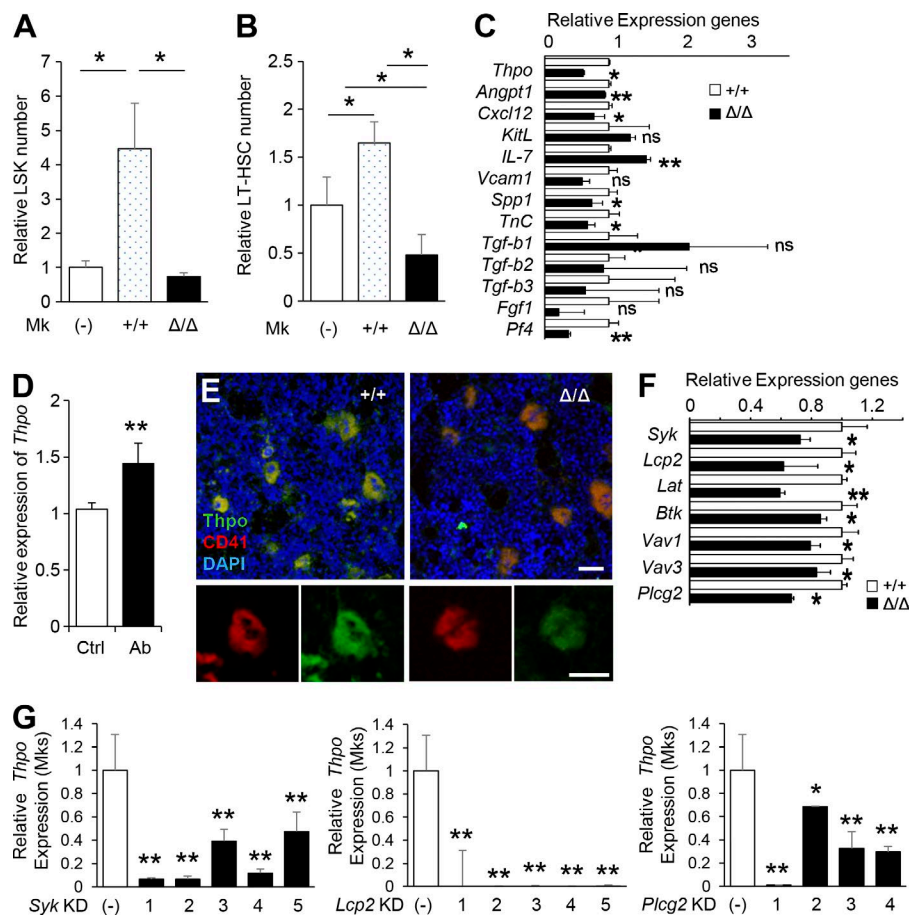


**Figure 2. Altered distribution of Mks in BM of *Clec2<sup>MkΔ/Δ</sup>* mice.** (A and B) Representative flow cytometric plots showing Mk progenitor cell population (MkP: Lin<sup>-</sup>cKit<sup>+</sup>Sca-1<sup>+</sup>[LKS<sup>-</sup>] CD150<sup>+</sup>CD41<sup>+</sup>; Pre-MegE: LKS<sup>-</sup>FcyR<sup>-</sup>CD105<sup>+</sup>CD150<sup>+</sup>) in *Clec2<sup>MkΔ/Δ</sup>* (Δ/Δ) and *Clec2<sup>+/+</sup>* (+/+) mice. (C–E) Number of MkP (C), Pre-MegE (D), and CD41<sup>+</sup> Mks (E) in Δ/Δ and +/+ mice. Means ± SD. *n* = 5; two independent experiments. ns, *P* = 0.46 for C, *P* = 0.24 for D, and *P* = 0.24 for E by Student's *t* test. (F and G) Representative flow cytometric plot showing Mks (F) and Mk frequency within the total Mk population (G), based on ploidy analysis of +/+ and Δ/Δ mice. Means ± SD. *n* = 4; two independent experiments. \*, *P* < 0.05 by Tukey's test. (H) IHC of BM, staining for ECs (VE-cadherin), Mks (CD41), and DAPI in the metaphysis and diaphysis of +/+ and Δ/Δ mice. Bar, 100 μm.

recipients. HSCs from *Clec2<sup>MkΔ/Δ</sup>* mice had lower engraftment rates, as assessed by chimerism in PB and BM of various HSPC fractions 4 mo after primary and secondary BMT (Fig. 5, A–D). No significant difference was noted in the lineage differentiation of engrafted HSCs from *Clec2<sup>+/+</sup>* or *Clec2<sup>MkΔ/Δ</sup>* mice (Fig. 5 E). Limiting dilution analysis revealed a significantly lower frequency of repopulating HSCs in the BM of *Clec2<sup>MkΔ/Δ</sup>* mice (Fig. 5 F). HSCs from *Clec2<sup>MkΔ/Δ</sup>* mice did not exhibit defective homing to the BM (Fig. 5 G). In addition, HSCs from *Clec2<sup>MkΔ/Δ</sup>* mice did not exhibit higher rates of apoptosis, as assessed by Annexin V staining (Fig. 5 H). Gene set enrichment analysis of LT-HSCs from *Clec2<sup>MkΔ/Δ</sup>* mice revealed altered HSC-related gene expression profiles, notably an enrichment in progenitor cell-related genes, relative to LT-HSCs from *Clec2<sup>+/+</sup>* mice, suggesting

enhanced differentiation and a loss of stem cell character of HSCs from *Clec2<sup>MkΔ/Δ</sup>* mice (Fig. 5 I). These results indicate a loss of stem cell potential in *Clec2<sup>MkΔ/Δ</sup>* mice.

*Clec2<sup>MkΔ/Δ</sup>* mice have major developmental vascular defects, chiefly in the lymphatic system (Suzuki-Inoue et al., 2010; Finney et al., 2012). To verify that the loss of stem cell potential resulted specifically from *Clec2* deletion in BM Mk lineages, we transplanted BM mononuclear cells (MNCs) from *Clec2<sup>+/+</sup>* or *Clec2<sup>MkΔ/Δ</sup>* mice (Ly5.2) along with BM MNCs from Ly5.1 mice at a 4:1 ratio into lethally irradiated Ly5.1 wild-type mice to test the effect of *Clec2*-deleted Mks on wild-type HSCs (Fig. 6 A). We observed a high frequency of Ly5.2<sup>+</sup> Mks in recipient BM (Fig. 6 B). The number of engrafted Ly5.1 LT-HSCs (CD34<sup>+</sup>Flt-3<sup>-</sup> HSCs and CD150<sup>+</sup>CD48<sup>-</sup>CD41<sup>-</sup> [SLAMF] HSCs) was significantly lower



**Figure 3. CLEC-2-positive Mks produce Thpo.** (A and B) Effect of 3 d of co-culture with Mks from Δ/Δ or +/+ mice on LSK cells (A) and LT-HSCs (B). Means ± SD. *n* = 4; two independent experiments. \*, *P* < 0.05 by Tukey's test. (C) Relative levels of niche factor (*Thpo*, *Angpt1*, *Cxcl12*, *Kitl*, *Il-7*, *Vcam1*, *Spp1*, *Tnfrsf1*, *Tgfb1*, *Tgfb2*, *Tgfb3*, *Fgf1*, and *Pf4*) transcripts in Mks from Δ/Δ mice compared with +/+ mice. (D) Relative levels of *Thpo* transcripts were significantly up-regulated in cultured Mks treated with a CLEC-2 stimulatory antibody. (E) IHC of BM from +/+ and Δ/Δ mice. Mks, which are CD41, exhibit lower *Thpo* levels in CLEC-2-deficient Mks. Magnification of a representative Mks is shown in the bottom panels. Bars: (top) 50 μm; (bottom) 100 μm. (F) Gene expression of CLEC-2 downstream pathway molecules in Mks from +/+ and Δ/Δ mice. (G) Knockdown of *Syk*, *Lcp2*, and *Plcg2* results in reduced expression of *Thpo* transcript in Mks. (C, D, F, and G) Means ± SD. *n* = 4; two independent experiments. ns, *P* > 0.05; \*, *P* < 0.05; and \*\*, *P* < 0.01 by Student's *t* test.

in transplants from *Clec2<sup>Mkd/Δ</sup>* mouse donors (Fig. 6 C). Furthermore, the percentage of Ly5.1<sup>+</sup> Pylonin Y-negative HSCs was significantly lower when *Clec2<sup>Mkd/Δ</sup>* mice served as donors (Fig. 6, D and E). Also, both Ly5.1<sup>+</sup> and Ly5.2<sup>+</sup> HSCs in the recipient of *Clec2<sup>Mkd/Δ</sup>* mouse donors stained higher levels of Ki67 (Fig. 6 F), indicating loss of cell cycle quiescence. These data suggest that the HSC defects exhibited by *Clec2<sup>Mkd/Δ</sup>* mice are a direct consequence of *Clec2* depletion in Mks.

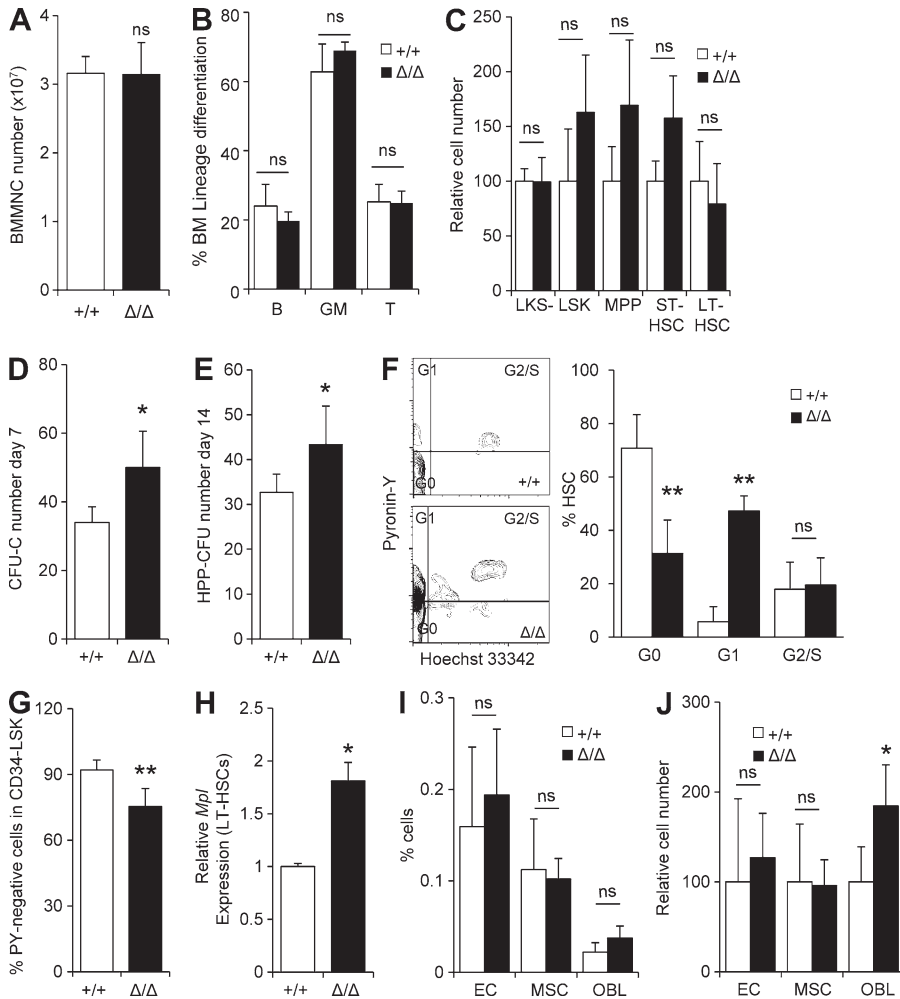
### Mk-specific CLEC-2 depletion results in extramedullary hematopoiesis

We next asked whether CLEC-2 deficiency affected HSC retention in the BM. *Clec2<sup>Mkd/Δ</sup>* mice exhibited massive splenomegaly (Fig. 7, A and B). Both HSC and HSPC number and frequency were elevated in *Clec2<sup>Mkd/Δ</sup>* mice (Fig. 7, C–E), indicating that the splenomegaly was caused by extramedullary hematopoiesis. PB of HSC *Clec2<sup>Mkd/Δ</sup>* mice exhibited increased numbers of LSKs and LT-HSCs (Fig. 7 F). Furthermore, a significant increase in CFU-C and HPC-CFC was observed in the PB of *Clec2<sup>Mkd/Δ</sup>* mice (Fig. 7, G and H), and the distance between LT-HSCs and Mks was significantly higher in *Clec2<sup>Mkd/Δ</sup>* mice than in *Clec2<sup>+/+</sup>* mice (Fig. 7 I). These data show that chronic loss of CLEC-2-expressing

Mks decreased HSC retention in BM and induced extramedullary hematopoiesis in the spleen.

### Administration of Thpo rescues HSC phenotypes in *Clec2<sup>Mkd/Δ</sup>* mice

To determine whether the functional defects in HSCs from *Clec2<sup>Mkd/Δ</sup>* mice could be attributed to reduced *Thpo* levels, we administered recombinant *Thpo* (PEG-rHuMGDF) to both *Clec2<sup>+/+</sup>* and *Clec2<sup>Mkd/Δ</sup>* mice. Intravenous *Thpo* injection for four consecutive days restored the number and frequency of polyploid Mks in *Clec2<sup>Mkd/Δ</sup>* mice (Fig. 8 A). In agreement with a previous study (Walter et al., 2015), administration of *Thpo* stimulated proliferation of HSCs, as shown by the increased the percentage of BrdU-positive HSCs and the percentage of Pylonin Y-negative cells in CD34-LSK cells in both *Clec2<sup>+/+</sup>* and *Clec2<sup>Mkd/Δ</sup>* mice (Fig. 8, B and C). However, as *Thpo* administration instigates self-renewal of HSCs, the absolute number and frequency of CD34<sup>+</sup> Pylonin Y-negative HSCs within the BM were restored (Fig. 8, D and E). *Thpo* administration also significantly increased the number of Mks within the BM in both *Clec2<sup>+/+</sup>* and *Clec2<sup>Mkd/Δ</sup>* mice (Fig. 8 F). The repopulation potential of HSCs from *Clec2<sup>Mkd/Δ</sup>* mice significantly increased with *Thpo* injection, as confirmed by competitive BMT (Fig. 8, G and H). Thus,



**Figure 4. HSCs from *Clec2*<sup>MkΔ/Δ</sup> mice exhibit reduced stem cell quiescence.** (A–C) Number of BM MNCs (A), lineage composition in the BM (B), and relative number of BM HSPCs (C) in +/+ and Δ/Δ mice. (D and E) CFU-C number (D) and HPP-CFC number (E) of 500 HSCs from Δ/Δ and +/+ mice. (A–E) Means  $\pm$  SD.  $n = 6$ ; two independent experiments. ns,  $P > 0.05$ ; and \*,  $P < 0.05$  by Student's *t* test. (F) Cell cycle analysis using Pyronin Y and Hoechst 33342 staining of CD34<sup>−</sup> LSK cells in +/+ and Δ/Δ mice. (G) Cell cycle state of CD34<sup>−</sup> LSK cells with Pyronin Y staining in +/+ and Δ/Δ mice. (H) Relative levels of *Mpl* transcripts in LT-HSCs from +/+ and Δ/Δ mice. (I and J) Number (I) and frequency (J) of ECs (CD45<sup>−</sup>Ter119<sup>−</sup>CD31<sup>+</sup>), MSCs (CD45<sup>−</sup>Ter119<sup>−</sup>Sca-1<sup>+</sup>ALCAM<sup>−</sup>), and OBLs (CD45<sup>−</sup>Ter119<sup>−</sup>CD31<sup>−</sup>Sca-1<sup>−</sup>ALCAM<sup>+</sup>) in Δ/Δ and +/+ mice. (F–J) Means  $\pm$  SD.  $n = 4$ ; two independent experiments. ns,  $P > 0.05$ ; \*,  $P < 0.05$ ; and \*\*,  $P < 0.01$  by Student's *t* test.

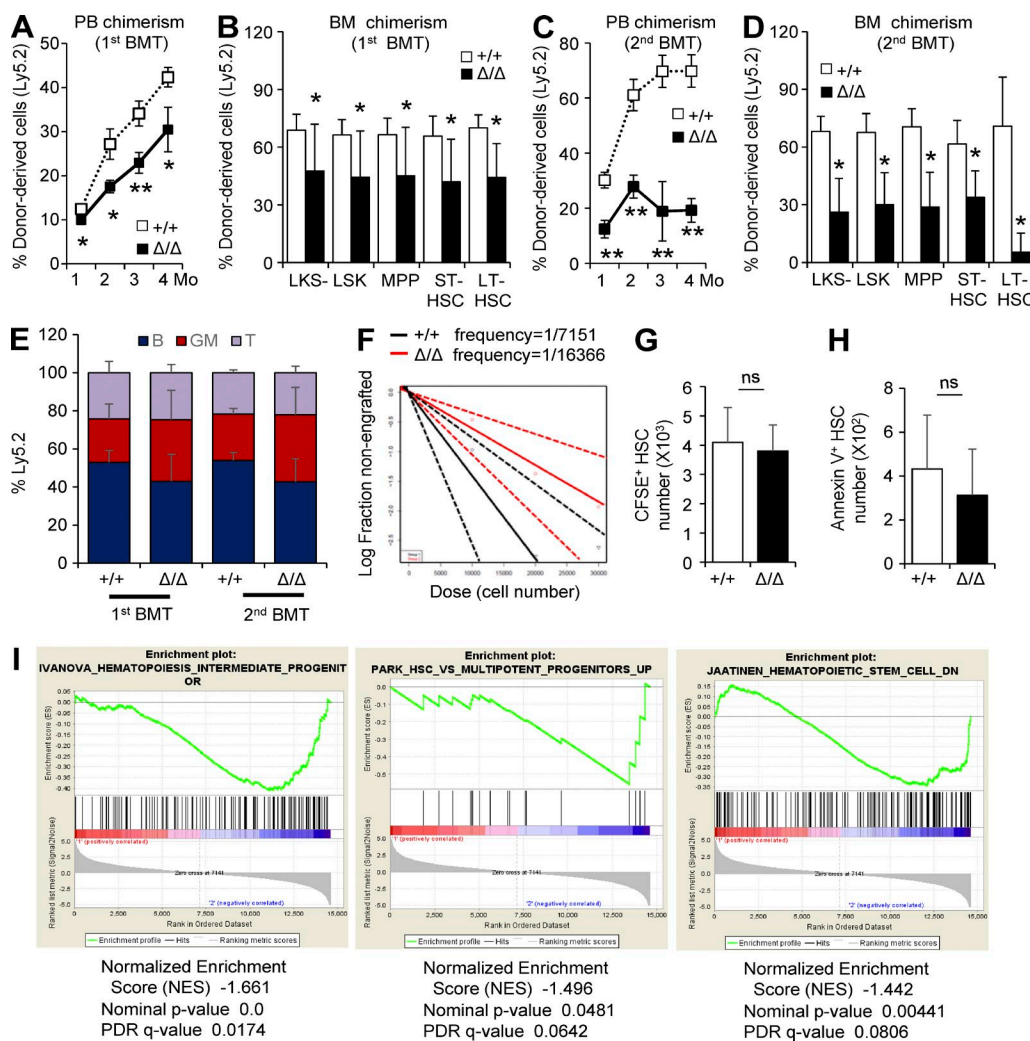
the loss of HSC potential in *Clec2*<sup>MkΔ/Δ</sup> mice depended on the defective Thpo production of Mks.

## DISCUSSION

In this study, we characterized the niche function of a C-type lectin-like family member, CLEC-2, in Mks. Mks from *Clec2*<sup>MkΔ/Δ</sup> mice exhibited reduced Thpo expression at both the gene and protein level. Knockdown of the CLEC-2 downstream pathway affected the expression of a broad range of niche factors in Mks, characteristically one being Thpo. Mk-specific deletion of CLEC-2 caused a subtle reduction in Mk number but significantly affected HSC quiescence and repopulation potentials. Administration of recombinant Thpo rescued HSC defects in *Clec2*<sup>MkΔ/Δ</sup> mice, indicating that defective Thpo production impaired HSC function in *Clec2*<sup>MkΔ/Δ</sup> mice. Thus, we demonstrated that CLEC-2 is an upstream factor essential for Mk-mediated maintenance of HSCs.

Our results identify Mk CLEC-2 signaling as a novel signaling pathway involved in the production of Thpo in the BM. Systemic Thpo levels depend on sequestration of Thpo by myeloproliferative leukemia protein (c-Mpl), a Thpo

receptor, expressed on platelets (Kuter and Rosenberg, 1995). However, Mks in *Clec2*<sup>MkΔ/Δ</sup> mice produced lower levels of Thpo, despite the presence of thrombocytopenia, indicating that systemic Thpo levels determined by the availability of c-Mpl did not affect Mk-produced Thpo for the maintenance of HSCs. It remains controversial whether liver production of Thpo can compensate for reduced systemic levels of this cytokine (McCarty et al., 1995; Qian et al., 1998). *Thpo* transcript levels in the liver increase with inflammatory stress and accumulation of desialylated platelets (Wolber et al., 2001; Grozovsky et al., 2015) but does not change in thrombocytopenic mouse models (Cohen-Solal et al., 1996). *Clec2*<sup>MkΔ/Δ</sup> mice exhibited no change in liver *Thpo* transcript levels (unpublished data), indicating that CLEC-2 deficiency did not stimulate liver Thpo production. Therefore, our data indicate that the BM Thpo level is loosely associated with serum and liver Thpo levels and that Mks supply a critical amount of Thpo for maintenance of HSCs in the BM. However, our data do not eliminate the possibility that Thpo production by the BM stroma is necessary to maintain HSCs in the BM. In fact, OBLs produce Thpo (Yoshihara



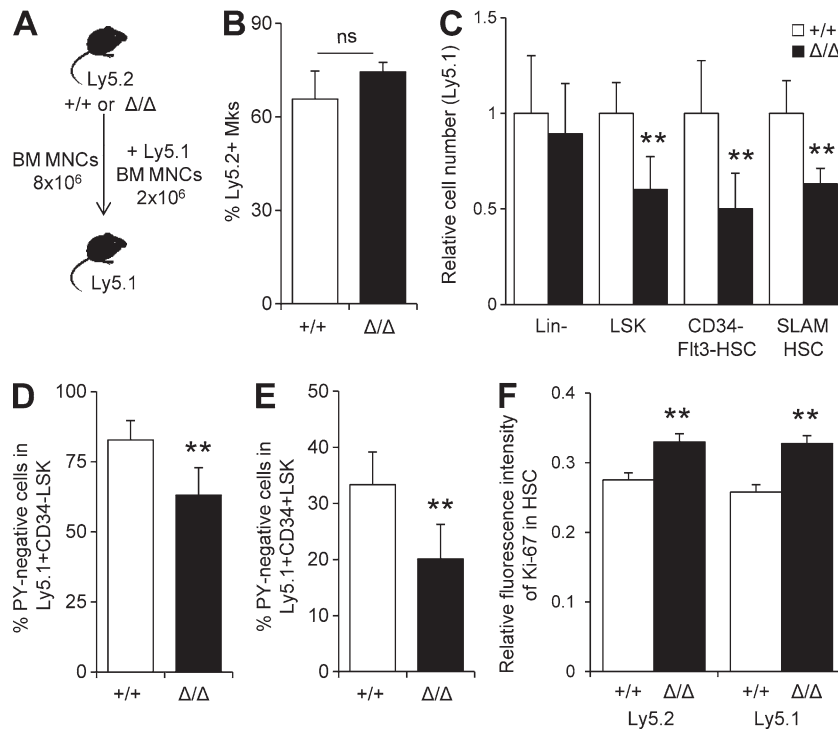
**Figure 5. HSCs from *Clec2*<sup>MkΔ/Δ</sup> mice exhibit reduced repopulation capacity.** (A and C) Percentage of donor-derived cells (Ly5.2) in PB after the first and second BMT at the indicated time intervals (in months). (B and D) Percentage of donor-derived cells (Ly5.2) in BM HSPCs after the first and second BMT at the indicated time intervals (in months). (A–D) Means ± SEM. *n* = 6; two independent experiments. \*, *P* < 0.05; and \*\*, *P* < 0.01 by Student's *t* test. (E) Lineage composition of Ly5.2 MNCs in recipient mice. Means ± SEM. *n* = 6; two independent experiments. *P* > 0.05 in all groups by Student's *t* test. (F) Extreme limiting dilution assay of BM MNCs from Δ/Δ and +/+ mice. *n* = 7; two independent experiments. *P* = 0.0432 by Pearson's  $\chi^2$  test. (G) Number of CFSE staining LT-HSCs from Δ/Δ and +/+ mice homing to the BM 24 h after BMT. Note that no significant difference was present in homing capacity of HSCs from Δ/Δ and +/+ mice. (H) Number of Annexin V<sup>+</sup> HSCs in Δ/Δ and +/+ mice. (G and H) Means ± SD. *n* = 4; two independent experiments. ns, *P* = 0.677 (G) or *P* = 0.48 (H) by Student's *t* test. (I) Gene set enrichment analysis of LT-HSCs from Δ/Δ and +/+ mice (*n* = 10). Genes expressed by intermediate and multipotent progenitors were significantly up-regulated in LT-HSCs from Δ/Δ mice (plots are second and third from left). Statistical analysis is as shown in figure.

et al., 2007), and the platelet  $\alpha$ -granule proteins PDGF and FGF-2 can stimulate expression of *Thpo* in BM stromal cells in vitro (Sungaran et al., 2000). Further studies are necessary to clarify the degree to which HSCs rely on locally or systemically produced *Thpo*.

Although HSCs from *Clec2*<sup>MkΔ/Δ</sup> mice were responsive to exogenous *Thpo*, indicating that the loss of stem cell potential of HSCs in *Clec2*<sup>MkΔ/Δ</sup> mice was caused by deficiency of *Thpo*, our data cannot differentiate whether *Thpo* directly or indirectly affected HSCs through the modulation of Mk number and function. Indeed, exogenous *Thpo* administration

robustly enhances Mk proliferation and Mk can produce various factors other than *Thpo* that can affect HSC stem cell potential (Bruns et al., 2014; Zhao et al., 2014). Supporting this, although our analysis revealed *Thpo* as a prominent niche factor affected by CLEC-2 depletion, CLEC-2 deficiency also affected other niche-related gene expressions (such as *Cxcl12*, *Angpt1*, *Vcam1*, and *Pf4*) in Mk. In addition, CLEC-2-deficient Mk exhibited decrease in various CLEC-2 downstream pathways. These data may indicate that CLEC-2 signaling may be involved in the modulation of a broad range of Mk functions. Our data further position Mk





**Figure 6. Loss of HSC quiescence in HSCs in *Clec2<sup>MkΔ/Δ</sup>* mice is Mk specific.** (A) Scheme of the experimental model for BMT.  $8 \times 10^6$  BM MNCs from  $\Delta/\Delta$  and  $+/+$  mice were transplanted into lethally irradiated (Ly5.1) mice along with Ly5.1-positive  $2 \times 10^6$  BM MNCs. (B) Percentage of Ly5.2-positive Mks in BMs of recipient mice. (C) Relative number of Ly5.1-positive HSPCs in recipient mice. (B and C) Means  $\pm$  SD.  $n = 10$ ; two independent experiments. ns,  $P = 0.167$ ; \*,  $P < 0.05$ ; and \*\*,  $P < 0.01$  by Student's  $t$  test. (D) Ly5.1<sup>+</sup> Pyronin Y-negative cells in CD34<sup>+</sup> LSK cells in the BMs of recipient mice. (E) Ly5.1<sup>+</sup> Pyronin Y-negative cells in CD34<sup>+</sup> LSK cells in the BMs of recipient mice. (D and E) Means  $\pm$  SD.  $n = 5$ ; two independent experiments. \*\*,  $P < 0.01$  by Student's  $t$  test. (F) Ratio of fluorescence intensity of Ki67 and TOTO-3 on single sorted cells in Ly5.1<sup>+</sup> or Ly5.2<sup>+</sup> HSCs in chimeric mice. Means  $\pm$  SEM.  $n = 50$ ; two independent experiments. \*\*,  $P < 0.01$  by Student's  $t$  test.

as a potent niche cell but emphasize the need to elucidate the complex niche regulation on HSCs exerted by Mks.

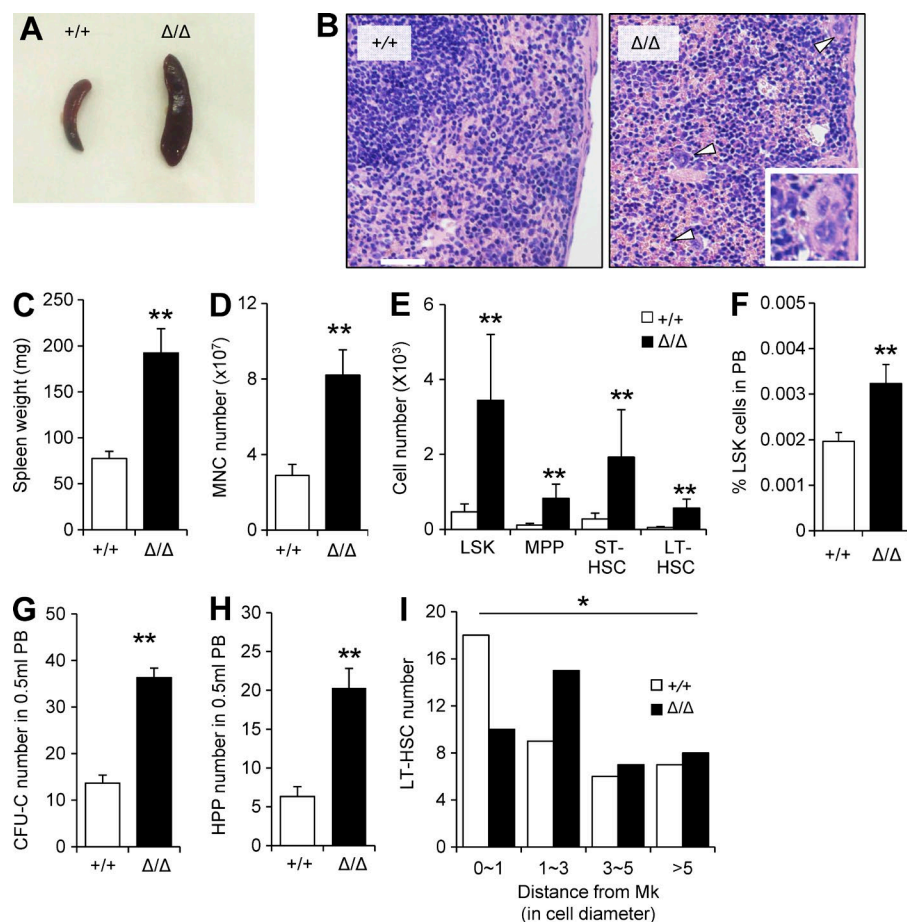
Recent studies showed that specific depletion of Mks disrupts HSC quiescence through different mechanisms. Induced depletion of Mks for 7 d in *PF4-Cre:iDTR* mice chronically activates HSCs to proliferate and self-renew, through either platelet factor 4 (PF4; Bruns et al., 2014) or transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1; Zhao et al., 2014). In contrast, we showed that acute depletion of Mks in *PF4-Cre:iMos-Csp* mice resulted in disruption of HSC quiescence and repopulation potentials without HSC self-renewal (Nakamura-Ishizu et al., 2014b). These three studies all reported direct Mk regulation of HSCs, yet portrayed Mks as acting in different modes, according to the time point after induction of Mk deletion. Confusing matters further, Mk depletion affected different subsets of HSCs in conflicting ways: CD34<sup>+</sup>Flt3<sup>+</sup>LSK cells exhibit modest or no increase in number, whereas CD150<sup>+</sup>CD105<sup>+</sup>LSK cells expanded up to 15-fold (Bruns et al., 2014; Zhao et al., 2014). The differences in the HSC niche functions of Mks may be attributed to the fact that depletion of Mks and platelets dramatically changes the levels of multiple factors. CLEC-2 depletion in Mks did not instigate self-renewal of HSCs or significant changes in platelet lineage-biased CD150<sup>+</sup>CD105<sup>+</sup>LSK cells (Pronk et al., 2007; unpublished data), but it did reduce the stem cell potential of LT-HSCs (CD34<sup>+</sup>Flt3<sup>+</sup>LSK; Christensen and Weissman, 2001). Our study indicates that CLEC-2-mediated Mk regulation of HSCs is not committed to the self-renewal of Mk lineage-biased HSCs, but instead specifically influences the quiescence and stem cell potential of a broader

spectrum of HSCs. CLEC-2 may be an Mk-specific niche regulatory factor that shows the long-term effect of Mk regulation on HSC maintenance.

Constitutive deletion of CLEC-2 and conditional deletion of CLEC-2 from Mk/platelet lineages reportedly results in blood and lymphatic vessel dis-separation phenotype (Bertozzi et al., 2010; Suzuki-Inoue et al., 2010). Recently, the role of CLEC-2 in lymph node development and adult lymph node maintenance was reported (Herzog et al., 2013; Bénézech et al., 2014). Chimeric mice in which BMs of WT mice were reconstituted with BMs from *Clec2<sup>MkΔ/Δ</sup>* mice have been reported to present hemorrhage in the lymph nodes, indicating that platelet CLEC-2 maintains the integrity of vessels in the adult lymph node. No apparent hemorrhages in the lymph nodes were observed in the chimeric mice in which BMs of *Clec2<sup>MkΔ/Δ</sup>* mice and *Clec2<sup>+/+</sup>* mice was transplanted in a 4:1 ratio, presumably because of the mixture of *Clec2<sup>+/+</sup>* mouse-derived BM (unpublished data). The gross morphology of vasculature in the BM was unaffected in *Clec2<sup>MkΔ/Δ</sup>* mice, and no hemorrhage was observed in the BMs (unpublished data). Moreover, EC-specific deletion of CLEC-2 did not affect hematopoiesis. However, whether deficiency of CLEC-2 in platelets affected the function of BM ECs, especially in association to immune response, should be investigated in the future.

A characteristic finding in the *Clec2<sup>MkΔ/Δ</sup>* mice was the presence of extramedullary hematopoiesis. Thpo is known as a cytokine to induce HSC mobilization (Murray et al., 1998). Accordingly, administration of exogenous Thpo to *Clec2<sup>MkΔ/Δ</sup>* mice increased mobilization of HSCs to PB and





**Figure 7. *Clec2*<sup>MkΔ/Δ</sup> mice exhibit extramedullary hematopoiesis and elevated mobilization of HSCs into PB.** (A and B) Gross appearance (A) and hematoxylin and eosin staining (B) of spleens from +/+ and Δ/Δ mice. Arrowheads indicate the presence of Mks in *Clec2*<sup>Mk+/+</sup> spleens. Magnification of an Mk is shown in the inset. Bar, 200 μm. (C–E) Analysis of spleen of Δ/Δ and +/+ mice. Spleen weight (C), total MNC number (D), and absolute populations of various HSPC fractions (E) are significantly higher in spleens of *Clec2*<sup>MkΔ/Δ</sup> compared with *Clec2*<sup>Mk+/+</sup> mice. (F–H) Percentage of LSK cells (F), CFU-C number (G), and HPP-CFC number (H) in PB of +/+ and Δ/Δ mice. (C–H) Means ± SD. *n* = 6; two independent experiments. \*\*, *P* < 0.01 by Student's *t* test. (I) Quantitation of distance of LT-HSCs from Mks in +/+ and Δ/Δ mice. Means ± SD. *n* = 6; two independent experiments. \*, *P* = 0.038 by Tukey's test.

did not rescue the extramedullary hematopoiesis phenotype. Therefore, decreased levels of Thpo in *Clec2*<sup>MkΔ/Δ</sup> mice are not causative of the extramedullary hematopoiesis phenotype. The mechanism of how Mks retain HSCs within the BM and how CLEC-2 signaling associates with HSC retention is yet to be investigated.

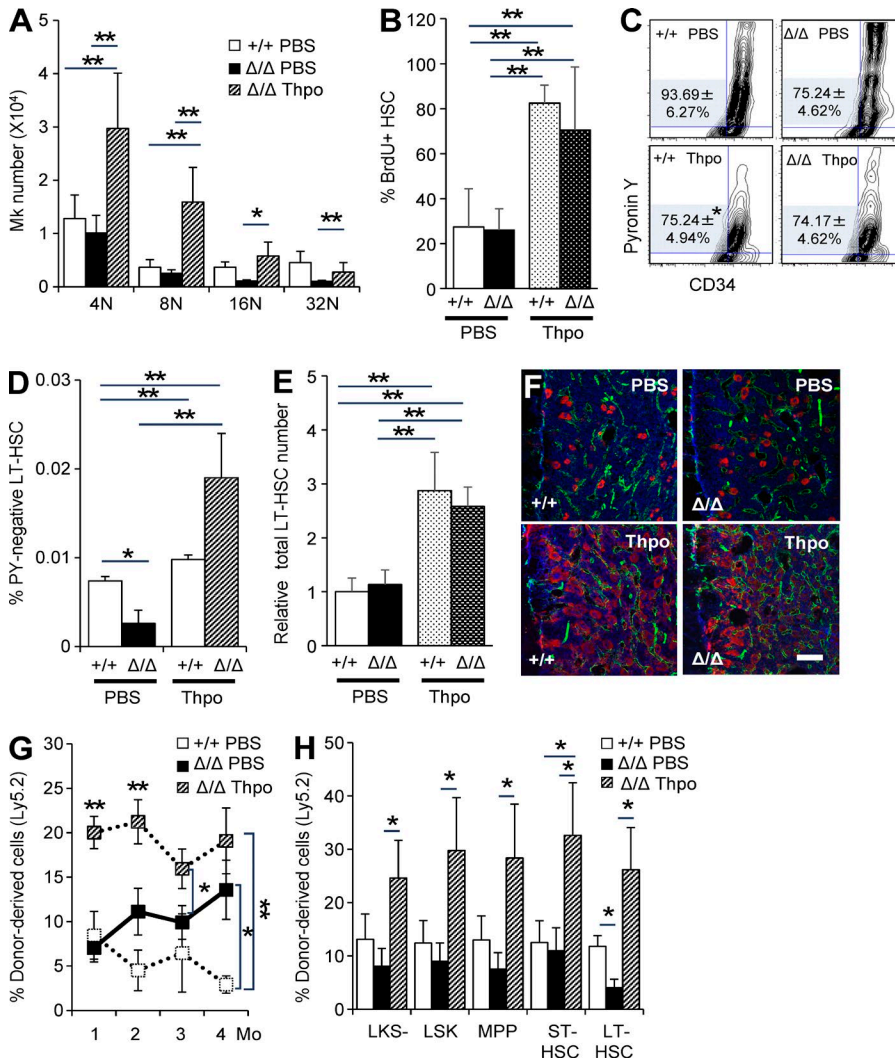
Mks indirectly regulate HSCs by stimulating OBLs after transplantation (Olson et al., 2013). Mk numbers in *Clec2*<sup>MkΔ/Δ</sup> mice were reduced preferentially in the metaphyseal region of the BM. Podoplanin, an activating ligand of CLEC-2 (Bertozzi et al., 2010), was highly expressed in osteo-lineage cells (Schacht et al., 2005). Displacement of Mks in *Clec2*<sup>MkΔ/Δ</sup> mice from the bone-rich metaphysis suggests that CLEC-2 deficiency in Mks may impair Mk and OBL interactions. Furthermore, it indicates that OBLs may indirectly regulate Thpo production in Mks via their expression of podoplanin. Moreover, our findings suggest that CLEC-2–podoplanin signaling may be a novel molecular pathway for niche cell function. Indeed, CLEC-2–deficient Mks exhibited decreased gene expression of CLEC-2 downstream molecules, and knockdown of signals downstream of the CLEC-2–podoplanin interaction (*Syk*, *Lcp2*, and *Plcg2*) confirmed that CLEC-2 signaling is crucial for Mk Thpo production. Loss of sialic acid expression on platelets con-

tributes to Thpo production in the liver (Grozovsky et al., 2015). Because the lectin-like properties of CLEC-2 allow it to interact with sialic acid residues on podoplanin (Pan et al., 2014), our findings provide insight into the involvement of glycosylation in Thpo production.

In summary, our study strongly indicates that Mks function as a niche to maintain HSC quiescence through CLEC-2. These findings could enable manipulation of HSCs and Mks for clinical applications, as well as therapies against diseases related to defects in HSCs and Mks.

## MATERIALS AND METHODS

**Mice.** All mice were in the C57BL/6 background. *Clec2*<sup>flox/flox</sup> mice were described previously (Osada et al., 2012). *PF4-Cre* transgenic mice were provided by R.C. Skoda (University Hospital, Basel, Switzerland; Tiedt et al., 2007). *Clec2*<sup>flox/flox</sup> mice were crossed with either *PF4-Cre* or *VE-cadherin-Cre* (*VEC-Cre*) transgenic mice (stock 006137 purchased from The Jackson Laboratory) to obtain *PF4-Cre:Clec2*<sup>flox/flox</sup> mice (*Clec2*<sup>MkΔ/Δ</sup>) or *VEC-Cre:Clec2*<sup>flox/flox</sup> mice (*Clec2*<sup>ECΔ/Δ</sup>), respectively. C57BL/6–Ly5.1 or C57BL/6–Ly5.2 mice were used for competitive repopulation assays. Unless specified, 10–12-wk-old mice were used in all experiments. All animal experiments were approved by Keio University and performed



**Figure 8. Maintenance of HSC quiescence requires Thpo produced by CLEC-2-positive Mk.** (A) Changes in Mk number based on ploidy analysis after intravenous Thpo injection in *Clec2<sup>MkΔ/Δ</sup>* ( $\Delta/\Delta$ ) and *Clec2<sup>Mk+/+</sup>* ( $+/+$ ) mice. Controls were injected with PBS. (B) Change in percentage of BrdU-positive HSCs after intravenous Thpo injection in  $\Delta/\Delta$  and  $+/+$  mice. (A and B) Means  $\pm$  SD.  $n = 4$  (B) or 5 (A); two independent experiments. \*\*,  $P < 0.01$  by Tukey's test. (C) Representative flow cytometry plot of Pyronin Y and Hoechst 33342 for CD34-LSK cells from  $\Delta/\Delta$  and  $+/+$  mice after administration of PBS or Thpo. Numbers indicate percentage of Pyronin Y-negative cells in CD34-LSK cells. Means  $\pm$  SD.  $n = 4$ ; two independent experiments. \*,  $P < 0.05$  by Student's  $t$  test. (D and E) Total number (D) and frequency (E) of Pyronin Y-negative CD34-LSK cells. Means  $\pm$  SD.  $n = 4$ ; two independent experiments. \*\*,  $P < 0.01$  by Tukey's test. Note that Pyronin Y-negative CD34-LSK cell number is restored in *Clec2<sup>MkΔ/Δ</sup>* ( $\Delta/\Delta$ ) BM after Thpo injection. (F) IHC of BMs from  $\Delta/\Delta$  and  $+/+$  mice after administration of PBS or Thpo. Bar, 100  $\mu$ m. (G and H) Percentage of donor-derived cells in PB (G) and BM HSPCs (H) at the indicated intervals after BMT. Means  $\pm$  SEM.  $n = 6$ ; two independent experiments. \*,  $P < 0.05$ ; and \*\*,  $P < 0.01$  by Tukey's test.

in accordance with the Guidelines of Keio University for Animal and Recombinant DNA experiments.

**PB analysis and colony assays.** PB was collected from the tail vein in a heparinized microtube (Drummond Scientific) and analyzed using CellTac (NIHON KOHDEN). For colony assays and assessment of PB mobilization of HSCs, PB was collected from the inferior vena cava of anesthetized mice using a 27G needle. MNCs from 0.5 ml PB were obtained by centrifugation using Lymphoprep (Axis-Shield) and then used for colony assays. Colony counts for CFU-C and HPP-CFC were assessed on days 7 and 14, respectively.

**Antibodies.** Primary antibodies used for IHC and flow cytometry were as follows: c-Kit (2B8; eBioscience), CD16/32 (93; eBioscience), VE-cadherin (eBioscience), c-Kit (R&D Systems), Sca-1 (E13-161.7; BioLegend), CD48 (HM48-1; BioLegend), CD150 (TC15-12F12.2; BioLegend), IL-7R $\alpha$  (SB/199; BioLegend), endoglin (MJ7/18; BioLegend), CD4 (L3T4; BD), CD8 (53-6.72; BD), B220 (RA3-6B2; BD),

TER-119 (BD), Gr-1 (RB6-8C5; BD), CD34 (RAM34; BD), Mac-1 (M/70; BD), Flt-3 (A2F10.1; BD), CD41 (MWR $\alpha$ g30; BD), CD45.2 (104; BD), CD45.1 (A20; BD), GPIIb $\alpha$  (Xia G5; Emfret), Clec2 (AbD Serotec), and Thpo (Bioss). Secondary antibodies for IHC were Alexa Fluor 488-conjugated IgGs (Molecular Probes) or Cy3/Cy5/DyLight549/DyLight649-conjugated IgGs (Jackson ImmunoResearch Laboratories, Inc.). IHC specimens were treated with DAPI (Molecular Probes) for nuclear staining. Stimulatory rabbit anti-mouse CLEC-2 antibody was a gift of K. Suzuki-Inoue.

**Immunostaining of BM.** Decalcified BM sections were prepared and stained as described previously (Nakamura-Ishizu et al., 2012). Frozen sections prepared according to the Kawamoto method (Kawamoto, 2003) were used to stain Lin<sup>-</sup>CD41<sup>-</sup>CD48<sup>-</sup>CD150<sup>+</sup> cells in the BM.

**Confocal microscopy and quantification of fluorescent images.** Fluorescence images were obtained using a confocal laser-scanning microscope (FV1000; Olympus). Scanning

was performed in sequential laser emission mode to avoid scanning at other wavelengths. Images obtained from BM sections were analyzed using the TissueQuest image analysis software (TissueGnostics).

**Flow cytometric analysis, cell cycle analysis, and competitive repopulation assays.** Flow cytometric analysis and competitive repopulation assays were performed as described previously (Arai et al., 2004). Cell cycle analysis of hematopoietic cells was performed using Pyronin Y staining and short-term BrdU incorporation assays (Takubo et al., 2010). For Ki-67 staining, HSCs were sorted and attached to glass slides with sedimentation and subsequently stained for Ki-67 and TOTO-3. Cells were observed under a confocal laser-scanning microscope (FV1000) for measurement of single cell fluorescence intensity and calculated for their relative Ki-67 fluorescence against nuclear stain (TOTO-3).

**BMT.** BM MNCs ( $4 \times 10^5$  cells) from C57BL/6-Ly5.1 mice, together with LT-HSCs ( $5 \times 10^2$  cells) from the indicated mice (Ly5.2), were transplanted into lethally irradiated C57BL/6-Ly5.1 congenic mice. Secondary transplantations into lethally irradiated C57BL/6-Ly5.1 congenic mice were performed using  $2 \times 10^6$  BM MNCs from primary recipients. Recipient mice were sacrificed for analysis 4 mo after BMT. For extreme limiting dilution assays (Hu and Smyth, 2009), lethally irradiated Ly5.1 mice were transplanted with  $10^4$ ,  $2 \times 10^4$ , or  $3 \times 10^4$  BM MNCs from either *Clec2<sup>+/+</sup>* or *Clec2<sup>MkΔ/Δ</sup>* mice along with  $2 \times 10^5$  Ly5.1 competitor cells. PB chimerism for the limiting dilution assay was assessed 12 wk after transplantation.

**In vitro HSC and Mk co-cultures.** Mature Mks (B220<sup>+</sup>Mac-1<sup>+</sup>Gr-1<sup>+</sup>CD41<sup>+</sup>) were obtained from mouse BM as described previously (Heazlewood et al., 2013). LT-HSCs (Lin<sup>+</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup>Flt-3<sup>+</sup>CD34<sup>+</sup>) sorted from Ly5.1 mice were co-cultured for 3 d with Mks in SF-O3 medium (Sankyo Junyaku) supplemented with murine recombinant SCF (100 ng/ml) with or without human recombinant Thpo (100 ng/ml) and then analyzed (Kabaya et al., 1996). LT-HSCs and Mks were cultured at a 1:1 ratio. Mks were cultured for 2 d to obtain conditioned medium for the indicated experiments. To inhibit Thpo activity, a recombinant mouse ThpoR (Mpl) Fc chimera (0.4 μg/ml; R&D Systems) was added to the culture. An IgG Fc fragment (0.4 μg/ml; Jackson ImmunoResearch Laboratories, Inc.) served as a control. For knockdowns in Mks, MISSION custom vectors (Sigma-Aldrich) were used.

**In vivo Thpo assays.** For in vivo stimulation of Thpo signaling, recombinant human Thpo (PEG-rHuMGDF; Kabaya et al., 1996; donated by Kyowa Hakko Kirin Co., Ltd.) was administered. Mice were treated with either 100 μg/kg (i.v.) of PEG-rHuMGDF or human IgG Fc fragment (Jackson ImmunoResearch Laboratories, Inc.). For rescue experiments, *Clec2<sup>MkΔ/Δ</sup>* mice were treated for four consecutive days.

**Quantitative PCR assay.** Isolated RNA was reverse transcribed with Superscript VILO (Invitrogen). Quantitative PCR assays were performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems), SYBR Premix Ex Taq (Takara Bio Inc.), and primer sets for each gene (Takara Bio Inc.). Values obtained were normalized to β-actin expression and expressed as fold induction relative to control samples.

**Genomic PCR assay.** Genomic DNA was isolated from sorted cells using NucleoSpin (Takara Bio Inc.), and PCR was conducted using 5'-ACGTATCTCTGAA CATCCAAGAAAG-3' and 5'-CTGATCTTACCTG CATTCCATTAGT-3' as primers.

**Gene set enrichment analysis.** Total RNA was extracted from LT-HSCs (Lin<sup>+</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup>CD34<sup>+</sup>Flt-3<sup>+</sup>) from *Clec2<sup>Mk+/+</sup>* and *Clec2<sup>MkΔ/Δ</sup>* mice. Total RNA was purified using an RNeasy Mini kit (QIAGEN). Microarray processing was performed by DNA Chip Research Inc. Normalized expression data were assessed using GSEA v2.0.13 software (Broad Institute). Gene sets used were PARK\_HSC\_VS\_MULTIPOTENT\_PROGENITORS\_DN, PARK\_HSC\_VS\_MULTIPOTENT\_PROGENITORS\_UP, PARK\_HSC\_MARKERS, PARK\_HSC\_AND\_MULTIPOTENT\_PROGENITORS, IVANOVA\_HEMATOPOIESIS\_STEM\_CELL, GRAHAM\_CML\_QUIESCENT\_VS\_NORMAL\_QUIESCENT\_UP, GRAHAM\_NORMAL\_QUIESCENT\_VS\_NORMAL\_DIVIDING\_UP, BYSTRYKH\_HEMATOPOIESIS\_STEM\_CELL\_AND\_BRAIN\_QTL\_TRANS, BAKKER\_FOXO3\_TARGETS\_UP, IVANOVA\_HEMATOPOIESIS\_EARLY\_PROGENITOR, IVANOVA\_HEMATOPOIESIS\_INTERMEDIATE\_PROGENITOR, IVANOVA\_HEMATOPOIESIS\_LATE\_PROGENITOR, IVANOVA\_HEMATOPOIESIS\_STEM\_CELL\_LONG\_TERM, IVANOVA\_HEMATOPOIESIS\_STEM\_CELL\_SHORT\_TERM, JAATINEN\_HEMATOPOIETIC\_STEM\_CELL\_UP, and JAATINEN\_HEMATOPOIETIC\_STEM\_CELL\_DN, obtained from the Molecular Signatures Database v4.0 available at the GSEA web site. The number of permutations was set at 1,000. Gene sets with nominal p-value <0.05 and a false discovery rate q-value (FDR-q) <0.25 were considered statistically significant.

**Statistical analysis.** All results are expressed as means ± SD unless otherwise specified. Statistical significance was determined by Tukey's multiple comparison test. The two-tailed Student's *t* test was used for two-group comparisons. All experiments were conducted and confirmed in at least two replicates.

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Author contributions: T. Suda, K. Takubo, and A. Nakamura-Ishizu designed the project, analyzed the data, and wrote the manuscript. A. Nakamura-Ishizu and K. Takubo organized, performed, and analyzed all experiments. H. Kobayashi performed and analyzed the microarray gene expression experiment. K. Suzuki-Inoue provided mice and discussed and analyzed experiments. All authors read and approved the final manuscript.

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