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 (Clec2Mks/Δ) produced lower levels of Thpo in Mks. CLEC-2-deficient Mks showed down-regulation of CLEC-2–related signaling molecules Syk, Lcp2, and Pleg2. Knockdown of these molecules in cultured Mks decreased expression of Thpo. Clec2Mks/Δ 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 Clec2Mks/Δ mice, as administration of recombinant Thpo to Clec2Mks/Δ 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.

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; Mks, megakaryocytes; 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 (Brunt 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 Mks, which both support HSCs by producing Thpo (Yoshikawa 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–Moorel receptor) stimulate Thpo production in hepatocytes through recognition of desialylated platelets (Grozovsky et al., 2015). Platelets and Mks express CLEC-2 (Suzuki-Inoue et al., 2006, 2007), which is among the top 25 genes specifically expressed on Mks (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 Mks in BM hematopoiesis, and whether it is involved in Thpo production in Mks, 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 Mks. 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 Mks contribute to a niche that regulates HSC quiescence.

RESULTS

CLEC-2 is highly expressed on BM Mks

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 Mks 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+ Mks and ECs was significantly high.

We investigated mice deficient in CLEC-2 specifically in the Mk lineage (PF4-Cre:Clec2flox/flox mice [Clec2fl/fl/Δ/Δ]), 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+ (glycoprotein IIb+ ) cells, but not LSK (Lineage negative, Sca-1−, c-Kit+) cells, in the BM of Clec2fl/fl/Δ/Δ mice (Fig. 1 F). CLEC-2–protean level was significantly reduced in platelets from Clec2fl/fl/Δ/Δ mice but retained in their long-term HSCs (LT-HSCs; Fig. 1 G). Deletion of Clec2 from Mks was also confirmed with IHC (Fig. 1 H). Clec2fl/fl/Δ/Δ mice exhibited mild thrombocytopenia along with anemia (Fig. 1 I).

CLEC-2–deficient Mks 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 Mks and Mk progenitors (MkP and Pre MegE cells) were unaltered in Clec2fl/fl/Δ/Δ mice (Fig. 2 A, Δ/Δ, and total CD41+ Mk cell number was unaltered in Clec2fl/fl/Δ/Δ mice (Fig. 2 E). However, ploidy analysis revealed a reduced frequency of mature polyploid Mks with CLEC-2 deficiency (Fig. 2 F and G). IHC revealed a significant reduction in the number of Mks in the metaphyseal region of the BM in Clec2fl/fl/Δ/Δ mice (Fig. 2 H). These observations indicate that CLEC-2 deficiency modestly affects the maturation of Mks but drastically alters the distribution of Mks within the BM.

CLEC-2–deficient Mks exhibit impaired Thpo production and niche function

Mks 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 Mks to support HSCs in vitro. Addition of Mks to HSC cultures resulted in a Thpo–dependent increase in HSC number (Nakamura-Ishizu et al., 2014b). Accordingly, addition of Clec2–/− Mks to cultured HSCs promoted proliferation, as indicated by expansion of LSK cells and LT-HSCs (Fig. 3, A and B). Clec2–deficient Mks exhibited a reduced capacity to stimulate HSPC proliferation and could not maintain LT-HSC populations (Fig. 3 B).

Next, we assessed whether Clec2–deficient Mks exhibited changes in the production of various niche factors. Clec2–deficient Mks exhibited a broad range of decrease in the gene expression of various niche factors, including Angpt1, Cxcl12, IL-7, Spp1, TnC, and Pf4 (Fig. 3 C). Characteristically, Clec2–deficient Mks secreted less Thpo and altered Mk niche function. Clec2–deficient Mks exhibited significantly lower Thpo expression than Mks from Clec2+/− mice (Fig. 3 C). Furthermore, wild-type Mks 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 Mks was confirmed by IHC (Fig. 3 E).
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 Pclg2 (Suzuki-Inoue et al., 2011). Cultured LT-HSCs were transduced with lentiviral clones that inducibly express each shRNA and then cultured to obtain CD41+Ter119− Mks. Knockdown of Syk, Lcp2, and Pclg2 was highly efficient: transcripts of the targeted genes were nearly absent in sorted Mks (not depicted). Knockdown of Syk, Lcp2, or Pclg2 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 Clec2ΔΔ/Δ mice were affected in vivo. Lineage and HSPC differentiation were unchanged in the BM of Clec2ΔΔ/Δ mice (Fig. 4, A–C). Functionally, HSPCs from Clec2ΔΔ/Δ mice displayed a significant elevation 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− LSK cells) in Clec2ΔΔ/Δ mice (Fig. 4, F and G). c-Mpl expression was significantly up-regulated in LT-HSCs from Clec2ΔΔ/Δ mice, suggestive of deficient Thpo signaling (Fig. 4 H). Loss of nonhematopoietic niche cells (ECs, MSCs, and OBLs) was not observed in BM of Clec2ΔΔ/Δ 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-Clec2floxed/floxed 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 G0 phase of the cell cycle.

HSCs from Clec2ΔΔ/Δ mice exhibit reduced stem cell potential

To further investigate the stem cell potential of HSCs from Clec2ΔΔ/Δ mice, we performed a competitive repopulation assay, using BM transplantation (BMT) of LT-HSCs from Clec2+/+ or Clec2ΔΔ/Δ mice (Ly5.2) into C57BL/6-Ly5.1
recipients. HSCs from Clec2MkΔ/Δ 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+/+ or Clec2MkΔ/Δ mice (Fig. 5 E). Limiting dilution analysis revealed a significantly lower frequency of repopulating HSCs in the BM of Clec2MkΔ/Δ mice (Fig. 5 F). HSCs from Clec2MkΔ/Δ mice did not exhibit defective homing to the BM (Fig. 5 G). In addition, HSCs from Clec2MkΔ/Δ 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 Clec2MkΔ/Δ mice revealed altered HSC-related gene expression profiles, notably an enrichment in progenitor cell–related genes, relative to LT-HSCs from Clec2+/+ mice, suggesting enhanced differentiation and a loss of stem cell character of HSCs from Clec2MkΔ/Δ mice (Fig. 5 I). These results indicate a loss of stem cell potential in Clec2MkΔ/Δ mice.

Clec2MkΔ/Δ 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+/+ or Clec2MkΔ/Δ mice (Ly5.2) along with BM MNCs from 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+ Mks in recipient BM (Fig. 6 B). The number of engrafted Ly5.1 LT-HSCs (CD34−Flt-3− HSCs and CD150+CD48−[SLAM] HSCs) was significantly lower in recipients of Clec2MkΔ/Δ Mks compared to recipients of Clec2+/+ Mks (Fig. 6 C). These results indicate a loss of stem cell potential in Clec2MkΔ/Δ mice.
in transplants from Clec<sup>2Δ/Δ</sup> mouse donors (Fig. 6 C). Furthermore, the percentage of Ly5.1<sup>+</sup> Pyronin Y–negative HSCs was significantly lower when Clec<sup>2Δ/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 Clec<sup>2Δ/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 Clec<sup>2Δ/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. Clec<sup>2Δ/Δ</sup> mice exhibited massive splenomegaly (Fig. 7, A and B). Both HSC and HSPC number and frequency were elevated in Clec<sup>2Δ/Δ</sup> mice (Fig. 7, C–E), indicating that the splenomegaly was caused by extramedullary hematopoiesis. PB of HSC Clec<sup>2Δ/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 Clec<sup>2Δ/Δ</sup> mice (Fig. 7 G). The distance between LT-HSCs and Mks was significantly higher in Clec<sup>2Δ/Δ</sup> mice than in Clec<sup>2+/+</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 Clec<sup>2Δ/Δ</sup> mice

To determine whether the functional defects in HSCs from Clec<sup>2Δ/Δ</sup> mice could be attributed to reduced Thpo levels, we administered recombinant Thpo (PEG-rHuMGDF) to both Clec<sup>2Δ/Δ</sup> and Clec<sup>2+/+</sup> mice. Intravenous Thpo injection for four consecutive days restored the number and frequency of polyploid Mks in Clec<sup>2Δ/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 percentage of BrdU-positive HSCs and the percentage of Pyronin Y–negative cells in CD34<sup>-</sup>LSK cells in both Clec<sup>2+/+</sup> and Clec<sup>2Δ/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>Pyronin Y–negative cells within the BM were restored (Fig. 8, D and E). Thpo administration also significantly increased the number of Mks within the BM in both Clec<sup>2+/+</sup> and Clec<sup>2Δ/Δ</sup> mice (Fig. 8 F). The repopulation potential of HSCs from Clec<sup>2Δ/Δ</sup> mice significantly increased with Thpo injection, as confirmed by competitive BMT (Fig. 8, G and H). Thus,
the loss of HSC potential in Clec2<sup>Δ/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>Δ/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>Δ/Δ</sup> mice, indicating that defective Thpo production impaired HSC function in Clec2<sup>Δ/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 platelets (Kuter and Rosenberg, 1995). However, Mks in Clec2<sup>Δ/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>Δ/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 et al., 1998).
et al., 2007), and the platelet α-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 Clec2Δ/Δ mice were responsive to exogenous Thpo, indicating that the loss of stem cell potential of HSCs in Clec2Δ/Δ 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 Ms 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 Ms. In addition, CLEC-2-deficient Ms 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 Ms

Figure 5. HSCs from Clec2Δ/Δ 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 χ² 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+ 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.
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-β1 (TGF-β1; 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−Flt3−LSK cells exhibit modest or no increase in number, whereas CD150+CD105−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+CD105−LSK cells (Pronk et al., 2007; unpublished data), but it did reduce the stem cell potential of LT-HSCs (CD34−Flt3−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 Clec2MkΔ/Δ 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 Clec2MkΔ/Δ mice and Clec2+/+ mice was transplanted in a 4:1 ratio, presumably because of the mixture of Clec2+/+ mouse–derived BM (unpublished data). The gross morphology of vasculature in the BM was unaffected in Clec2MkΔ/Δ 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 Clec2MkΔ/Δ 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 Clec2MkΔ/Δ mice increased mobilization of HSCs to PB and
did not rescue the extramedullary hematopoiesis phenotype. Therefore, decreased levels of Thpo in Clec2<sup>Δ/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>Δ/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>Δ/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 contributes 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>flk/flk</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>flk/flk</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>flk/flk</sup> mice (Clec2<sup>flk/flk</sup>Cre) or VEC-Cre:Clec2<sup>flk/flk</sup> mice (Clec2<sup>flk/flk</sup>Cre), 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...
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-7Rα (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), Flk-3 (A2F10.1; BD), CD41 (MWR.eg30; BD), CD45.2 (A20; BD), GPIbα (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^−^CD34^−^LSK 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 TissueGnostics software (TissueGnostics).

Flow cytometric analysis, cell cycle analysis, and competitive repopulation assays. Flow cytometric analysis and competitive repopulation assays were performed as described previously (Araki 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 × 10^5) cells from C57BL/6-Ly5.1 mice, together with LT-HSCs (5 × 10^5) 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 × 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 × 10^4, or 3 × 10^4 BM MNCs from either Clec2^ΔΔ^ or Clec2MkΔΔΔ mice along with 2 × 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^−^Mac-1^−^Gr-1^−^CD41^−^) were obtained from mouse BM as described previously (Heazlewood et al., 2013). LT-HSCs (Lin^−^c-Kit^+^Sca-1^+^Flt-3^−^CD34^−^) sorted from Ly5.1 mice were co-cultured for 3 d with Mks in SF-O3 medium (Sanju-Ishizu) 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, Clec2MkΔΔΔ mice were treated for four consecutive days.

Quantitative PCR assay. Isolated RNA was reverse transcribed with SuperscriptVILO (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 CATCCAAAGAG-3′ and 5′-CTGATCTTACCTG CATTCCATTAGT-3′ as primers.

Gene set enrichment analysis. Total RNA was extracted from LT-HSCs (Lin^−^c-Kit^+^Sca-1^+^CD34^−^Flt-3^−^) from Clec2MkΔΔΔ and Clec2MkΔΔΔ 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, 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, BAKER_FOXY3_TARGETS_UP, IVA NOVA_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_DOWN, obtained from the Molecular Signatures Database v4.0 available at the GSEA website. 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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