Cell Stem Cell
Article

Thrombopoietin/MPL Signaling Regulates Hematopoietic Stem Cell Quiescence and Interaction with the Osteoblastic Niche

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DOI 10.1016/j.stem.2007.10.020

SUMMARY

Maintenance of hematopoietic stem cells (HSCs) depends on interaction with their niche. Here we show that the long-term (LT)-HSCs expressing the thrombopoietin (THPO) receptor, MPL, are a quiescent population in adult bone marrow (BM) and are closely associated with THPO-producing osteoblastic cells. THPO/MPL signaling upregulated β1-integrin and cyclin-dependent kinase inhibitors in HSCs. Furthermore, inhibition and stimulation of THPO/MPL pathway by treatments with anti-MPL neutralizing antibody, AMM2, and with THPO showed reciprocal regulation of quiescence of LT-HSC. AMM2 treatment reduced the number of quiescent LT-HSCs and allowed exogenous HSC engraftment without irradiation. By contrast, exogenous THPO transiently increased quiescent HSC population and subsequently induced HSC proliferation in vivo. Altogether, these observations suggest that THPO/MPL signaling plays a critical role of LT-HSC regulation in the osteoblastic niche.

INTRODUCTION

HSCs self-renew and differentiate to produce multilineage blood cells throughout an individual's lifetime. Maintenance of such stem cell activities requires both intrinsic and extrinsic factors. For HSCs, these factors are provided by the microenvironment called the stem cell niche. Cell-cell, cell-extracellular matrix, and receptor-ligand interactions occur between HSCs and the niche (Li and Xie, 2005; Adams and Scadden, 2006; Li and Li, 2006; Moore and Lemischka, 2006; Wilson and Trumpp, 2006). Such interaction of HSCs with the niche is critical to maintaining the balance between proliferation and quiescence, which is a common stem cell property with respect to the cell cycle. Cell-cycle quiescence is essential for long-term preservation of stem cell properties, including self-renewal capacity and the ability to differentiate into multiple lineages (Cheng et al., 2000).

We recently clarified a molecular mechanism whereby the cell cycle of HSCs is regulated by the osteoblastic niche in BM (Arai et al., 2004). Cell-cycle regulation by the niche is critical for HSC fate. We previously reported that $LIN^{-}SCA1^{hi}KIT^{hi}$ (LSK) cells in peripheral blood and spleen mobilized by G-CSF were not in the side population (SP) (Arai et al., 2004), indicating that HSCs exited from the BM niche are not in G0. We found that quiescent HSCs adhere to osteoblastic cells in the BM niche and showed that interaction of the Tie2 receptor with its ligand angiopoietin-1 (Ang-1) leads to tight adhesion of HSCs to the osteoblastic niche, resulting in maintenance of quiescence (Arai et al., 2004). Currently, various molecules involved in the signaling and adhesive interaction between HSCs and the BM niche are identified (Yin and Li, 2006). In this study, we showed that THPO/MPL signaling is a candidate niche factor.

Signaling via THPO and its receptor, MPL, regulates megakaryogenesis (Kaushansky, 1995). MPL was identified as the product of the *c-MPL* gene, the wild-type homolog of the oncogene *v-MPL*, the transforming gene of murine myeloproliferative leukemia virus (Vigon et al., 1992). THPO acts as both a megakaryocyte colony-stimulating and maturation factor in megakaryopoiesis (de Sauvage et al., 1994; Lok et al., 1994; Wendling et al., 1994). In megakaryopoiesis, THPO/MPL interaction primarily stimulates late megakaryocytic colony-forming units, consequently increasing the number of mature megakaryocytes with the capacity to form proplatelets. Initially, THPO/MPL signaling was identified as the primary regulator of platelet production (Kaushansky, 1995), and

MPL later revealed its role closely related to HSC function (Kimura et al., 1998; Solar et al., 1998; Buza-Vidas et al., 2006). *THPO* or *MPL* knockout mice exhibit not only significantly fewer megakaryocytes and circulating platelets (Gurney et al., 1994; de Sauvage et al., 1996) but also show fewer HSCs in the BM (Carver-Moore et al., 1996; Alexander et al., 1996; Kimura et al., 1998). $MPL^{-/-}$ mice showed the decrease of numbers of progenitor cells in multilineage and reduction of competitive repopulating capacity (Carver-Moore et al., 1996; Alexander et al., 1996; Kimura et al., 1998). In addition, hyperresponsiveness to THPO seen in mice lacking the adaptor protein Lnk results in HSC expansion (Buza-Vidas et al., 2006). Like other hematopoietic cytokines, THPO activates three major signaling pathways: p42/44 mitogen-activated protein kinase (MAPK) (Rojnuckarin et al., 1999), phosphoinositide 3-kinase-Akt (Geddis et al., 2001), and Janus kinase (JAK)-signal transducer, and activator of transcription (STAT) 3 and STAT5 (Dorsch et al., 1995; Drachman et al., 1995). Although these signaling pathways have been broadly studied in the context of megakaryopoiesis, the role of THPO/MPL signaling in the interaction of quiescent HSCs with the niche is still unknown.

Here we identified that $LSKCD34^{-}MPL^{+}$ cells and $LSKCD34^{+}MPL^{+}$ cells are regarded as quiescent and active HSCs, respectively. In addition, both $LSKCD34^{-}MPL^{+}$ and $LSKCD34^{+}MPL^{+}$ cells had the ability for long-term BM reconstitution (LTR). Further, we found that quiescent MPL^{+} HSCs adhered to THPO-producing osteoblastic cells on the endosteal surface. Such positional association between MPL^{+} HSC and $THPO^{+}$ osteoblastic cells suggests that THPO/MPL signaling contributes to regulation of quiescent LT-HSCs in the osteoblastic niche. We also investigated the physiological role of THPO/MPL signaling in regulating HSCs in the niche using an anti-MPL neutralizing antibody (Ab) and recombinant THPO. Inhibition of the THPO/MPL pathway with a neutralizing anti-MPL Ab reduced the number of quiescent HSCs and HSC-niche interaction. On the other hand, exogenous THPO transiently increased the proportion of quiescent HSCs in vivo. These observations indicate that THPO and MPL in HSCs play a role in the maintenance of quiescence of HSCs in the niche.

RESULTS

Quiescent HSCs Express MPL

Although MPL expression in HSCs has been demonstrated (Berardi et al., 1995; Solar et al., 1998), the function of MPL in this context remains unclear. To analyze that function, we examined first the MPL expression in subsets of LSK cells. It has been shown that LT-HSCs are enriched in the $CD34^{-}FLT3^{-}Tie2^{+}$ or $CD48^{-}CD150^{+}$ fractions in LSK cells (Osawa et al., 1996; Hsu et al., 2000; Adolfsson et al., 2001; Christensen and Weissman, 2001; Arai et al., 2004; Kiel et al., 2005). Therefore, we analyzed the proportion of MPL^{+} cells in LT-HSCs (Figure 1A). $MPL^{+}Tie2^{+}$ cells were enriched in $LSKCD34^{-}FLT3^{-}$ cells. In $LSKCD48^{-}$ cells, MPL^{+} cells were divided into $CD150^{+}$ and $CD150^{-}$ cells. The percentages of MPL^{+} cells in $LSKCD34^{-}FLT3^{-}Tie2^{+}$ and $LSKCD48^{-}CD150^{+}$ cells were 96.4% ± 1.7% and 97.2% ± 3.6%, respectively, suggesting that MPL is expressed in LT-HSCs and may play a role in LTR activity.

Next, we analyzed the expression of MPL in SP cells in LSK. SP and non-SP phenotypes in LSK cells represent quiescent and cycling HSCs, respectively (Arai et al., 2004). FACS analysis showed that 85.6% ± 2.3% of LSK-SP cells express MPL (Figure 1B). To investigate the potential relevance of MPL expression to HSC cell-cycle status, we analyzed the proportion of LSK-MPL^{+} cells after 5-fluorouracil (5-FU)-induced myelosuppression (Figure 1C). After 2 days of 5-FU treatment, the remaining LSK cells were MPL positive (82.1% ± 6.1% of LSK cells), whereas LSK-MPL^{-} cells were susceptible to 5-FU treatment, which are similar findings to the case of LSK-SP cells, as previously reported (Arai et al., 2004). After 6 days of 5-FU treatment, the proportion of LSK-SP cells was reduced, and the population of LSK cells shifted from SP to non-SP. Similar to the change of SP, MPL expression was downregulated (44.6% ± 5.1%) after 6 days of 5-FU treatment.

Next, to clarify the changes of cell-cycle regulators, we examined the expression of cyclin-dependent kinase inhibitors (Cdkns), c-Myc, and Tie2 in $LSKCD34^{+\text{ or }-}MPL^{+\text{ or }-}$ cells. As shown in Figure 1D, $p57^{Kip2}$ (*Cdkn1c*) was markedly expressed in $CD34^{-}MPL^{+}$ fraction in LSK, and *Tie2* was expressed in MPL^{+} fraction in LSK regardless of CD34 expression. In addition, $p57^{Kip2}$ was highly expressed in LSK-SP cells (see Figure S1 in the Supplemental Data available with this article online). Altogether, these data suggest that MPL is expressed in the quiescent LT-HSCs in adult BM.

MPL^{+} HSCs Adhere to the Endosteal Surface, and Osteoblastic Cells Produce THPO in Adult BM

We previously reported that Tie2 is a marker for LT-HSCs and that 5-FU-resistant $Tie2^{+}$ cells are quiescent HSCs in the osteoblastic niche (Arai et al., 2004). Thus, we examined expressions of MPL in 5-FU-treated BM to define the localization of MPL^{+} HSCs along with Tie2 in BM (Figure 2A). 5-FU-resistant $Tie2^{+}MPL^{+}$ cells were localized to the endosteal surface, where osteoblasts reside, suggesting that MPL^{+}HSCs are localized in the osteoblastic niche. To further confirm whether 5-FU-resistant MPL^{+} cells are in quiescence, we performed BrdU long-term label-retaining (BrdU-LTR) cell assay. BrdU-LTR is a marker for identification of quiescent cells in tissue (Zhang et al., 2003). As shown in Figure 2B, BrdU-LTR cells expressed MPL and adhered to bone-lining cells in 5-FU-treated BM. These data suggest that 5-FU-resistant MPL^{+} HSCs are quiescent HSCs in the osteoblastic niche.

Osteoblastic cells are the key component of the HSC niche, and quiescent HSCs specifically localize to the endosteal surface of adult BM (Nilsson et al., 2001; Calvi et al., 2003; Zhang et al., 2003; Arai et al., 2004). Therefore, we analyzed THPO expression in BM. Immunohistochemical staining of THPO in BM showed that a subset of bone-lining alkaline phosphatase (ALP)-positive osteoblastic cells express THPO (Figure 2C), suggesting that THPO is

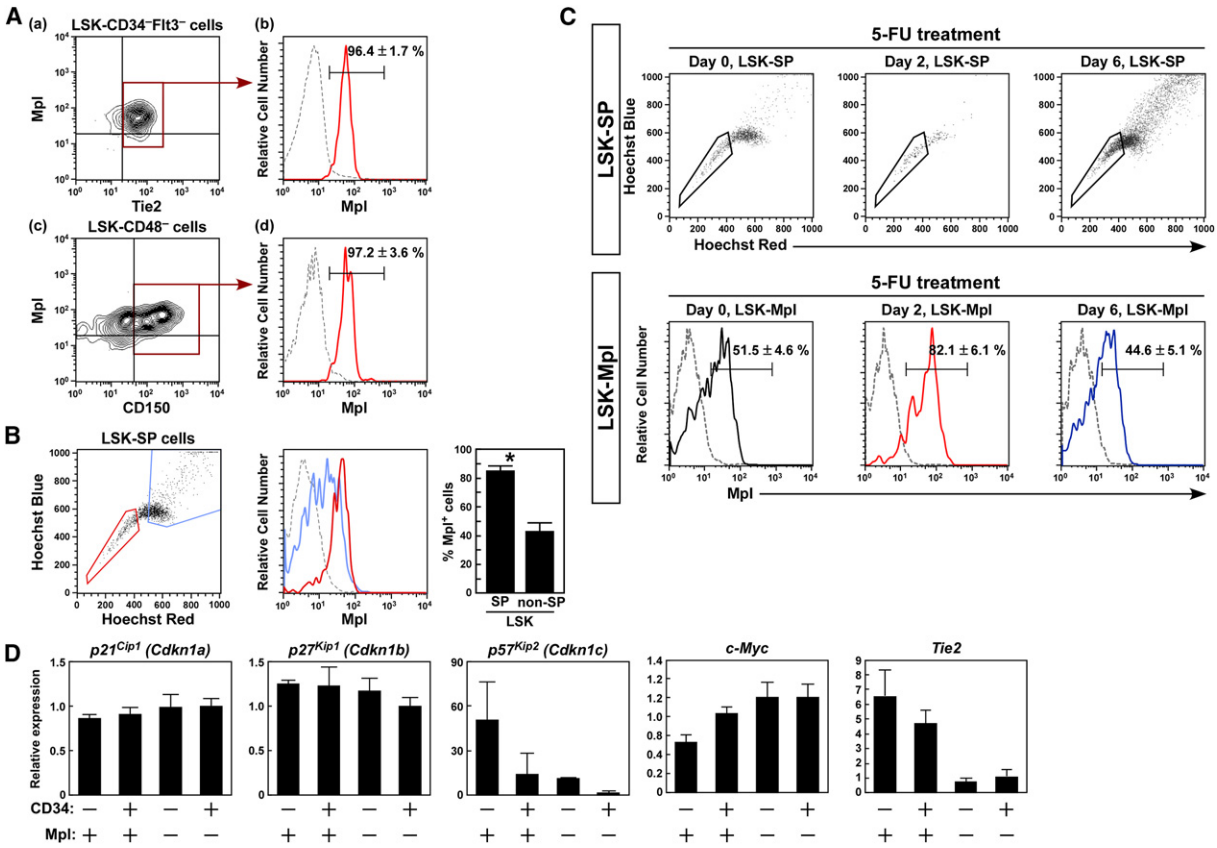

Figure 1. MPL Is Highly Expressed on Quiescent LT-HSCs

(A) The proportion of MPL$^+$ cells in the fraction of LSKCD34$^-$FLT3$^-$Tie2$^+$ cells and LSKCD48$^-$CD150$^+$ cells. Expression of MPL and Tie2 in LSKCD34$^-$FLT3$^-$ cells (Aa). LSKCD34$^-$FLT3$^-$Tie2$^+$ cells were gated and analyzed for the frequency of MPL$^+$ cells (Ab). Expression of MPL and CD150 in LSKCD48$^-$ cells (Ac). LSKCD48$^-$CD150$^+$ cells were gated and analyzed the frequency of MPL$^+$ cells (Ad). Most CD34$^-$FLT3$^-$Tie2$^+$ and CD48$^-$CD150$^+$ fractions in LSK cells express MPL. Dotted lines indicate isotype-matched control. Data represent mean ± SD (n = 4).

(B) The proportion of MPL$^+$ cells in quiescent and active states. Expression of MPL in LSK-SP (red) and LSK-non-SP cells (light blue) was analyzed. LSK-SP cells showed a high percentage of the MPL$^+$ fraction compared to LSK-non-SP cells. Dotted lines indicate isotype-matched control. Data represent mean ± SD (n = 4).

(C) Changes in the proportion of LSK-SP (upper) and LSK-MPL$^+$ (lower) cells after 5-FU treatment. Both of the SP and MPL$^+$ cells in LSK were enriched 2 days after 5-FU injection, while non-SP and MPL$^-$ cells were reduced. On day 6 after 5-FU treatment, a shift in the LSK population from SP to non-SP was observed. At the same time, MPL expression in LSK cells was downregulated on day 6 after 5-FU injection. Dotted lines indicate isotype-matched control. Data represent mean ± SD (n = 4). Representative data of a series of experiments are shown.

(D) Expression of *p21^{Cip1}*, *p27^{Kip1}*, *p57^{Kip2}*, *c-Myc*, and *Tie2* in LSKCD34$^{+\text{ or }-}$MPL$^{+\text{ or }-}$ fractions. Data represent mean ± SD from two independent experiments.

produced in the microenvironment of osteoblastic niche cells. In addition, 5-FU-resistant MPL$^+$ cells adhered to the THPO-producing osteoblastic cells on the bone surface (Figure 2D). Quantitative PCR (qPCR) confirmed that THPO was expressed in the BM-derived osteoblastic cell clones (Figure 2E). These data suggest that THPO/MPL signaling between osteoblastic cells and HSCs is involved in the niche regulation of HSCs, and the cell adhesion of MPL$^+$ HSCs to THPO$^+$ osteoblastic cells might contribute to the maintenance of quiescent HSCs in the osteoblastic niche.

MPL in LSK Cells Is a Specific Marker for LT-HSCs

Since MPL expression in a subset of LSK cells corresponded to the known LT-HSC population, we hypothesized that THPO/MPL signaling was physiologically involved in the HSC function. Then we examined whether expression of MPL in HSCs contributes to LTR activity. First we compared BM reconstitution capacity between MPL$^+$ and MPL$^-$ fraction in LSK cells. As shown in Figure 3A, the cells that have LTR activity were enriched in MPL$^+$ fraction in LSK fraction. In addition, MPL$^+$ cells showed remarkable repopulation after the second BM transplantation (BMT) (Figure 3B). For further evaluation of the role of MPL in LT-HSC and ST-HSC population, we subdivided LSK cells into four fractions (CD34$^-$MPL$^+$, CD34$^+$MPL$^+$, CD34$^-$MPL$^-$, and CD34$^+$MPL$^-$) and examined LTR activity (Figure 3C). It has been reported that LT-HSCs were enriched in CD34$^-$ fraction in LSK cells (Osawa et al., 1996). We found that MPL$^+$ fraction had

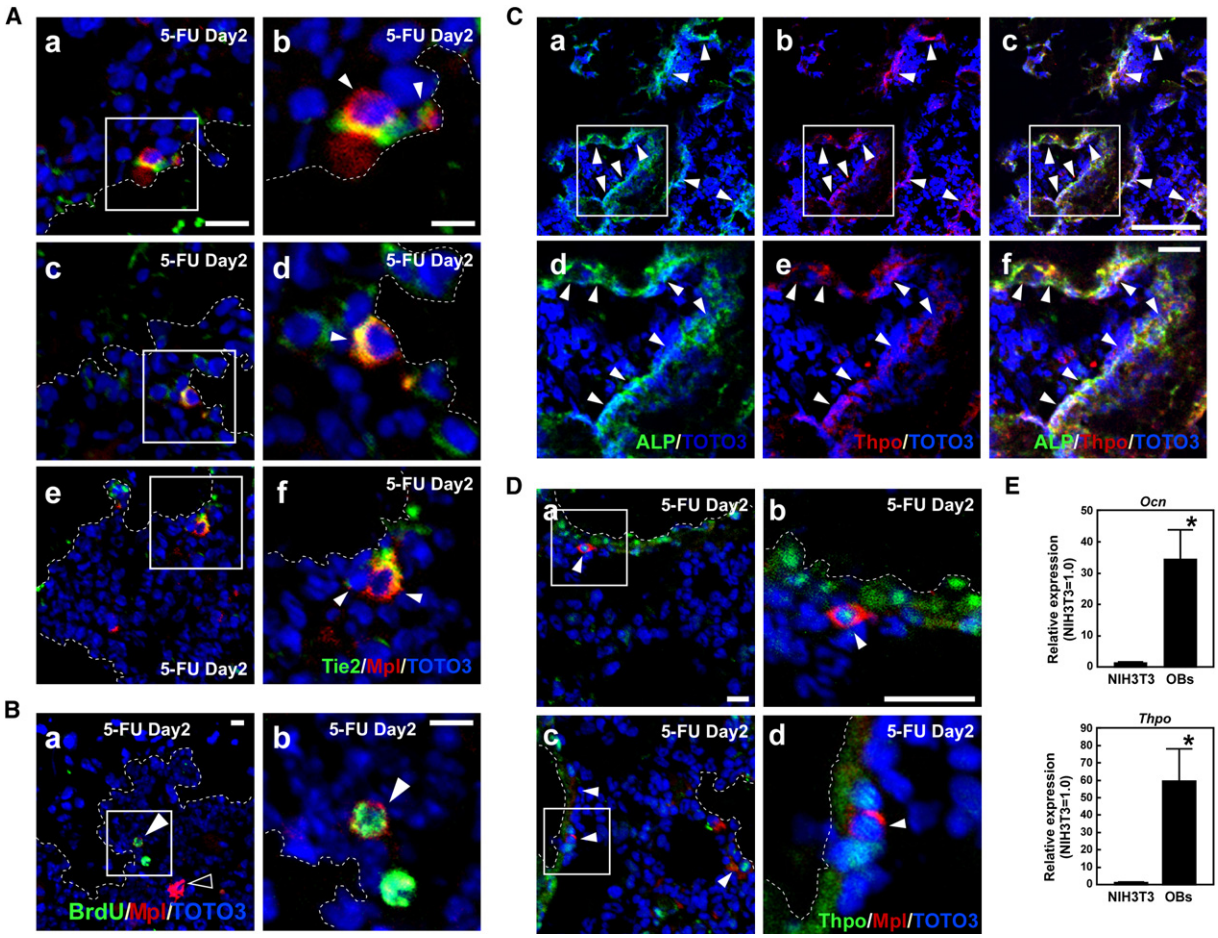

Figure 2. Localization of MPL$^+$ HSCs and THPO-Expressing Osteoblastic Cells in the Trabecular Bone Area of BM

(A) Immunohistochemical staining of Tie2 (green) and MPL (red) in 5-FU treated BM. (Aa, Ac, Ae) Multiple views of the trabecular bone surface. (Ab, Ad, Af) Higher magnifications of enclosed boxes in (Aa), (Ac), and (Ae), respectively. MPL$^+$Tie2$^+$ HSCs adhered to the endosteal surface (arrowheads). Scale bars, 10 μm (Aa, Ac, Ae), 3 μm (Ab, Ad, Af).

(B) Expression of MPL in BrdU-LTR cells. (Ba) Lower magnification. (Bb) Higher magnifications of enclosed boxes in (Ba). Scale bar, 3 μm.

(C) Expression of THPO in trabecular bone area of adult mouse BM. (Ca–Cc) Lower magnification. (Cd–Cf) Higher magnifications of enclosed boxes in (Ca)–(Cc), respectively. (Ca and Cd) Expression of ALP (green). (Cb and Ce) Expression of THPO (red). (Cc and Cf) Merged images of (Ca) and (Cb) and (Cd) and (Ce), respectively. THPO expression was detected in the ALP$^+$ bone-lining osteoblastic cells (arrowheads). Scale bar, 100 μm (Ca–Cc), 25 μm (Cd–Cf).

(D) Expression of THPO (green) and MPL (red) in 5-FU treated BM. (Da and Dc) Multiple views of the trabecular bone surface. (Db and Dd) Higher magnifications of enclosed boxes in (Da) and (Dc), respectively. 5-FU-resistant MPL$^+$ HSCs were adhered to THPO-producing bone-lining cells (arrowheads). Scale bar, 15 μm.

(E) qPCR analysis of *osteocalcin* (*Ocn*) and *THPO* expression in NIH 3T3 cells and BM-derived osteoblastic cells. *THPO* expression was expressed highly in *osteocalcin*-expressing osteoblastic cells compared to NIH 3T3 cells. Data represent mean ± SD from three independent experiments.

significantly higher LTR activity than MPL$^-$ fraction in LSKCD34$^-$ cells (Figure 3C). Peripheral blood analyses showed that the donor-derived LSKCD34$^-$MPL$^+$ cells differentiated into myeloid, B cell, and T cell lineages (Figure 3D). These data suggest that LT-HSC was predominantly enriched in LSKCD34$^-$MPL$^+$ cells. Unexpectedly, MPL$^+$ cells in LSKCD34$^+$ cells, which were thought short-term (ST)-HSCs, clearly showed lymphomyeloid reconstitution (Figures 3C and 3D), even though the peripheral blood chimerism was lower than LSKCD34$^-$MPL$^+$ cells. These data indicate that MPL is an effective marker for LT-HSCs. This result can contribute to establishment of a novel cell purification strategy of LT-HSCs, with which higher purity is achievable than with currently available protocol.

THPO/MPL Signaling Maintains an Immature Phenotype in HSCs

To investigate the function of THPO/MPL signaling in HSC maintenance in vitro, we performed long-term colony-initiating cell (LTC-ICs) assays and cobblestone area-forming cell (CAFCs) assays with or without the anti-MPL neutralizing Ab, AMM2. AMM2 did not inhibit colony formation of freshly isolated LSK cells (Figure 4A), while AMM2

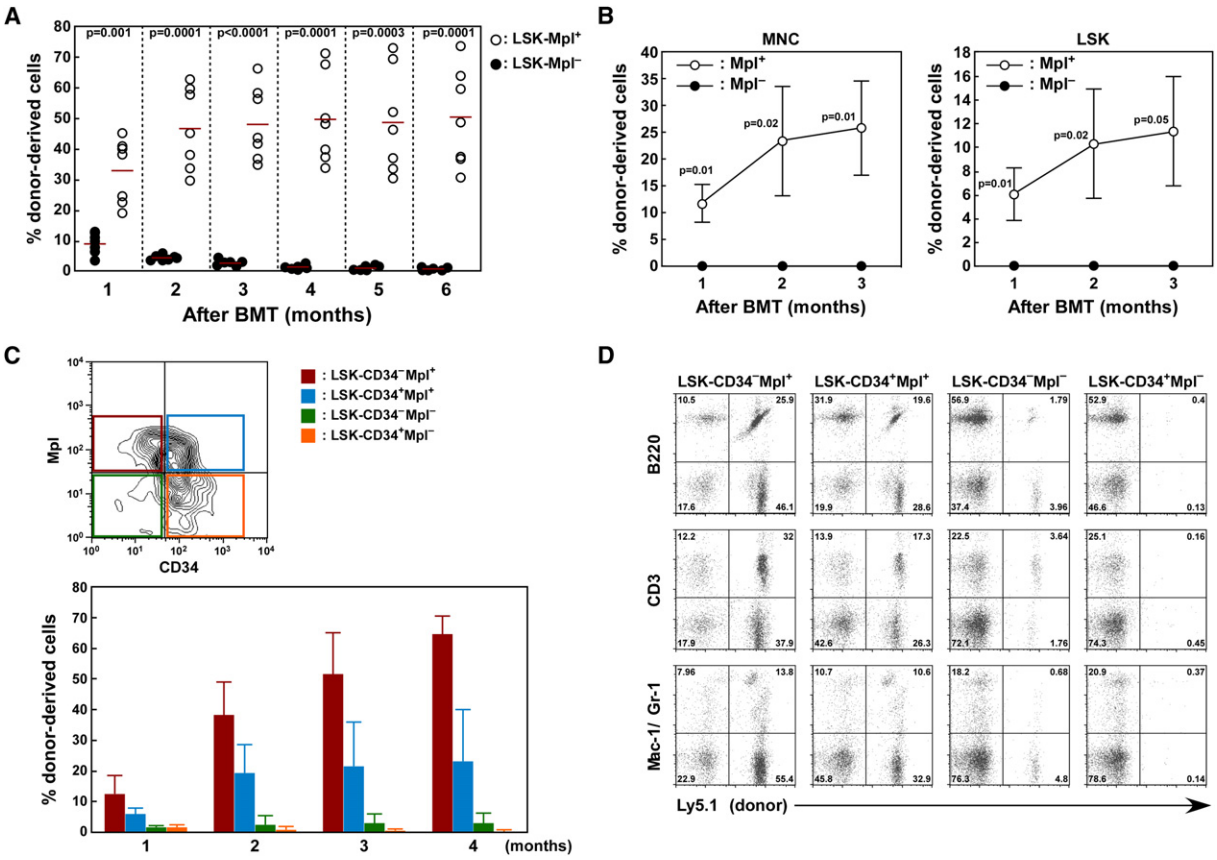

Figure 3. LT-HSCs Were Enriched in MPL$^+$ Fraction

(A) MPL$^+$ (○) and MPL$^-$ (●) fractions of LSK cells were sorted from 8-week-old mice BMMNCs and transplanted into lethally irradiated mice. Percentages of donor-derived (Ly5.1$^+$) cells in recipient mice 1–6 months after BMT. Red lines indicate mean percentages of donor-derived cells. The data shown represent mean ± SD (n = 7 per group).

(B) BMMNCs (left) or LSK cells (right) were isolated from LSK-MPL$^+$ (○) or MPL$^-$ (●) transplanted primary recipient mice and secondary transplanted into lethally irradiated mice. Percentages of donor-derived (Ly5.1$^+$) cells in secondary recipient mice 1–3 months after BMT.

(C) Eight-week-old mice derived LSKCD34$^{+\text{ or }-}$MPL$^{+\text{ or }-}$ cells (upper panel) were transplanted into lethally irradiated recipient mice. Remarkable long-term reconstitution was seen in LSKCD34$^-$MPL$^+$ cells (lower panel). Data represent mean ± SD (n = 10 per group).

(D) Peripheral blood analysis of B, T, and myeloid cell lineages 4 months after BMT of LSKCD34$^{+\text{ or }-}$MPL$^{+\text{ or }-}$ cells.

inhibited the maintenance of LTC-IC on OP9 stromal cells (Figure 4B). These data suggest that THPO/MPL signaling maintains immature HSC phenotypes in vitro.

We analyzed the function of THPO/MPL signaling in HSC cobblestone formation. To do so, we cocultured LSK-MPL$^+$ cells and OP9 stromal cells in the presence of THPO or AMM2 and evaluated CAFCs. As shown in Figure 4C, exogenous THPO enhanced cobblestone formation 3-fold in LSK-MPL$^+$ cells compared to stem cell factor (SCF) alone. By contrast, AMM2 inhibited adhesion of LSK-MPL$^+$ cells and reduced CAFCs in a dose-dependent manner. In addition, THPO upregulated *β1-integrin* expression in LSK-MPL$^+$ cells compared to SCF alone (Figure 4D). It has been shown that THPO activated β1-integrin in a human progenitor cell line and CD34$^+$CD38$^-$ cells (Cui et al., 1997). Altogether, these data suggest that THPO/MPL signaling preserves immature phenotypes in HSCs via interaction with stromal cells.

Effects of THPO/MPL Signaling in the Regulation of Cell-Cycle-Related Genes in HSCs

Next, we examined the effects of THPO/MPL signaling on the expression of cell-cycle regulators. First, we examined effects of administration of AMM2 or THPO in vivo on expression of *Cdkns* (*p21^{Cip1}*, *p27^{Kip1}*, and *p57^{Kip2}*), *c-Myc*, and *Tie2* in LSK cells. AMM2 and THPO treatment revealed a reciprocal correlation with expression of *p57^{kip2}* (increased by THPO, decreased by AMM2), *c-Myc* (decreased by THPO, increased by AMM2), and *Tie2* (increased by THPO, decreased by AMM2) (Figure 5A). In addition, *p21^{Cip1}* expression was slightly upregulated by THPO. Next, we examined the effects of THPO treatment on LSKCD34$^-$MPL$^+$ and LSKCD34$^+$MPL$^+$ cells in vitro and found that THPO maintained the expression levels of *p57^{Kip2}*, *c-Myc*, and *Tie2* in LSKCD34$^-$MPL$^+$ cells (Figure 5B). Although THPO treatment upregulated *p57^{Kip2}* and *Tie2* in LSKCD34$^+$MPL$^+$ cells, the stimulation

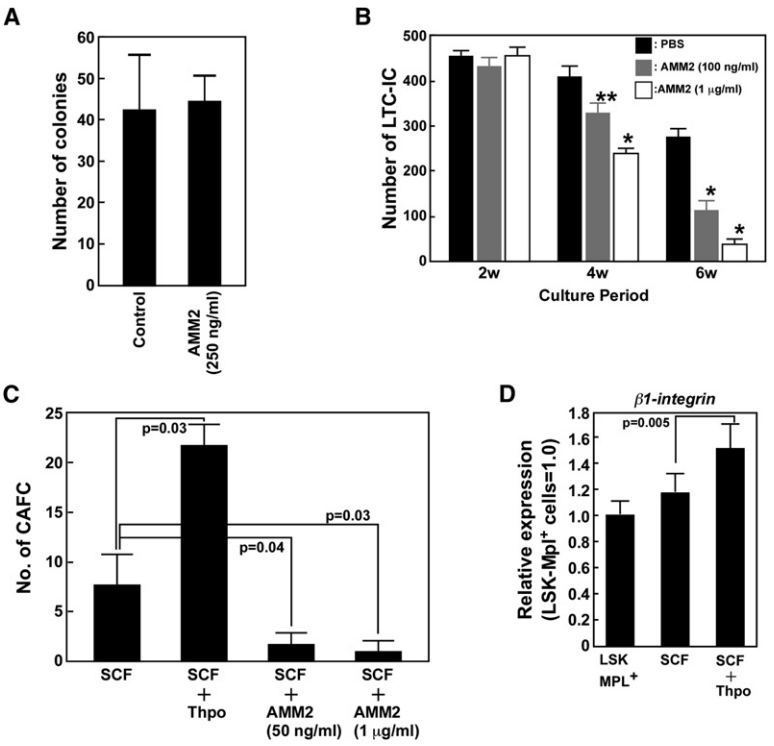

Figure 4. Role of THPO/MPL Signaling in HSC Maintenance In Vitro

(A) Numbers of CFU-C in the culture with or without AMM2.

(B) Numbers of LTC-IC. LSK cells (500 cells per well) were cocultured with OP9 stromal cells in the presence or absence of AMM2 (100 ng/ml or 1 μg/ml). Colony assays were performed after 2–6 weeks of coculture. Data represent mean ± SD (*$p < 0.01$, **$p < 0.05$).

(C) Numbers of CAFCs. LSK-MPL$^+$ cells were sorted and cultured on OP9 stromal cells in the presence of SCF with or without THPO or AMM2. After 7 days of cultivation, numbers of CAFCs were counted. THPO increased the number of CAFCs, whereas AMM2 decreased CAFCs in a dose-dependent manner. Data represent mean ± SD.

(D) Relative expression of *β1-integrin* after in vitro culture of LSK-MPL$^+$ cells with SCF or SCF and THPO was analyzed by qPCR. Expression of *β1-integrin* in freshly isolated LSK-MPL$^+$ cells (before culture) was set at 1.0. Data represent mean ± SD. Data are representative of three independent experiments (A–D).

of THPO/MPL signaling did not enhance LTR activity of LSKCD34$^+$MPL$^+$ cells after 2 days of cultivation (data not shown). We speculate that self-renewal capacity and the efficient LTR activity might be enhanced, provided the adequate cell-adhesion molecules and interaction with extracellular matrix had existed in culture condition. If so, optimization of culture condition including artificial extracellular matrix and other niche factors must be necessary to reproduce niche environment in vitro that maximizes the intrinsic capability of HSCs. Combinational treatment of SCF with THPO suppressed *p57^{Kip2}* and *Tie2* in LSKCD34$^-$MPL$^+$ cells, while THPO suppressed SCF-induced upregulation of *c-Myc*. These data suggest that THPO/MPL signaling is associated with the regulation of cell-cycle-regulator expression in LT-HSCs. We hypothesized from these findings that THPO/MPL signaling is involved in the maintenance of quiescence of LT-HSCs.

THPO/MPL Signaling Is Required for the Maintenance of Quiescence of LT-HSCs

In order to examine the role of THPO/MPL signaling in the maintenance of quiescent HSC, we treated mice with AMM2 and analyzed the cell-cycle status of LT-HSCs. This treatment slightly decreased the platelet counts on day 6 (Figure S2) but did not induce apoptosis of LSK cells (data not shown). It was previously reported that pyronin Y (PY)$^{low/-}$ and PY$^+$ cells were in G0 and G1 phases of cell cycle, respectively (Hüttmann et al., 2001). Then, we investigated the effect of AMM2 treatment on the cell-cycle status of LT-HSCs by PY staining. Consistent with previous reports (Yamazaki et al., 2006), most LSKCD34$^-$ cells are PY$^{low/-}$, suggesting that these cells are in the G0 phase of the cell cycle in the steady state. On day 6 of AMM2 injection, AMM2 treatment dose-dependently increased the PY$^+$ fraction in LSKCD34$^-$ cells (Figures 6A and 6B). These data suggest that the inhibition of THPO/MPL signaling results in reduction of the quiescent HSC population. In addition, the proportion of SP cells in LSK cells was reduced concomitantly with the increase of PY$^+$ cells after 6 days of AMM2 injection (Figure 6C, Figure S3). In contrast to the AMM2, the inhibition of SCF/c-Kit signaling by anti-c-Kit neutralizing Ab, ACK2, did not affect PY$^-$ population in LSKCD34$^-$ cells but reduced PY$^+$ fraction in LSKCD34$^+$ cells (Figure S4). In addition, ACK2 treatment reduced non-SP in LSK cells on day 3 (Figure S4).

From these data above, we hypothesized that blocking THPO/MPL signals influence not only the cell cycle but also HSC-niche interactions. To clarify this issue, we examined whether AMM2 administration enables HSCs to engraft under nonmyeloablative conditions (Figures 6D and 6E). Since it is likely that a transition of the cell population from non-SP to SP cells would disturb engraftment of the donor HSCs, we administered a low dose of 5-FU to deplete the endogenous cycling HSCs. As shown in Figure 6E, only combined administration of AMM2 and 5-FU enabled donor-derived cells to be engrafted in recipient BM (recipient mice positive for lymphomyeloid reconstitution, five of five), while efficient engraftment of donor LSK cells was not observed with other pretreatments (PBS alone, zero of five; 5-FU alone, one of five; AMM2 alone, zero of five). These data suggest that inhibition of THPO/MPL signaling reduced the number of quiescent LT-HSCs and allowed exogenous HSC engraftment

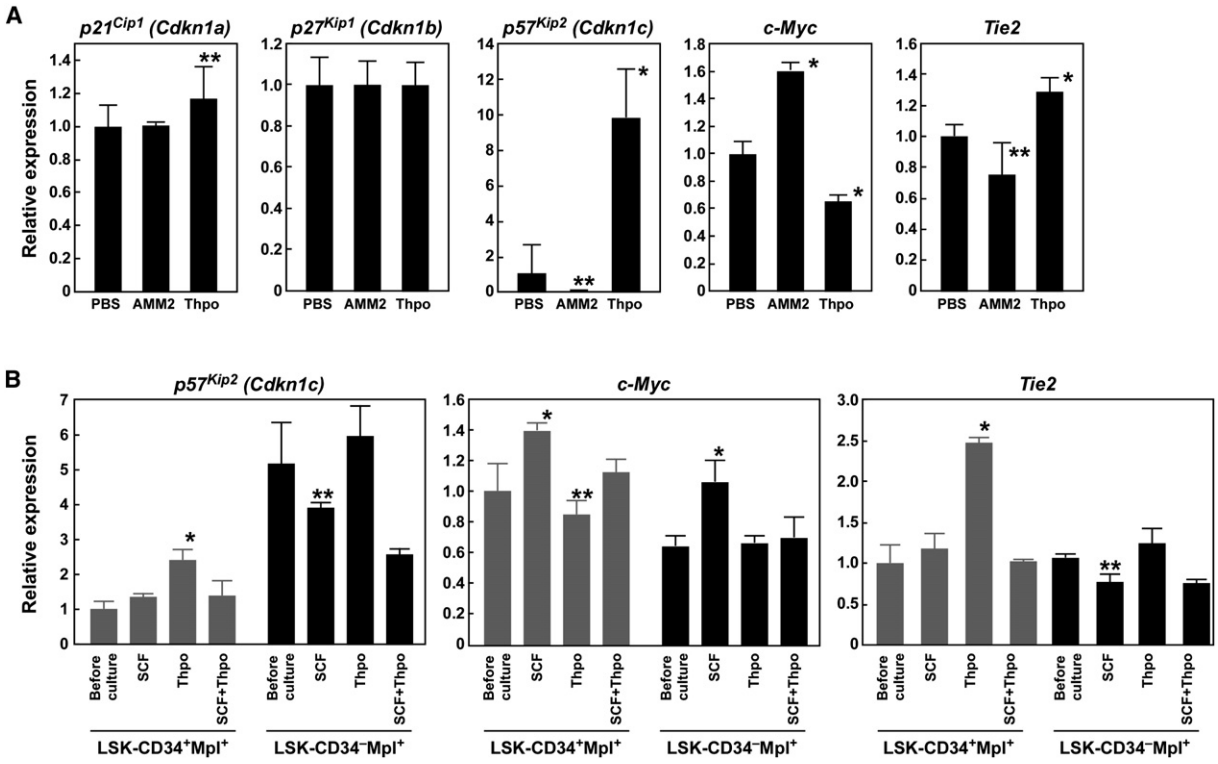

Figure 5. Expression of Cell-Cycle-Related Genes

(A) LSK cells were sorted 6 days after AMM2 injection and after 4 days of daily THPO injection. Relative expression of *Cdkns* ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$), *c-Myc*, and *Tie2* was analyzed. Data represent mean ± SD from separate experiments (*$p < 0.01$, **$p < 0.05$, compared to PBS).

(B) $LSKCD34^{-}MPL^{+}$ and $LSKCD34^{+}MPL^{+}$cells were sorted and cultured and analyzed the expression of $p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$, *c-Myc*, and *Tie2*. Data represent mean ± SD (*$p < 0.01$, **$p < 0.05$, compared to before culture in each group).

without irradiation. These findings support the idea that THPO/MPL signaling is important for maintaining quiescent HSC population in the steady state in vivo.

On the other hand, a continuous administration of AMM2 did not enable the engraftment of donor-derived LSK cells (Figure 6E). In serum-free culture with SCF and THPO in vitro, AMM2 transiently increased BrdU incorporation of LSK cells, while the number of expanded cells was eventually reduced by AMM2 on day 4 of culture (Figure S5). We assume that residual AMM2 in the peripheral circulation of recipient mice negatively affected post-BMT expansion of donor cells, as shown in Figure 6E.

Stimulation of THPO/MPL Signaling Transiently Increased the Proportion of Quiescent HSCs

Next, we analyzed the proportion of LSK-SP cells after THPO injection in mice. After 2 days of THPO injection, the proportion of SP increased, and the proportion of non-SP decreased in LSK cells. The ratio of SP/non-SP in LSK increased 2-fold by the THPO treatment (Figures 7A and 7B). In particular, the ratios of tip to basal SP increased 2.4-fold and 2.0-fold in LSK-SP cells on days 2 and 4 after THPO treatment, respectively (Figure 7B). Increases of the ratio of SP to non-SP and tip to basal-SP by THPO treatment indicates the possibility that some cycling HSCs convert to quiescent phenotype. In addition, pretreatment with THPO (12 hr prior to 5-FU injection) enhanced 5-FU resistance of $LSK\text{-}MPL^{+}$ cells on day 2 post-5-FU injection, compared to PBS treatment (Figure S6). The higher number of $LSK\text{-}MPL^{+}$ cells survived when a higher dose of THPO (100 μg/kg) was administrated. In particular, MPL^{high} fraction in LSK survived preferentially by THPO. THPO treatment increased the number of LSK cells 4 days after injection, and the ratio of SP to non-SP cells was decreased on day 4. On day 6, the ratio of SP/non-SP and tip/basal SP and the number of LSK cells were returned to the normal level (Figure 7B and data not shown). Continuous daily injection of THPO also increased the proportion of LSK-SP cells (Figure 7C). The ratio of SP to non-SP in LSK cells increased 2.3-fold on day 4 after THPO injection compared to PBS treatment (Figure 7D). On day 6 of daily THPO treatment, the ratio of SP to non-SP on LSK cells was decreased due to the increase of the number of LSK cells (Figure 6D). Platelet counts also increased following THPO injection (Figure S2). To examine whether the SP cells after THPO treatment maintained quiescence, we analyzed the cell cycle of LSK-SP and $LSKCD34^{-}$ cells. Both SP and $CD34^{-}$ fraction in LSK cells maintained quiescence 2 days after 100 μg/kg of THPO injection (Figure 7E). On the other hand, a higher dose of THPO administration increased the proportion of PY^{+} fraction in $LSKCD34^{-}$ and

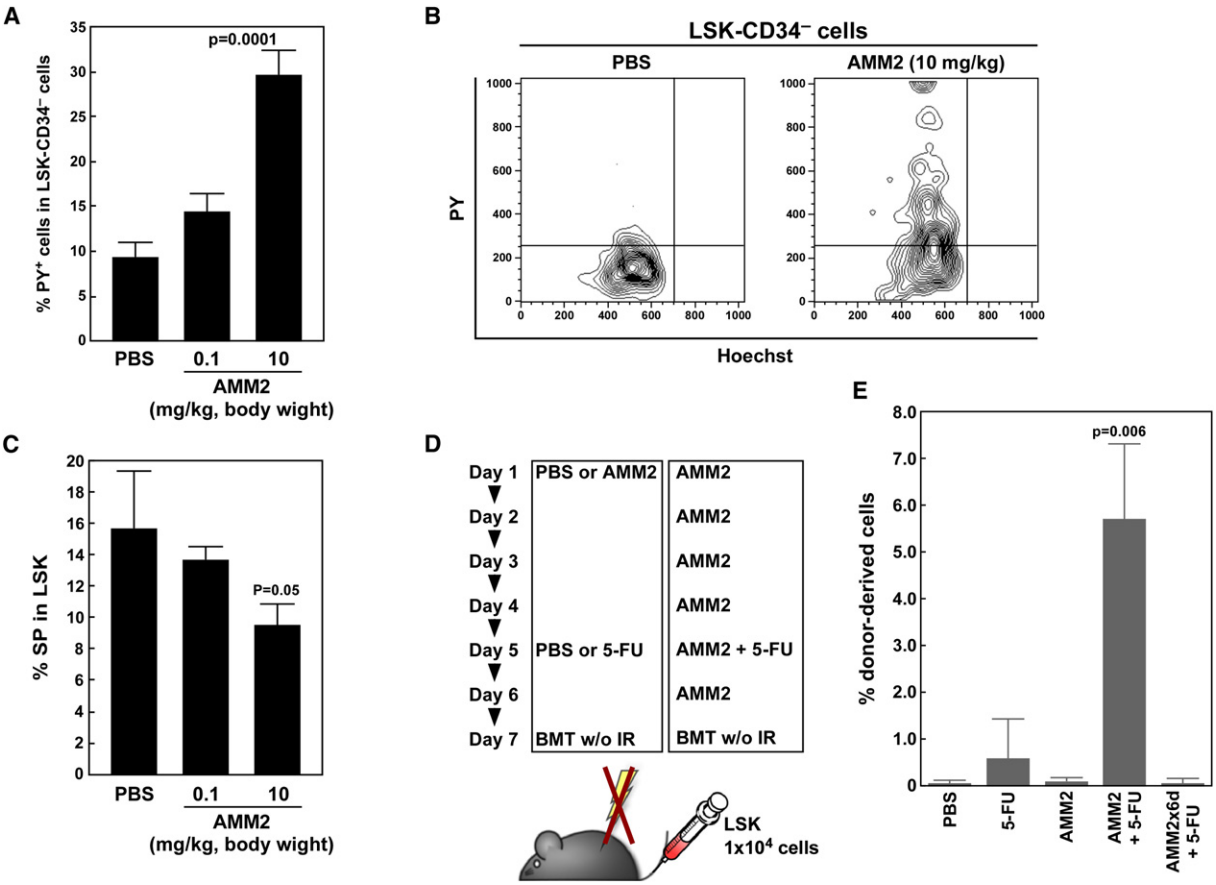

Figure 6. Inhibition of MPL Signaling Reduced the Number of Quiescent LT-HCSs

(A) PY analysis of LSKCD34$^-$ cells after AMM2 treatment. Inhibition of MPL increased the frequency of PY$^+$ cells in LSKCD34$^-$ fraction. Data represent mean ± SD (n = 6 per group).

(B) Hoechst and PY staining emission patterns of LSKCD34$^-$ cells.

(C) Percentages of SP cells in LSK cells after 6 days of AMM2 treatment. AMM2 decreased SP cells in LSK cells in a dose-dependent manner. Data represent mean ± SD (n = 5 per group).

(D) Experimental procedure of BMT without irradiation. Left box shows a single injection of AMM2 or PBS 6 days before BMT. Right box indicates daily administration of AMM2. On day 5, mice were treated with 5-FU or PBS injection. On day 7, Ly5.1$^+$ 1x10^4 LSK cells were injected into each group without X-ray irradiation.

(E) Inhibition of THPO/MPL signaling by AMM2 permits BMT without irradiation. Percentages of donor-derived cells in peripheral blood are shown. Data represent mean ± SD (n = 5 per group).

LSK-SP cells on day 2 (Figure 7F and data not shown), indicating that a high dose of THPO rapidly induced proliferation of HSCs.

DISCUSSION

THPO is known to be the primary cytokine regulating megakaryocyte development and platelet production, and it is involved in regulation of survival and proliferation of HSCs (Alexander et al., 1996; Borge et al., 1996; Kimura et al., 1998; Solar et al., 1998; Fox et al., 2002). In addition, THPO/MPL signaling synergistically induced HSC proliferation with other cytokines (Sitnicka et al., 1996; Ema et al., 2000). In this study, we demonstrate that THPO/MPL signaling is involved in the niche regulation of LT-HSCs, maintaining quiescent population of HSCs in the osteoblastic niche. Currently, the stem cell niches for hematopoietic system were identified in two sites in the BM: one at the endosteal surface and the other in sinusoidal vascular areas (Calvi et al., 2003; Zhang et al., 2003; Arai et al., 2004; Kiel et al., 2005), and it has been proposed that the osteoblastic niche is thought to maintain HSC quiescence over the long term (Yin and Li, 2006). In addition, it was reported that CXCL12-abundant reticular cells associated with HSCs in both the vascular and the osteoblastic niche (Sugiyama et al., 2006).

Quiescence of HSCs is critical to ensure lifelong hematopoiesis and to protect the HSC pool from myelotoxic insult and premature exhaustion under conditions of hematopoietic stress (Cheng et al., 2000). Here we showed that MPL was expressed in the LT-HSC fractions (Figures 1 and 3), and MPL expression in HSCs is closely correlated

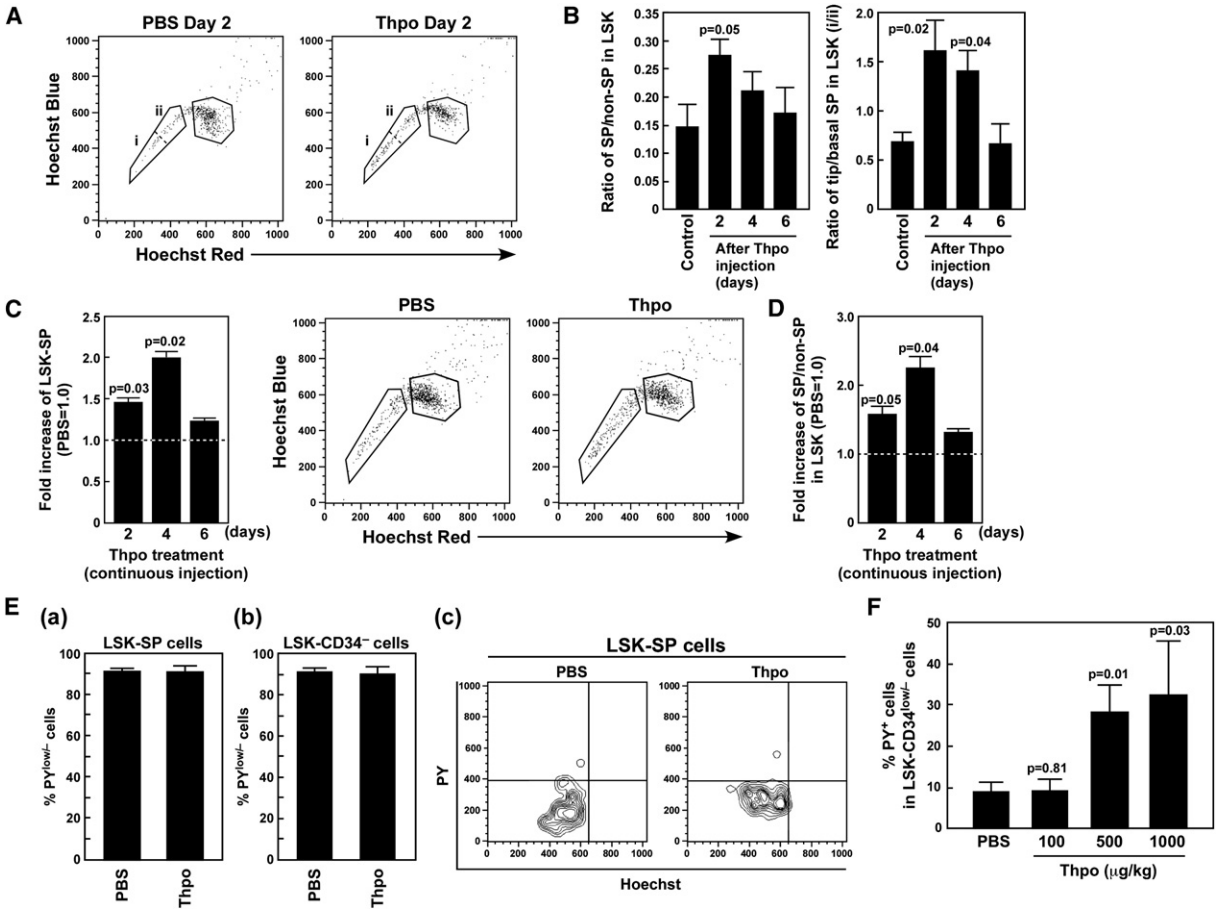

Figure 7. Effects of Exogenous THPO in the Regulation of LT-HSCs

FACS analysis of the frequency of quiescent HSCs after stimulation of THPO/MPL signaling by exogenous administration of THPO.

(A) Representative FACS profiles of LSK-SP cells after 2 days of THPO injection.

(B) Ratio of SP to non-SP fraction in LSK after THPO injection (left). Ratio of tip (gate i) to basal (gate ii) portion of LSK-SP fraction after THPO injection (right). Data represent mean ± SD (n = 4 per group).

(C) Proportion of LSK-SP cells after continuous daily THPO injection. THPO transiently increased the proportion of SP cells 2–4 days after the first injection (left). Data shown are the fold increase of the SP fraction in LSK cells mediated by THPO compared to PBS treatment. Data represent mean ± SD (n = 4 per group). Representative FACS profiles of LSK-SP after 4 days of daily THPO treatment are shown (right).

(D) Fold increase in the ratio of SP/non-SP between PBS and THPO treatments. Data represent mean ± SD (n = 4 per group).

(E) PY analysis of LSK-SP and LSKCD34$^-$ cells after 2 days of THPO administration. LSK-SP (Ea) and LSKCD34$^-$ cells (Eb) maintained the quiescence after 2 days of THPO treatment. Data represent mean ± SD (n = 4 per group). Hoechst and PY staining emission pattern of LSK-SP cells (Ec).

(F) The percentages of PY$^+$ fraction in LSKCD34$^-$ cells. High dose of THPO increased PY$^+$ fraction in LSKCD34$^-$ cells. Data represent mean ± SD (n = 4 per group).

with cell-cycle status (Figure 1). MPL$^+$ HSCs closely contacted to THPO-producing osteoblastic cells at the endosteal surface in the trabecular bone area (Figure 2), indicating that THPO/MPL signaling is involved in the niche regulation of LT-HSCs in the osteoblastic niche, such as Ang-1/Tie2 (Arai et al., 2004), Jagged1/Notch1 (Calvi et al., 2003), N-cadherin (Zhang et al., 2003), or osteopontin (Nilsson et al., 2005; Stier et al., 2005). In addition, we found that THPO/MPL signaling upregulated β1-integrin (Figure 4D). These data suggest that THPO/MPL signaling-induced cell adhesion of LT-HSCs to the osteoblastic niche may contribute to the maintenance of quiescent HSCs. Moreover, AMM2 and THPO treatment revealed that THPO/MPL signaling is involved in the regulation of cell-cycle-regulator expression, such as *p21^{Cip1}*, *p57^{Kip2}*, *c-Myc*, and *Tie2* (Figure 5). Especially, we found that THPO treatment increased and AMM2 treatment decreased the expression of *p57^{Kip2}*, which is highly expressed in the quiescent population in LT-HSCs (Figure 1D and 5 and Figure S1). In the accompanying paper, Qian et al. (2007) clearly show that *p57^{Kip2}* is dramatically downregulated in *THPO$^{-/-}$* HSCs. The reduction of *p57^{Kip2}* expression in HSCs by AMM2 treatment is consistent with the data of *THPO$^{-/-}$* HSCs (Qian et al., 2007). It has been reported that p57^{Kip2} forms a complex with cyclin D1, which is essential for HSC expansion, in the

cytoplasm of HSCs and inhibits cyclin D activity (Yamazaki et al., 2006). Scandura et al. (2004) reported a result of microarray analysis using primary human hematopoietic cells, showing that $p57^{Kip2}$ is the only Cdkn induced by TGF-β. Unexpectedly, TGF-β receptor null BM did not show significant abnormalities in HSC function (Larsson et al., 2003). Therefore, we hypothesize that the combined activities of cell-cycle-regulator and cell-adhesion molecules play an important role in regulation of THPO/MPL-mediated HSC maintenance. It remains to elucidate the function of $p57^{Kip2}$ in quiescent HSCs.

Administration of AMM2 decreased the number of quiescent population in LT-HSCs. In contrast, ACK2 decreased the cycling HSCs and progenitor cells (Figure S4). It is known that dormant primitive stem cells are resistant to ACK2 (Kodama et al., 1992; Nishimura et al., 2002). Therefore, we hypothesize that THPO/MPL signaling takes part in the maintenance of a quiescent population, and the postnatal reduction of the HSC pool in *MPL*- or *THPO*-deficient mice might be due to continuing defects in quiescence of LT-HSCs. Indeed, Qian et al. (2007) clearly show the age-progressive loss of LT-HSC in $THPO^{-/-}$ mice. This reduction in the LT-HSCs in $THPO^{-/-}$ mice is accompanied by the accelerated HSC cell cycling. In the serum-free culture, AMM2 initially enhanced BrdU incorporation of HSCs, whereas it reduced proliferation of cells on day 4 of culture (Figure S5). The enhanced BrdU incorporation in response to AMM2 initially might reflect the ability of THPO to promote quiescence of HSCs; however, on day 4, most of the cells in cultures are not stem cells, and the reduced proliferation might reflect that the effect of THPO on more committed progenitors is to enhance proliferation rather than quiescence. We speculate that THPO-induced quiescence is HSC specific. Furthermore, by administrations of AMM2 and 5-FU together in recipient mice, donor LSK cells were able to engraft without lethal radiation (Figure 6), indicating that inhibition of THPO/MPL signaling reduces HSC-niche interactions. It suggests that inhibiting the HSC-niche interaction represents a novel technique for BMT without irradiation. To attain a better engraftment without myeloablative conditioning, it is critical to inhibit the interaction of endogenous HSC with the niche effectively after AMM2 treatment. One prediction would be that simultaneous use of G-CSF with AMM2 has a potential to induce efficient mobilization of HSCs to peripheral blood and enable higher engraftment. Further investigations are necessary to clarify this issue. On the other hand, we found that the continuous injection of AMM2 did not allow nonmyeloablative BMT. We hypothesize that THPO/MPL signaling is also involved in HSC proliferation for replenishment of BM cellularity during an initial rapid cycling and expansion phase of BMT. Fox et al. (2002) previously reported that THPO expands HSCs after BMT. In addition, Qian et al. (2007) also clearly demonstrated that $THPO^{-/-}$-recipient mice showed the defects in posttransplantation expansion of donor HSCs and clarified that the expansion of HSCs in the first weeks after transplantation is highly THPO dependent.

We also found that THPO administration transiently increased a quiescent population of HSCs (Figure 7). The regulation of HSC itself and reproduction of the niche is of considerable importance for establishment of optimal condition of the ex vivo expansion of LT-HSCs.

Altogether, our data suggest that the THPO/MPL pathway is a component of the HSC osteoblastic niche and propose a novel biphasic mechanism of THPO/MPL signaling for the regulation of LT-HSCs in the niche. THPO and MPL interaction not only regulates self-renewal but also quiescence of HSCs in adult BM. In the steady condition, THPO/MPL pathway participates in the maintenance of quiescent HSCs and HSC-niche interactions.

It has been discussed lately that stem cell niche is the potential target of the stem cell-based therapy (Adams et al., 2007). Our data show the possibility that THPO/MPL pathway becomes an attractive target to niche therapy. The result of our nonmyeloablative BMT assay allows us to foresee the possibility that the manipulation of the components of leukemic stem cell (LSC) niche contributes to a new strategy for leukemic therapy. It is possible that possession of the stem cell niche by LSCs causes the resistance to anticancer drugs. Therefore, inhibition of niche factors may induce the detachment of LSC from the niche and enhance sensitivity to the anticancer therapy. Identifications of LSC niche and their niche component are important issues for this end.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 (B6-Ly5.2) mice were purchased from Japan SLC (Shizuoka, Japan). C57BL/6 mice congenic for the Ly5 locus (B6-Ly5.1) were purchased from Sankyo-Lab Service (Tsukuba, Japan). Animal care in our laboratory was in accordance with the guidance of Keio University for animal and recombinant DNA experiments.

Antibodies

The following mAbs were used for FACS analysis and cell sorting: anti-c-Kit (2B8, BD Biosciences), anti-Sca-1 (E13-161.7, BD Biosciences), anti-CD4 (L3T4, BD Biosciences), anti-CD8 (53-6.72, BD Biosciences), anti-B220 (RA3-6B2, BD Biosciences), anti-TER-119, Gr-1 (RB6-8C5, BD Biosciences), anti-Mac-1 (M1/70, BD Biosciences), anti-FLT3 (A2F10.1, BD Biosciences), anti-CD34 (RAM34, eBioscience), anti-Tie2 (TEK4), and anti-MPL (AMM2). A mixture of CD4, CD8, B220, TER-119, Mac-1, and Gr-1 was used as the lineage mix (Lin). The following Abs were used for immunohistochemistry: anti-MPL (AMM2), anti-c-Kit polyclonal Ab (R & D Systems), anti-Tie2 (BD Biosciences), anti-THPO (donated from Kirin Brewery Co., Ltd), and anti-ALP (B4-48, R & D Systems). AMM2 and anti-c-Kit Abs (ACK2, eBioscience) were used for in vivo administration to inhibit THPO/MPL and SCF/c-Kit signaling, respectively.

Preparation of Rat Monoclonal Abs against Mouse c-MPL

Male Wistar rats (7-week-old) were first immunized intraperitoneally and via the hindfoot pad with 100 μg of soluble, recombinant extracellular domain of mouse c-MPL and received three further intraperitoneal injections of 10 μg of the same protein with 2 week intervals. Four days after the final injection, spleen cells from immunized rats were fused with P3X63-Ag.8.653 cells and a murine myeloma cell line and cultured in hybridoma selection medium. Hybridomas were screened for production of rat antibodies against mouse c-MPL with a cell-based enzyme-linked immunosorbent assay using FDCP2 cells overexpressing

mouse *c-MPL*. Six clones producing specific antibodies were selected and designated AMM1-6. Antibodies were tested for their ability to inhibit mouse THPO-induced growth of FDCP2 cells expressing mouse MPL and megakaryocyte colony formation (Figure S7). AMM2 had the most potent neutralizing activity.

Cell Preparation and Flow Cytometry

Eight-week-old mouse-derived BM cells were fully flushed from femurs and tibias, and mononuclear cells (MNCs) were isolated using density centrifugation with Lymphoprep (Axis-Shield). Procedures for SP staining, PY staining, and immunofluorescence staining were previously described (Arai et al., 2004). Stained cells were analyzed and sorted by FACSvantage DiVa (Becton Dickinson). The expression of MPL in LSK cells was analyzed at 2 and 6 days after 5-FU treatment (150 mg/kg body weight, i.v.).

Immunohistochemistry

Procedure for preparation of BM sections, BrdU-LTR assay, and immunohistochemistry of tissue sections has been previously described (Arai et al., 2004). For nuclear staining, specimens were treated with TOTO3 (Molecular Probes). Fluorescence images were obtained by using a confocal laser-scanning microscope (Olympus, model FV1000).

Culture of Cell Lines and Primary Osteoblastic Cells

OP9 stromal cells were maintained as previously described (Arai et al., 2004). NIH 3T3 were maintained in DMEM (Sigma) supplemented with 10% FCS at 37°C in humidified 5% CO_2 air. Isolation and maintenance of adult mouse BM-derived primary osteoblastic cells were described previously (Arai et al., 2004).

In Vitro Cell Cultivation

LSKCD34$^+$MPL$^+$ cells and LSKCD34$^-$MPL$^+$ cells were sorted and cultured on fibronectin-coated plate. Cultures were maintained in SF-O3 medium (Sanko Junyaku Co., Ltd.) containing 1.0% BSA 100 ng/ml SCF and/or 100 ng/ml THPO. After 2 days of cultivation, cells were collected, and total RNA was isolated using the RNeasy Mini Kit (QIAGEN). Isolated RNA was reverse transcribed using Advantage RT-for-PCR Kit (Clontech).

BM Reconstitution Assay

Cells (2×10^3) of LSK-MPL$^+$ and LSK-MPL$^-$ cells were sorted from 8-week-old Ly5.1 mice BM and transplanted into lethally irradiated Ly5.2 mice with 2×10^5 competitor cells (Ly5.2). Cells (1×10^2) of LSKCD34$^-$MPL$^+$, LSKCD34$^+$MPL$^+$, LSKCD34$^-$MPL$^-$, and LSKCD34$^+$MPL$^-$ cells were also sorted from 8-week-old Ly5.1 mice and transplanted into Ly5.2-recipient mice with 2×10^5 competitor cells. Percentages of donor-derived cells were analyzed monthly by FACS. Reconstitution of donor (Ly5.1) myeloid and lymphoid cells was monitored by staining blood cells with antibodies against Ly5.2, Ly5.1, CD3, B220, Mac-1, and Gr-1. For serial BMT assay, BMMNCs (5×10^5) or LSK cells (5×10^3) were isolated from four primary recipient mice after 6 months of the first BMT and were transplanted to lethally irradiated Ly5.2-recipient mice. For BMT assays without X-ray irradiation, recipient mice were treated with single injection of AMM2 (1 mg/kg body weight, i.v.) or PBS 6 days before BMT. Simultaneously, another group of recipient mice was treated with 6 days of daily administration of AMM2. On day 5, recipient mice were treated with 5-FU (150 mg/kg body weight, i.p.) or PBS injection. On day 7, Ly5.1$^+$ 1×10^4 LSK cells were injected into each group without X-ray irradiation (Figure 6D).

In Vivo Assay of THPO/MPL Signaling

To inhibit THPO/MPL signaling in vivo, mice were treated with AMM2 (0.1 or 10 mg/kg body weight, i.v.). To stimulate THPO/MPL signaling in vivo, mice were treated with single or continuous daily injection of THPO (100 μg/kg body weight, i.v.). After 2, 4, and 6 days of AMM2 or THPO treatment, LSK-SP cells were analyzed. In order to analyze the gene expression induced by AMM2 or THPO treatment, LSK cells were sorted after 6 days of AMM2 (1 mg/kg body weight, i.v.) injection or after 4 days of daily THPO (100 μg/kg body weight, i.v.) injection. mRNA expressions were analyzed by qPCR.

Quantitative Real-Time PCR Analysis

A quantitative real-time PCR (qPCR) assay was performed on an ABI 7500 Fast Real-Time PCR System using TaqMan Fast Universal PCR master mixture (Applied Biosystems, Foster City, CA). Probes and primers used in qPCR assay are listed in the Supplemental Data. Data were analyzed by 7500 Fast System SDS Software 1.3.1. All experiments were done in triplicates.

Statistical Analysis

The significance of differences among groups was determined by two-tailed Student's t test.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and seven figures and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/1/6/685/DC1/.

ACKNOWLEDGMENTS

This work was supported by Grant-in-Aid for Specially Promoted Research and Grant-in-Aid for Young Scientists (A) from The Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan; and by "High-Tech Research Center" Project for Private Universities, matching fund subsidy from MEXT, by a Takeda Science Foundation, and by The Sumitomo Foundation. We acknowledge Ms. Ayako Kumakubo for preparation of tissue sections of BM. We also thank Ms. Ayami Ono for technical assistance.

Received: May 8, 2007
Revised: September 4, 2007
Accepted: October 29, 2007
Published online: November 15, 2007

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