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Critical Role of Thrombopoietin in Maintaining Adult Quiescent Hematopoietic Stem Cells

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SUMMARY

The role of cytokines in regulation of hematopoietic stem cells (HSCs) remains poorly understood. Herein we demonstrate that thrombopoietin (THPO) and its receptor, MPL, are critically involved in postnatal steady-state HSC maintenance, reflected in a 150-fold reduction of HSCs in adult *Thpo*$^{-/-}$ mice. Further, whereas THPO and MPL proved not required for fetal HSC expansion, HSC expansion posttransplantation was highly MPL and THPO dependent. The distinct role of THPO in postnatal HSC maintenance is accompanied by accelerated HSC cell-cycle kinetics in *Thpo*$^{-/-}$ mice and reduced expression of the cyclin-dependent kinase inhibitors *p57*^{Kip2} and *p19*^{INK4D} as well as multiple *Hox* transcription factors. Although also predicted to be an HSC viability factor, BCL2 failed to rescue the HSC deficiency of *Thpo*$^{-/-}$ mice. Thus, THPO regulates posttransplantation HSC expansion as well as the maintenance of adult quiescent HSCs, of critical importance to avoid postnatal HSC exhaustion.

INTRODUCTION

A compartment of rare pluripotent hematopoietic stem cells (HSCs) is both required and sufficient to sustain blood cell production for life. The ability of HSCs to self-renew is a requisite for their extensive expansion during fetal development and posttransplantation (Iscove and Nawa, 1997; Pawliuk et al., 1996) as well as to sustain HSCs at relatively constant numbers in adult bone marrow (BM) (Morrison et al., 1996). Postnatal HSC self-renewal is closely coupled to the cells' unique cell-cycle kinetics. Although cycling with very slow kinetics, adult HSCs are predominantly quiescent (Bradford et al., 1997; Cheshier et al., 1999), and it is likely that maintenance of HSC quiescence and slow cell-cycle progression could be critically involved in sustaining a self-renewing HSC compartment for life.

Our knowledge about physiological intrinsic and extrinsic cues regulating HSC maintenance and expansion remains limited (Molofsky et al., 2004; Oostendorp et al., 1999; Sauvageau et al., 2004; Wilson and Trumpp, 2006). Notably, of the few essential intrinsic regulators of HSC maintenance identified, PTEN and GFI-1 have been found at least in part to act through restriction of HSC proliferation (Hock et al., 2004; Yilmaz et al., 2006b), as has the cyclin-dependent kinase (CDK) inhibitor p21^{Cip1} (Cheng et al., 2000b). Similarly, angiopoietin-1 has been identified as an extrinsic regulator of HSC quiescence (Arai et al., 2004). In striking contrast, a number of classical hematopoietic cytokines have, largely based on in vitro findings, been suggested to rather act as potent stimulators of HSC proliferation (Sauvageau et al., 2004; Zhu and Emerson, 2002). However, with the exception of thrombopoietin (THPO), efforts to confirm their proposed roles as physiological regulators of HSC maintenance and expansion have failed (Sauvageau et al., 2004; Sorrentino, 2004), as have extensive attempts to use combinations of cytokines as proliferative factors to expand HSCs ex vivo (Oostendorp et al., 1999).

Adult mice deficient in the genes encoding the hematopoietin cytokine *Thpo* or its receptor, *Mpl*, have been suggested to have reduced HSC function and numbers (Buza-Vidas et al., 2006; Kimura et al., 1998; Solar et al., 1998), but the extent of this deficiency and the mechanism of action remain unexplored. Based on in vitro studies, THPO has been proposed to be a potent stimulator of HSC proliferation when acting in synergy with other cytokines (Sitnicka et al., 1996). However, most cytokines synergizing with THPO to enhance proliferation, unlike THPO, do not appear to play a physiological role in HSC maintenance or expansion, and efforts to expand HSCs ex vivo through such cytokine combinations have largely failed (Oostendorp et al., 1999). When acting alone, THPO appears

to support in vitro survival and maintenance of highly enriched HSCs (Borge et al., 1996), and in agreement with this, *Mpl* is most highly expressed in quiescent long-term (LT)-HSCs rather than more rapidly cycling short-term (ST)-HSCs and multipotent progenitors (MPPs) (Buza-Vidas et al., 2006). Thus, rather than promoting HSC cycling, THPO might play a crucial role in maintenance of quiescent HSCs, either acting as an antiapoptotic factor, as has been suggested for several other cytokines (Cross and Dexter, 1991; Maraskovsky et al., 1997), or potentially through sustaining adult HSC quiescence. Importantly it would be predicted, if critically involved in regulation of HSC quiescence, that $Thpo^{-/-}$ mice might not have an HSC deficiency in fetal development when HSCs cycle rapidly and undergo extensive expansion (Pawliuk et al., 1996) but that they postnatally would develop a progressive HSC deficiency, as HSCs normally would acquire a more quiescent phenotype. Investigation of this would be of considerable relevance for regulation of human HSCs, as patients with congenital amegakaryocytic thrombocytopenia (CAMT), who are born with lack-of-function mutations in *Mpl*, with exception to thrombocytopenia appear to be born with normal hematopoiesis but within few years develop a multilineage BM failure (Ballmaier et al., 2003).

Here we investigated the dynamics of HSC expansion and maintenance in $Thpo^{-/-}$ mice and demonstrate that THPO and its receptor MPL are essential for postnatal HSC maintenance, but not for fetal HSC expansion, as both $Thpo^{-/-}$ and $Mpl^{-/-}$ fetal livers (FLs) in fact rather have slightly increased HSC numbers. In contrast, $Thpo^{-/-}$ mice progressively lose HSC numbers with age, resulting in a more than 150-fold reduction in 1-year-old mice, translating into slightly reduced BM cellularity and a multilineage hematopoietic defect, less extensive but similar to what is observed in CAMT patients (Ballmaier et al., 2003). This critical role of THPO in postnatal HSC maintenance does not appear to primarily reflect the proposed antiapoptotic effects of THPO, as overexpression of *BCL2* failed to correct the HSC phenotype of $Thpo^{-/-}$ mice. Rather, lineage (LIN)$^-$SCA1^{hi}KIT^{hi} (LSK) CD34$^-$FLT3$^-$ cells in $Thpo^{-/-}$ mice display accelerated cell-cycle kinetics and reduced transcriptional expression of the negative cell-cycle regulators $p57^{Kip2}$ and $p19^{INK4D}$ and different Homeobox (*Hox*) transcription factors. Additionally, we also found that HSC expansion posttransplantation is highly THPO dependent, implicating a distinct role of THPO in postnatal rather than fetal HSC expansion. Thus, the cytokine THPO is a critical regulator of postnatal maintenance of quiescent HSCs and posttransplantation HSC expansion.

RESULTS

Expansion of Fetal HSCs in $Thpo^{-/-}$ Mice

Whereas previous studies have implicated an important role of THPO in regulation of adult multipotent stem/progenitor cells (Buza-Vidas et al., 2006; Kimura et al., 1998; Solar et al., 1998), its role in the extensive HSC expansion observed during fetal development (Pawliuk et al., 1996) had not been investigated. Surprisingly, we found embryonic day (E)14.5 FL cellularity to be significantly and reproducibly increased in $Thpo^{-/-}$ mice (Figure 1A). Further, as the frequencies of LSKFLT3$^-$ HSCs and LSKFLT3$^+$ MPPs were similar in wild-type (WT) and $Thpo^{-/-}$ FLs (Figure 1B), their absolute numbers were in fact slightly but significantly enhanced in $Thpo^{-/-}$ FLs (Figure 1C). Importantly, a similar increase in FL cellularity (Figure 1E) and trend toward elevated LSKFLT3$^-$ and LSKFLT3$^+$ cells (Figures 1F–1G) was observed in $Mpl^{-/-}$ mice.

As self-renewing LT-HSCs can only be identified through their unique ability to sustain multilineage hematopoiesis in vivo (Iscove and Nawa, 1997), limiting numbers (2×10^4/mouse) of unfractionated E14.5 FL cells (CD45.2) from WT or $Thpo^{-/-}$ mice were transplanted into myeloablated recipients in competition with 2×10^5 WT BM cells (CD45.1). In agreement with the phenotypic analysis, a similar frequency of recipients of WT (25/34; 73%) and $Thpo^{-/-}$ (23/35; 65%) FL cells were positive for donor-derived long-term peripheral blood (PB) multilineage (B, T, and myeloid) reconstitution (Figure 1D). Combined with the enhanced FL cellularity, these data further substantiated the conclusion that THPO and MPL are not required to promote and in fact if anything rather appear to somewhat restrict fetal HSC expansion.

Selective and Age-Progressive Loss of $Thpo^{-/-}$ HSCs

We next investigated the role of THPO in postnatal BM HSC maintenance. Unlike in FL, the total number of BM mononucleated cells in 2-week-old $Thpo^{-/-}$ mice was equivalent to that in WT mice (Figure 2A), whereas the frequencies of LSKFLT3$^-$ HSCs and LSKFLT3$^+$ MPPs (Adolfsson et al., 2005) were clearly reduced (4.0-fold and 2.7-fold, respectively). This demonstrates a distinctly different role of THPO in regulation of fetal and early postnatal HSCs.

We further investigated adult WT and $Thpo^{-/-}$ mice for phenotypically defined LSKCD34$^-$FLT3$^-$ LT-HSCs, LSKCD34$^+$FLT3$^-$ ST-HSCs, and LSKCD34$^+$FLT3$^+$ MPPs (Adolfsson et al., 2005; Yang et al., 2005) (Figure 2B). At 3 months of age, LSKCD34$^-$FLT3$^-$ and LSKCD34$^+$FLT3$^-$ cells were further reduced in $Thpo^{-/-}$ mice by as much as 7.9-fold and 7.5-fold, respectively, whereas LSKCD34$^+$FLT3$^+$ cells were less reduced (3.5-fold). When using CD150 as an additional and documented marker for phenotypic identification of HSCs (Kiel et al., 2005; Yilmaz et al., 2006a), a 16-fold reduction in LSKCD34$^-$FLT3$^-$CD150$^+$ cells was observed in 3-month-old $Thpo^{-/-}$ mice (Figure S1A in the Supplemental Data available with this article online), and a similar (12-fold) reduction of LSKCD150$^+$ cells was observed in 3-month-old $Mpl^{-/-}$ mice (Figure 2C). In 1-year-old mice, the frequencies of LSKCD34$^-$FLT3$^-$ and LSKCD34$^+$FLT3$^-$ cells were further reduced (14.5-fold and 9.1-fold, respectively), whereas the reduction in LSKCD34$^+$FLT3$^+$ cells was similar to that seen at 3 months (2.3-fold reduction; Figure 2B). Thus, whereas 57.6% of LSK cells are FLT3$^-$ in

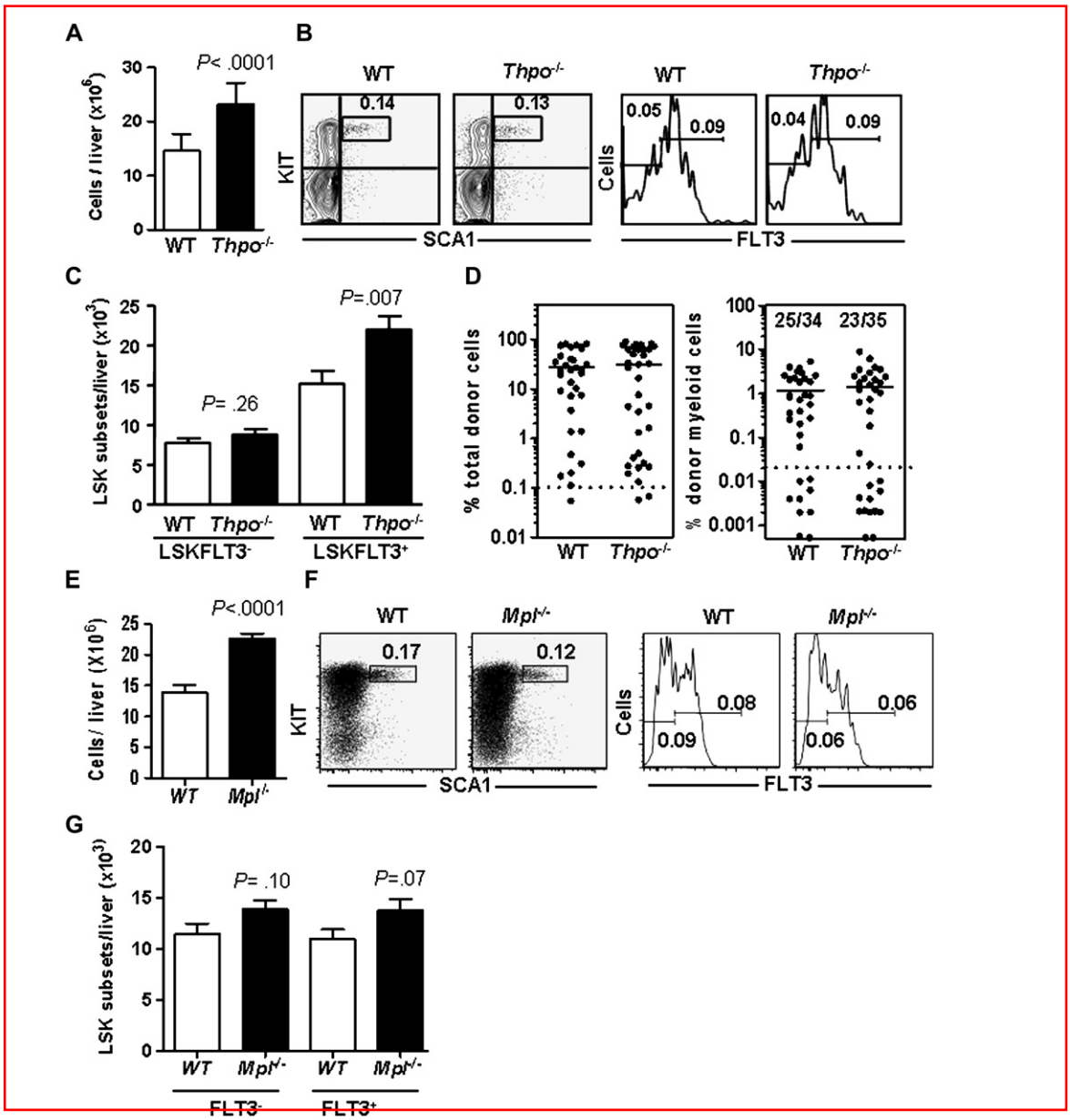

Figure 1. Increased Numbers of Fetal Liver HSCs in *Thpo*$^{-/-}$ and *Mpl*$^{-/-}$ Mice

(A) Total nucleated cells in *Thpo*$^{-/-}$ E14.5 FL (mean ± SD).

(B) Representative FACS plots of LSK analysis of E14.5 FL in WT and *Thpo*$^{-/-}$ mice. The panels show cells already gated as LIN$^-$ and subsequently as LSK and then FLT3$^-$ and FLT3$^+$ subsets (LSKFLT3$^-$, LSKFLT3$^+$). Numbers shown are mean percentages of total cells in 15–17 mice of each genotype.

(C) Number of LSKFLT3$^-$ and LSKFLT3$^-$ cells in WT and *Thpo*$^{-/-}$ E14.5 FL (mean ± SD).

(D) Total donor PB reconstitution (left) and donor-derived myeloid cells (right) in myeloablated WT recipients (CD45.1 or CD45.1/2) at 16 weeks after transplantation of limiting doses (2 × 10^4 cells/mouse) of WT or *Thpo*$^{-/-}$ E14.5 FL cells (CD45.2) in competition with 1–2 × 10^5 WT (CD45.1) BM cells from 10-week-old mice. Each symbol represents an individual recipient, and horizontal bars indicate mean values of reconstitution in positive mice. The numbers shown in the panels are the fraction of mice positive for lympho-myeloid reconstitution, using the threshold level of 0.1% total cells (dashed line, left) and of 0.02% each of the B, T, and myeloid lineages (dashed line, right). Data shown are from two independent experiments.

(E) Total nucleated cells in *Mpl*$^{-/-}$ E14.5 FLs (mean ± SEM).

(F) Representative FACS plots of LSK analysis of E14.5 FL in WT and *Mpl*$^{-/-}$ mice.

(G) Numbers (mean ± SEM) of LSKFLT3$^-$ and LSKFLT3$^+$ cells in WT and *Mpl*$^{-/-}$ E14.5 FL.

All data in (E)–(G) represent mean values of eight mice analyzed of each genotype.

1-year-old WT mice, only 23.6% have this phenotype in *Thpo*$^{-/-}$ mice. This demonstrates a selective role of THPO in maintenance of adult LT- and ST-HSCs with less effect on MPPs, consistent with the high expression of MPL and THPO responsiveness in HSCs and downregulation in LSKCD34$^+$FLT3^{hi} MPPs (Buza-Vidas et al., 2006).

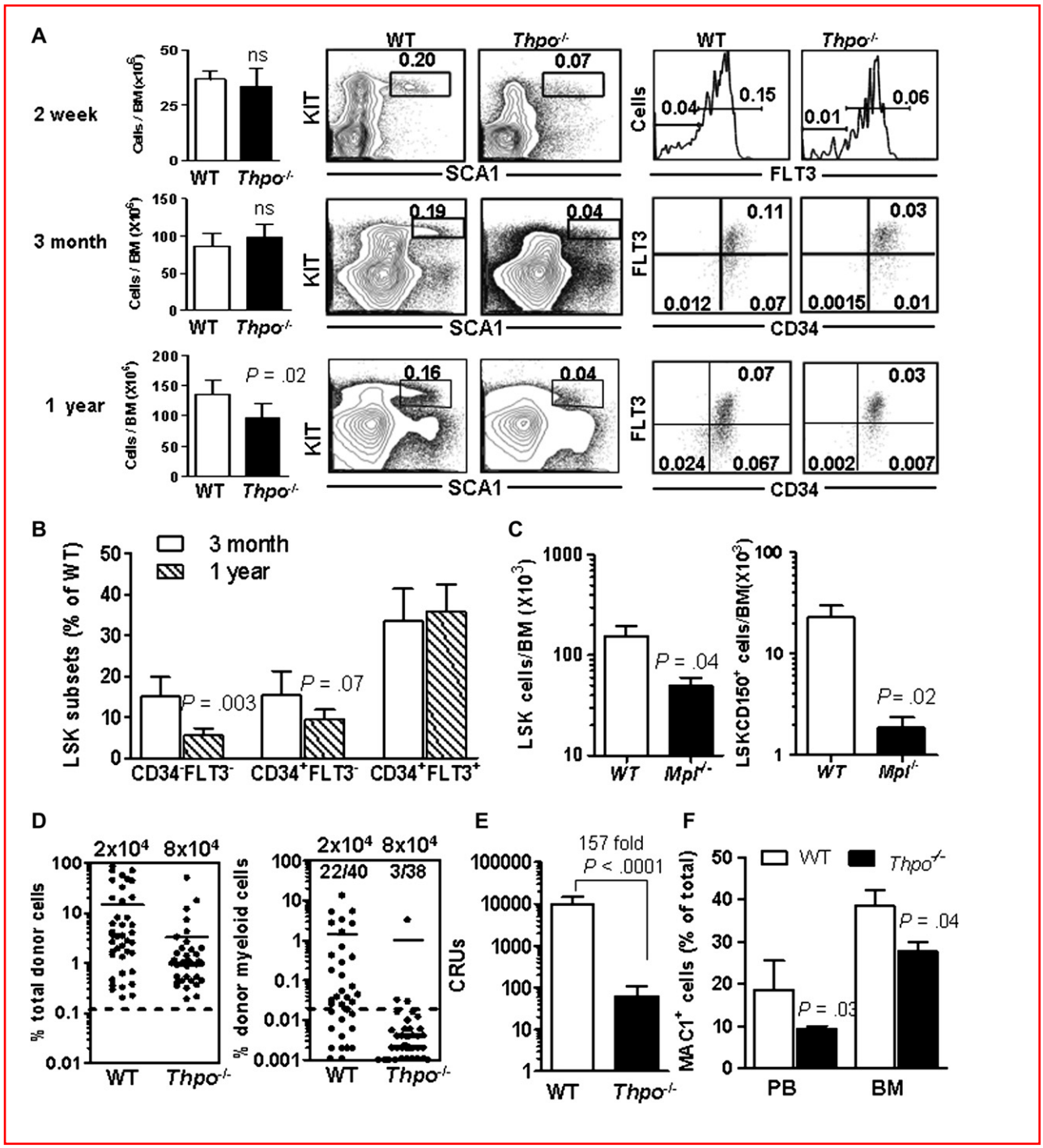

Figure 2. Selective and Progressive Postnatal Reduction of LT-HSCs in *Thpo*$^{-/-}$ Mice

(A) Total nucleated BM cells (mean ± SD) from two tibias and two femurs and representative FACS plots of LSK subsets (LSKCD34$^-$FLT3$^-$, LSKCD34$^+$FLT3$^-$, and LSKCD34$^+$FLT3$^+$) from 2 week, 3 month (12–14 week), and 1-year-old WT and *Thpo*$^{-/-}$ mice. Numbers shown are mean percentages of total cells in 5–12 mice of each genotype (2 week, n = 12; 3 month, n = 5; and 1 year, n = 5).

(B) Mean (±SD) numbers of LSKCD34$^-$FLT3$^-$, LSKCD34$^+$FLT3$^-$, and LSKCD34$^+$FLT3$^+$ cells in BM of 3 month (n = 5–6), and 1 year (n = 5) old *Thpo*$^{-/-}$ mice relative to age-matched WT controls.

(C) Mean (±SEM) numbers of LSK and LSKCD150$^+$ cells in BM (two femurs and two tibias) of 3-month-old WT and *Mpl*$^{-/-}$ mice (n = 6), analyzed in two separate experiments.

(D) Total donor-derived PB reconstitution and donor-derived myeloid contribution 16 weeks after transplantation of limiting doses (2 × 10^4 WT or 8 × 10^4 *Thpo*$^{-/-}$ BM cells) from 3-month-old mice. Each circle represents an individual recipient, and horizontal bars indicate mean levels of reconstitution among positive mice. Frequencies shown are the fraction of mice positive for lympho-myeloid reconstitution, using the threshold level of 0.1% total cells (dashed line, left) and of 0.02% each of the B, T, and myeloid lineages (dashed line, right). Data shown are from two independent experiments.

To evaluate the frequencies of functionally defined LT-HSCs in 3-month-old WT and *Thpo*-deficient mice, a limited dose of BM cells from WT or *Thpo*$^{-/-}$ mice was transplanted competitively into myeloablated WT recipients (for experimental design, see Figure S2). Based on the frequency of recipient mice positive for donor-derived long-term multilineage reconstitution (Figure 2D), a 40-fold reduction of LT-HSCs in *Thpo*$^{-/-}$ mice was calculated (see Experimental Procedures). In agreement with phenotypic data, LT-HSCs were further reduced at 1 year of age, where limiting dilution experiments demonstrated a 157-fold reduction of competitive repopulating units (CRUs) in *Thpo*$^{-/-}$ mice (Figure 2E). Thus, phenotypic as well as functional evaluation of HSC numbers demonstrated an age-progressive reduction of HSCs in *Thpo*$^{-/-}$ mice (summarized in Figure S1B).

Previous studies have demonstrated that young adult (up to 12 week old) *Thpo*$^{-/-}$ and *Mpl*$^{-/-}$ mice, although having reduced levels of progenitors of multiple lineages, sustain normal levels of mature myeloid cells with exception of the megakaryocyte lineage (de Sauvage et al., 1996; Gurney et al., 1994). Notably, in the PB and BM of 1-year-old *Thpo*$^{-/-}$ mice, we could demonstrate a slight but significant reduction in the frequency of mature cells of the short-lived granulocyte lineage (MAC1^{+}; Figure 2F). Because BM cellularity also became significantly reduced in *Thpo*$^{-/-}$ mice at this age (Figure 2A), the absolute reduction in BM MAC1^{+} myeloid cells was more extensive. These results implicate that the severe reduction in HSCs in *Thpo*$^{-/-}$ mice at 1 year of age results in a BM multilineage deficiency, in agreement with what is observed in CAMT patients, typically a few years after birth (Ballmaier et al., 2003).

Enhanced Cycling of HSCs in *Thpo*$^{-/-}$ Mice

The cellular and possible molecular mechanisms by which THPO ensures postnatal and adult HSC maintenance and expansion while not affecting fetal HSC expansion were next explored. We first performed a series of genetic experiments to investigate whether regulation of the HSC compartment by THPO might involve suppression of apoptosis, as it has been shown to be a potent in vitro survival factor for highly enriched HSCs (Borge et al., 1996). First, we demonstrated that THPO potently promotes in vitro survival of functionally defined LT-HSCs (see Figures S3A–S3D). Next, we intercrossed *Thpo*$^{-/-}$ mice with transgenic mice in which the potent antiapoptotic regulator *BCL2* is expressed under control of the *H2K* promoter, resulting in high pan-hematopoietic (including HSC) expression (Domen et al., 1998). Importantly, these mice have previously been used to implicate permissive actions of multiple cytokines through BCL2-mediated rescue of the hematopoietic phenotype of cytokine-deficient mice (Akashi et al., 1997; Lagasse and Weissman, 1997; Maraskovsky et al., 1997). However, in the case of the LT-HSC, ST-HSC, and MPP phenotypes of *Thpo*$^{-/-}$ mice, introduction of BCL2 had little or no effect (Figures 3A and 3B), failing to support a major involvement of antiapoptotic pathways in THPO-mediated regulation of postnatal HSCs.

Based on an initial screening by global (affymetrix-based) gene profiling of LSKCD34^{-}FLT3^{-} LT-HSCs and LSKCD34^{+}FLT3^{-} ST-HSCs, purified from adult BM of WT and *Thpo*$^{-/-}$ mice, we observed only small changes in the expression of some key apoptotic regulators and in genes implicated as regulators of self-renewal such as *Bmi1*, *Hif1α*, *ATM*, and *Tal1* (H.Q., R.M., and S.E.W.J., unpublished data). Although most cell-cycle regulators, including also the CDK inhibitors, showed little or no change in expression, *p57*^{Kip2} was downregulated as much as 6- to 7-fold in LSKCD34^{-}FLT3^{-} LT-HSCs and 2- to 3-fold in LSKCD34^{+}FLT3^{-} ST-HSCs from *Thpo*$^{-/-}$ BM (H.Q., S.E.W.J., unpublished data). The downregulation of *p57*^{Kip2} in LSKFLT3^{-} cells was confirmed by quantitative real-time-PCR (Q-PCR) (Figure 4A), and also expression of the CDK inhibitor *p19*^{INK4D} (Tschan et al., 1999) was somewhat reduced, whereas *p21*^{Cip1}, *p27*^{Kip1}, and *p18*^{INK4C} were not significantly affected (Figure 4A). Reduced *p57*^{Kip2} expression was observed in LSKCD34^{-}FLT3^{-} LT-HSCs as well as LSKCD34^{+}FLT3^{-} ST-HSCs (Figure 4B). Notably, we found *p57*^{Kip2} to be selectively expressed in LSKCD34^{-}FLT3^{-} LT-HSCs, whereas the CDK inhibitors *p21*^{Cip1} and *p27*^{Kip1} implicated in HSC and progenitor regulation (Cheng et al., 2000a; Cheng et al., 2000b), showed more comparable expression levels in LT-HSCs, ST-HSCs, and MPPs (Figure 4C).

As several Hox transcription factors have been implicated as potent promoters of HSC self-renewal (Antonchuk et al., 2002; Lawrence et al., 2005; Sauvageau et al., 2004), we here investigated whether their expression was potentially altered in *Thpo*$^{-/-}$ HSCs. Notably, the expression levels of *Hoxb4*, *Hoxa5*, *Hoxa9*, and *Hoxa10* were all reduced (2.1- to 4.3-fold) in *Thpo*$^{-/-}$ LSKFLT3^{-} cells (Figure 4D) with a preferential reduction in the most primitive LSKCD34^{-}FLT3^{-} cells (Figure 4E).

Based on the downregulation of the CDK inhibitor *p57*^{Kip2}, we next investigated the potential requirement of THPO in regulation of quiescent HSCs. In support of such a role, we found accelerated bromodeoxyuridine (BrdU) incorporation kinetics in cells enriched for HSCs in *Thpo*$^{-/-}$ mice (Figures 5A and 5B). In keeping with this, reduced numbers of *Thpo*$^{-/-}$ LSK cells were in the quiescent G_0 state, and increased numbers were in G_1 and $S/G_2/M$ (Figures 5C and 5D). The same pattern of reduced quiescence and enhanced cycling was seen in LSKCD34^{-}FLT3^{-} and LSKCD34^{+}FLT3^{-} cells (Figure 5E). Furthermore, we also found a markedly reduced fraction of *Thpo*$^{-/-}$ LSK and LSKCD34^{-}FLT3^{-} cells with a side

(E) CRUs (±95% confidence intervals) in WT and *Thpo*$^{-/-}$ BM, as calculated for two femurs and two tibias from five 1-year-old mice. For each genotype, cells at different doses (10^4, 2×10^4, 5×10^4 of WT cells/mouse, and 8×10^4, 2×10^5, 5×10^5 *Thpo*$^{-/-}$ cells/mouse) were transplanted into 16–20 recipients.

(F) Mean (±SD) percentages of myeloid (MAC1^{+}) cells in PB and BM of 1-year-old *Thpo*$^{-/-}$ mice (n = 4–6 mice/group).

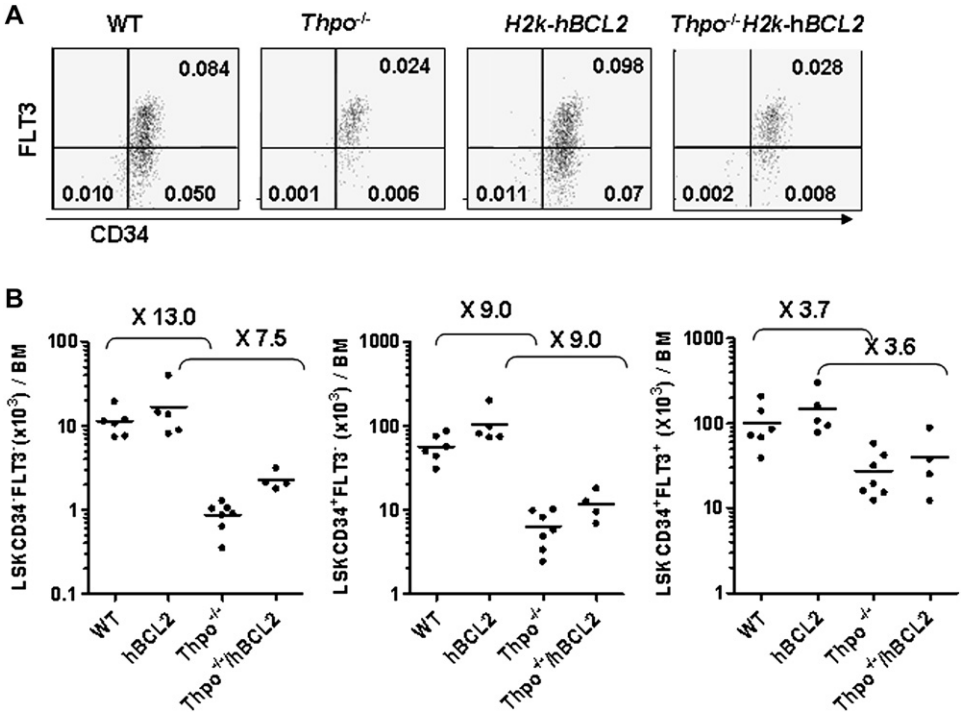

Figure 3. Forced Expression of hBCL2 Fails to Correct the HSC Deficiency in *Thpo*$^{-/-}$ Mice

(A) Representative FACS analysis of LSK subsets in BM from 11- to 14-week-old WT, *Thpo*$^{-/-}$, *H2k-hBCL2*, and *Thpo*$^{-/-}$*H2k-hBCL2* mice (n = 4–7). Mean percentages of total BM cells in each subpopulation are shown.

(B) Absolute numbers of LSKCD34^{-}FLT3^{-} LT-HSCs (left) and LSKCD34^{+}FLT3^{-} ST-HSCs (right) per BM (two femurs and two tibias) in 11- to 14-week-old WT, *Thpo*$^{-/-}$, *H2k-hBCL2*, or *Thpo*$^{-/-}$*H2k-hBCL2* mice (n = 4–7 each). Numbers represent fold reduction in LT-HSCs, ST-HSCs, and MPPs in *Thpo*$^{-/-}$ mice. When comparing the LSKCD34^{-}FLT3^{-} cells in *Thpo*$^{-/-}$ mice, the reduction was not significantly different ($p = 0.14$) on a WT and *H2k-hBCL2* background.

population (SP) phenotype (Figure 5F). Specifically, a 41-fold reduction in the number of LSKCD34^{-}FLT3^{-} SP (2.4% of WT counterparts) was observed (Figure 5G), a phenotype proposed to be tightly coupled to quiescent HSCs (Arai et al., 2004). Thus, THPO promotes postnatal maintenance of quiescent HSCs.

Role of THPO in Posttransplantation HSC Expansion

Unlike in steady-state adult BM, where HSCs are mostly quiescent or slowly cycling (Bradford et al., 1997; Cheshier et al., 1999), HSCs transplanted into myeloablated recipients are stimulated to expand and rapidly replenish HSCs and downstream progenitor cells (Pawliuk et al., 1996). In a typical experimental BM transplantation setting, only a small fraction of the normal HSC compartment is transplanted, resulting in an initial rapid cycling and expansion phase, followed by reestablishment of steady state where HSCs re-enter a quiescent or slow cycling status (Pawliuk et al., 1996). Thus, we hypothesized that the dynamic posttransplantation expansion of HSCs might also be deficient in *Thpo*$^{-/-}$ recipients. To investigate this, we transplanted 4×10^3 WT BM LSK cells into myeloablated WT or *Thpo*$^{-/-}$ recipient mice (experimental design, see Figure S4). In agreement with previous studies (Fox et al., 2002), the survival of *Thpo*$^{-/-}$ recipients was reduced to only 43% (6 out of 14), whereas all WT recipients survived, and this correlated with a dramatic thrombocytopenia in *Thpo*$^{-/-}$ recipients (Figure 6A). Despite the high number of LSK cells transplanted, total donor-derived blood cell reconstitution, as well as myeloid and B cell lineage reconstitution, was significantly reduced in *Thpo*$^{-/-}$ recipients after 16 weeks (Figure 6B). Moreover, donor-derived LSK stem cells as well as LIN^{-}SCA1^{-}KIT^{hi} myeloid progenitors were reduced 6.1- and 4.6-fold, respectively, in primary *Thpo*$^{-/-}$ compared to WT recipients (Figures 6C and 6D). Most notably, when BM cells from *Thpo*$^{-/-}$ primary recipients were transplanted into secondary myeloablated *Thpo*$^{-/-}$ recipients, the hematopoietic defect became even more severe (Figures 6E and 6F), and more than 50% of *Thpo*$^{-/-}$ recipients died by 8 weeks posttransplantation, whereas all WT recipients survived (see Figures S5A and S5B). Total BM cellularity was reduced as much as 3-fold (Figure 6E), donor-derived LSK stem cells were undetectable, and LIN^{-}SCA1^{-}KIT^{hi} myeloid progenitors also almost completely depleted in the surviving secondary *Thpo*$^{-/-}$ recipients already 8 weeks posttransplantation (Figures 6F and 6G). Thus, THPO plays a critical role in HSC maintenance in steady state as well as in regenerating adult BM.

Although the long-term reconstitution experiments described above established a critical role of THPO in

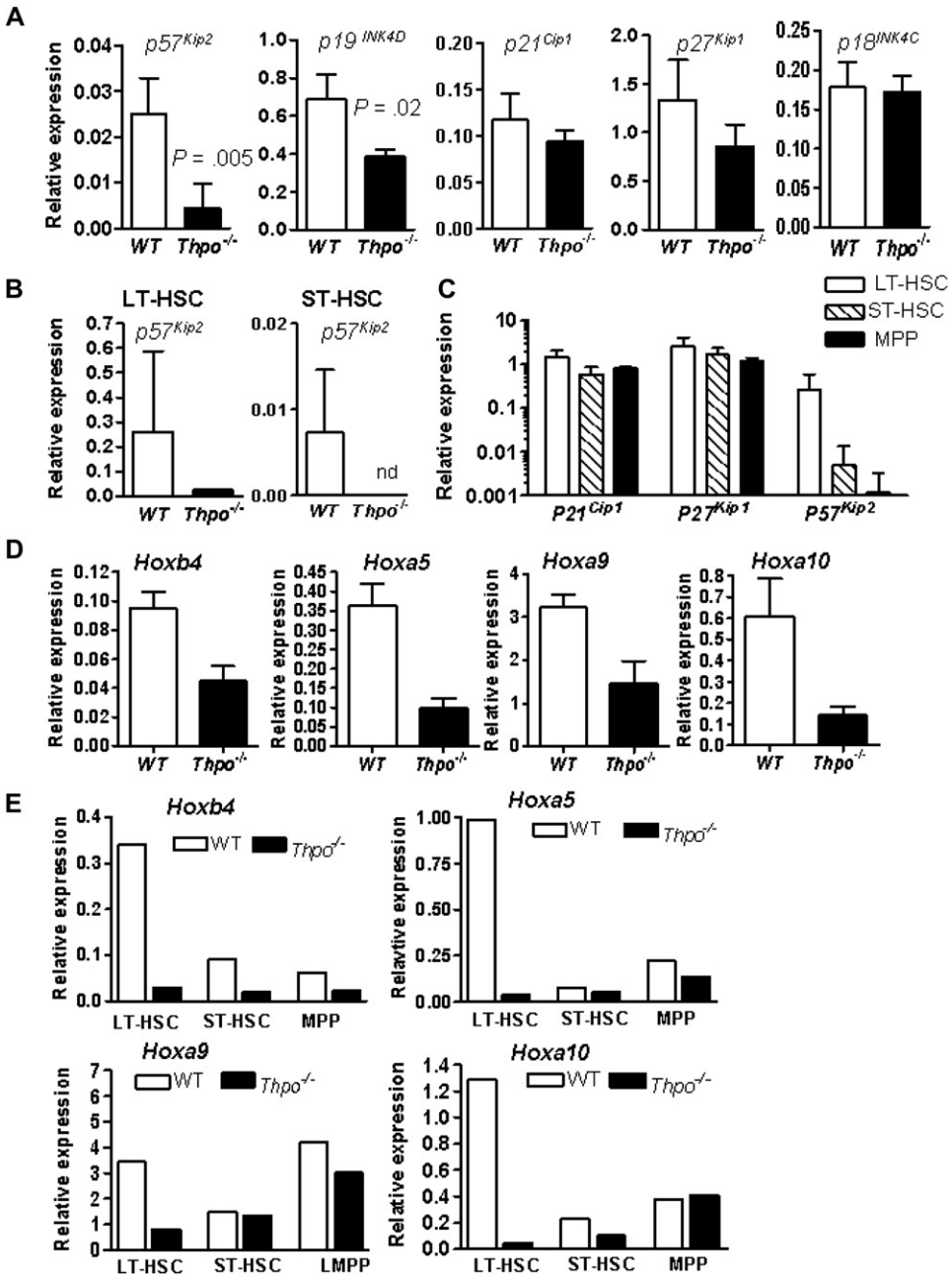

Figure 4. Transcriptional Expression of CDK Inhibitors and *Hox* Transcription Factors in *Thpo*$^{-/-}$ LSK Subsets

(A) Quantitative PCR expression analysis of *p57*^{Kip2}, *p19*^{INK4D}, *p21*^{Cip1}, *p27*^{Kip1}, *and p18*^{INK4C} in LSKFLT3^{-} cells from WT and *Thpo*$^{-/-}$ mice.

(B) Expression of *p57*^{Kip2} in *Thpo*$^{-/-}$ LSKCD34^{-}FLT3^{-} and LSKCD34^{+}FLT3^{-} cells (mean ± SD).

(C) Expression of *p21*^{Cip1}, *p27*^{Kip1}, and *p57*^{Kip2} in WT LSK subsets. Data are mean (±SD) values from two independent experiments. nd, not detectable.

(D) Expression of *Hoxb4*, *Hoxa5*, *Hoxa9*, and *Hoxa10* in LSKFLT3^{-} cells from WT and *Thpo*$^{-/-}$ mice. Data represent mean (±SEM) values of triplicate determinations from two independent experiments.

(E) Expression of *Hoxb4*, *Hoxa5*, *Hoxa9*, and *Hoxa10* in LSK subsets from WT and *Thpo*$^{-/-}$ mice. Data represent mean values from one to two independent experiments. All results (A–E) are shown relative to *hprt* expression levels.

replenishment and/or maintenance of HSCs posttransplantation, they could not separate between a role of THPO in maintenance of HSCs in the BM after transplanted HSCs go back into steady-state cycling and/or during the extensive HSC expansion that occurs in the initial phase after transplantation in ablated recipients (Pawliuk et al., 1996). In fact, as THPO proved to not be required for fetal HSC expansion, it appeared likely that

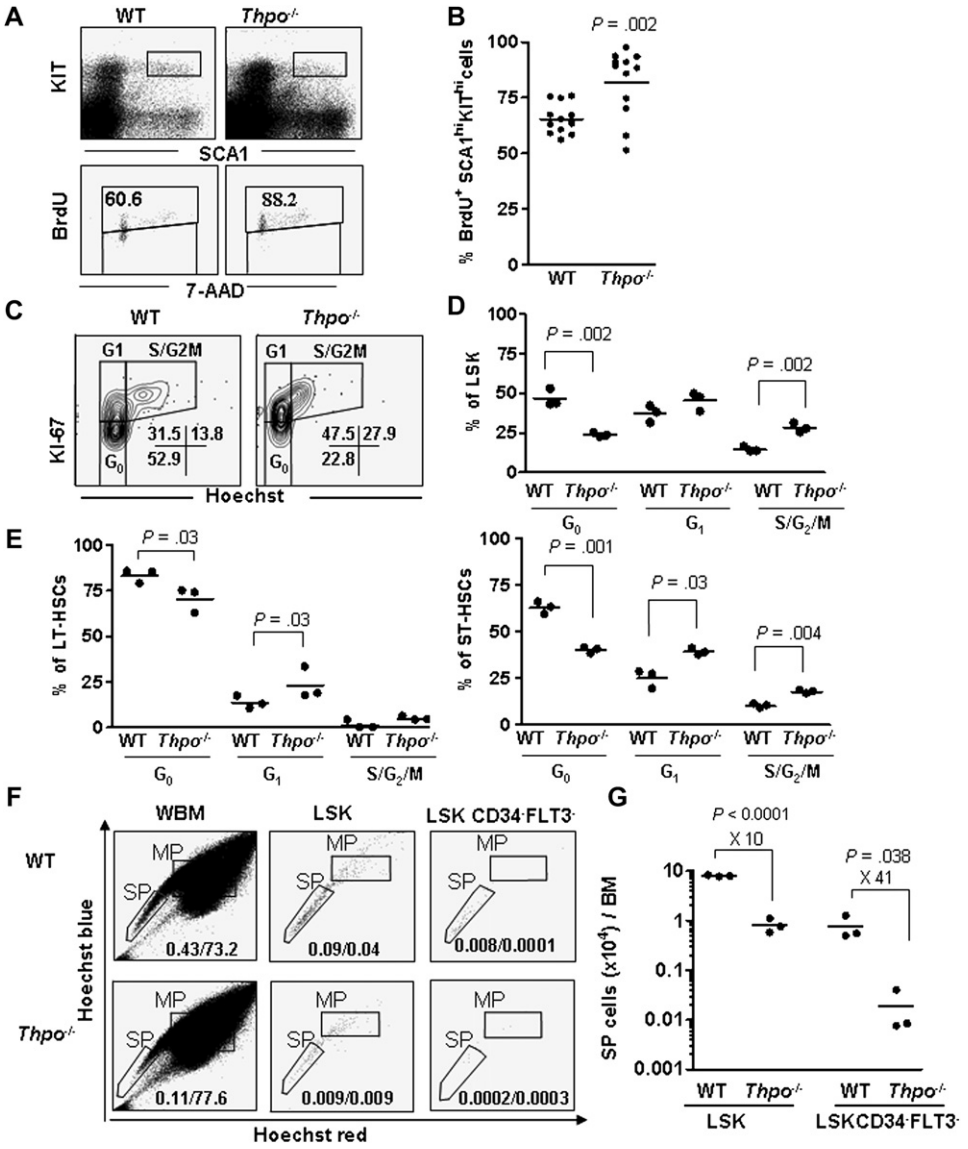

Figure 5. THPO Regulates Quiescent HSCs

(A) Analysis of BM SCA1^{hi}KIT^{hi} cells from WT and *Thpo*$^{-/-}$ mice exposed to BrdU for 3 days. Panels show gating strategy of SCA1^{hi}KIT^{hi} cells (top) being BrdU^{+} (low). The numbers in the profiles show percentages of BrdU^{+}SCA1^{hi}KIT^{hi} cells.

(B) Percentages of BrdU^{+}SCA1^{hi}KIT^{hi} cells in 14-week-old WT and *Thpo*$^{-/-}$ mice. Data are from two separate experiments (n = 12 mice of each genotype).

(C–E) Cell-cycle analysis of *Thpo*$^{-/-}$ LSK cells. Data are from one of two independent experiments with similar results (three mice of each genotype in each experiment). Cell-cycle status within defined LSK subsets (LSKCD34^{-}FLT3^{-}, LSKCD34^{+}FLT3^{-}) was determined by simultaneous two-parameter analysis with DNA content versus KI-67 expression. (C) Numbers in quadrants show the percentage of gated LSK cells in each of the cell-cycle phases (G_0, G_1, and $S/G_2/M$). (D) Mean cell-cycle distribution of total LSK cells from one representative experiment. (E) Mean cell-cycle distribution of LSKCD34^{-}FLT3^{-} (left) and LSKCD34^{+}FLT3^{-} (right) cells from one representative experiment.

(F and G) Proportion and numbers of BM LSK subsets in 9-week-old WT and *Thpo*$^{-/-}$ mice with SP phenotypes. (F) Representative FACS profiles of SP and MP analysis in whole BM (WBM), total LSK, and LSKCD34^{-}FLT3^{-} cells in WT and *Thpo*$^{-/-}$ mice. The numbers in the panels show percentage of SP/percentage of MP out of total nucleated cells. (G) Numbers of gated LSK and LSKCD34^{-}FLT3^{-} (n = 3 of each genotype) with SP phenotype in BM (two femurs and two tibias). Each dot represents a single mouse. Horizontal bars show mean numbers of SP cells within LSK and LSK CD34^{-}FLT3^{-} cells. Also shown are p values comparing WT and *Thpo*$^{-/-}$ mice.

THPO might also not be required for the initial phase of HSC expansion posttransplantation. To specifically investigate this, we next analyzed the reconstitution of BM LSK and LSKFLT3^{-} cells at 3 and 6 weeks after transplantation of WT LSK cells in WT and *Thpo*$^{-/-}$ recipients. Notably, reconstitution of total LSK as well as LSKFLT3^{-} cells was severely impaired after 3 as well as 6 weeks posttransplantation (Figure 7), in agreement with THPO playing

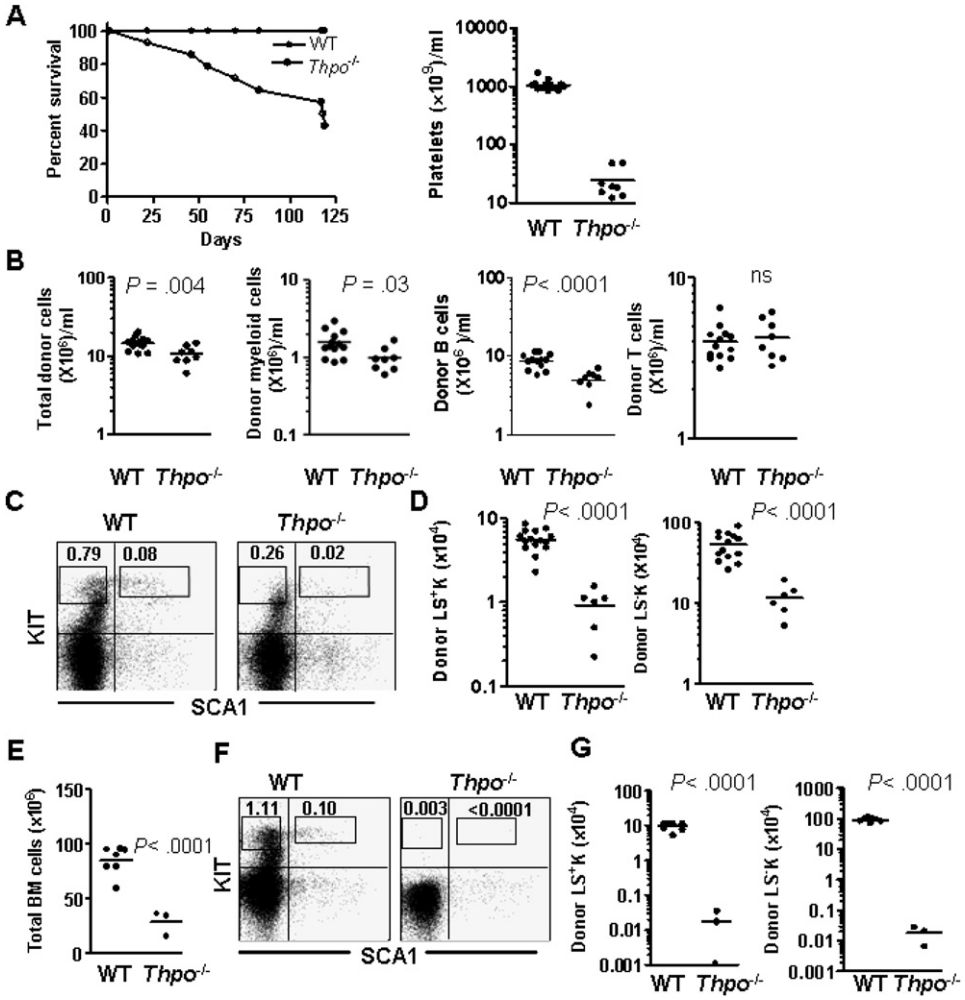

Figure 6. Critical Role of THPO in Long-Term Replenishment of HSCs in Posttransplantation BM

Myeloablated WT or *Thpo*$^{-/-}$ mice (CD45.2) were transplanted with 4 × 10^3 purified WT LSK (CD45.1) from 10- to 12-week-old mice. PB reconstitution was analyzed at 16 weeks after primary transplantation.

(A) Survival curves (left) and platelets (right) in transplanted mice.

(B) Total PB donor cells (CD45.1) and donor-derived myeloid, B, and T cells. Horizontal bars show the mean values.

(C) Representative FACS plots of LIN$^-$SCA1$^+$KIT^{hi} (LS$^+$K) and LIN$^-$SCA1$^-$KIT^{hi} (LS$^-$K) analysis in WT and *Thpo*$^{-/-}$ recipient mice 17 weeks after transplantation. Numbers shown are mean percentages relative to total BM cells (n = 6–14).

(D) Total donor-derived BM LS$^+$K cells (left) and LS$^-$K cells per femur and tibia in WT and *Thpo*$^{-/-}$ recipients 17 weeks after transplantation. Each dot represents a single recipient, and bars show mean cell numbers per both femurs and tibias. Data are from two independent experiments.

(E) Total BM cells in *Thpo*$^{-/-}$ and WT secondary recipients at 8 weeks after transplantation.

(F) Representative FACS plots for LS$^+$K and LS$^-$K analysis in *Thpo*$^{-/-}$ and WT secondary recipients 8 weeks after transplantation. Numbers shown are mean percentages of LS$^+$K and LS$^-$K cells of total BM cells in all recipients of each group (n = 3–7).

(G) Total donor-derived LS$^+$K and LS$^-$K cells in *Thpo*$^{-/-}$ and WT recipients 8 weeks after transplantation. Each dot represents single recipients, and bars show mean values from two femurs and tibias.

a critical role in promoting HSC expansion early in posttransplantation.

DISCUSSION

The importance of the classical hematopoietin family of cytokines as key regulators of blood lineage development has been recognized for decades, and their utility translated into clinical medicine (Kaushansky, 2006). It was also early appreciated that many of these cytokines, when acting in synergy with ligands for receptors of the cytokine tyrosine kinase (FLT3 and KIT) and gp130 (IL-6 and IL-11) families, could potently promote the proliferation of primitive progenitors and stem cells (Lyman and Jacobsen, 1998; Metcalf, 1993; Ogawa, 1993). Thus, it was expected that hematopoietin cytokines would also play important physiological roles in HSC regulation, but providing evidence for this as well as translating their potent synergistic activities into a means to ex vivo expand HSCs has proven to be a daunting task (Oostendorp

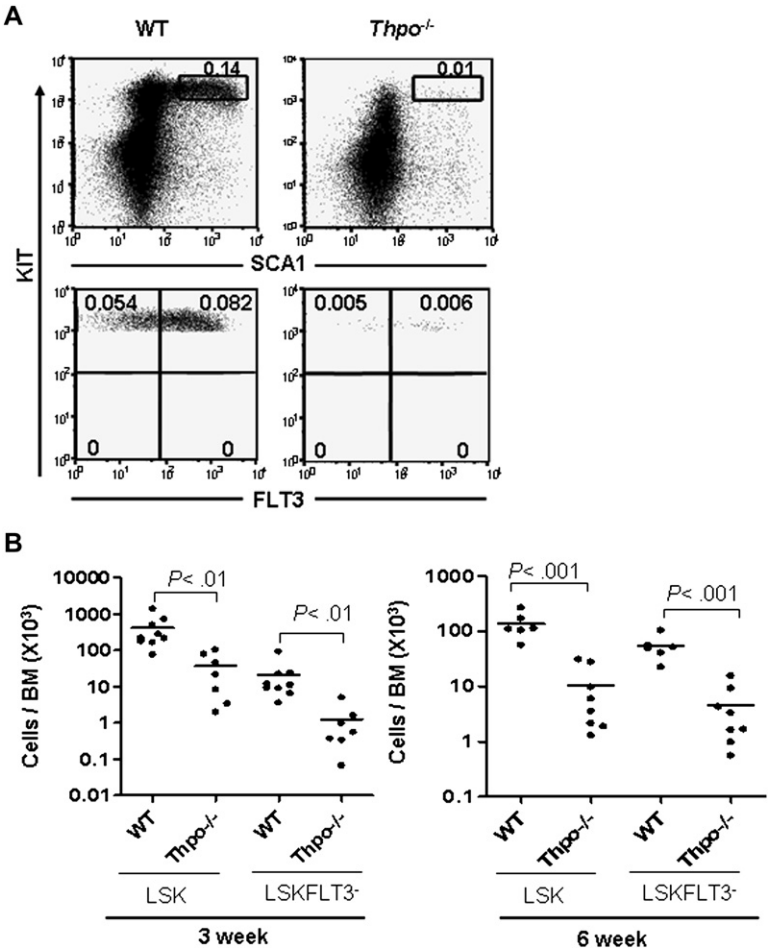

Figure 7. Initial HSC Expansion Post-transplantation Is THPO Dependent

Myeloablated WT or *Thpo*$^{-/-}$ mice (CD45.2) were transplanted with 4 × 10^3 purified WT LSK (CD45.1) from 10- to 12-week-old mice.

(A) Representative donor-derived (CD45.1) LSK and LSKFLT3$^-$ analysis in WT and *Thpo*$^{-/-}$ recipient mice 3 weeks after transplantation. Numbers shown are mean percentages relative to total BM cells (n = 7–8).

(B) Total donor-derived BM LSK (left) and LSKFLT3$^-$ (right) cells per two femurs and tibias in WT and *Thpo*$^{-/-}$ recipients 3 and 6 weeks after transplantation. Each dot represents a single recipient, and bars show mean cell numbers per both femurs and tibias. Data are from two independent experiments.

et al., 1999; Sorrentino, 2004). However, more recent observations have suggested that THPO, initially identified as the primary regulator of megakaryocyte and platelet formation (Kaushansky, 1995), might also be involved in regulation of HSCs (Buza-Vidas et al., 2006; Fox et al., 2002; Kimura et al., 1998; Solar et al., 1998). Although initially having been identified through in vitro studies as a survival factor (Borge et al., 1996) and a synergistic factor for HSC proliferation (Sitnicka et al., 1996), efforts to promote HSC expansion with cytokine combinations including THPO proved to only marginally improve HSC maintenance (Bryder and Jacobsen, 2000; Ema et al., 2000).

In the present studies, we establish through complementary phenotypic and functional analysis a key role of THPO and MPL in regulation of HSC maintenance in steady-state adult BM and through cell-cycle analysis provide findings suggesting that a key physiological role of THPO in promoting maintenance of adult HSCs might be to suppress or prolong their cell-cycle transit, and thereby protect the steady-state HSC compartment from premature exhaustion. In contrast, we show that the extensive HSC expansion that occurs in the fetal liver (Pawliuk et al., 1996) is THPO independent, and in fact, analysis of *Thpo*$^{-/-}$ as well as *Mpl*$^{-/-}$ mice demonstrated that FL HSCs were slightly expanded. The contrasting findings in FL and adult steady-state BM could be compatible with THPO and MPL playing a distinct role in regulation of quiescent or slowly cycling HSCs, but not in regulation of rapidly expanding HSCs. However, as THPO also proved to be of considerable importance for short-term expansion of HSCs after transplantation into lethally irradiated recipients (Pawliuk et al., 1996), as well as during the first few weeks postnatally when HSCs continue to cycle (Bowie et al., 2006), our findings are more compatible with a distinct role of THPO in maintenance and expansion of HSCs in postnatal and adult hematopoiesis, but not during fetal development. The mechanism by which THPO is critically involved in promoting HSC expansion after transplantation remains to be established, although it appears likely that it also might be to prolong cell-cycle transit, as rapidly cycling HSCs have recently also been shown to have a protracted cell-cycle transit when compared to downstream progenitors (Dykstra et al., 2006; Nygren et al., 2006).

Although we cannot exclude a potential role of THPO also in homing of HSCs after transplantation, the reduced HSC numbers in *Thpo*$^{-/-}$ mice established through limiting dilution experiments could not be entirely due to a homing defect of *Thpo*$^{-/-}$ HSCs, because phenotypic analysis of the HSC compartment in *Thpo*$^{-/-}$ mice

showed a similar (although less extensive) age-progressive postnatal loss of HSCs in steady-state *Thpo*$^{-/-}$ mice. Furthermore, in the limiting dilution experiments, *Thpo*$^{-/-}$ BM cells were intentionally transplanted into WT recipients along with WT competitor cells, providing a source of normal THPO expression and function in the transplant as well as in the recipient microenvironment.

In agreement with a distinct role of THPO in sustaining quiescent HSCs in steady-state adult BM, LSKCD34^{-}FLT3^{-} cells in *Thpo*$^{-/-}$ BM had clearly accelerated cell-cycle kinetics. Most notably, we found the CDK inhibitor *p57*^{Kip2} to be clearly downregulated in *Thpo*$^{-/-}$ LSKFLT3^{-} cells. Although the role of p57^{Kip2} in HSC regulation has not been established, it has, in agreement with our studies, recently been reported to be preferentially expressed in LT-HSCs (Yamazaki et al., 2006). Notably, in the accompanying paper by Yoshihara et al. (2007) in this issue of *Cell Stem Cell*, in vivo administration of MPL blocking antibodies resulted in a similar reduction in *p57*^{Kip2} expression in HSCs as we observed in *Thpo*$^{-/-}$ mice. An important role of THPO in promoting HSC maintenance and expansion was further supported by reduced expression in *Thpo*$^{-/-}$ LSKFLT3^{-} cells of several *Hox* genes proposed to promote HSC self-renewal (Antonchuk et al., 2002; Lawrence et al., 2005; Sauvageau et al., 2004), including *Hoxb4*, *Hoxa5*, *Hoxa9*, and *Hoxa10*. The finding of altered transcriptional expression of CDK inhibitors and Hox transcription factors provides insight into potential mechanisms of action for how THPO might promote in vivo HSC maintenance and expansion, but further studies will be needed to establish whether these observations represent more than an association. This could be a considerable challenge, as the expression of several of these genes appears to be downregulated in *Thpo*$^{-/-}$ HSCs and because considerable redundancy has been proposed among these regulators. With regard to the downregulated expression of *Hox* genes in *Thpo*$^{-/-}$ LSKFLT3^{-}, it is of interest that THPO has been shown to promote nuclear transport of Hoxa9 in primitive hematopoietic cells (Kirito et al., 2004).

Although the clear association of the enhanced cycling with downregulation of *p57*^{Kip2} and *p19*^{INK4D} in *Thpo*$^{-/-}$ HSCs implicated that THPO regulates HSC cell-cycle quiescence, an alternative scenario would be that THPO would simply promote survival of high MPL expressing quiescent HSCs (Borge et al., 1996; Buza-Vidas et al., 2006). However, whereas overexpression of BCL2 has been used to successfully demonstrate the permissive actions of other cytokine functions in hematopoiesis (Akashi et al., 1997; Cross and Dexter, 1991; Lagasse and Weissman, 1997; Maraskovsky et al., 1997), introduction of high levels of BCL2 failed to rescue the HSC deficiency in *Thpo*$^{-/-}$ mice. Importantly, although these findings failed to support a role of antiapoptotic pathways in mediating the effects of THPO on HSC maintenance, they do not preclude involvement of antiapoptotic regulators distinct from BCL2.

Ample evidence suggests that HSC quiescence is specifically regulated and secured in anatomically and molecularly defined stem cell niches in the BM (Calvi et al., 2003; Zhang et al., 2003). In agreement with this, the only previously identified extrinsic regulator of HSC quiescence, angiopoietin-1, has been demonstrated to be expressed by osteoblasts in the BM niche (Arai et al., 2004) and is exclusively important for regulation of postnatal HSCs (Puri and Bernstein, 2003). Although THPO is primarily produced in the liver, BM stroma cells express *Thpo* (Dormady et al., 2001), and in the accompanying paper from Yoshihara et al. (2007), THPO is demonstrated to be expressed in osteoblasts in the BM stem cell niche. They also demonstrate through transitory gain and lack-of-function approaches in adult mice that THPO promotes HSC quiescence and maintains HSC interactions with their niche components, in agreement with our finding of accelerated cell cycling of HSCs in *Thpo*$^{-/-}$ mice. In light of these findings, it is a possible scenario that the enhanced cycling of HSCs in *Thpo*$^{-/-}$ mice might be secondary to their egress out of the stem cell niche, which might result in release from their quiescent state in the niche.

Upon BM transplantation of ablated recipients, adult HSCs undergo extensive self-renewal divisions to expand the number of HSCs and to replenish progenitors of multiple lineages (Iscove and Nawa, 1997; Pawliuk et al., 1996). Herein we demonstrate that this dynamic process is perturbed in *Thpo*$^{-/-}$ mice, resulting in reduced HSC expansion in the short term after transplantation and eventually in a complete exhaustion of HSCs in the absence of THPO.

The importance of THPO and MPL in human HSC regulation has been implicated in patients with CAMT with lack-of-function mutations in *Mpl* (Ballmaier et al., 2003). Although the HSC compartment has not been studied directly in these patients, they might have an identical HSC phenotype as here reported for *Thpo*$^{-/-}$ mice. As for mice, THPO might not be critically involved in regulation of human fetal HSCs, as all CAMT patients are born with a pure megakaryocyte/platelet deficiency and an otherwise normocellular BM (Ballmaier et al., 2003). However, during the course of CAMT, most patients develop a progressive multilineage BM failure, in support of a critical role of THPO in regulation of postnatal human HSC maintenance, but not fetal HSC expansion.

In conclusion, our studies establish a unique role of THPO in postnatal HSC expansion and maintenance of quiescent HSCs in the adult BM.

EXPERIMENTAL PROCEDURES

Mice

Congenic CD45.1 and CD45.2 C57BL/6 WT mice were used as controls and as transplant recipients. *Thpo*$^{-/-}$ (CD45.1 or CD45.2) mice have been described (de Sauvage et al., 1996) and were kindly provided by Dr. John W. Adamson, (Blood Center of Southeastern Wisconson). *Mpl*$^{-/-}$ mice were generated as described (Alexander et al., 1996). H2k-hBCL2 mice (Domen et al., 1998) were kindly provided by Dr. Albrecht Muller, Wuertzburg, Germany. The H2k promoter ensures high pan-hematopoietic levels of human BCL2, including HSCs (Domen et al., 1998). Both strains had been backcrossed to

C57BL/6 mice for more than ten generations. All animal protocols were approved by the animal ethical committees at Lund University and the Walter and Eliza Hall Institute.

PB Cell Analysis

PB was counted in an automated cell counter (Sysmex) and prepared for fluorescence-activated cell sorting (FACS) analysis as previously described (Yang et al., 2005).

FACS of HSC Subsets

BM cellularities were calculated by using the total number of cells collected from two femurs and two tibias after crushing in a mortar (*Thpo*$^{-/-}$ mice) or flushing (*Mpl*$^{-/-}$ mice). FLs were obtained from time-matched pregnant WT and *Thpo*$^{-/-}$ or *Mpl*$^{-/-}$ mice at E14.5, where the time of the vaginal plug was set as E0.5. Modifications of previously described procedures were used to evaluate the distribution or to sort cells within the LSK compartment (Adolfsson et al., 2005; Yang et al., 2005). Briefly, FL or BM cells from 2-week-old mice were incubated with lineage (LIN)-cocktail (purified rat antibodies against B220, CD5, CD8α, GR-1, TER-119, plus CD4 and MAC1 in BM only). Subsequently, the cells were stained with goat anti-rat–tricolor, anti-mouse-FLT3-PE, -SCA1-FITC/SCA1-Pacific blue/SCA1-PE-CY5.5, -KIT-APC/KIT-APC-Alexa 750, or isotype-matched control antibodies. BM cells from adult mice were then stained with anti-mouse-CD34-biotin, in addition to FLT3 or CD150, SCA1, KIT, and subsequently with SAv-PE-Cy7. All samples were stained with the vital marker Fluorogold or 7-amino actinomycin (7-AAD; Sigma-Aldrich Co.) prior to analysis on FACSCalibur or FACSDiva (BD). Data analysis was carried out with FlowJo software (TreeStar Inc.). The purities of all LSK subsets were reproducibly higher than 99%. For specific details regarding antibodies used for FACS see the Supplemental Experimental Procedures.

SP Analysis

To evaluate the distribution of SP and main population (MP) cells within the LSKCD34$^-$FLT3$^-$ compartment, unfractionated BM cells were suspended at 1 million cells/ml and stained with 2.5 μg/ml Hoechst 33342 (Invitrogen, Molecular Probes) followed by cell surface stainings and analysis on a FACSDiva as previously described (Nadin et al., 2003; Yang et al., 2005). The gatings of SP and MP were based on negative controls in which Verapamil (100 μM) (ABBOT Scandinavia) was used when cells were incubated with Hoechst 33342.

Competitive Repopulation Assay

The competitive reconstitution assay using the congenic CD45.1/CD45.2 mouse model has been described previously (Yang et al., 2005). Briefly, different doses of CD45.1 or CD45.2 donor BM or FL cells were injected intravenously along with 2 × 10^5 competitor CD45.1 or CD45.2 BM cells into myeloablated (900 cGy) CD45.1 hosts. PB was analyzed at 3 and 16 weeks after transplantation for donor reconstitution by FACS. Briefly, nucleated blood cells were stained with antibodies against CD45.1, CD45.2, MAC1, B220, CD4, and CD8α. Positively reconstituted mice were defined as having a minimum of 0.1% CD45.2$^+$ total cells and 0.02% CD45.2$^+$ cells of each of the myeloid (MAC1$^+$), B (B220$^+$), and T (CD4/CD8$^+$) lineages after analysis of a minimum of 5 × 10^4 PB cells (Buza-Vidas et al., 2006). The specificity of the FACS staining is controlled by analyzing PB of untransplanted CD45.1 mice with the same anti-CD45.2 and lineage antibodies, always finding a background CD45.2 staining of less than 0.01% of total PB cells and less than 0.002% of CD45.2$^+$MAC1$^+$ cells. When multiple limiting dilution transplant doses were used, and CRUs were calculated based on the frequencies of positive mice at each dose (Szilvassy et al., 1990), using Limit Dilution Analysis software (StemCell Technologies, Inc.). In experiments where a single low dose of BM cells was used, the frequency of repopulating cells was calculated based on the formula p = 1 − 10^ (log [frequency of negative mice]/cell dose) (Buza-Vidas et al., 2006).

Noncompetitive Repopulation Assay

Briefly, 4 × 10^3 sorted WT BM LSK cells (CD45.2) were injected intravenously into myeloablated (900 cGy) WT or *Thpo*$^{-/-}$ CD45.1 hosts. PB at 3, 6, and 16 weeks after transplantation was analyzed for donor BM LSK and PB lineage reconstitution by FACS as described above.

BrdU and Cell-Cycle Status Analysis of HSCs

Age-matched WT and *Thpo*$^{-/-}$ mice (12 weeks old) were given an intraperitoneal injection with 1 mg BrdU (Sigma-Aldrich) per 6 g body weight and allowed to freely drink water containing BrdU (1 mg / ml) for 72 hr prior to isolation of BM cells for analysis. Cells were stained with lineage markers, rat anti-mouse-SCA1-PECy5.5 (eBioscience), -KIT-APC, -FLT3-PE, -CD34-biotin, and subsequently with SAv-PE-Cy7. BrdU incorporation was evaluated by using a BrdU and 7-AAD intracellular staining kit (BD PharMingen). Cell-cycle status within defined LSK subsets (LSKCD34$^-$FLT3$^-$, LSKCD34$^+$FLT3$^-$, and LSKCD34$^+$FLT3$^+$) was determined by simultaneous two-parameter analysis using DNA content versus KI-67. KI-67 expression was detected with anti-KI-67-FITC (BD PharMingen), and DNA was stained with Hoechst 33342 (Sigma, 2.5 μg/ml).

Q-PCR

RNA extraction and Q-PCR of adult BM LSKCD34$^-$FLT3$^-$, LSKCD34$^+$FLT3$^-$, and LSKCD34$^+$FLT3$^+$ cells or LSKFLT3$^-$ cells were performed as previously described (Adolfsson et al., 2005; Månsson et al., 2007). For specific TaqMan Assays-on-Demand probes used, see the Supplemental Experimental Procedures. The assay was performed on an ABI 7900 Fast Real-Time PCR System with TaqMan Fast Universal PCR master mixture (Applied Biosystems). Data were analyzed by 7900 Fast System SDS Software 2.2.1. All experiments were performed in triplicates, and differences in cDNA input were compensated for by normalization against Hprt expression levels.

Statistics

Data are expressed as mean (±SD or ±SEM). Unless otherwise stated, the statistical significance of differences between WT and *Thpo*$^{-/-}$ mice was determined by using the two-tailed Mann-Whitney t test or an unpaired t test.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and five figures and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/1/6/671/DC1/.

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