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Hepatic thrombopoietin is required for bone marrow hematopoietic stem cell maintenance

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Abstract

Hematopoietic stem cell (HSC) maintenance depends on extrinsic cues. Currently, only local signals arising from the bone marrow niche have been shown to maintain HSCs. However, it is not known whether systemic factors also sustain HSCs. We assessed the physiological source of thrombopoietin (TPO), a key cytokine required for maintaining HSCs. Using *Tpo^{DsRed-CreER}* knock-in mice, we showed that TPO is expressed by hepatocytes but not by bone marrow cells. Deletion of *Tpo* from hematopoietic cells, osteoblasts, or bone marrow mesenchymal stromal cells does not affect HSC number or function. However, when *Tpo* is deleted from hepatocytes, bone marrow HSCs are depleted. Thus, a cross-organ factor, circulating TPO made in the liver by hepatocytes, is required for bone marrow HSC maintenance. Our results demonstrate that systemic factors, in addition to the local niche, are a critical extrinsic component for HSC maintenance.

Hematopoietic stem cells (HSCs) reside primarily in the bone marrow and are maintained by extrinsic cues that arise from supporting niche cells (1). Endothelial cells (2, 3) and perivascular mesenchymal stromal cells (2–6) are critical components of the bone marrow niche. Growing functional genetic evidence suggests that HSCs are maintained largely through signals arising directly from, or mediated through, these local niche cells (7). However, olfaction maintains hematopoietic progenitors through systemic γ -aminobutyric acid (GABA) levels in *Drosophila* (8), suggesting that long-range signals may be able to directly maintain mammalian HSCs.

No such distal maintenance factors have yet been identified in the mammalian hematopoietic system, although long-range cues, such as estrogen from the ovaries and erythropoietin from

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SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S10

References (35–40)

the kidneys, can acutely stimulate HSC proliferation and dictate HSC and progenitor differentiation (9, 10). Neurotransmitters from the nervous system can mobilize HSCs, but this effect is mediated through mesenchymal stromal cells in the niche (11). Therefore, evidence indicates roles for long-range cues that modify HSC behavior, but direct evidence for constant maintenance of HSCs by a cross-organ long-range systemic factor is lacking.

Signaling of the hematopoietic cytokine thrombopoietin (TPO) through its receptor c-MPL is essential for thrombopoiesis (12–14) and HSC maintenance (15–17). Patients with loss-of-function mutations in c-MPL or TPO develop congenital amegakaryocytic thrombocytopenia and subsequent bone marrow failure (18–20). *Tpo* mRNA is expressed by multiple cell types, including hepatocytes (14, 21), osteoblasts (17), megakaryocytes (22, 23), and stromal cells (21, 24). However, *Tpo* is under stringent translational control by inhibitory elements in the 5' untranslated region (25), so it is not clear whether any of the above-mentioned cell types actually synthesize TPO protein. *Tpo* has not been conditionally deleted from any cell types to assess its source for HSC maintenance. Thus, it is not clear how TPO maintains bone marrow HSCs in vivo. Loss of hepatic TPO leads to low platelet counts (26), showing that TPO from the liver regulates thrombopoiesis.

Using quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis, we found that *Tpo* transcripts were enriched in osteoblasts, mesenchymal stromal cells, and the liver (Fig. 1A and fig. S1, A and B), consistent with previous reports (14, 17, 21, 27). To systemically assess the expression of TPO protein, we generated *Tpo*^{DsRed-CreER} knock-in mice by replacing the stop codon of *Tpo* with a *P2A-DsRed-P2A-CreER* cassette (fig. S1, C to F). The P2A elements allow the translation of TPO, DsRed, and CreER recombinase under the control of *Tpo* endogenous regulatory elements. This arrangement enabled us to monitor the translational expression of TPO in vivo. We then generated *Tpo*^{DsRed-CreER}; *loxPZsGreen* mice (Fig. 1B). Consistent with the low expression level of *Tpo* in vivo (25), no DsRed fluorescence was detected (Fig. 1, C to F). However, upon tamoxifen (TMX) administration to 8-week-old mice, we detected broad and specific expression of ZsGreen in hepatocytes (Fig. 1, G to J, and fig. S1, G to O). We also observed rare ZsGreen⁺ cells in the kidney (fig. S1P). However, no ZsGreen⁺ bone marrow cells could be detected (Fig. 1, K to N, and fig. S1Q). Thus, TPO is generated by hepatocytes but not by cells in the bone marrow.

We generated a loss-of-function allele of *Tpo* (*Tpo*^{gfp}) by recombining a gene encoding enhanced green fluorescent protein (*gfp*) into the start codon of *Tpo* (fig. S2, A to C). As expected, *Tpo* transcripts were depleted from *Tpo*^{gfp/gfp} mouse livers (fig. S2D). Consistent with earlier reports (28, 29), whole-body loss of TPO led to reduced platelet counts (fig. S3, A to C) and reduced numbers of megakaryocytes (fig. S3, D to J). Bone marrow from *Tpo*^{gfp/gfp} mice had normal cellularity, but CD150⁺CD48[−]Lin[−]Sca1⁺cKit⁺ HSC frequency (the percentage of live whole bone marrow cells) decreased about 70-fold compared with the frequency in *Tpo*^{+/+} controls (fig. S3, K to M). CD150[−]CD48[−]Lin[−]Sca1⁺cKit⁺ multipotent progenitor (MPP) (30) and Lin[−]Sca1⁺cKit⁺ (LSK) hematopoietic progenitor frequencies declined by 10- and 3-fold, respectively (fig. S3, N and O). Lineage-restricted hematopoietic progenitors appeared normal, except that CD34⁺FcγR[−]Lin[−]Sca1[−]cKit⁺ common myeloid progenitors (CMPs) were reduced (fig. S3P). Bone marrow and spleen cells from *Tpo*^{gfp/gfp}

mice formed fewer colonies in methylcellulose (fig. S3Q). Spleen cellularity did not change, but spleen HSC frequency was reduced (fig. S3, R and S). *Tpo*^{+/*gfp*} heterozygous mice displayed intermediate phenotypes (fig. S3). Thus, TPO is a major factor required for HSC maintenance.

We generated a floxed allele of *Tpo* (*Tpo*^{*fl*}) by inserting *loxP* sequences flanking exons 2 to 4 of *Tpo* (fig. S4, A to C). Recombination of the *loxP* sites will lead to the deletion of the start codon and the generation of a frameshift. We recombined the *Tpo*^{*fl*} allele in the germ line to generate *Tpo*⁻ through mating with *Elia-cre* mice (fig. S4D). As expected, *Tpo* transcripts were absent from homozygous *Tpo*^{-/-} mice (fig. S4E). *Tpo*^{-/-} mice had significant reduction of HSCs, MPPs, and megakaryocytes (fig. S4, F to H) to levels nearly identical to those in the *Tpo*^{*gfp/gfp*} mice (fig. S3). Recombination of the *Tpo*^{*fl*} allele therefore gave a strong loss of *Tpo* function.

Megakaryocytes have been proposed to be a major source of TPO for HSCs (22, 23). However, we did not detect any *Tpo* expression in megakaryocytes by qRT-PCR (Fig. 1A) or by using reporter mice (Fig. 1, K to N). Nonetheless, we directly tested whether megakaryocytes (or any hematopoietic cells) are sources of TPO for HSC maintenance in vivo by generating *Vav1-cre;Tpo*^{*fl/gfp*} mice. *Vav1-cre* efficiently deleted *Tpo* from the hematopoietic system (fig. S5A). Eight-week-old *Vav1-cre;Tpo*^{*fl/gfp*} mice had normal blood cell counts (fig. S5B), cellularity, and HSC frequency (fig. S5, C and D) and normal restricted hematopoietic progenitors in the bone marrow (fig. S5, E to H). Spleen cellularity, HSC frequency, and megakaryocytic cells were also unaffected (fig. S5, I to K). Bone marrow cells from *Vav1-cre;Tpo*^{*fl/gfp*} mice formed normal numbers of colonies in methylcellulose and reconstituted irradiated recipients normally (fig. S5, L and M). Thus, hematopoietic cells, including megakaryocytes, are not a critical source of TPO for HSC maintenance.

Osteoblasts have also previously been proposed to be the main source of TPO in the bone marrow (17), and osteoblasts express *Tpo* transcripts (Fig. 1A); however, we could not detect any *Tpo* translational activity in osteoblasts from our reporter mice (Fig. 1, K to N). We directly tested whether deletion of *Tpo* from osteoblasts affects HSCs. Consistent with previous reports (2, 3), *Col2.3-cre* recombined efficiently in bone-lining osteoblasts (fig. S6, A to G). Eight-week-old *Col2.3-cre;Tpo*^{*fl/gfp*} mice had normal blood cell counts (fig. S6H), cellularity, and HSC frequency in the bone marrow and spleens (Fig. 2, A and B). Restricted hematopoietic progenitors were also normal (fig. S6, I to L). Spleen megakaryocytic cells were unaffected (fig. S6M), as were the capacities of bone marrow and spleen cells to form colonies in methylcellulose (Fig. 2C and fig. S6N). *Col2.3-cre;Tpo*^{*fl/gfp*} bone marrow cells had normal capacities to reconstitute irradiated recipients (Fig. 2D and fig. S6, O and P). Thus, osteoblasts are not a critical source of TPO for HSC maintenance.

Bone marrow *Lepr*⁺ mesenchymal stromal cells are a critical source of HSC niche factors, including stem cell factor (SCF) and CXCL12 (2, 3). These cells express *Tpo* transcripts (Fig. 1A), although we could not detect *Tpo* translational activity (Fig. 1, K to N). We conditionally deleted *Tpo* from mesenchymal stromal cells by generating *Lepr-cre;Tpo*^{*fl/gfp*} mice. *Tpo* was efficiently deleted from these cells (fig. S7, A to G). Eight-week-old *Lepr-*

cre;Tpo^{fl/gfp} mice had normal blood cell counts (fig. S7H), cellularity, and HSC frequency in the bone marrow and spleens (Fig. 2, E and F). Restricted hematopoietic progenitors were also normal (fig. S7, I to L). Spleen megakaryocytic cells were unaffected (fig. S7M). Bone marrow and spleen cells from *Lepr-cre;Tpo^{fl/gfp}* mice formed normal numbers of hematopoietic colonies in meth-ylcellulose (Fig. 2G and fig. S7N). Bone marrow cells from *Lepr-cre;Tpo^{fl/gfp}* mice reconstituted irradiated recipients normally (Fig. 2H and fig. S7, O and P). Thus, bone marrow mesenchymal stromal cells are not a critical source of TPO for HSC maintenance.

The above data suggest that TPO produced locally by osteoblasts or mesenchymal stromal cells is not required for HSC maintenance. To test whether systemic TPO generated by the liver is important for HSC maintenance, we generated *Alb-cre;Tpo^{fl/fl}* mice. As expected (31), *Alb-cre* recombined specifically and efficiently in hepato-cytes but not in the bone marrow (Fig. 3, A to D, and fig. S8, A to D). Eight-week-old *Alb-cre;Tpo^{fl/fl}* mice had a fivefold reduction in platelet count (Fig. 3E and fig. S8E) and a fivefold reduction in megakaryocytic cells in the bone marrow (Fig. 3F). *Alb-cre;Tpo^{fl/fl}* mice exhibited normal bone marrow and spleen cellularity (Fig. 3G). The frequency of bone marrow HSCs was reduced by a factor of 24 compared with that in controls (Fig. 3H). LSK progenitors and MPPs were similarly reduced (fig. S8, F and G). CMPs were reduced in the bone marrow, whereas other restricted hematopoietic progenitors appeared normal (fig. S8H). Bone marrow and spleen cells from *Alb-cre;Tpo^{fl/fl}* mice formed fewer colonies in methylcellulose (Fig. 3, I and J). Spleen HSC frequency was normal in *Alb-cre;Tpo^{fl/fl}* mice (Fig. 3H), suggesting that there was no compensatory extramedullary hem-atopoiesis. Spleen CD41⁺ cell frequency decreased (fig. S8I). Bone marrow cells from *Alb-cre;Tpo^{fl/fl}* mice had severe defects in their ability to reconstitute irradiated recipients (Fig. 3, K and L). Overall, these data show that hepatic TPO is critical for the maintenance of bone marrow HSCs.

During development, HSCs transiently reside in the fetal liver (32), where *Alb-cre* recombines (33). Thus, it is possible that bone marrow HSCs in *Alb-cre;Tpo^{fl/fl}* mice acquire a persistent defect during development, although global deletion of *Tpo* has no impact on fetal liver HSCs (16). Nonetheless, we conditionally deleted *Tpo* from adult hepatocytes by administering the hepatotropic Cre-bearing virus AAV8-TBG-cre (34). Consistent with a prior report (34), we observed specific and efficient recombination in hepatocytes after a single intravenous administration of AAV8-TBG-cre to adult mice (Fig. 4, A to D, and fig. S9, A to D).

We administered AAV8-TBG-cre virus once to 8-week-old *Tpo^{fl/fl}* mice and wild-type littermate control mice and analyzed the mice 4 to 8 weeks later. AAV8-TBG-cre-treated wild-type mice had phenotypes identical to those of buffer-treated *Tpo^{fl/fl}* mice. These mice were pooled together as controls. Deleting *Tpo* from adult hepatocytes (*AAV8-cre;Tpo^{fl/fl}*) significantly reduced platelet counts (fig. S9E). Bone marrow and spleen cellularity did not change (Fig. 4E). However, *AAV8-cre;Tpo^{fl/fl}* mice had significant reductions in bone marrow HSC, LSK, MPP, CMP, and CD41⁺ cell frequencies (Fig. 4F and fig. S9, G to I). The colony-forming capacities of bone marrow and spleen cells were also reduced (Fig. 4, H and I). Spleen HSC and CD41⁺ megakaryocytic cell frequencies trended lower (Fig. 4G and

fig. S9J). Bone marrow cells from *AAV8-cre;Tpo^{fl/fl}* mice 2 months after the virus treatment had a significant decrease in their ability to reconstitute irradiated recipients, whereas bone marrow cells from *AAV8-cre;Tpo^{fl/fl}* mice 1 month after the virus treatment had an intermediate phenotype (Fig. 4J and fig. S9K). To quantify the frequency of functional HSCs, we performed limit dilution assays. In these assays, the frequencies of long-term multilineage reconstituting cells in bone marrow cells from control and *AAV8-cre;Tpo^{fl/fl}* mice were 1/87,660 and 1/498,020, respectively, corresponding to a 6-fold reduction (Fig. 4K). *AAV8-cre;Tpo^{fl/fl}* mice had no significant difference in Annexin V⁺ bone marrow HSCs compared with controls (Fig. 4L and fig. S9L), but HSCs from *AAV8-cre;Tpo^{fl/fl}* mice cycled more (Fig. 4M and fig. S9M). These data indicate that adult HSCs lacking hepatocyte-derived TPO signal are depleted through loss of quiescence.

Tpo transcription is up-regulated in the bone marrow during hematopoietic stress (21). We injected TMX-treated *Tpo^{DsRed-CreER};loxPZsGreen* mice with 5-fluorouracil but failed to detect ZsGreen expression in the bone marrow (fig. S10), suggesting that bone marrow is not a major source of TPO in stress, at least under the conditions we tested. Our data show that hepatocytes are the major functional source of systemic TPO for bone marrow HSC maintenance under steady-state conditions. Further studies are required to determine the role of local and systemic TPO in nonhomeostatic conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. TPO is expressed by hepatocytes but not by bone marrow cells
(A) qRT-PCR analysis of *Tpo* transcript levels ($n = 3$ mice; error bars indicate SD). **(B)** Schema of TPO expression analysis in *Tpo*^{DsRed-CreER}; *loxpZsGreen* mice. LSL, loxp-Stop-loxp. **(C to J)** Confocal images of liver sections from sham-treated (+Sham) or TMX-treated *Tpo*^{DsRed-CreER}; *loxpZsGreen* mice. DAPI, 4',6-diamidino-2-phenylindole. **(K to N)** Confocal images of femur sections from sham- or TMX-treated *Tpo*^{DsRed-CreER}; *loxpZsGreen* mice.

Fig. 2. Osteoblasts and mesenchymal stromal cells are not critical sources of TPO for HSC maintenance

(A to C) Cellularity (A), HSC frequency (B), and bone marrow colony-forming cell frequency (C) were normal in *Col2.3-cre;Tpo^{fl/gfp}* mice ($n = 4$ to 5 mice). (D) Bone marrow cells (5×10^5) from *Col2.3-cre;Tpo^{fl/gfp}* mice gave normal donor cell reconstitution compared to controls (in two experiments with a total of eight to nine recipient mice per genotype). (E to G) Cellularity (E), HSC frequency (F), and bone marrow colony-forming cell frequency (G) were normal in *Lepr-cre;Tpo^{fl/gfp}* mice ($n = 5$ to 6 mice). (H) Bone marrow cells (5×10^5) from *Lepr-cre;Tpo^{fl/gfp}* mice gave normal levels of donor cell reconstitution compared to controls (in two experiments with a total of eight recipient mice per genotype). Controls (Con), *Col2.3-cre;Tpo^{+/gfp}*, *Lepr-cre;Tpo^{+/gfp}*, or *Tpo^{fl/gfp}* mice. /

gfp, *Col2.3-cre;Tpo^{fl/gfp}* or *Lepr-cre;Tpo^{fl/gfp}* mice. Data are means $\pm$ SD. ns, not significant ($P > 0.10$).

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Fig. 3. Hepatocyte-derived TPO is required for HSC maintenance

(A to D) Confocal images of liver sections from *Alb-cre;loxpTdTomato* (*Alb-cre;loxpTdT*) mice. HNF4α, hepatocyte nuclear factor 4-α. (E) Platelet counts were decreased in *Alb-cre;Tpo^{fl/fl}* mice ($n = 4$). (F) Bone marrow CD41⁺ cell frequency was decreased in *Alb-cre;Tpo^{fl/fl}* mice ($n = 5$ to 7). (G) Cellularity was normal in the bone marrow and spleens from *Alb-cre;Tpo^{fl/fl}* mice ($n = 5$ to 6). (H) HSCs were depleted in the bone marrow but not in spleens from *Alb-cre;Tpo^{fl/fl}* mice ($n = 6$ to 8). (I and J) Colony-forming cell numbers were decreased in the bone marrow and trended lower in spleens (SPL) from *Alb-cre;Tpo^{fl/fl}* mice ($n = 5$ to 6). E, erythroid burst-forming unit; Mk, megakaryocyte; GM, granulocyte-macrophage; GEMM, granulocyte-erythrocyte-monocyte-megakaryocyte. (K) Bone marrow cells (5×10^5) from *Alb-cre;Tpo^{fl/fl}* mice gave lower levels of donor cell reconstitution (in two experiments with a total of eight to nine recipient mice per genotype). (L) Recipient mice from (K) showed significant decrease in donor bone marrow HSC chimerism 16 weeks posttransplant ($n = 6$ mice per genotype). Con, *Alb-cre;Tpo^{+/+}*, *Tpo^{fl/fl}*, or *Tpo^{fl/+}* mice; /, *Alb-cre;Tpo^{fl/fl}* mice. Data are means $\pm$ SD. ns, not significant ($P > 0.10$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 4. TPO from adult hepatocytes regulates bone marrow HSC maintenance and quiescence (**A** to **D**) Confocal images of liver sections from AAV8-TBG-cre-treated *loxpTdT^{Tomato}* mice. (**E**) Bone marrow and spleen cellularity was normal in *Alb-cre; Tpo^{fl/fl}* mice ($n = 7$ to 8). (**F**) Bone marrow HSCs were depleted in AAV8-TBG-cre-treated *Tpo^{fl/fl}* mice ($n = 4$ to 5). (**G**) Spleen HSC frequency tended to decrease in AAV8-TBG-cre-treated *Tpo^{fl/fl}* mice. (**H** and **I**) Bone marrow and spleen colony-forming cell frequencies decreased in *AAV8-cre; Tpo^{fl/fl}* mice ($n = 6$ to 7). (**J**) Bone marrow cells (5×10^5) from *AAV8-cre; Tpo^{fl/fl}* mice gave lower levels of donor cell reconstitution than controls (in two to three experiments with a total of $n = 16$ for controls, $n = 9$ for 1 month after AAV8 treatment, and $n = 14$ for 2 months after AAV8 treatment). (**K**) Limit dilution assays showed a 6-fold reduction in functional HSCs in the bone marrow from *AAV8-cre; Tpo^{fl/fl}* mice compared with those in controls (in two independent experiments). (**L**) Annexin V⁺ DAPI⁻ bone marrow HSC frequency was not affected in *AAV8-cre; Tpo^{fl/fl}* mice ($n = 5$). (**M**) Bone marrow HSCs incorporated more bromodeoxyuridine (BrdU) in *AAV8-cre; Tpo^{fl/fl}* mice ($n = 4$). Con, *Tpo^{+/+}* mice treated with AAV8-TBG-cre or *Tpo^{fl/fl}* mice treated with phosphate-buffered saline;

/ , $Tpo^{fl/fl}$ mice treated with AAV8-TBG-cre. Data are means $\pm$ SD. ns, not significant ($P > 0.10$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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