Biochemical and Biophysical Research Communications 454 (2014) 353–357

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Megakaryocytes are essential for HSC quiescence through the production of thrombopoietin

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ARTICLE INFO

Article history:
Received 4 October 2014
Available online 24 October 2014

Keywords:
Megakaryocytes
Hematopoietic stem cells
Thrombopoietin
Niche
Bone marrow

ABSTRACT

Tissue homeostasis demands regulatory feedback, suggesting that hematopoietic stem cell (HSC) activity is controlled in part by HSC progeny. Yet, cell extrinsic HSC regulation has been well characterized only in niche cells of non-hematopoietic origin. Here we identify feedback regulation of HSCs by megakaryocytes (Mks), which are mature hematopoietic cells, through production of thrombopoietin (Thpo), a cytokine pertinent for HSC maintenance. Induced ablation of Mk cell population in mice perturbed quiescent HSCs in bone marrow (BM). The ablation of Mks resulted in decreased intra-BM Thpo concentration presumably due to Thpo production by Mks. Thpo administration Mk ablated mice restored HSC functions. Overall, our study establishes Mk as an essential cellular component of the HSC niche and delineates cytokine-oriented regulation of HSCs by their own progeny.

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1. Introduction

Consistent production of mature hematopoietic cells requires both stringent maintenance and appropriate differentiation of HSCs [1]. While intrinsic mechanisms govern HSC activity, HSCs reside in a niche composed primarily of non-hematopoietic cells, which provide direct and indirect regulatory cues for HSC integrity [2]. Upon differentiation, HSCs produce large numbers of lineage-committed progenitor cells, which give rise to mature hematopoietic cells [3]. Feedback among cells is a fundamental property of a hierarchical system of tissue stem cells [4,5]. As predominant residents of the BM, mature hematopoietic cells, such as macrophages, regulate HSC mobilization [6,7] or modulate activities of non-hematopoietic niche cells, such as osteoblasts (OB) [6]. However, it is not clear whether and how mature hematopoietic cells residing in the BM directly influence HSC cell cycle quiescence, a property that determines the fundamental stemness of HSCs [8]. Here we show that Mks, which are mature hematopoietic cells are essential for HSC maintenance. Our data implies that Mks produce and secrete of thrombopoietin (Thpo) to drive their own maturation and maintain HSC quiescence.

2. Methods

2.1. Mice

All mice were on a C57BL/6 background. *PF4-Cre* transgenic mice were kindly provided by Prof. Radek C. Skoda (University Hospital, Basel, Switzerland) [9]. *Mos-iCsp3* transgenic mice [10], a gift of Dr. Masato Fujioka of Keio University were crossed with *PF4-Cre* transgenic mice to obtain *PF4-Cre:Mos-iCsp3* mice. A chemical homodimerizer, AP20187, was intraperitoneally injected (10 mg/kg body weight) into *PF4-Cre:Mos-iCsp3* mice to ablate Mk and equivalently to control mice. C57BL/6-Ly5.1 or C57BL/6-Ly5.2 mice were used for competitive repopulation assays. Unless specified, 10–12-week-old mice were used in each experiment. All animal experiments were approved by Keio University and performed in accordance with the Guidelines of Keio University for Animal and Recombinant DNA experiments.

2.2. Antibodies

Primary antibodies used for immunohistochemistry (IHC) and flow cytometry are summarized in the Supplementary Table 1.

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http://dx.doi.org/10.1016/j.bbrc.2014.10.095
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Secondary antibodies for IHC were Alexa 488 fluorescence-conjugated IgGs (Molecular Probes) or Cy3/Cy5/DyLight549/DyLight649-conjugated IgGs (Jackson ImmunoResearch). IHC specimens were treated with DAPI (Molecular Probes) for nuclear staining.

2.3. Immunostaining of bone marrow

Frozen sections prepared according to the Kawamoto method [11] were used to stain $Lin^-CD41^-CD48^-CD150^+$ cells in the BM. Fluorescence images were obtained using a confocal laser-scanning microscope (FV1000; Olympus). Scanning was performed in sequential laser emission mode to avoid scanning at other wavelengths. Images obtained from BM sections were analyzed using TissueQuest imaging analysis software (Tissuegnostics).

2.4. Flow cytometric analysis, cell cycle analysis and competitive repopulation assays

Flow cytometric analysis and competitive repopulation assays were performed as described [12]. Cell cycle analysis of hematopoietic cells was performed using Pyronin Y staining and short term BrdU incorporation assays [13].

2.5. BM transplantation

BM MNCs (4×10^5 cells) from C57BL/6-Ly5.1 mice together with 500 LT-HSCs from indicated mice (Ly5.2) were transplanted into lethally-irradiated C57BL/6-Ly5.1 congenic mice. Recipient mice were sacrificed for analysis 4 months after BMT.

2.6. In vitro HSC and Mk co-cultures

Mature Mks ($Ter119^-CD41^+$) were obtained from mouse BMs as described [14]. LT-HSCs ($Lin^-c\text{-}Kit^+Sca\text{-}1^+Flt\text{-}3^-CD34^-$) sorted from Ly5.1 mice were co-cultured with Mks in SF-O3 medium supplemented with murine recombinant SCF (100 ng/ml) and with or without human recombinant Thpo (100 ng/ml) for 3 days and analyzed [12]. LT-HSCs and Mks were cultured at 1:1 ratio. To inhibit Thpo, a recombinant mouse Thpo receptor (Mpl)-Fc fusion protein (0.4 μg/ml) (R&D systems) was added to the culture. An IgG Fc fragment (0.4 μg/ml) (Jackson ImmunoResearch) served as a control. For Thpo knock down in Mks, MISSION custom vectors (Sigma) were used.

2.7. In vivo Thpo assays

Thpo concentrations of obtained BM and blood serum were measured using a mouse Thpo ELISA kit (R&D systems). For in vivo stimulation of Thpo signaling, recombinant human thrombopoietin (PEG-rHuMGDF) [15] (donated from Kyowa Hakko Kirin Co., Ltd.) was administered. Mice were treated either with 100 μg/kg (i.v.) PEG-rHuMGDF or a human IgG Fc fragment (Jackson ImmunoResearch). For rescue experiments, mice were treated for 2 consecutive days starting on the day of dimerizer administration.

2.8. Quantitative PCR assay

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

2.9. Statistical analysis

All results are expressed as the mean ± SD unless otherwise specified. Statistical significance was determined by Tukey's multiple comparison test. The two-tailed Student's *t*-test and log rank test were used for two-group comparisons.

3. Results and discussion

3.1. Long-term reconstituting HSCs (LT-HSCs) reside closely to Mks

LT-HSCs [16] and mature Mks [17] reportedly reside close to blood vessels within the BM. We utilized immunohistochemical staining to analyse the location of mature Mks and LT-HSCs within the BM. LT-HSCs ($CD150^+CD41^-CD48^-Lineage(Lin)^-Sca\text{-}1^+cKit^+$) (Fig. 1A) were located significantly closer to Mks (average: 2.33 cell diameters apart) than were BM mononuclear cells (MNCs) (average: 5.17 cell diameters apart), and more than half of all LT-HSCs resided near Mks (<3 cell diameters apart) (Fig. 1B).

3.2. Acute Mk depletion affects quiescent HSCs in BM

We next evaluated Mk maintenance of HSCs by inducing Mk depletion in transgenic *PF4-Cre:Mos-iCsp3* mice, which express a genetically modified dimerizable caspase gene upon latelet factor 4 (PF4) induced Cre expression [10]. Although it has been reported that PF4 is expressed on HSCs [18], gene expressions of *Pf4* in LT-HSCs was significantly lower that Mks (Supplementary Fig. 1a) and PF4 expression was not detected in HSCs from *PF4-Cre:flox-CAG-GFP* mice (Supplementary Fig. 1b). GFP expressions were prominent in platelets of *PF4-Cre:flox-CAG-GFP* mice (Supplementary Fig. 1b). Mk depletion was induced through activation of caspase upon administration of AP20187 to *PF4-Cre:Mos-iCsp3* (herein Mk-depleted mice). Mk-depleted mice analyzed 36 h after AP20187 injection exhibited significant decrease in BM Mk numbers compared to controls (Fig. 1C, Supplementary Fig. 2a and b). Caspase-induced apoptosis did not alter the number of anuclear platelets (Supplementary Fig. 2c) there by circumvented the effect of thrombocytopenia. Acute Mk depletion significantly lowered the number of BM LT-HSCs (Fig. 1D), while hematopoietic cell populations in the BM, spleen and thymus were unaffected (data not shown). Functionally, HSPCs from Mk-depleted BMs exhibited higher colony formation activity (Supplementary Fig. 3a and b), indicative of increased cell proliferation capacity. Mk depletion increased HSPC mobilization to PB (Supplementary Fig. 4a–d), suggesting that defective retention of HSCs in the BM underlies LT-HSC loss. Cell cycle analysis of HSCs from Mk-depleted mice exhibited higher fractions of Pyronin-Y positive HSCs signifying a loss of HSC quiescence (Fig. 1E and F). Competitive BM transplantation of HSCs from Mk-depleted mice (Ly5.2) resulted in significantly lower chimerisms in both PB and BM hematopoietic cells compared to HSC from control mice (Ly5.2) (Fig. 1G and H). Taken together, acute Mk depletion revealed that Mks regulated both retention in and quiescence of BM HSCs.

3.3. Mks produce Thpo for the maintenance of HSCs

We next assessed Mk production of niche factors. While Mks expressed genes of various niche factors (including *KitL*, *SPP*, *Tgfb1*, *Tgfb3* [19] and *Pf4*) (data not shown), Mks exhibited significant expression of *Thpo* expression (Fig. 2A). Compared to other non-hematopoietic niche cells (osteoblasts (OB), endothelial cells (EC) or mesenchymal stem cells (MSC)), especially OBs which have been reported to produce Thpo in the BM [20], Mks expressed significantly high amounts of *Thpo* transcripts (Fig. 2A). *Thpo* transcript

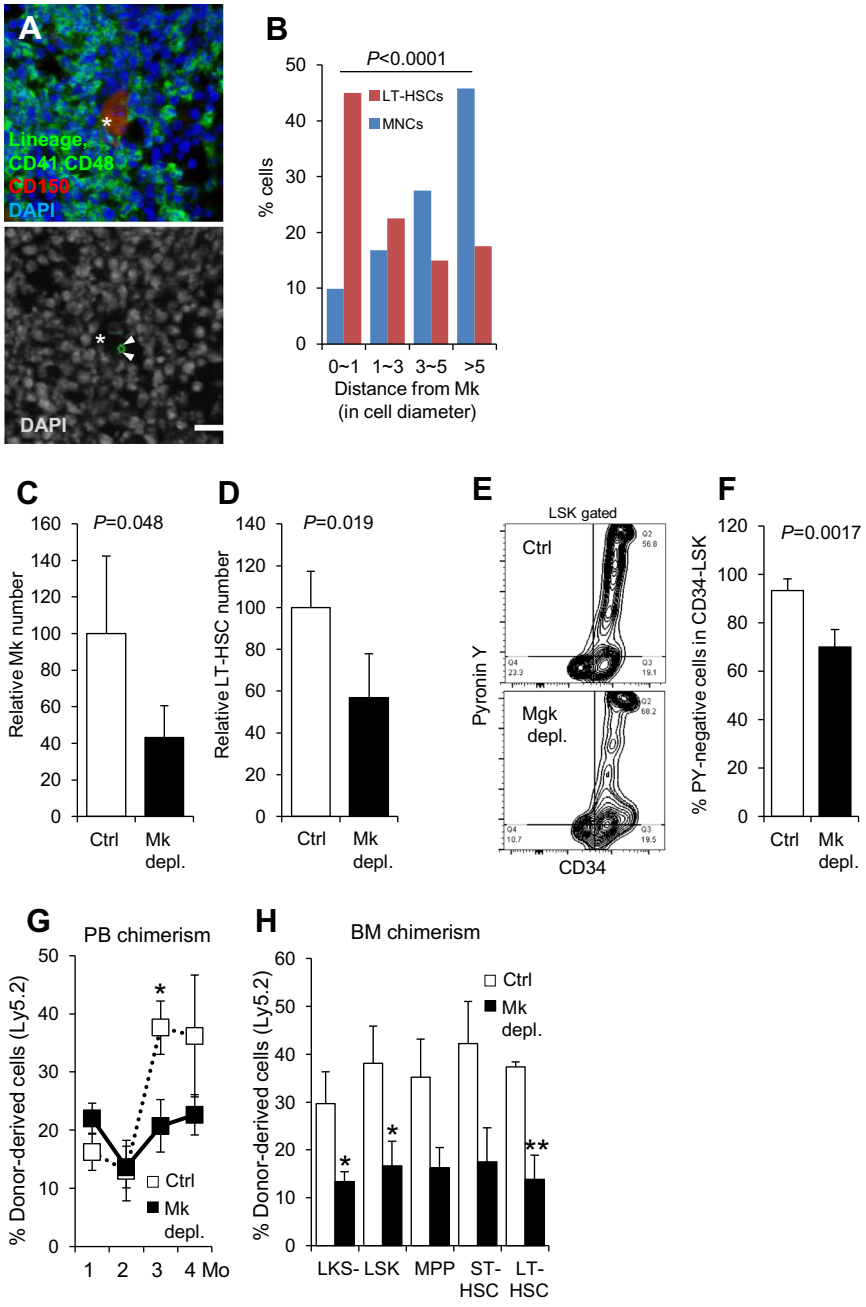

Fig. 1. Mk-specific depletion cause a decline in HSC number and function. (A) Lineage (Lin)$^-$CD41$^-$CD48$^-$CD150$^+$ HSCs (arrowheads) residing adjacent to an Mk (asterisk). HSCs were identified using TissueQuest software. Scale bar = 50 μm. (B) Distribution of the distances of LT-HSCs and BM MNCs from Mks. Relative number of Mks (C) and LT-HSCs (D) in Mk-depleted (Mk depl.) mice compared to control mice (mean ± SD) (either *PF4-Cre*$^-$*Mos-iCsp*$^+$ or *PF4-Cre*$^+$ *Mos-iCsp*$^-$) (mean value of control set at 100) 36 h after AP20187 injection (n = 5). (E) Representative flow cytometric plot of Pyronin-Y staining for CD34$^{+/-}$LSK cells in Mk-depleted and control mice (n = 4). (F) Percentage of Pyronin-Y-negative cells in CD34$^-$ LSK cells (mean ± SD) (n = 4). Percentage of donor-derived cells in PB (g) and BM HSPCs (h) at indicated intervals after BMT (mean ± SEM) (n = 6). $^*p < 0.05$, $^{**}p < 0.01$.

levels also increased with Mk ploidy (Fig. 2B). Thpo protein expression in Mks was confirmed by IHC (Fig. 2C and D).

ELISA assays indicated remarkably low Thpo concentrations in the BM but not serum of Mk-depleted mice (Fig. 2E and F). To determine whether functional defects seen in HSCs from Mk-depleted mice are attributable to decreased Thpo levels, we administered recombinant Thpo (PEG-rHuMGDF) to both Mk-depleted and control mice. Thpo administration restored the number and frequency of quiescent HSCs (Fig. 2G and H). *In vitro* analysis confirmed the effect of Mk-derived Thpo on HSCs: addition of Mks to HSCs cultured in the absence of Thpo increased the number of LT-HSCs (Fig. 2I). Addition of a recombinant Mpl-Fc protein that inhibits Thpo function to the Mk culture abolished this effect, indicating that HSCs require Mks for Thpo production (Fig. 2I). Furthermore, Mks knocked down of *Thpo* with shRNA, could not maintain the number of co-cultured LT-HSC (Fig 2J and Supplementary Fig. 5).

In summary, we have identified Mks as an active cellular component of the BM niche. Mks reportedly co-localize with HSCs [14] and indirectly regulate HSCs by stimulating OBs post-transplantation [21]; however, our findings identify direct Mk regulation of HSCs in steady state hematopoiesis. The decline in HSC number and their loss of quiescence upon acute Mk-specific ablation in the BM indicates that retention and maintenance of quiescent HSCsin the BM requires the presence of Mks. We show that Mk

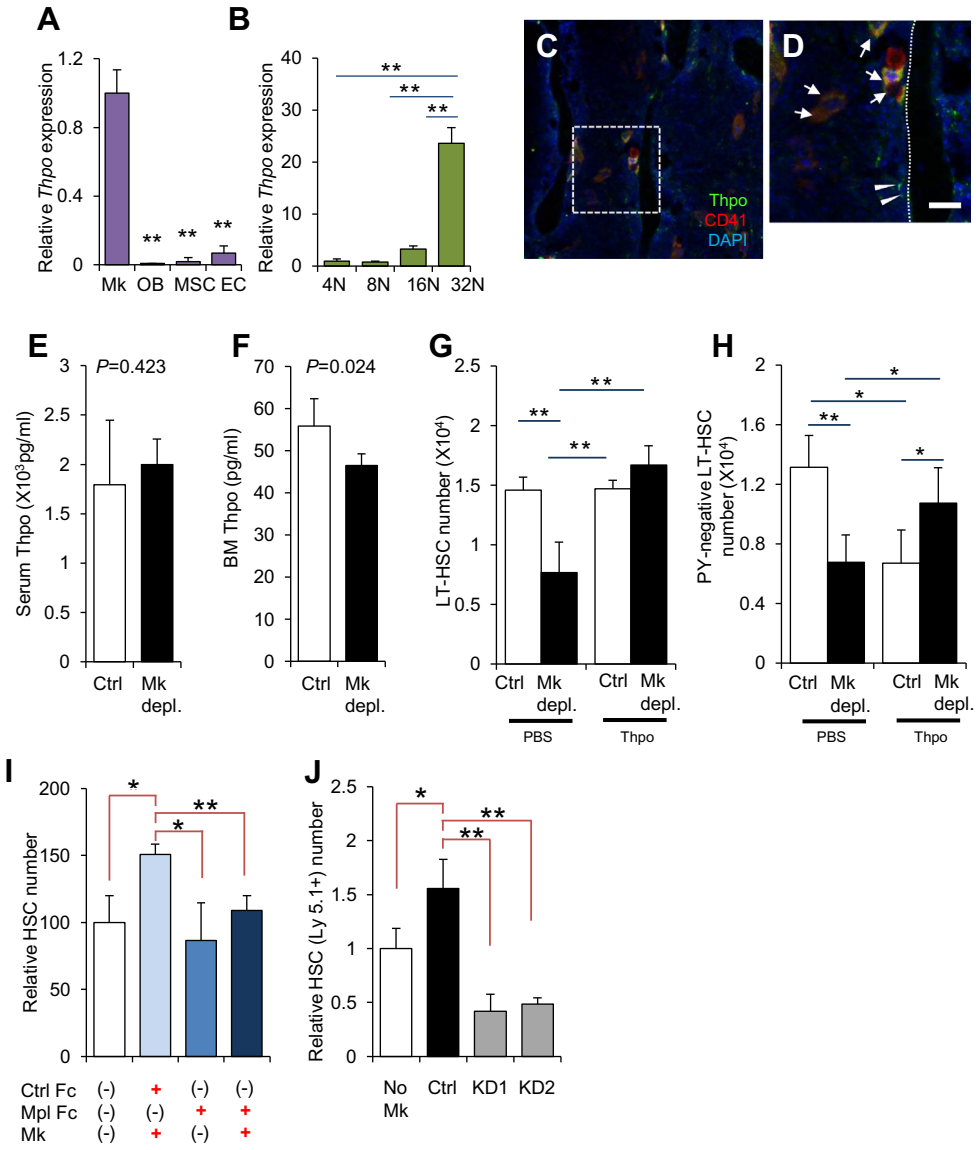

Fig. 2. Mks produce Thpo for the maintenance of quiescent HSCs. (A) Relative levels of *Thpo* transcripts are higher in Mks compared to non-hematopoietic niche cells (OBs ($CD45^-Ter119^-CD31^-Sca\text{-}1^-ALCAM^+$), ECs ($CD45^-Ter119^-CD31^+$) and MSCs ($CD45^-Ter119^-CD31^-Sca\text{-}1^+ALCAM^-$) (mean ± SD) ($n = 4$). (B) Relative *Thpo* expression is high in polyploid Mgks ($n = 4$). (C,D) IHC of BM shows Mks (arrows) expressing Thpo (green). Thpo expression in osteoblasts lining the bone surface (arrowheads) is seen. (D) Enlargement of dotted area in (C). Scale bar = 100 μm for (C) and 50 μm for (D). Serum (E) and BM (F) concentration of Thpo from control and Mk-depleted mice (mean ± SD) ($n = 5$). Number of LT-HSCs (G) and Pyronin-Y negative LT-HSCs (H) with or without Thpo administration in control and Mk-depleted mice (mean ± SD) ($n = 4$). (I) Changes in the relative number of LT-HSCs following addition of Mks to LT-HSC culture in the absence of Thpo (mean ± SD) ($n = 4$). Culture containing only HSCs (far left bar) is set at 100. Note that the presence of Mks in the culture (second bar from left) increases the number of MNCs and HSCs, an effect attenuated by Thpo inhibition by Mpl Fc (bar on far right) (mean ± SD) ($n = 4$). (J) Knockdown (KD1 and KD2) of *Thpo* resulted in significant decrease in the number of co-cultured HSCs (Ly5.1+) with Mks. The bar on far left indicates culture without Mks. (mean ± SD) ($n = 4$). $^*p < 0.05$, $^{**}p < 0.01$.

production of Thpo is one mechanism for Mk regulation on HSCs. Our study strongly indicates that Mks function as niche cells and confirms that HSC quiescence is directly regulated by HSC progeny.

Author contributions

T.S., K.T. and A.N-I designed the project, analyzed the data and wrote the manuscript. A.N-I and K.T. organized, performed and analyzed all experiments. All authors read and approved the final manuscript.

Conflicts of interest disclosure

The authors declare no competing financial interests.

Acknowledgments

The authors thank T. Muraki and T. Hirose for technical assistance. A.N-I. was supported in part by a fellowship from JSPS, Grants-in-Aid for JSPS Fellows (25-40030 and 05-045-0166/4096) a research grant from the SENSHIN medical research foundation, a research grant from the Takeda Foundation and a grant from the Naito Foundation. K. T. was supported in part by a MEXT Grant-in-Aid for Young Scientists (A), a Grant of National Center for Global Health and Medicine and a research grant from the Astellas Foundation for Research on Metabolic Disorders. T.S. and K.T were supported in part by a MEXT Grant-in-Aid for Scientific Research (A) and a MEXT Grant-in-Aid for Scientific Research on Innovative Areas.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.095.

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