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REVIEW

Multifaceted roles of thrombopoietin in hematopoietic stem cell regulation

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Thrombopoietin (Thpo) and its receptor myeloid proliferative leukemia (Mpl) were initially identified as the cytokine signaling that stimulates megakaryopoiesis and platelet production. However, Thpo–Mpl signaling has also been widely characterized as one of the few cytokine systems that directly regulates hematopoietic stem and progenitor cells. The ability of Thpo signaling to stimulate hematopoietic stem cell (HSC) self-renewal has led to the development and utilization of Thpo mimetic drugs to treat hematopoietic diseases with restricted function of HSCs, such as aplastic anemia. This review will cover the mechanisms by which Thpo–Mpl signaling regulates HSCs.

Keywords: hematopoietic stem cell; thrombopoietin; cytokine signaling

Introduction

Thrombopoietin (Thpo) was initially identified during the 1950s as a major regulator of platelet production.[1,2] The discovery of its receptor, myeloid proliferative leukemia (Mpl), preceded the identification of Thpo. Mpl was successfully cloned through the identification of a human homolog to the oncogene *v-mpl* of myeloproliferative leukemia virus.[3,4] Mpl was classified as an orphan receptor of the hematopoietin receptor superfamily, and its function was characterized as stimulating the proliferation of hematopoietic cells and inducing megakaryopoiesis.[5,6] Subsequently, the ligand for Mpl, Thpo, was identified and cloned.[7–9]

A large amount of research has confirmed the function of Thpo–Mpl signaling as the major stimulator of megakaryopoiesis and platelet production. While Thpo does not affect terminal proplatelet production and release from megakaryocytes, Thpo affects both early megakaryocyte formation from progenitor cells and megakaryocyte maturation.[10–12] Thpo can act alone or in combination with other cytokines, such as interleukin (IL-)3, IL-11, and stem cell factor (SCF), to increase the formation of megakaryocyte colony forming units *in vitro*.[13] Moreover, mice with conventional gene knockout of either *Thpo* and *Mpl* exhibit impaired megakaryocyte formation and are severely thrombocytopenic.[14,15] However, unlike other hematopoietin receptor superfamily members that stimulate a single lineage of hematopoiesis, Thpo–Mpl signaling affects hematopoietic stem and progenitor cells (HSPCs) in addition to megakaryopoiesis.

Thpo stimulates HSPC proliferation

Since the identification of Mpl as a human homolog of the oncogene *v-mpl*, Thpo–Mpl signaling has been implicated in enhancing the proliferation of hematopoietic cells.[6] v-Mpl is an oncoprotein consisting of a portion of the Friend murine leukemia virus envelope sequence fused with a truncated form of Mpl lacking the extracellular domain of the full Mpl sequence. Infection of hematopoietic cells by v-Mpl immortalizes them.[6] Thpo, in combination with SCF and IL-3, stimulates dormant hematopoietic progenitor cells to expand *in vitro*.[16,17] Furthermore, Thpo can

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sustain the viability of $Lin^{-}Sca1^{+}$ hematopoietic progenitor cells for a longer period (>40 h) compared with SCF *in vitro*.[18] Moreover, both $Thpo^{-/-}$ and $Mpl^{-/-}$ mice have reduced numbers of erythroid and myeloid progenitors.[14] Thpo-responsive bipotential erythroid-megakaryocyte progenitors ($CD150^{+}CD9^{hi}$ progenitor cells) have also been identified.[19]

Advances in methods to isolate and purify bone marrow (BM) hematopoietic stem cell (HSCs) have further demonstrated that Thpo–Mpl signaling not only induces proliferation of hematopoietic progenitor cells, but also HSCs. Along with SCF, Thpo is one of the few cytokines that directly stimulates HSCs. Both $Thpo^{-/-}$ and $Mpl^{-/-}$ mice exhibit a severe loss of HSCs. A 16-fold reduction of $CD34^{-}Flt3^{-}CD150^{+}Lin^{-}Sca1^{+}cKit^{+}$ (LSK) cells and a 12-fold reduction of $CD150^{+}$ LSK cells have been noted in $Thpo^{-/-}$ and $Mpl^{-/-}$ mice, respectively.[20] Moreover, in humans, patients with congenital amegakaryocytic thrombocytopenia suffer a progressive decline in HSC numbers and subsequent BM failure due to a loss-of-function mutation in *MPL*.[21] HSCs with defective Thpo–Mpl signaling not only are reduced in number due to impaired proliferation, but also exhibit impaired reconstitution of lethally irradiated mice when competing with wild-type cells. Approximately 10-fold more BM cells from $Mpl^{-/-}$ mice were required to compete equally with $Mpl^{+/+}$ BM cells.[22] $Thpo^{-/-}$ BM cells exhibited up to a 157-fold decrease in competitive repopulating units using limiting dilution experiments.[20] However, it has been argued that Mpl does not control BM HSC numbers, but rather stimulates the expansion of differentiating clones.[23] Wild-type BM cells did not outcompete $Mpl^{-/-}$ BM cells when competitively transplanted to $Thpo^{-/-}$ mice. The data indicate that the number of HSCs in $Mpl^{-/-}$ BM is equivalent to that of wild-type mice and suggest that Thpo–Mpl signaling may rather support the expansion of hematopoietic progenitor cells after transplantation. The previous studies that assessed stem cell potential in Thpo signaling deficiency mainly utilized competitive transplantation of total BM cells due to the limitation in the number of HSCs in $Thpo^{-/-}$ or $Mpl^{-/-}$ mice. We are currently using assays based on small sample number or single-cell HSCs to investigate the alterations in HSCs upon Thpo deficiency. Studies that compare the HSC potential using highly purified HSCs may provide new insights into revealing the true nature of HSCs from $Thpo^{-/-}$ or $Mpl^{-/-}$ mice.

The role of Thpo–Mpl signaling in enhancing HSC proliferation has also been demonstrated through exogeneous administration of Thpo mimetic drugs. Administration of Thpo mimetic drugs *in vivo* can stimulate HSCs to exit quiescence.[24] High-resolution single HSC divisional tracking indicated that, among various receptor agonists, only Mpl receptor agonists stimulated self-renewal divisions of HSCs.[25] Although enhanced cell cycling corresponds to the loss of HSC stem cell potential,[26] the mechanism by which Thpo enhances cell proliferation specifically to self-renew HSCs and maintain their stem cell potential is largely unknown. Upon stimulating cell proliferation, Thpo signaling enhances DNA repair pathways to alleviate replication stress,[27] which may be linked to the preservation of HSC stemness. Thpo may also sustain HSC stem cell potential through metabolic regulation. Thpo stabilizes Hif1a expression in normoxic cultured primary murine HSCs and also affects the expression of Meis1, which is crucial for regulating HSC metabolism via Hif1a.[28–30] As HSCs largely reside in a hypoxic environment and rely on glycolysis for energy production, Thpo may modulate metabolic changes in HSCs to maintain HSC stem cell potential. Nevertheless, studies are necessary to investigate the mechanisms by which Thpo–Mpl signaling can facilitate efficient self-renewal divisions, possibly through single cell-based experiments.

Thpo regulates HSC quiescence and stemness

Cell cycle quiescence is a fundamental property of HSCs that maintains a sufficient reserve pool of stem cells within the BM.[26] However, contradicting the effect of excessive Thpo to stimulate HSC proliferation, HSCs from $Thpo^{-/-}$ mice exhibit an increase in proliferation and a loss of cell cycle quiescence.[20] Inhibition of Thpo signaling using *in vivo* injection of AMM2, an antibody that inhibits Mpl, into wild-type mice resulted in an increase in pyronin-Y staining in $CD34^{-}$LSK cells.[31] Thpo–Mpl signaling was associated with changes in the expression of the cell cycle regulator gene *p57Kip2* (*Cdkn1c*) in HSCs. However, it has still not been resolved how Thpo–Mpl signaling can balance its actions on HSCs for both quiescence and cell

proliferation. Contrary to what has been shown using Thpo mimetics to expand and proliferate HSCs, our group has reported an increase in the percentage of quiescent side-population LSK cells after 2 days of recombinant Thpo injection into wild-type mice.[31] Moreover, *in vitro* studies have shown that cultures of low Hoescht 33342/low Rhodamine 123 HSCs with only Thpo sustained cell number but did not induce proliferation.[17] These contradictory results may indicate that HSC response to Thpo is stringently time- and dose-dependent and is influenced by the presence of other cytokines. Indeed, it has been suggested that the time and dose of Thpo administration can interfere with the response of hematopoietic recovery after myelosuppression.[32] Further studies are necessary to analyze the kinetics of Thpo function to maintain HSC quiescence.

Another aspect in need of investigation is the effect of Thpo–Mpl signaling on specific HSC subsets. While numerous attempts have been made to purify and isolate the most potent HSCs within the BM, no study has succeeded in enriching a single uniform subset of highly potent HSCs. The difficulty of HSC purification may be due to the fact that HSCs are heterogeneous in surface marker expression and function.[33] While Thpo–Mpl pathway is implicated in the regulation of HSCs, the effect of the pathway on the expression of specific HSC-related genes seems to vary, suggesting that a specific subset of HSCs may respond to Thpo. HSCs from *Thpo*$^{-/-}$ mice exhibited a significant decline in the expression of the *Hoxb4*, *Hoxa5*, *Hoxa9*, and *Hoxa10* genes, but only small changes in *Bmi1*, *Hif1a*, *Atm*, and *Tal1*, which are all genes crucial for HSC self-renewal.[20] Interestingly, the emergence of HSCs highly expressing the von Willebrand factor (Vwf) gene, which are highly potent HSCs with a strong skewing to megakaryocytic differentiation, depended on Thpo signaling.[34] However, while the delineation and characterization of HSC subsets and heterogeneity advance, it will be interesting to investigate whether specific HSCs respond to Thpo–Mpl signaling, especially using single cell-based experiments.

Thpo determines lineage differentiation of HSCs

Along with the identification of heterogeneity in stem cell potential, recent studies have highlighted the heterogeneity in lineage differentiation, especially the megakaryocyte/platelet lineage, of HSCs.[33] Similar to Vwf^{+} HSCs, HSCs expressing high levels of c-Kit differentiated with a bias to the megakaryocyte lineage.[35] The physiological relevance of HSCs preferentially differentiating to megakaryocytes lies possibly with the necessity for rapid action to stimulate platelet production under acute hematopoietic stress conditions that are likely complicated by life-threatening thrombocytopenia. Related to this, megakaryocyte lineage-committed progenitor cells have been identified[36] along with CD41^{hi} HSC-like megakaryocyte progenitors (stem-like megakaryocyte progenitor), which expand and differentiate upon inflammation.[37] However, whether Thpo–Mpl signaling alters the capacity of HSCs to differentiate to megakaryocytes has been largely unknown.

We recently reported that Thpo is related to megakaryocyte lineage skewing in HSCs via upregulation of mitochondria signaling.[38] Administration of an Mpl agonist (romiplostim) to wild-type mice rapidly upregulated mitochondrial metabolism. BM transplantation of HSCs from romiplostim-treated mice exhibited a higher reconstitution of platelets compared with other lineages. Single-cell RNA sequencing of HSCs from mice treated with romiplostim exhibited an increase in the expression of mitochondria metabolism–associated gene sets. Romiplostim increased HSC staining by tetramethylrhodamine ethyl ester (TMRE), which indicates an increase in mitochondrial membrane potential. Supporting the increase in TMRE staining, romiplostim-treated HSCs exhibited an increase in oxidative phosphorylation and mitochondria-based energy production. It was further confirmed that HSCs from *Thpo*$^{-/-}$ mice exhibited a decrease in TMRE staining and were reduced in energy production (unpublished data).

Although HSCs reside in a hypoxic niche and rely on glycolysis for immediate energy production,[39] HSCs harbor considerable amounts of mitochondria. HSCs with high mitochondrial activity have been associated with low stem cell potential.[30,40] Nonetheless, recently, it has been reported that HSCs have higher mitochondrial mass compared to hematopoietic progenitor cells as assayed through the expression of Dendra2 fluorescence in hematopoietic cells from mice that express a mitochondrially targeted Dendra2.[41] The varying

amounts of mitochondria mass in hematopoietic cells question the role of mitochondria in HSC lineage differentiation. We therefore analyzed megakaryocyte-lineage differentiation in unstimulated, steady-state HSCs according to mitochondria activity. Competitive BM transplantation of a minimal number of HSCs demonstrated that TMRE^{hi} HSCs reconstituted recipient mice with a megakaryocyte bias when compared with HSCs with low mitochondrial activity. Megakaryocyte lineage bias in HSCs is associated with the expression of the platelet marker vWF.[34] We therefore analyzed the protein expression of up to 30 megakaryocyte and metabolic regulators in single HSCs using mass cytometry.[38] t-SNE analysis showed a drastic shift in expression patterns when HSCs were subjected to romiplostim stimulation. In line with past reports, we observed a sharp downregulation of Mpl expression on HSCs with romiplostim treatment. Among various megakaryocyte markers, the expression of CD9 was significantly associated with Thpo–Mpl signaling as well as mitochondrial activity. We also noted the upregulated expression of various megakaryocyte markers, such as CD41, PF4, vWF, and CD105, on HSCs treated with romiplostim (unpublished data). Collectively, our data indicated that Thpo–Mpl signaling influenced mitochondrial metabolism and suggested that metabolic regulation may alter HSC lineage differentiation (Fig. 1). We are currently analyzing the molecular mechanisms of how Thpo–Mpl signaling alters mitochondrial metabolism and lineage differentiation.

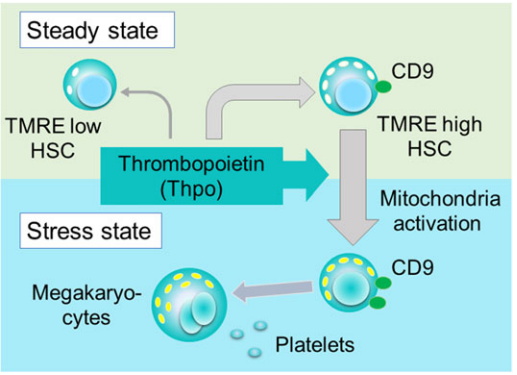

Figure 1. **Schematic representation of Thpo–Mpl signaling in the megakaryocyte lineage bias differentiation of HSCs. During steady-state hematopoiesis, HSCs can be subdivided according to mitochondrial membrane potential (TMRE staining). HSCs staining high with TMRE (TMRE^{hi} HSCs) express CD9 and preferentially differentiate to megakaryocytes. Upon exogenous Thpo stimulation, HSCs further enhance mitochondrial activity and differentiate to megakaryocytes.**

Thpo receptor agonists in the treatment of BM failure

Due to accumulating evidence that Thpo–Mpl signaling maintains HSCs, Thpo receptor agonists have been tested for the treatment of hematopoietic diseases involving impaired BM HSCs.[42] The two major Thpo mimetic drugs, romiplostim and eltrombopag, have been approved for clinical use for the treatment of immune thrombocytopenia.[43] Both drugs do not stimulate the production of auto-antibodies toward endogenous Thpo; autoantibody production was a side effect observed with the therapeutic administration of recombinant Thpo.[44] Romiplostim is a synthetic fusion protein of a Thpo-receptor binding domain and an Fc fragment of IgG1.[45] Eltrombopag is a nonpeptide small molecule that binds to the transmembrane domain of Mpl.[45] Despite high endogenous Thpo levels,[46] the effect of eltrombopag on HSCs can be sustained even after the termination of the drug in aplastic anemia patients.[47] Recently, it was reported that, in addition to its effect on activating Thpo–Mpl signaling in HSCs, eltrombopag enhances the stem cell potential of human HSCs through the reduction of intracellular iron, due to its structure containing a chelator motif.[48] Eltrombopag effectively lowered cellular labile iron concentration, as analyzed by enhanced staining to calcein AM in HSCs. These data suggest that the action of Thpo mimetic drugs on HSCs may differ. As eltrombopag does not bind to murine Mpl, we utilized romiplostim for the stimulation of Thpo–Mpl signaling in HSCs.[38] Similar to eltrombopag, both romiplostim and recombinant Thpo enhanced HSC staining to calcein AM when supplemented into *in vitro* HSC cultures (unpublished data). As eltrombopag does not affect murine HSCs, it is difficult to directly compare the degree by which the agents affect intracellular iron levels. Also, as calcein AM is commonly used for the detection of live/dead cells, the use of a specific probe for the detection of intracellular iron is necessary. Nonetheless, further investigation is necessary to clarify the difference between the action of endogenous Thpo and Thpo-receptor agonists on HSCs.

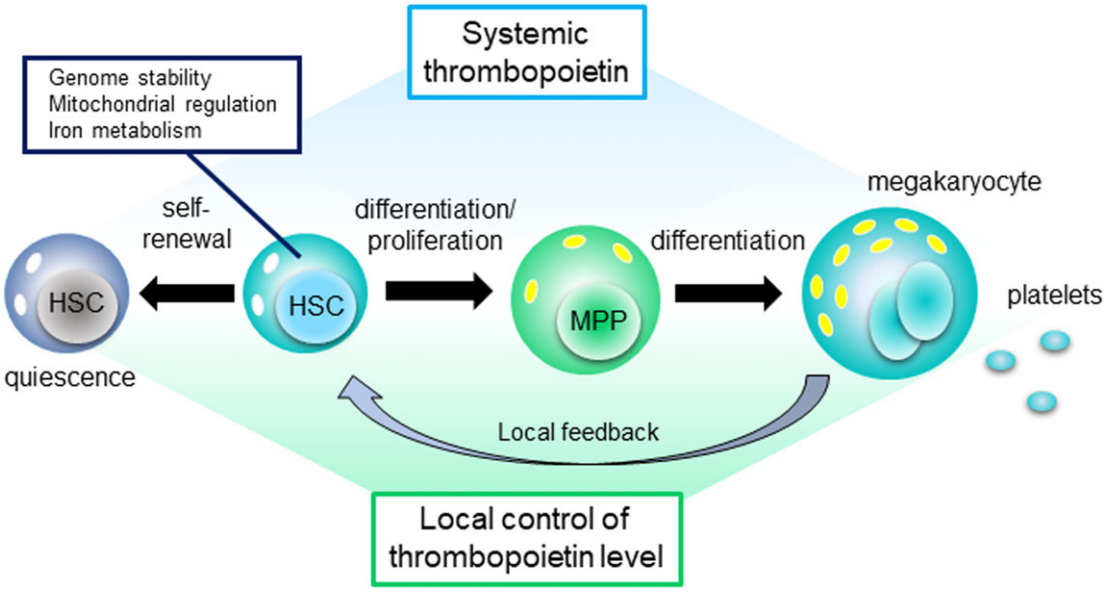

Figure 2. Schematic representation of the functions of Thpo. Thpo influences HSC quiescence, self-renewal, proliferation, and differentiation. Thpo affects genome stability, mitochondrial metabolism, and possibly iron metabolism in HSCs, which may influence the outcome of Thpo–Mpl signaling in HSCs. Both systemic and local Thpo levels regulate BM HSCs.

Regulation of Thpo production and Mpl expression for HSC maintenance

While Thpo is mainly produced by the liver, expression of *Thpo* has been identified in other organs, such as the kidney, and in other cells, such as smooth muscle cells and macrophages.[7,49] Mpl can also be expressed in cells other than hematopoietic cells, such as neurons.[50,51] A large amount of evidence shows that circulating Thpo levels are regulated through the absorbance of Thpo by Mpl-expressing platelets.[52,53] Moreover, during thrombocytopenia, liver and kidney *Thpo* mRNA levels are unaffected.[54] The availability of Thpo for HSC maintenance can also be affected by platelet mass. Mice with mutations in the genes for the transcription factor Myb and coregulator p300 ($Myb^{Plt4/Plt4}$) mice exhibit abnormal thrombocytosis accompanied by a decline in circulating Thpo levels.[55] HSC phenotypes in $Myb^{Plt4/Plt4}$ mice mimic that of Thpo-deficient mice; that is, they enter the cell cycle and are reduced in numbers.[55]

The regulation of HSCs relies on both intrinsic and extrinsic factors, with the BM HSC niche being the main extrinsic element.[56] The HSC niche consists of nonhematopoietic stromal cells and mature hematopoietic cells, which produce various niche factors for the maintenance of HSCs. Past reports have shown that *Thpo* mRNA levels of BM stromal cells increase in response to thrombocytopenia.[54] *Thpo* mRNA expression in BM stromal cells is also induced by platelet-derived growth factor BB and fibroblast growth factor 2 and is suppressed by platelet factor 4, thrombospondin, and transforming growth factor-β.[57] We have also shown that Thpo is produced in the osteoblastic niche and that Mpl^{+} HSCs reside closely to osteoblasts.[31] Mature hematopoietic cells, such as macrophages, have also been reported as a source of Thpo production.[58] Within the BM, we reported that mature megakaryocytes may produce Thpo and function as a niche.[59] Both studies identified Thpo expression in osteoblasts and megakaryocytes using quantitative PCR and antibody detection. However, it was recently shown that HSCs did not rely on local Thpo production by the BM niche.[60] The cell-specific deletion of *Thpo* in megakaryocytes, osteoblasts, and mesenchymal stromal cells did not alter HSC stem cell potential, while *Thpo* depletion in liver cells had similar effects in HSCs as in $Thpo^{-/-}$ mice. Although the data indicate that the main source of Thpo to maintain steady-state hematopoiesis is the liver, changes in Thpo production in the local milieu under various stress conditions have not

been investigated. Also, Mpl has been reported to be expressed on endothelial cells[61] and stromal cells (unpublished data), this expression may interact with the availability of Thpo for HSC maintenance in the BM. Megakaryocytes can be produced *in vitro* from human adipose-derived mesenchymal stromal/stem cells that secrete Thpo in a paracrine manner for trans-differentiation.[62] Moreover, megakaryocytes express Mpl and internalize Thpo-bound Mpl, a process that will influence the availability of Thpo within the niche. Further studies are necessary to elucidate the precise local regulation of Thpo concentrations in the BM niche.

Conclusion

From the discovery of Thpo through its binding to a viral oncoprotein, the study of Thpo–Mpl signaling has uncovered its multifaceted roles in hematopoiesis. The most intriguing aspect of Thpo–Mpl signaling is its contradictory effects on HSCs: from differentiation to self-renewal and from proliferation to quiescence (Fig. 2). As the application of Thpo mimetic drugs expands from the treatment of thrombocytopenia to BM failure and prospectively to hematopoietic malignancies, unraveling the mechanisms and dose-related outcomes of Thpo–Mpl signaling in HSCs is crucial. Furthermore, studies to clarify the regulation of systemic and local production of Thpo during stress hematopoiesis are necessary. In-depth study of Thpo–Mpl signaling in HSCs is promising for the treatment of hematopoietic diseases and life-long retention of healthy BM HSCs.

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Competing interests

The authors declare no competing interests.

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