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The Thrombopoietin/MPL Pathway in Hematopoiesis and Leukemogenesis

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ABSTRACT

Hematopoietic stem cells (HSC) comprise a small percentage of total hematopoietic cells. Their ability to self-renewal is key to the continuous replenishment of the hematopoietic system with newly formed functional blood cell types while maintaining their multipotential capacity. Understanding the extrinsic signals that are essential to HSC maintenance will provide insights into the regulation of hematopoiesis at its most primitive stage, and with the knowledge applied, will potentially lead to improved clinical transplantation outcomes. In this review, we will summarize the current understanding of the role of the thrombopoietin/MPL signaling pathway in HSC maintenance during adult and fetal hematopoiesis. We will also speculate on the downstream key players in the pathway based on published data, and summarize the role of this pathway in leukemia. J. Cell. Biochem. 112: 1491–1498, 2011.

KEY WORDS: THPO; MPL; HSC; HEMATOPOIETIC DEVELOPMENT; LEUKEMIA

The hematopoietic system consists of hematopoietic stem cells (HSC), hematopoietic progenitors, and differentiated cells of multiple lineages that carry out their various functions of supplying oxygen, defending against microorganism insults, and maintaining homeostasis. The late-stage functional cells have a limited lifespan, so the system relies on primitive stem and progenitor cells to continuously proliferate and differentiate in order to replace the lost cells. It is generally accepted that HSC have the ability to self-renew, meaning that upon cell division, at least one daughter cell inherits the characteristics of the mother cell and retains "stemness." Furthermore, HSC are predominantly quiescent and reside in osteoblastic niches near the trabecular bones, protecting them from extrinsic insults. HSC have been further categorized into long- and short-term HSC based on cell surface immunophenotypic markers and functional correlates in serial transplantation assays [Wilson et al., 2009].

The maintenance of the HSC pool is critical to support a healthy organism. Loss of HSC from cell death (e.g., irradiation) or from intrinsic or extrinsic stimuli that disturb their ability to self-renew will cause inevitable bone marrow failure, leading to pancytopenia, and eventual death. Therefore, understanding the signals that are essential for HSC homeostasis is of significant interest to the field of hematology and stem cell biology. In addition to self-renewing proliferation, cellular viability may also be involved in determining the HSC pool size. Circulating cytokine signals as well as paracrine cytokine signals from the bone marrow niche microenvironment, including but not limited to stem cell factor, Flt3 ligand, interleukin-3, and angiopoietin have been found to participate in HSC homeostasis. In this review, we will focus on the effect of the thrombopoietin (THPO)/MPL signaling pathway on HSC biology.

DISCOVERY OF THE THPO/MPL PATHWAY

Two decades ago, *v-mpl* was identified as a viral oncogene responsible for transforming myeloproliferative leukemia virus (MPLV)-infected hematopoietic progenitors [Souyri et al., 1990]. *v-mpl* is an envelope protein that shares striking structural similarities with members of the cytokine receptor superfamily. Two years later, molecular cloning identified the human homolog, *MPL*, as a hematopoietic growth factor receptor [Vigon et al., 1992]. Two isoforms (MPL-P and MPL-K) were identified that differed at their 3′ ends due to alternative splicing, leading to distinct cytoplasmic domains (Fig. 1). Later findings suggested that the two isoforms are co-expressed, with MPL-P being the dominant form [Horikawa et al., 1997]. MPL-P contains the intracellular domain that is highly homologous to *v-mpl* in sequence, suggesting MPL-P is the isoform involved in signal transduction [Vigon et al., 1992]. Similar to other members of the type I cytokine receptor family, MPL contains the characteristic extracellular domain structure that

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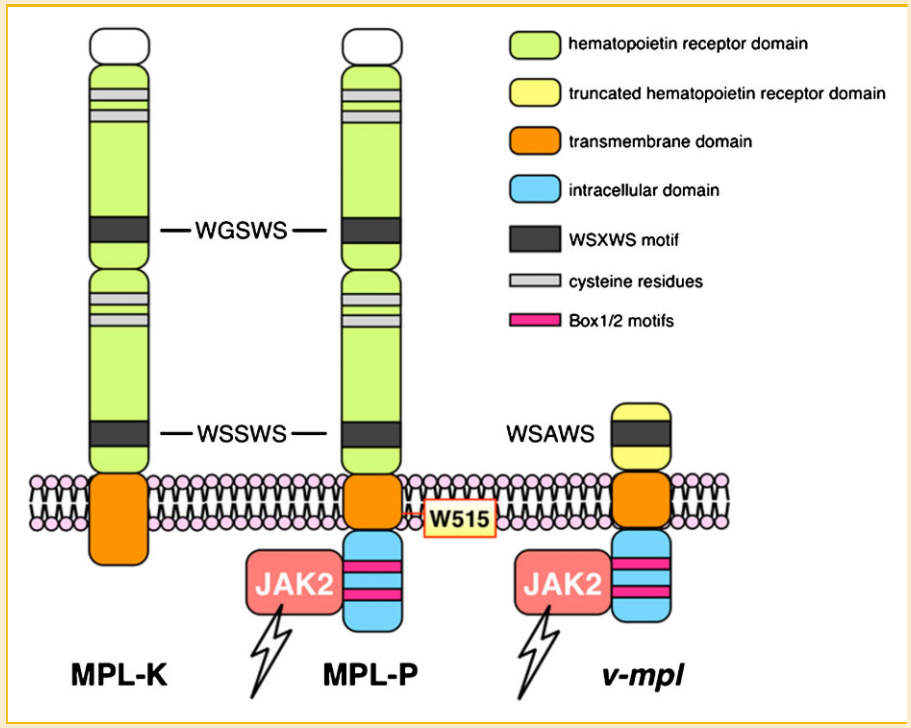

Fig. 1. Schematic representation of human MPL-K, MPL-P, and v-mpl.

includes two pairs of cysteine residues and a WSXWS motif for directing proper folding, with highest homology to interleukin-3 and erythropoietin receptors [Vigon et al., 1992; Alexander and Dunn, 1995]. However, MPL is unique among family members in that it contains two of these motifs [Alexander and Dunn, 1995]. The cytoplasmic domain of MPL contains the Box 1 and Box 2 membrane-proximal motifs that are essential for binding of the Janus kinase 2 (JAK2) protein to initiate signaling (Fig. 1) [Gurney et al., 1995].

Expression of MPL is largely confined to tissues that support hematopoiesis, including bone marrow, spleen, and fetal liver. MPL has been found on immature human $CD34^{+}CD38^{-}$ stem and progenitor cells, megakaryocyte progenitors and platelets, and was recently shown to be expressed primarily in the most primitive $CD34^{+}CD38^{-}CD90^{+}CD45RA^{-}$ cell compartment [Goardon et al., 2011]. Initial characterization of MPL-deficient mice found severe thrombocytopenia but relatively normal levels of other hematological cell types [Gurney et al., 1994]. Subsequently, multiple groups identified THPO as the primary ligand for the MPL receptor [Lok et al., 1994; Wendling et al., 1994]. Upon THPO binding, MPL receptors undergo homodimerization to initiate intracellular signaling, including activation of the JAK2/signal transducers and activators of transcription (STAT) pathway (Fig. 2) [Ezumi et al., 1995]. THPO/MPL signal transduction was shown to play critical roles in thrombopoiesis, from *ex vivo* megakaryocyte progenitor expansion and differentiation to *in vivo* platelet production in BALB/c mice [Kaushansky et al., 1994; Lok et al., 1994].

THE ROLE OF THPO/MPL SIGNALING IN MURINE ADULT HEMATOPOIESIS

In addition to its role in thrombopoiesis, THPO was also found to play a role in expanding erythroid and granulocytic–monocytic progenitors [Kaushansky et al., 1996]. In addition, *ex vivo* hematopoietic progenitor studies of $MPL^{-/-}$ mice revealed a more than 50% decrease in total colony-forming unit cells (CFU-C) as well as significant reductions in myeloid, erythroid, megakaryocytic, and blast CFU-C [Alexander et al., 1996]. Similar findings were observed in cells derived from $THPO^{-/-}$ mice [Carver-Moore et al., 1996]. Surprisingly, no reduction in peripheral blood counts other than platelet number was observed, implying that maturation of progenitors into differentiated and functional blood cells remained largely unaffected [Alexander et al., 1996]. These data indicate a potential defect in the multipotent cell compartment or even in the stem cell compartment due to loss of THPO/MPL signaling. Subsequently, *ex vivo* studies successfully defined the role of THPO in expanding or maintaining the pool of transplantable HSC, further establishing the responsiveness of cells in the primitive hematopoietic compartment to this cytokine signaling pathway [Matsunaga et al., 1998; Yagi et al., 1999].

To further characterize HSC defects in $MPL^{-/-}$ mice, Kimura et al. first performed *in vivo* CFU-spleen (CFU-S) assays by injecting bone marrow cells derived from $MPL^{+/+}$ or $MPL^{-/-}$ (on the 129/Sv background) mice into $MPL^{+/+}$ or $MPL^{-/-}$ recipients, followed by irradiation of the recipient mice at 11Gy [Kimura et al., 1998]. The results showed a significant loss of CFU-S from $MPL^{-/-}$ donor cells.

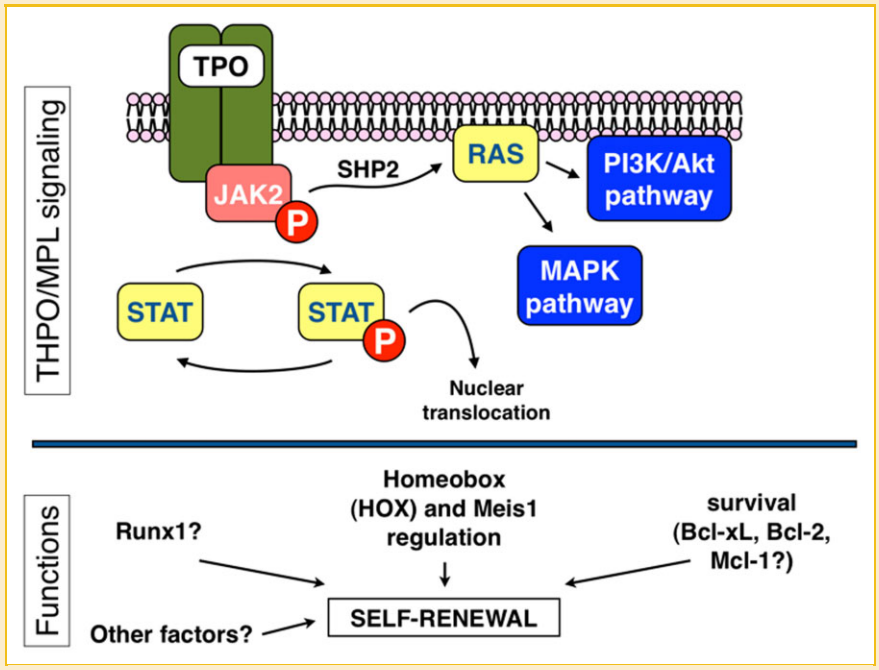

Fig. 2. THPO/MPL downstream signaling pathways and proposed regulatory roles in self-renewal.

Interestingly, $MPL^{+/+}$ donor cells gave rise to equal frequency of CFU-S in both $MPL^{+/+}$ and $MPL^{-/-}$ recipients, suggesting that the CFU-S defect is cell-intrinsic. Subsequently, a long-term repopulating cell assay was performed using increasing numbers of test cells from $MPL^{+/+}$ and $MPL^{-/-}$ mice to compete with a fixed number of $MPL^{+/+}$ competitor cells [Kimura et al., 1998]. Analyses of relative engraftment in the primary transplants at 9–10 months as well as in the secondary and tertiary transplants at 3-month time points all showed $MPL^{-/-}$ test cells significantly lost competitiveness, indicating a repopulating defect of $MPL^{-/-}$ long-term HSC. Alternatively, this pronounced phenotype could also result from the total absence of long-term repopulating cells in the $MPL^{-/-}$ donor mice.

In vivo studies on $THPO^{-/-}$ mice also led to similar conclusions. One elegant experiment demonstrated that transplantation of normal bone marrow cells into lethally irradiated $THPO^{-/-}$ recipient mice resulted in a 10- to 20-fold reduction in the expansion of transplantable HSC when compared to the $THPO^{+/+}$ recipient group, as 7.5×10^6 donor cells from the $THPO^{-/-}$ primary transplant is required to reconstitute and rescue 80% of lethally irradiated recipient mice in the secondary transplantation while 3×10^5 cells from the $THPO^{+/+}$ primary recipient mice was sufficient to rescue all irradiated secondary recipient mice [Fox et al., 2002]. Exogenous administration of a physiological dose of THPO to the $THPO^{-/-}$ primary recipient mice completely rescued the phenotype. One puzzling finding from this same report was that short-term repopulating ability (5 weeks post-transplantation) of normal bone marrow cells was also compromised in $THPO^{-/-}$ recipient mice, leading to significantly reduced rescue of the lethally irradiated recipient mice. Intriguingly, exogenous THPO administration did not reverse this phenotype. Although a detailed progenitor analysis was not done, this phenotype may be due to ineffective production of sufficient numbers of megakaryocytic progenitors from the transplanted cells, even with THPO rescue. Further examination of why $THPO^{-/-}$ recipient mice failed to support hematopoietic reconstitution by wild-type $Lin^-Sca\text{-}1^+Kit^+$ cells revealed that there was more than a 10-fold decrease in $Lin^-Sca\text{-}1^+Kit^+$ and $Lin^-Sca\text{-}1^+Kit^+Flt3^-$ cells in the $THPO^{-/-}$ recipient mice 3 weeks after transplantation when compared to their wild-type counterpart, suggesting that the THPO/MPL pathway is required to maintain this compartment in the engrafted marrow [Qian et al., 2007]. It is also possibly due to loss of more primitive hematopoietic cells that would have given rise to these cells in the absence of THPO immediately after transplantation.

THE THPO/MPL PATHWAY IN PRIMATE AND HUMAN

The study of the influence of the THPO/MPL pathway on the maintenance of HSC has been extended to non-human primates and humans. THPO injection rescued irradiation-induced loss of multilineage hematopoietic progenitors in primates, including GEMM-CFU, GM-CFU, BFU-E, and MEG-CFU [Farese et al., 1996]. This was associated with faster recovery of peripheral blood counts following irradiation. Human $CD34^+CD38^-$ hematopoietic cells derived from umbilical cord blood can be further separated into MPL^+ and MPL^- cells using an anti-MPL antibody against the surface receptors. $CD34^+CD38^-MPL^+$ cells, but not $CD34^+CD38^-MPL^-$ cells, were able to efficiently engraft transplanted human fetal bone marrow fragments in immunodeficient mice [Solar et al., 1998].

Interestingly, a MPL point mutation in the extracellular ligand-binding domain was identified in a rare human disease called congenital amegakaryocytic thrombocytopenia (CAMT) [Ballmaier et al., 2001; Steinberg et al., 2007]. Patients with CAMT present with isolated thrombocytopenia in infancy, and develop pancytopenia later in their childhood. Progenitor assays using bone marrow mononuclear cells showed a significant reduction in clonogenic cells not only in the megakaryocytic lineage but also in myeloid and erythroid lineages when compared to healthy donors. Due to its phenotypic similarities with the findings of thrombocytopenia and the stem cell defect in $MPL^{-/-}$ mice, the authors proposed that both thrombocytopenia and pancytopenia observed in CAMT patients result from MPL loss-of-function mutations.

Moreover, a retrospective clinical study on patients undergoing bone marrow transplantation found two independent factors that predict poor transplantation prognosis—one is post-transplantation platelet engraftment of $<150{,}000/\mu l$; the other is development of idiopathic secondary transplant thrombocytopenia (ISPT) [Ninan et al., 2007]. Interestingly, the former is associated with significantly lower dose of $CD34^+$ cells/kg upon transplant, and the latter with a low dose of $CD34^+CD38^-$ cells/kg, suggesting defects in short- and long-term engraftment, respectively. The utilization of thrombopoiesis to predict transplantation outcome implies that the THPO/MPL signaling pathway has a significant influence on HSC homeostasis.

THE ROLE OF THPO/MPL SIGNALING DURING HEMATOPOIETIC DEVELOPMENT

Due to multi-lineage progenitor defects observed in $MPL^{-/-}$ and $THPO^{-/-}$ mice, it is reasonable to hypothesize that the defects may already be evident during embryonic development. *In situ* hybridization revealed MPL mRNA expression in Aorta-Gonad-Mesonephros (AGM), fetal liver, and yolk sac as early as E10.5. In the AGM, MPL is exclusively expressed in the hematopoietic clusters [Petit-Cocault et al., 2007]. It is possible that the earliest hematopoietic cells that emerge from the aortic endothelium are responsive to THPO signaling. A transplantation assay showed defects in long-term repopulation and engraftment in secondary recipients repopulated with $MPL^{-/-}$ E11.5 AGM cells, suggesting loss of MPL-mediated signaling has a negative impact on the quality or quantity of the earliest transplantable hematopoietic cells. A colony formation assay also showed reduced CFC, mostly due to a reduction in BFU-E and CFU-Mk, in E11.5 $MPL^{-/-}$ AGM. A closer look at the absolute number of $CD45^+Kit^+$ and $CD34^+Kit^+$ cells in the E10.5 AGM showed a slight increase in $MPL^{-/-}$ AGM, but no difference between E11.5 $MPL^{+/+}$ and $MPL^{-/-}$ AGM cells, suggesting a quantitative defect transiting from E10.5 to E11.5 in the $MPL^{-/-}$ AGM [Fleury et al., 2010]. Interestingly, quantitative PCR showed a slight, yet significant, reduction in the expression levels of anti-apoptotic Bcl-2 and Bcl-xL [Fleury et al., 2010]. It is possible that the reduced expression of survival factors is responsible for the observed defect of $MPL^{-/-}$ AGM cells in the transplantation assay. Whether it is responsible for the observed defects in AGM remain to be further investigated. Moreover, reductions in the mRNA expression of Meis1 and HoxB4, homeobox proteins that are involved in leukemia formation, were detected in $MPL^{-/-}$ cells [Fleury et al., 2010]. Future studies on the identification of the key downstream factors should take advantage of the inducible murine genetic models to best examine the effects at a specific developmental stage.

An early study separated fetal liver $AA4^+Sca1^+kit^+$ cells into MPL^+ and MPL^- populations, and found that $AA4^+Sca1^+kit^+MPL^+$ cells were capable of engrafting lethally irradiated mice but $AA4^+Sca1^+kit^+MPL^-$ cells were not in a competitive repopulation assay [Solar et al., 1998]. Surprisingly, initial analysis of E12.5 fetal liver hematopoietic progenitor cells revealed no differences in the frequency of progenitors at various developmental stages between $MPL^{+/+}$ and $MPL^{-/-}$ embryos [Alexander et al., 1996]. However, a recent time course study was performed on fetal liver $lin^-AA4^+Sca1^+$ (highly enriched in HSC), $lin^-CD34^+Kit^+$ [enriched in long-term hematopoietic reconstituting cells, (LTR)], and hematopoietic progenitors, where the respective functional readouts for each cell type were secondary transplantation (primary transplantation $\geq$20 weeks, secondary transplantation of 7 weeks), primary transplantation at week 20, and colony formation [Petit-Cocault et al., 2007]. Fetal liver cellularity was also examined. Results showed that total cellularity was low in E10.5 $MPL^{-/-}$ fetal liver when compared to the $MPL^{+/+}$ counterpart, yet no difference in cellularity was observed at E12.5 and E14.5, suggesting a delayed migration of hematopoietic cells from the AGM to the fetal liver. The difference in cellularity between E10.5 and E12.5 may account for reduced CFC in $MPL^{-/-}$ at E10.5 but the lack of differences between the two groups at E12.5. Surprisingly, CFC frequencies of all types were reduced again in E14.5 $MPL^{-/-}$ fetal liver, possibly indicating the existence of defects in more primitive hematopoietic cells at earlier time points that led to reduced progenitor output. Indeed, higher numbers of E14.5 fetal liver cells, but not E12.5, were required to establish engraftment in recipient mice at week 20, suggesting decreased frequency of LTR in $MPL^{-/-}$ fetal liver. Moreover, significantly more donor cells were required from $MPL^{-/-}$ fetal liver to establish engraftment in the secondary recipients, further indicating reduced HSC frequency. Altogether these data suggest that loss of THPO/MPL signaling negatively impacts on the pool size of early hematopoietic cells, predisposing to a defect in sustaining hematopoiesis later in adult life.

Notably, another study comparing E14.5 wild-type and $THPO^{-/-}$ fetal liver cells did not find any repopulating defects [Qian et al., 2007]. In this study, 2×10^4 $Lin^-Sca^+Kit^+CD34^-Flt3^-$ (defined as LT-HSC) cells were injected into wild-type recipient mice. This lack of effect could possibly be due to the significantly higher number of cells used in the study. Alternatively, because cells from $THPO^{-/-}$ mice express MPL, it is also possible that the cells regain their repopulating ability when injected into wild-type mice. Moreover, the different combinations of immunophenotypic markers used for defining the HSC also make the comparison and interpretation extremely difficult.

SPECULATION ON THE ROLE OF THE THPO/MPL PATHWAY IN PROMOTING HSC SURVIVAL

It has been proposed that apoptosis plays a role in the regulation of the size of the HSC compartment. Overexpression of Bcl-2 in the

hematopoietic system using the H-2K^{b} promoter led to increased numbers of Lin^{-}Kit^{high}Sca-1^{high}Thy1.1^{low} LT-HSC, with more cells in the G_0 phase. This is associated with increased repopulating potential in a competitive transplantation assay [Domen et al., 2000]. Moreover, Bcl-2 overexpression under the control of Sca-1 transcriptional regulatory elements also led to increased numbers of Sca-1^{+} cells in the AGM, leading to enhanced long-term (4 months) engraftment in a competitive transplantation assay when limiting cell numbers (1×10^4 cells) were injected into the recipient mice [Orelio et al., 2004]. Interestingly, in a serial transplantation assay using E12 AGM cells and E12 fetal liver cells, Bcl-2 overexpressing cells engrafted 100% and 50% of the secondary recipients, respectively (the primary recipient mice with 75–100% engraftment of E12 cells were used as donors for secondary transplantation). In contrast, none of the secondary recipients injected with wild-type cells had any detectable engraftment. These data indicate that apoptosis/cell survival plays a significant role in regulating the quantity or quality of HSC both during development and in adult life.

Although THPO by itself does not support *ex vivo* growth of hematopoietic cells, it is capable of maintaining the viability of these cells [Sitnicka et al., 1996]. Furthermore, one experiment showed that Bcl-2 overexpression (under the control of H-2K promoter) in MPL$^{-/-}$ mice led to partial rescue of Lin^{-}Sca^{+}Kit^{+}CD34^{-}Flt3^{-} cells, suggesting that the THPO/MPL pathway may have a survival-promoting downstream component for maintaining the HSC pool size [Qian et al., 2007]. It is therefore of interest to determine which anti-apoptotic molecules and pathways are responsible for the regulation of HSC homeostasis and whether they are regulated by the THPO/MPL-mediated signaling cascades.

In the study of thrombopoiesis, Bcl-xL is a critical downstream survival protein of the THPO/MPL pathway to sustain the viability of developing megakaryocytes [Kozuma et al., 2007; Sanz et al., 2001]. As Bcl-xL knock-out leads to embryonic lethality at E13, and since Bcl-xL, but not Bcl-2, is expressed in human CD34^{+}CD38^{-} cells, it is possible that the THPO/MPL/Bcl-xL pathway may be functionally important in these more primitive cells [Motoyama et al., 1995; Park et al., 1995]. It is worth noting, however, that data from both mouse and human studies indicate that Mcl-1 functionally plays a critical role in promoting survival of the early compartment (Lin^{-}Sca-1^{+}Kit^{+} in mice and CD34^{+}CD38^{-} in human cells) [Opferman et al., 2005; Campbell et al., 2010]. Whether the THPO/MPL pathway regulates Mcl-1 protein levels in cells included in these populations, and whether Bcl-xL and Mcl-1 play non-redundant survival roles in primitive HSC, remain to be investigated.

THE THPO/MPL PATHWAY SUPPORTS HSC IN THEIR QUIESCENT STATE AND INTERACTION WITH THE OSTEOBLASTIC NICHE

In addition to survival, THPO/MPL signaling has also been shown to regulate the quiescent state of Lin^{-}Sca^{+}Kit^{+}CD34^{-}Flt3^{-} cells in the THPO$^{-/-}$ genetic mouse model [Qian et al., 2007]. This is associated with reduced expression of p57KIP2 in Lin^{-}Sca^{+}Kit^{+}CD34^{-}Flt3^{-} and Lin^{-}Sca^{+}Kit^{+}CD34^{+}Flt3^{-} cells as well as decreased expression of p19INK4D and p21CIP1 in the Lin^{-}Sca^{+}Kit^{+}Flt3^{-} population in THPO$^{-/-}$ mice, suggesting that THPO/MPL signaling may be involved in the regulation of cell cycle progression. Another study showed that MPL-expressing hematopoietic cells in the bone marrow are in close proximity to THPO-producing osteoblastic niche cells, suggesting that the THPO/MPL signal is highly enriched in that microenvironment to maintain HSC in their quiescent state [Yoshihara et al., 2007]. Interestingly, as mentioned above, Bcl-2 ectopic expression in the HSC compartment also led to an increased percentage of cells in the G_0 phase, raising the question as to whether THPO-mediated quiescence is a consequence of improved survival.

HOMEOBOX GENES AS DOWNSTREAM EFFECTORS OF THE THPO/MPL PATHWAY IN HSC MAINTENANCE

Homeobox (Hox) transcription factors have been implicated in HSC self-renewal. In addition to downregulation of HoxB4 in MPL$^{-/-}$ E10.5 AGM cells, as mentioned above, HoxB4, HoxA5, HoxA9, and HoxA10, which were highly expressed in Lin^{-}Sca^{+}Kit^{+}CD34^{-}Flt3^{-} long-term repopulating cells in wild-type mice, were detected at very low levels in the equivalent population in THPO$^{-/-}$ mice [Qian et al., 2007]. An *in vitro* assay using the UT-7/THPO human leukemic cell line found that THPO signaling stimulates expression of HoxB4 via the p38 mitogen-activated protein kinase (MAPK) pathway [Kirito et al., 2003]. Moreover, THPO stimulation also promotes Meis1 mRNA expression as well as nuclear translocation of HoxA9 without affecting its total protein levels in UT-7/THPO and Sca-1^{+}Kit^{+}Gr-1^{-} cells [Kirito et al., 2004]. Perhaps the next important question is whether any (or all) of the Hox proteins play a key role in determining the cell fate in response to THPO stimulation. It would also be critical to directly examine whether THPO/MPL signaling plays a role in determining symmetric versus asymmetric division as well as polarity formation during development and in adult bone marrow.

CROSSTALK BETWEEN THE THPO/MPL PATHWAY AND RUNX1

Runx1, a master transcription factor in hematopoiesis, was also found to be significantly downregulated in MPL$^{-/-}$ AGM when compared to wild-type AGM cells [Petit-Cocault et al., 2007]. It is not known whether THPO/MPL signaling directly regulates Runx1 or whether these results may be due to comparison of different cell population as a consequence of loss of THPO/MPL signaling. Runx1 is critical for the emergence of hematopoietic clusters from the aortic endothelium during embryonic development [Chen et al., 2009]. Intriguingly, no obvious defect in HSC emergence was described in MPL$^{-/-}$ embryos, and the AGM cellularity (CD45^{+}c-Kit^{high}) at E10.5 was paradoxically higher than that in WT embryos [Petit-Cocault et al., 2007; Fleury et al., 2010]. Moreover, Runx1 is viewed as a differentiation promoter that guides HSC emergence from hemogenic endothelium, yet THPO/MPL signaling seems to be responsible for the maintenance of HSC (and long-term repopulating

hematopoietic cells) by promoting cell survival and/or maintaining quiescence. In addition, Runx1 inducible knock-out in the adult hematopoietic system showed no effect on HSC or increased HSC activity, while THPO or MPL knock-out is associated with continuous loss of HSC phenotypes [Ichikawa et al., 2004]. Notably, the technical aspects of the studies may need to taken into consideration, as Cre recombinase inducible knock-out was used in the Runx1 study but germline knock-out was used in the THPO/MPL studies. Nonetheless, similar to MPL knock-out, Runx1 knock-out in adult also leads to insufficient platelet production [Growney et al., 2005]. On the other hand, expression of a Runx1 dominant-negative mutant in $Lin^{-}Sca\text{-}1^{+}Kit^{+}$ cells, or in embryonic stem cells induced for hematopoietic cell development, led to increased expression of MPL, suggesting that endogenous Runx1 plays a role in the repression of the MPL gene in these early hematopoietic cells [Satoh et al., 2008]. The authors further proposed that the repressive effect results from recruitment of mSin3A, a co-repressor, by Runx1 to the promoter region of the MPL gene, although the chromatin immunoprecipitation assay was performed on $Lin^{-}Sca\text{-}1^{+}$ cells, a different population than the cells in which the phenotypes were shown. Overall, the significance of Runx1 downregulation in $MPL^{-/-}$ mice and the reciprocal regulation between MPL and Runx1 remain to be further investigated.

THE ROLE OF THPO/MPL IN LEUKEMOGENESIS

When human MPL was cloned, it was known that *v-mpl* was the truncated form of the cytokine receptor lacking the extracellular ligand-binding domains. As *v-mpl* is by itself sufficient to promote transformation of murine bone marrow cells, and given that the THPO/MPL pathway is active in the HSC compartment, it is reasonable to postulate that activation of the THPO/MPL pathway may play a role in leukemogenesis. An early study found that MPL mRNA expression is detectable by Northern blotting in 26 out of 50 cases of acute myeloid leukemia (AML). Subsequent *in vitro* assay showed that blast cells from 22 out of the 26 MPL+ cases responded to exogenous THPO for proliferation [Matsumura et al., 1995]. Except for French-American-British M7 (megakaryoblastic) AML that all express MPL, MPL expression does not correlate with FAB classification (M0–M6). Another study showed 47% (53 out of 114) of AML and 14% (2 out of 14) of acute lymphoblastic leukemia (ALL) expressed surface MPL (detected by biotinylated THPO). Interestingly, both ALL cases showed *in vitro* proliferative response to THPO [Takeshita et al., 1998]. Yet another study showed that, although 37 out 60 ALL cases expressed surface MPL, none of them responded to THPO stimulation [Corazza et al., 2006]. On the other hand, six out of 11 AML samples stimulated with THPO responded with either improved survival or proliferation. Serum THPO levels were significantly lower in patients with MPL-expressing AML when compared to AML without MPL expression or ALL, implicating uptake of THPO by the blast cells in the former group [Corazza et al., 2006].

MPL mutation was detected in 5–9% of $JAK2^{V617F}$-negative myelofibrosis with myeloid metaplasia (MMM) cases and in 1% of essential thrombocythemia (ET), two classes of myeloproliferative disorders [Pikman et al., 2006]. Although patients with MMM or ET may evolve into myelodysplastic syndrome or AML, MPL mutations were not found in the latter two secondary diseases [Pardanani et al., 2006]. The most common mutation was W515L or W515K, which resides in the transmembrane domain (Fig. 1). Expression of MPLW515L in 32D, UT7, or Ba/F3 cells led to cytokine-independent growth. This was associated with constitutive phosphorylation of JAK2, STAT3, STAT5, AKT, and ERK, suggesting constitutive activation of the mutant receptors. Transplantation of MPL^{W515L}-overexpressing bone marrow cells, but not $MPL^{wildtype}$-overexpressing cells, into lethally irradiated recipient mice leads to development of myeloproliferative disorders with full penetrance, further verifying the disease-causing role of the mutation [Pikman et al., 2006]. Indeed, MPLW515L mutation was found in three out of 12 acute megakaryoblastic leukemia that are either associated with trisomy 21 or t(9;22), further implying a role of such mutation in pathogenesis [Hussein et al., 2009]. On the other hand, while other mutations of MPL in the transmembrane domain have also been identified, *in vitro* and *in vivo* assays failed to show their growth-promoting role [Chaligne et al., 2008].

Given the role of the THPO/MPL pathway in maintaining the repopulating ability of normal HSC, it would be interesting to investigate whether the THPO/MPL pathway plays a role in the regulation of leukemia initiating cells and whether blocking the pathway would aid in combating disease relapse. Delivery of a MPL inhibitor would be relatively straightforward because of surface expression of the receptors. However, potential adverse effects such as thrombocytopenia or premature bone marrow failure may hinder its clinical use. Recently, one intriguing report showed that eltrombopag, a non-peptide MPL agonist licensed for use in the treatment of chronic idiopathic thrombocytopenic purpura, paradoxically decreased *ex vivo* growth of primary AML blasts while recombinant THPO did not [Kalota and Gewirtz, 2010]. This could be due to overwhelming stress induction following receptor activation by eltrombopag in blast cells, or due to aberrant structure or function of the MPL protein in malignancy, leading to a cytotoxic effect upon eltrombopag binding. In any case, this report demonstrates the potential use of targeting this pathway as a novel therapeutic strategy in leukemia treatment, and merits further studies.

CONCLUDING REMARKS

Since its discovery 20 years ago, researchers have been rigorously investigating the role of the THPO/MPL pathway in hematopoiesis. It is now clear that this pathway is not only critical for thrombopoiesis but also for the regulation of HSC. However, how the THPO/MPL pathway affects HSC homeostasis and at what stage of differentiation the pathway plays a critical role is still not fully understood. It is also an open question as to how to validate the germline THPO and MPL knock-out models in the study of adult HSC without conditionally targeted alleles. A tissue-specific inducible mouse genetic model system may solve this issue. With the advancement of scientific and imaging tools and the expansion of our knowledge regarding HSC biology in general, future studies should focus on a

closer look at the effect of the pathway on self-renewing cell divisions at a single-cell level, as well as its downstream effector molecules that direct polarity establishment and niche interaction. Moreover, as there is clearly a survival-promoting component of the THPO/MPL pathway in the long-term repopulating HSC, a closer look at how the pro-apoptotic and anti-apoptotic signals achieve their balance and what molecules are involved will allow a more thorough understanding of how the HSC pool size is maintained by the THPO/MPL pathway. Equally important is how we translate our understanding of the pathway in normal HSC into leukemia stem cells. So far, most of the clinical studies are merely correlative, and the sample sizes are too small. Although some *ex vivo* functional experiments were performed to demonstrate the responsiveness of blast cells to THPO stimulation, a more relevant biological system is needed to facilitate our understanding of the influence of this pathway on disease pathogenesis. The current biggest obstacle is the lack of an inducible knock-out mouse model to study the effect of the THPO/MPL pathway in leukemia formation and progression. Moreover, the development of xenotransplantation models in immunodeficient mice will allow us to study the molecular effect of the THPO/MPL pathway on patient-derived blast cells and leukemia repopulating cells, and to test investigational inhibitors in a pre-clinical setting.

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