Thrombopoietin is required for full phenotype expression in a JAK2V617F transgenic mouse model of polycythemia vera

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Abstract

The myeloproliferative neoplasms, polycythemia vera, essential thrombocythosis and primary myelofibrosis are hematopoietic stem cell disorders and share driver mutations that either directly activate the thrombopoietin receptor, MPL, or activate it indirectly through gain-of-function mutations in the gene for JAK2, its cognate tyrosine kinase. Paradoxically, MPL surface expression in hematopoietic stem cells is also reduced in the myeloproliferative neoplasms due to abnormal post-translational glycosylation and premature destruction of JAK2, suggesting that the myeloproliferative neoplasms are disorders of MPL processing since MPL is the only hematopoietic growth factor receptor in hematopoietic stem cells. To examine this possibility, we genetically manipulated MPL expression and maturation in a JAK2V617F transgenic mouse model of polycythemia vera. Elimination of MPL expression completely abrogated the polycythemia vera phenotype in this JAK2V617F transgenic mouse model, which could only be partially restored by expression of one MPL allele. Most importantly, elimination of thrombopoietin gene expression abrogated the polycythemia vera phenotype in this JAK2V617F transgenic mouse model, which could be completely restored by expression of a single thrombopoietin allele. These data indicate that polycythemia vera is in part a thrombopoietin-dependent disorder and that targeting the MPL-thrombopoietin axis could be an effective, nonmyelotoxic therapeutic strategy in this disorder.

Introduction

The myeloproliferative neoplasms (MPN), polycythemia vera (PV), essential thrombocythosis (ET) and primary myelofibrosis (PMF) are clonal hematopoietic stem cell (HSC) disorders...
that share gain of function mutations which directly or indirectly constitutively activate JAK2 [1–4], the cognate tyrosine kinase of the erythropoietin (EPO) and thrombopoietin (THPO) receptors [5], and also utilized by the granulocyte colony-stimulating factor receptor [6]. Constitutive JAK2 activation accounts for increased blood cell production in the MPN because JAK2 is responsible for the proliferation and survival of committed hematopoietic progenitor cells (HPC) [7,8]. Ruxolitinib, a JAK1/2 inhibitor, is effective in controlling unregulated MPN HPC proliferation [9]. The MPN, however, are HSC disorders and JAK2\( ^{V617F} \) did not alter MPN HSC pool size nor did JAK2 inhibition significantly reduce the MPN HSC burden in animal [10] or human studies [11], suggesting mechanisms other than JAK2 activation are also involved in MPN pathophysiology.

HSC express only one hematopoietic growth factor receptor, the THPO receptor, MPL. JAK2 is the obligatory chaperone for MPL cell-surface expression and stability [12]. THPO promotes HSC survival [13] and megakaryocytic progenitor cell proliferation [14] but is not required for megakaryocyte maturation or platelet production [15,16]. Its major role in adult hematopoiesis is maintenance of HSC quiescence within the bone marrow osteoblastic niche [17,18]. Adult mice lacking the MPL or THPO gene, appear normal except for thrombocytopenia but have a marked increase in plasma THPO and a decrease in marrow HSC [19]. In humans with congenital amegakaryocytic thrombocytopenia (CAMT), MPL loss of function mutations, usually in the MPL distal extracellular cytokine receptor homology domain (CRHD) [20], cause thrombocytopenia, an elevated plasma THPO level, and progressive marrow aplasia [21].

With respect to the MPN, MPL is a proto-oncogene since the retrovirus MPLV, which encodes an MPL gene truncated in its extracellular domain, caused an acute, fatal PV-like syndrome in mice [22], and in vitro, immortalized murine HPC [23]. Ectopic THPO-producing murine bone marrow cells caused a fatal transplantable myeloproliferative disorder with splenomegaly, osteomyelofibrosis, pancytopenia and leukemic transformation [24,25]. In contrast, ectopic EPO expression in murine marrow cells [26] or erythroid progenitor cell-specific expression of JAK2\(^{V617F} \) caused erythrocytosis without significant extramedullary hematopoiesis (EMH) and failed to propagate the erythrocytosis phenotype in secondary recipients [10], emphasizing the primary role of HSC, MPL and THPO in MPN pathophysiology.

In humans, hereditary or acquired MPL mutations involving the transmembrane domain or distal CRHD are associated with an ET or PMF phenotype [2,27,28]. Furthermore, mutated CALR binds and activates MPL causing an ET or PMF phenotype [29–31]. Importantly, germline single nucleotide polymorphisms (SNP) involving the MPL distal CRHD were associated with a variably penetrant, benign thrombocytosis phenotype with an elevated plasma THPO level, were ethnic group-specific [32,33] and could be modeled in the mouse [34,35]. Hereditary THPO mutations permitting unregulated THPO production caused thrombocytosis alone [36], but in one family were associated with leukemic transformation or myelofibrosis [37].

In contrast to MPL mutations, JAK2\(^{V617F} \) causes PV, ET and PMF. However, like hereditary or acquired MPL [2,38] and CALR mutations [29], impaired MPL cell-surface expression is a feature of JAK2\(^{V617F} \), positive PV, ET and PMF [34–42] and presumably responsible for increased plasma THPO in these disorders [34,43,44]. But how impaired expression of the hematopoietic growth factor receptor responsible for HSC maintenance, expansion and thrombopoiesis could cause myeloproliferation has been a conundrum. To examine this issue, we genetically manipulated MPL and THPO expression in a JAK2\(^{V617F} \) transgenic mouse model of PV [45]. Our results indicate that despite expression of constitutively-active JAK2\(^{V617F} \), the PV phenotype in this mouse model still required THPO signaling and suggest that interfering with the MPL-THPO interaction could have therapeutic value treating the MPN.
Materials and methods

Generation of murine models

This research project involved the use of mice and was approved by the Johns Hopkins University School of Medicine IACUC under protocol number M013M467. Isofluorane was used for anesthesia and CO2 narcosis and cervical dislocation was used for euthanasia.

Generation of transgenic mice expressing 13 copies of the entire coding region of human JAK2 V617F plus the 3’ noncoding region cloned into the HS321/45-vav vector and crossed into a C57Bl/6 background has previously been described [45]. Wild-type C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). MPL knockout mice [46] and THPO knockout mice [15] created by homologous recombination using a targeting vector containing a neomycin-resistance (neo') cassette in C57Bl/6 mice were obtained from Genentech. Experiments were performed using mice 6–9 weeks of age or older. Mice were raised in approved housing and all experimental protocols were approved by our institutional ACUC (Protocol #M013M467).

Genotyping

Mice were genotyped using tail snips obtained at 3–5 weeks of age. The genotyping primers are shown in the S1 Table in the Supplemental information.

Hematological analysis

Mice were anesthetized with isofluorane (Baxter NDC 10019-360-60) and tail vein blood (100 μL) was collected in K-EDTA. Complete blood and differential counts were performed with a Hemavet 950FS (Drew Scientific) using the manufacturer’s mouse program and controls (Mouse Multi-trol 600065).

Histopathology

Mice were sacrificed and the spleen and femurs were removed and placed in formalin, paraffin-embedded and stained with hematoxylin and eosin for morphology, and silver-stained for analysis of reticulin formation.

Thrombopoietin assay

The plasma thrombopoietin concentration was measured using an ELISA assay (Quantikine ELISA; catalogue # MTP00, R&D Systems, Minneapolis, MN) according to the manufacturer’s specifications.

Hematopoietic progenitor cell (HPC) colony-forming analysis

CFU-GEMM, CFU-GM and BFU-E colony formation was assessed in vitro using washed suspensions of 5 X 10^4 marrow cells or 3 X 10^5 spleen cells suspended in IMDM and plated in 1% methylcellulose with 30% FBS (Methocult medium, catalogue #3534, STEMCELL Technologies, Vancouver, BC Canada) containing 10 ng/μL of mIL3 (catalogue #02733, STEMCELL), 10 ng/μL of rhIL6 (#206-IL-010, R&D SYSTEMS, Minneapolis, MNUSA), 50ng/μL of THPO (#288-TP-005, R&D) and 3 U/μL of rhEPO (Johnson and Johnson, New Brunswick, NJ USA). All colony-forming assays were performed between weeks 15–18. Colony-forming assays were performed in triplicate with at least 3 replicate experiments and colony formation was assessed at 7 days. CFU-Mk colony formation was assessed using 5 X 10^4 washed marrow cells or 3 X 10^5 spleen cells suspended in IMDM and plated in methylcellulose (Megacult-C medium,
04850, STEMCELL) containing 10 ng/μL of mIL3, 10 ng/μL of rhIL6 and 50ng/μL of THPO, R&D). Colony number was assessed at 7 days after staining with acetylcholinesterase activity as described in the Megacult-C protocol for murine cells.

**Bone marrow flow cytometry**

Bone marrow from three to four mice per experimental group was flushed from the femurs and tibias with staining medium (RPMI with 2% FBS), filtered and suspended at a concentration of 10^8 cells/mL in staining medium. The antibodies used for staining were: CD34 (Clone: RAM34)-allophycocyanin (APC) or FITC; FcRγ (Clone: 93)-PE or Flt3 (clone: A2F10)-PE; c-Kit (Clone: 2B8)-APC-Alexa Fluor 750; Sca1 (Clone: D7)-PE-cyanin 7; CD150 (Clone: TC15-12F12.2)-APC (BioLegend, San Diego, CA USA); CD48 (Clone: HM48.1)-PE (BD Biosciences, San Jose, CA USA) and biotin-streptavidin-peridinin -chlorophyll-protein complex-cyanin 5.5 (PerCP-Cy5.5) or eFluor450-labeled lineage cocktail (CD3e, Gr1, B220 and Ter119) (all from Thermo Fisher Scientific, Waltham, MA USA). Apoptotic cells were identified with the FITC Annexin V Apoptosis Kit (BD Biosciences). Labeled cells were analyzed on a 9 laser LSRII (BD Biosciences) [47].

**Statistical analysis**

Pairwise individual significance was determined using either Student’s T-test or, if the normality or equal variance test failed, the Mann-Whitney Rank Sum test. In addition, an all pairwise multiple comparison procedure (Dunn’s Method) was performed on each group of age-matched hemoglobin, neutrophil and platelet levels from the four genotypes resulting from each cross (e.g. wild-type, JAK2^{V617F}, MPL^{del/del}, MPL^{del/+}, JAK2^{V617F}/MPL^{del/del}, JAK2^{V617F}/MPL^{del/+}). Calculations were performed using Sigma Plot (Systat Software, San Jose, CA).

**Results**

**Breeding strategy**

To study the role of MPL in the PV phenotype, we used a JAK2^{V617F} transgenic mouse, which develops erythrocytosis, leukocytosis and thrombocytosis with an eventual decline in erythropoiesis associated with EMH and osteomyelofibrosis over a time course of 6–46 weeks without leukemic transformation [45]. To manipulate MPL expression, we bred JAK2^{V617F} transgenic mice with MPL^{del/del} mice to obtain JAK2^{V617F} transgenic mice either null or heterozygous for the MPL gene. The breeding strategy (S1 Fig) yielded the expected Mendelian allelic ratios with the exception of slightly fewer than expected JAK2^{V617F}/MPL^{del/del} mice. However, all mice were robust, had weights compatible with their sex and there was no excess mortality or sex-related differences with respect to blood counts in any genotype (S2 Table).

**Effect of the JAK2^{V617F} transgene on the blood counts, marrow and spleen HPC colony formation, marrow and spleen histology, spleen weights and the plasma THPO level**

There were slight but consistent differences in the hemoglobin level and neutrophil counts in MPL^{del/del} mice as well as the expected marked difference in their platelet counts. In JAK2^{V617F} transgenic mice, the hemoglobin level was initially higher than in the wild-type mice, rose further by 14–16 weeks and fell to the level of wild-type mice by 33 weeks (Fig 1A). The neutrophil count was initially normal, a feature seen in many PV patients, but increased thereafter, while the platelet count was elevated at 6–9 weeks and progressively increased. HPC colony-forming assays at 16 weeks revealed that JAK2^{V617F} enhanced the number of marrow (Fig 2A–2D) and
Fig 1. 

**MPL**del and **THPO**del genotypes mitigate increased hemoglobin, neutrophil and platelet levels in a **JAK2**V617F transgenic mouse model of PV. (A) Age-matched hemoglobin, neutrophil and platelet levels in wild-type (WT) mice compared to **MPL**del/del and **JAK2**V617F transgenic mice over a period of 6 to 46 weeks. The symbols indicate statistically significant differences. The number of mice studied and complete statistical analysis for all genetic crosses is in the S3 Table in the Supporting information. (B) Age-matched hemoglobin, neutrophil and platelet levels in **JAK2**V617F transgenic mice compared to **JAK2**V617F/MPL**del/del** and **JAK2**V617F/MPL**del/+** transgenic mice. (C) Age-matched hemoglobin, neutrophil and platelet levels in **JAK2**V617F transgenic mice compared to **JAK2**V617F/THPO**del/del** and **JAK2**V617F/THPO**del/+** transgenic mice. *p < 0.001; † p < 0.05.

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spleen (Fig 3A–3D) CFU-GEMM, CFU-GM, BFU-E and CFU-Mk compared to wild-type mice. Splenomegaly developed after 33 weeks of age (Fig 4), along with osteomyelofibrosis and splenic EMH (Fig 5), while plasma THPO was reduced relative to the wild-type mouse, indicating increased THPO utilization (Fig 6).

Loss of MPL expression mitigated the PV phenotype of the JAK2\(^{V617F}\) transgenic mouse

\(\text{MPL}\) gene elimination in the JAK2\(^{V617F}\) transgenic mouse (JAK2\(^{V617F}/\text{MPL}\)del/del) reduced the platelet count to the level of \(\text{MPL}\)del/del mice, prevented an increase in the neutrophil count, and reduced but did not completely normalize the hemoglobin level (Fig 1B). JAK2\(^{V617F}/\text{MPL}\)del/del mice had reduced numbers of marrow (Fig 2A–2D) and spleen (Fig 3A–3D) CFU-GEMM, CFU-GM, BFU-E and CFU-Mk. Spleen weight was also reduced in these mice after...
33 weeks (Fig 4) and, most importantly, there was complete reversal of the osteomyelofibrosis and splenic EMH associated with JAK2 V617F expression (Fig 5), while plasma THPO was markedly elevated (Fig 6).

Expression of one MPL allele (JAK2 V617F/MPL del/+ ) restored erythrocytosis but not the neutrophilic leukocytosis or thrombocytosis (Fig 1B). Marrow and spleen histology (Fig 5) associated with the JAK2 V617F phenotype was partially restored, as was spleen size at 33 weeks (Fig 4). The plasma THPO level was markedly reduced compared to the JAK2 V617F/MPL del/del mouse but was still higher than in the JAK2 V617F transgenic mouse (Fig 6), supporting increased THPO utilization with JAK2 V617F expression.

Loss of THPO expression abrogated the PV phenotype in the JAK2 V617F transgenic mouse

Since plasma THPO was not elevated in the JAK2 V617F transgenic mouse compared to wild-type mouse, suggesting that THPO contributed to the PV phenotype, we bred JAK2 V617F mice with THPO del/del mice. In the absence of THPO in JAK2 V617F/THPO del/del mice, erythrocytosis was partially suppressed at 6–12 weeks, the neutrophil count was suppressed until after 15–18 weeks, and the platelet count was reduced to normal at all-time points (Fig 1C); marrow (Fig 2A–2D) and spleen (Fig 3A–3D) CFU-GEMM, CFU-GM, and CFU-Mk numbers were reduced but not marrow BFU-E, there was a reduction in spleen weight, though not to normal, (Fig 4) and, most strikingly, reversal of the osteomyelofibrosis and splenic EMH (Fig 5).

Fig 3. MPL del, and THPO del genotypes mitigate spleen HPC colony formation in a JAK2 V617F transgenic mouse model of PV. (A) In vitro colony formation by spleen CFU-GM from JAK2 V617F transgenic mice was increased compared to wild-type mice and JAK2 V617F/MPL del/del and JAK2 V617F/THPO del/del transgenic mice (* P < 0.001). (B) In vitro colony formation by spleen CFU-GEMM from JAK2 V617F transgenic mice was increased compared to wild-type mice and JAK2 V617F/MPL del/del and JAK2 V617F/THPO del/del transgenic mice (* P < 0.001). (C) In vitro colony formation by spleen BFU-E from JAK2 V617F transgenic mice was increased compared wild-type mice and JAK2 V617F/MPL del/del and JAK2 V617F/THPO del/del transgenic mouse. (* P < 0.001) (D) In vitro colony formation by spleen CFU-Mk from JAK2 V617F transgenic mice was increased compared to wild-type mice and JAK2 V617F/MPL del/del and JAK2 V617F/THPO del/del transgenic mice (* P < 0.001). The horizontal lines of the boxes indicate the 25th percentile, the median and 75th percentile respectively and the error bars indicate the 10th and 90th percentiles. * P < 0.001. The number of mice of each genotype used in all the experiments is the same as in Fig 2.

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In contrast to restoration of one MPL allele in the JAK2V617F/MPL\textsuperscript{del+} mouse (Fig 1B), restoration of one THPO allele in the JAK2V617F mouse (JAK2V617F/THPO\textsuperscript{del+}) delayed the erythrocytosis until 15 weeks, completely restored the neutrophil leukocytosis and thrombocytosis (Fig 1C), spleen weight (Fig 4) and marrow and spleen histology (Fig 5). Plasma THPO was reduced to below the level of the THPO\textsuperscript{del+} mouse, indicating increased THPO utilization in the presence of JAK2V617F (Fig 6). Thus, despite the presence of MPL with a constitutively-active JAK2, THPO-mediated signaling through MPL was still required for full expression of the PV phenotype in this JAK2V617F transgenic mouse model.

The marrow CD150+CD48- HSC compartment was expanded in the JAK2V617F transgenic mouse and reduced in the absence of the MPL or THPO genes

MPL-THPO signaling is critical for HSC function and survival [48], therefore, we examined by flow cytometry the effect of loss of MPL or THPO on the number of marrow LT-HSC (CD150+CD48-) in the JAK2V617F transgenic mouse. As shown in Fig 7, after week 16, the LT-HSC population was expanded 2 fold in the JAK2V617F transgenic mouse compared to the wild-type mouse, but in the absence of either the MPL or THPO gene, the LT-HSC population in JAK2V617F transgenic mice was reduced to the level of MPL\textsuperscript{del+} or THPO\textsuperscript{del+} mice. These results demonstrate that marrow LT-HSC in the JAK2V617F transgenic mouse were dependent on THPO for marrow function.
Discussion

The discovery that MPL protein expression was impaired in \textit{JAK2^{V617F}}-positive PV, PMF [39] and ET [40] was counterintuitive because MPL is the only hematopoietic growth factor receptor in HSC and the MPN are characterized by apparently autonomous myeloproliferation [49]. Moreover, impaired MPL protein expression appeared to be universal in the MPN since it was also associated with \textit{MPL} [38,50] and \textit{CALR} [29] mutations as well as with germline \textit{MPL} SNP in the MPL distal CRHD causing familial thrombocytosis [32,33].

The potential mechanisms for impaired MPL expression include \textit{MPL} mutations, increased MPL turnover, or incomplete post-translational processing. CAMT is due to \textit{MPL} mutations [21], usually in the distal CRHD, while all three mechanisms are responsible for impaired...
MPN MPL expression [2,27,41,51]; germline SNP causing impaired MPL expression, which are also located in the distal CRHD, appear to involve impaired post-translational processing [51,52].

MPL is produced as an incompletely-glycosylated 80 kDa protein, which is fully glycosylated in the Golgi to a 95 kDa mature protein with JAK2 as its obligate chaperone [12]. Normally, both immature and mature MPL proteins are expressed at the cell-surface and both are THPO-responsive [41,53]. All MPL SNP or MPN driver mutations, however, result in impaired terminal MPL glycosylation in the distal CRHD; JAK2\textsuperscript{V617F} imposes an additional defect. JAK2 is responsible for enhancing MPL stability and recycling [12,41] but JAK2\textsuperscript{V617F} increases MPL ubiquitination and proteasomal degradation, resulting in decreased MPL recycling and half-life, predominantly involving mature MPL [41].

Importantly, impaired MPL expression does not affect megakaryocyte maturation or platelet production [15,16] but impairs plasma THPO clearance by these cells [54], increasing the stimulus for HPC proliferation, either unrestricted (MPL\textsuperscript{S505N}, MPL\textsuperscript{W515K/L}, CALR\textsuperscript{del/del} and JAK2\textsuperscript{V617F}), or restricted to megakaryopoiesis (MPL\textsuperscript{K39N} and MPL\textsuperscript{P106L}) because sufficient cell-surface MPL is still expressed in HCP for this purpose [53]. Indeed, the MPN phenotype partly mirrors that of mice [24,25] or humans with constitutive THPO production [36], a phenotype reversible in the mice by abrogation of THPO production [25]. Since MPL is essential to maintain HSC quiescence and survival in the marrow osteoblastic niche [17,18,55], while it is also responsible for THPO catabolism [54], we postulated that impaired MPL expression was central to MPN phenotypic behavior, causing myeloproliferation by HPC and eventually myelofibrosis due to increased circulating THPO, depending on the MPN driver mutation allele burden, while paradoxically permitting loss of HSC from marrow.
To test this hypothesis, we chose a JAK2V617F transgenic mouse model that recapitulated the natural history of PV [45]. Not surprisingly, when bred to an MPLdel/del mouse, there was abrogation of the PV phenotype and a reduction in marrow HSC, which could be partially restored with expression of one MPL allele. This confirms a central role for MPL in this transgenic mouse model of PV, and indicates that the lower plasma THPO level compared to the wild-type mouse was due to increased THPO consumption by the JAK2V617F-mediated expansion of the megakaryocyte and circulating platelet pools, a feature also seen with MPL and CALR mutations [56].

To examine the role of THPO on the JAK2V617F transgenic mouse phenotype, we bred this mouse with a THPOdel/del mouse. Surprisingly, there was modification of the PV phenotype including reversal of the osteomyelofibrosis and reduction in marrow HSC despite the fact
there was biallelic expression of a functional MPL with JAK2\textsuperscript{V617F} as its tyrosine kinase. Resto-
ration of a single THPO allele was sufficient to restore the PV phenotype, in contrast to incom-
plete restoration with a single MPL (JAK2\textsuperscript{V617F}/MPL\textsuperscript{del/\textsuperscript{+}}). This observation indicates that
constitutive MPL signaling alone through JAK2\textsuperscript{V617F} was insufficient to support the full PV
phenotype in this transgenic mouse model.

Our observations of abrogation of the PV phenotype by THPO gene deletion appear at
odds with the results of the study of Sangkhae et al \cite{57}. That study, however, employed a dif-
ferent JAK2\textsuperscript{V617F} transgenic mouse model with an ET phenotype \cite{35} and only 16 weeks of
observation, rendering their results not comparable to our JAK2\textsuperscript{V617F} transgenic mouse
model, which recapitulated the natural history of PV, but required over 33 weeks of observa-
tion for full expression of the disease phenotype. Furthermore, Sangkhae et al claimed that
THPO was not necessary for expression of the ET phenotype in their mouse model. How-
ever, in agreement with our observations, thrombocytosis was abrogated and in vitro
HPC proliferation, megakaryocyte number and size, and spleen size were reduced in their
JAK2\textsuperscript{V617F}/THPO\textsuperscript{del/del} mice, indicating THPO dependence in their JAK2\textsuperscript{V617F} transgenic
mouse model.

An important consideration is whether observations in a JAK2\textsuperscript{V617F} transgenic mouse
model of PV with normal mouse MPL expression, can be extrapolated to human PV. In the
wild-type mouse, the average platelet count is \(~1,000\times 10^{9}/L\) and the plasma THPO level is
\(~400\, \text{pg/mL}\), while in humans, the average platelet count is \(~250\, \times 10^{9}/L\) and the plasma THPO
level is \(~55\, \text{pg/m}\) \cite{58}. Furthermore, mice, unlike humans, can survive without expressing
MPL \cite{46}. These differences, however, may be deceiving.

Humans with the benign germline SNP, K39N (MPL Baltimore \cite{32}), however, actually
recapitulate mouse hematopoiesis with thrombocytosis, an elevated plasma THPO level and
impaired MPL cell-surface expression. Conversely, when the asparagine at residue 39 in wild-
type mice is removed, their platelet counts fall into the human normal range as do their plasma
THPO levels \cite{59}, indicating that mouse hematopoiesis can also in part recapitulate human
hematopoiesis.

Similarly, THPO metabolism in the JAK2\textsuperscript{V617F} transgenic mouse model of PV also repre-
sents a difference between this model and the human disease since plasma THPO is elevated
in human PV. It does, however, emphasize that the MPN are hematopoietic growth factor-
dependent disorders, particularly in the JAK2\textsuperscript{V617F} heterozygous state. For example, in human
PV, BFU-E heterozygous for JAK2\textsuperscript{V617F} were responsive in vitro to erythropoietin in a manner
similar to normal BFU-E \cite{60} and HPC hematopoietic growth factor-responsiveness was also
observed in vitro with MPL \cite{38} and CALR \cite{4,31} mutations. In this regard, our in vitro HPC
colony-forming assays indicate that JAK2\textsuperscript{V617F} expression alone accounted for approximately
50\% of marrow GFU-GM colony formation (Fig 2A) and 30\% of CFU-Mk colonies (Fig 2D),
with the rest dependent on THPO stimulation.

The reduction in marrow LT HSC in the absence of MPL or THPO in our studies also
supports the contention that the MPN are hematopoietic growth factor-dependent-diseases.
Importantly, an MPL small molecule antagonist preferentially inhibited JAK2\textsuperscript{V617F}-positive
PV HSC proliferation both in vitro and in vivo compared to normal HSC \cite{61}, while in vitro,
PV HPC\textsuperscript{68} and murine cell lines expressing activating MPL \cite{38} or CALR \cite{4,31} mutations
were still THPO-responsive despite the presence of constitutively-activated JAK2. Addition-
ally, in vivo exposure of wild-type mice to an MPL antagonist antibody permitted nonmyelo-
blastic bone marrow transplantation, substantiating the need for THPO to maintain LT-HSC
in their marrow niches \cite{18}. Importantly, while MPL absence leads to CAMT in humans,
MPL expression is impaired but not absent in MPN HSC and HPC and as demonstrated
experimentally \cite{61}, should give normal HSC and HCP a survival advantage in the presence
of a THPO antagonist. Finally, the development of biologically effective in vivo silencers of THPO production [62, 63] makes testing this therapeutic approach feasible.

From our observations, therefore, we postulate that impaired MPL expression in the MPN results in an inappropriately high plasma THPO level through failure of THPO clearance by MPN platelets and megakaryocytes, which augments activated JAK2 signaling in HPC, while weakening the ability of HSC to remain in the marrow osteoblastic niche (Fig 8). With time, marrow HSC loss due to differentiation or migration and sequestration in the spleen, and continued megakaryocyte stimulation by the elevated plasma THPO, could produce a PMF phenotype, regardless of the MPN driver mutation. From this perspective, the MPN are in part hematopoietic growth factor-dependent disorders and targeting the MPL-THPO axis could be an effective, nonmyelotoxic therapeutic strategy.

**Supporting information**

S1 Fig. Breeding strategy to obtain JAK2<sup>V617F</sup> transgenic mice in the desired background. JAK2<sup>V617F</sup> transgenic mice were crossed into the MPL knockout background as shown with the ratios of each genotype obtained over the total number of progeny from the matings. Identical
breeding schemes were used to obtain the $JAK2^{V617F}/THPO^{del/del}$ and $JAK2^{V617F}/THPO^{del/+}$ transgenic mouse genotypes.

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