Incomplete restoration of Mpl expression in the mpl−/− mouse produces partial correction of the stem cell–repopulating defect and paradoxical thrombocytosis

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Expression of Mpl is restricted to hematopoietic cells in the megakaryocyte lineage and to undifferentiated progenitors, where it initiates critical cell survival and proliferation signals after stimulation by its ligand, thrombopoietin (TPO). As a result, a deficiency in Mpl function in patients with congenital amegakaryocytic thrombocytopenia (CAMT) and in mpl−/− mice produces profound thrombocytopenia and a severe stem cell–repopulating defect. Gene therapy has the potential to correct the hematopoietic defects of CAMT by ectopic gene expression that restores normal Mpl receptor activity. We rescued the mpl−/− mouse with a transgenic vector expressing mpl from the promoter elements of the 2-kb region of DNA just proximal to the natural gene start site. Transgene rescued mice exhibit thrombocytosis but only partial correction of the stem cell defect. Furthermore, they show very low-level expression of Mpl on platelets and megakaryocytes, and the transgene-rescued megakaryocytes exhibit diminished TPO-dependent kinase phosphorylation and reduced platelet production in bone marrow chimeras. Thrombocytosis is an unexpected consequence of reduced Mpl expression and activity. However, impaired TPO homeostasis in the transgene-rescued mice produces elevated plasma TPO levels, which serves as an unchecked stimulus to drive the observed excessive megakaryocytopoiesis. (Blood. 2009;113:1778-1785)

Introduction

Thrombopoietin (TPO) plays a dual role in hematopoiesis, acting as both the primary stimulus for megakaryocytopoiesis and as a critical factor supporting hematopoietic stem cell (HSC) survival and proliferation.1 TPO-induced homodimerization of its cognate receptor, Mpl, induces activation of the Jak/Stat family of tyrosine kinases, leading to the generation of multiple downstream proliferative and antiapoptotic signals.2 Both TPO-deficient (Tpo−/−) and Mpl-deficient (mpl−/−) mice exhibit severe thrombocytopenia,3,4 and marrow cells transplanted from mpl−/− mice demonstrate a profound repopulating defect.5,8 In humans, loss-of-function mutations in Mpl are the cause for the clinical disorder congenital amegakaryocytic thrombocytopenia (CAMT).9,10 Individuals with CAMT present with isolated thrombocytopenia in early childhood and eventually develop marrow failure with pancytopenia. A more rapid progression has been described in patients who have complete loss of Mpl function than in those with missense mutations that permit some residual receptor activity.11,12 CAMT has the potential to be cured by gene therapy through the addition of a normal mpl transgene into the HSCs of affected individuals. Furthermore, because mpl-transduced stem cells would be endowed with proliferative and survival advantages, it should be possible to cure CAMT by correction of a small percentage of diseased stem cells and with submyeloablative conditioning.

One of the major challenges to the restoration of physiologic TPO/Mpl signaling is the development of vectors that will restrict Mpl expression to undifferentiated hematopoietic cells and those committed to the megakaryocyte lineage. TPO is constitutively produced, mainly by the liver and to a lesser extent by the kidneys and skeletal muscle.2 Its lineage-specific proliferative and survival effects are due to the tissue-restricted pattern of Mpl expression. Furthermore, because TPO is internalized and destroyed after binding to Mpl, plasma TPO levels are primarily regulated by megakaryocyte and platelet mass.13-20 In murine hematopoietic stem cell gene transfer studies, ectopic expression of an Mpl transgene under the direction of a constitutive promoter produced dys hematopoiesis with both inappropriate expansion of erythroid cells and the development of thrombocytopenia due to depressed plasma TPO levels.21-24

Successful gene therapy of CAMT necessitates ectopic expression of Mpl in a manner that faithfully mimics its pattern in nature. Using a transgenic mouse model, Ziegler et al demonstrated that the 2-kb portion of noncoding DNA just upstream of the Mpl start site is capable of directing alkaline phosphatase reporter gene expression limited to platelets and megakaryocytes of adult mice and cells of early embryonic hematopoiesis in fetal mice.25 In this report, we describe rescue of the mpl−/− mouse by ectopic expression of an mpl transgene under control of the same 2-kb promoter. The transgene-rescued mice show low-level expression of Mpl in the megakaryocyte lineage and only partial recovery of the stem cell–repopulating defect. Surprisingly, the rescued mice also exhibit high peripheral blood platelet counts due to excessive megakaryocytopoiesis driven by elevated plasma TPO levels. These results demonstrate a paradoxical mechanism for the development of thrombocytosis. Decreased Mpl expression on platelets and megakaryocytes may be a more common etiology for idiopathic thrombocytosis than is currently recognized.


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Methods

Mice

All mice were maintained in a specific pathogen–free facility and studied under a protocol approved by the University of Washington Institutional Animal Care and Use Committee. The mpl−/− mice backcrossed into the C57BL/6J background were generously provided by Dr Warren Alexander (The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia). The wild-type (WT) congenic control mice used were C57BL/6J (Ly5.2), B6.SJL-Ptprca Pepcb/J (Ly5.1), and green fluorescent protein (GFP)–expressing C57BL/6-Tg(UBC-GFP)30Scha/J, all purchased from The Jackson Laboratory (Bar Harbor, ME). The mpl transgenic correction vector was generated by cloning an mpl minigene, containing the murine mpl coding region with its first natural intron, into pBluescript just distal to the previously described 2-kb mpl promoter (a gift from Radek Skoda, University Hospital Basel, Basel, Switzerland). The vector was linearized and microinjected into single-cell mouse embryos from a mpl−/− mouse, which were then implanted into pseudopregnant females. Offspring were screened by polymerase chain reaction (PCR) and Southern blot which were then implanted into pseudopregnant females. 27 Offspring were analyzed by PCR and Southern blot for the presence of the mpl transgene using primers specific for the murine mpl cDNA region proximal to the murine mpl endogenous promoter. Southern blots were performed using standard methods.28 Blots were airdried and probed with a 562-bp EcoRI/XhoI fragment containing sequence from exons 3 to 5 of mpl, generated by PCR of the mpl cDNA with the primers CATAAGAATTCTCTGGGAACCTGATTGTGTT and TCACATCTCGAGATGACACACAGGCAGGGAAGG and cloned into the-amplon of the pBluescript. Vector copy numbers were quantified by PhosphorImager analysis (GE Healthcare, Little Chalfont, United Kingdom).

DNA preparation/Southern blotting

DNA was prepared from the splenocytes of WT and Tg(mpl)–corrected mice using the Puregene DNA isolation kit (QIAGEN, Valencia, CA) per manufacturer’s instructions. Southern blots were performed using standard techniques on 10 µg NsiI-digested genomic DNA from Tg(mpl) mice and control samples made by dilution of the transgenic vector into genomic mpl−/− DNA.28 Blots were exposed to 32P-labeled probes using the manufacturer’s instructions. 

RNA preparation and quantitative real-time PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) per the manufacturer’s instructions. Reverse transcription was performed with the SuperScript III First-Strand Synthesis System (Invitrogen) using 1 µg RNA in a 20-µL reaction per manufacturer’s instructions. Quantitative real-time PCR (qPCR) was performed on a Light Cycler 2.0 machine (Roche Applied Science, Indianapolis, IN) using LightCycler FastStart DNA MasterPLUS SYBR Green I. The following primer pairs were used to detect: (1) murine mpl—TGTCTTGGGATGAGGAAG (located in exon 2) and AGGGCCCACTATCCAC (located in exon 3) and (2) murine Gapdh—AGAGCTGAACGGGAAG and CTGTTGAATGCCGAGG. Results were normalized using a standard curve generated by dilutions of a plasmid containing murine Gapdh.

Western blot analysis

For platelet lysate preparation, mice were anesthetized and blood was drawn from the retro-orbital sinus. Blood was diluted with 3.2% Na2C6H5O7 (sodium citrate) and centrifuged at 300g for 15 minutes. Platelet-rich plasma (PRP) was isolated and centrifuged at 1550g for 10 minutes. Liquid culture megakaryocyte progenitors were washed once with ice-cold PBS; megakaryocytes and platelets were lysed in a buffer composed of 50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaVO4, 1 µg/mL leupeptin, and 1 µg/mL aprotinin. Whole-cell lysates were obtained by centrifugation at 14 000g for 15 minutes at 4°C. The protein concentrations were determined using the Protein/DC Assay (Bio-Rad, Hercules, CA) to assure equal loading between lanes. For each sample, 45 µg total protein was denatured by boiling for 10 minutes in loading buffer containing a final concentration of 63 mM Tris/base (pH 6.8), 1% β-mercaptoethanol, 1% sodium dodecyl sulfate, and 10% glycerol. Proteins were size-separated on 4% to 20% denaturing polyacrylamide gel, transferred to nitrocellulose, blocked with PBS containing 6% BSA, probed with antibodies, and visualized by chemiluminescence as previously described.29 Membranes were probed with the following primary antibodies: polyclonal anti-phospho-Stat3 (Tyr705), polyclonal anti-Stat3, polyclonal anti-phospho-Stat5 (Tyr694), polyclonal anti-Stat5, polyclonal anti-phospho-mitogen-activated protein kinase (MAPK; Thr202/Tyr204), and polyclonal anti-MAPK (Cell Signaling Technology, Beverly, MA); polyclonal anti-phospho-Jak2 (Tyr1007/1008) and polyclonal anti-Jak2 (Upstate Biotechnology, Lake Placid, NY); and polyclonal Mpl antiserum (kindly provided by Amgen, Thousand Oaks, CA). Secondary antibodies, horseradish peroxidase–coupled goat anti-mouse IgG or goat anti–rabbit IgG, were purchased from Bio-Rad. Quantification of immunoreactive bands was performed by using the Kodak Image Station 440cf (Kodak, Rochester, NY).

Flow cytometric analysis

All studies on peripheral blood and marrow specimens were performed on single-cell suspensions stained in cold fluorescence-activated cell sorter (FACS) buffer (PBS supplemented with 1% FBS) with the indicated anti–mouse antibodies and appropriate isotype-matched controls using concentrations recommended by the manufacturer. After staining on ice for 30 minutes, cells were washed twice in FACS buffer and fluorescence was analyzed using a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA). Analysis of peripheral blood cells was performed with gates set to identify platelets, white blood cells (WBCs), and red blood cells (RBCs) according to their forward scatter (FSC) and side scatter (SSC) characteristics.

Peripheral blood counts and TPO levels

In determining peripheral blood counts, platelet activation and clumping were minimized by drawing blood from the inferior vena cava of anesthetized mice into syringes containing 1.5% EDTA (pH 8.0) at a final ratio of 1 part anticoagulant to 9 parts blood. Platelet counts were determined on a flow cytometer on samples diluted 1:600 in PBS by quantitating the number of particles falling into a FSC and SSC platelet gate in the volume of fluid analyzed in 20 seconds. To validate the platelet gate, some blood samples were diluted 1:50 in PBS and stained with anti-GP Ib–FITC (clone Xia.G5; EMFRET Analytics, Eibelstadt, Germany) prior to making a 1.600 dilution in PBS and analysis of GP Ibα particles by flow cytometry. Hemoglobin concentrations and WBC counts were determined using a Coulter T890 (Beckman Coulter, Fullerton, CA). TPO assays were performed on 1.5 diluted platelet-poor plasma from inferior cava–drawn blood using a TPO ELISA kit (R&D Systems, Minneapolis, MN) per the manufacturer’s instructions.

Bone marrow cell isolation, progenitor cell culture, and purification

Bone marrow cells were flushed from the femurs and tibiae of 6- to 10-week-old mice with Iscove-modified Dulbecco medium (IMDM) containing 5% heat-inactivated FBS. The dilute bone marrow was filtered through 70-µm mesh to remove bone particles; RBCs were lysed in hypotonic buffer (155 mM NH4Cl, 7.3 mM NaHCO3, and 126 µM EDTA), and nucleated cells were resuspended in IMDM. Marrow mononuclear cells were stained with anti-CD41–FITC (BD Pharmingen) and analyzed by flow cytometry. Erythroid (E) and granulocyte-macrophage (GM) progenitors (burst-forming unit [BFU]–E, colony-forming unit [CFU]–GM, and CFU–granulocyte, erythroid, macrophage, megakaryocyte) (GEMM) were cultured in methylcellulose-based media (Methocult GF M3434; StemCell Technologies, Vancouver, BC) and megakaryocyte progenitors (CFU-Mk) were cultured in collagen-based media (MegaCult-C; StemCell Technologies) supplemented with 50 ng/mL recombinant murine TPO and 10 ng/mL recombinant murine IL-3 (PeproTech, Rocky Hill, NJ) per the manufacturer’s instructions. Liquid culture megakaryocyte progenitors were generated by culturing bone marrow mononuclear cells in IMDM supplemented with...
The repopulating ability for all Ly5.2 donors was reported as a percentage of the normal Ly5.1 competitor cell repopulating ability at 16 weeks after transplantation by staining buffy coat cells with anti-CD45.2-FITC, anti-CD14–PE, and isotype-matched control antibodies. The percentage of cells staining CD41+/CD14− is shown in the bottom right of each histogram and is representative of 3 independent experiments (4 mice from each strain were pooled in each experiment).

LSK cell isolation

After harvesting bone marrow cells, RBCs were lysed and committed hematopoietic cells were removed using a lineage cell depletion kit (Miltenyi Biotec, Auburn, CA) per the manufacturer’s instructions. The remaining cells were subsequently stained with anti–Sca-1–PE (eBioscience), and then the double-positive lineage-negative/Sca-1+ cells were sorted using a FACSVantage SE II cell sorter (Becton Dickinson, San Jose, CA).

Transplantation studies

Transplant recipients were 6- to 10-week-old mice irradiated using a Mark I-68 137Cs irradiator (J. L. Shepherd & Associates, San Fernando, CA). In studies comparing Ly5.1 and Ly5.2 repopulating cells, 3 × 10^5 marrow mononuclear cells from each donor source were transplanted into 10^7 0 cGy–irradiated B6.SJL-Ptprca PeperB/D2S (Ly5.1) recipients. Flow cytometric evaluation of repopulating ability was performed on peripheral blood cells at 16 weeks after transplantation by staining buffy coat cells with anti-CD45.1–PE (BD Pharmingen) and anti-CD45.2–FITC (BD Pharmingen). The repopulating ability for all Ly5.2 donors was reported as a percentage of the normal Ly5.1 competitor cell repopulating ability (% Ly5.2/CD14−/CD41+ peripheral blood leukocytes/% Ly5.1/CD14−/CD41+ peripheral blood leukocytes × 100). In the other repopulating studies, 3 × 10^5 marrow mononuclear cells from Tg(mpl) mice and 10^5 marrow mononuclear cells from Tg(GFP) mice were transplanted into 850 cGy–irradiated WT and mpl−/− recipients. GFP expression on peripheral blood cells was performed at 16 weeks after transplantation. Platelets were analyzed by flow cytometry on FSC- and SSC-defined platelet populations of anticoagulated whole blood, and leukocytes were analyzed after staining buffy coat cells with anti-CD45.2–PerCP-Cy5.5 (BD Pharmingen).

Histologic analysis

Femurs harvested from mice were fixed in buffered formalin and embedded in paraffin. Sections (4-μm thickness) were stained with hematoxylin-eosin and examined by light microscopy. Samples were visualized using a Leica DMLS microscope (Bannockburn, IL) with a 20× objective (n=0.17/B), and images were captured using a QImaging camera (Retiga 1300R) and QCapture Pro 60 imaging software (QImaging, Surrey, BC). Images were imported into and processed using Photoshop 4.6 software (Adobe, San Jose, CA).

Statistics

Data are reported as mean plus or minus standard error (SE). Comparisons of interest were made via t test, paired and unpaired, as appropriate.

Results

Transgene copy number, peripheral blood, and marrow

We have maintained a colony of Tg(mpl) mice for more than 7 years. They breed normally, show no gross pathology, and have a normal life expectancy. By Southern blotting they are shown to carry 38 copies of the ectopic Mpl transgene (Figure 1A). WT,
mpl<sup>−/−</sup>, and Tg(mpl) mice have equivalent peripheral blood hemoglobin levels and WBC counts (Table 1). Platelet counts in the mpl<sup>−/−</sup> mice are 15% of WT levels, a finding that is consistent with other reports. In contrast, Tg(mpl) mice exhibit thrombocytosis, with platelet counts that are approximately double those of WT mice (Table 1). The relative numbers of morphologically recognizable megakaryocytes in histologic femur sections parallels the peripheral blood platelet counts (Figure 1B,C). Furthermore, quantitation of the percentage of megakaryocyte lineage-committed cells in the marrows of all 3 mouse strains (Figure 1D) confirmed an increase in the percentage of megakaryocyte lineage-committed cells in the Tg(mpl) mice. These results demonstrate an increase in marrow megakaryocytopoiesis in the Tg(mpl) mice.

**Hematopoietic progenitors and repopulating cells**

As expected, an examination of bone marrow progenitors revealed a more generalized defect in hematopoiesis in the mpl<sup>−/−</sup> mice. Compared with WT mice, they have reduced numbers of marrow CFU-GM, CFU-GEMM, and CFU-Mk progenitors (Figure 2A). Though all progenitor numbers are restored to normal in the Tg(mpl) mice (Figure 2A), they still show evidence of a megakaryocyte maturation defect. Compared with WT mice, the marrows of mpl<sup>−/−</sup> mice have equivalent peripheral blood counts (Table 1). The CFU-Mk colonies (total counts shown in panel A) were further characterized by size. There were significantly fewer large (> 20 cells) colonies in Tg(mpl) than WT marrow (P < .005). Means plus or minus SE are shown.

**Impaired TPO homeostasis drives the increased megakaryocytopoiesis**

As expected, plasma TPO levels in mpl<sup>−/−</sup> mice were found to be significantly elevated. Plasma Tpo levels were also elevated in Tg(mpl) mice (Figure 4A), in spite of their high platelet counts. However, by Western blotting, the mpl<sup>−/−</sup> mouse generates overcorrection of the platelet count but incomplete recovery of stem cell–repopulating activity.

**Table 1. Peripheral blood counts**

<table>
<thead>
<tr>
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<th>WT</th>
<th>mpl&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Tg(mpl)</th>
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</thead>
<tbody>
<tr>
<td>Hemoglobin, g/L</td>
<td>154 ± 4</td>
<td>154 ± 5</td>
<td>154 ± 2</td>
</tr>
<tr>
<td>WBC, ×10&lt;sup&gt;3&lt;/sup&gt;/L</td>
<td>6.0 ± 0.5</td>
<td>6.7 ± 1.3</td>
<td>6.3 ± 1.4</td>
</tr>
<tr>
<td>Platelets, ×10&lt;sup&gt;9&lt;/sup&gt;/L</td>
<td>706.6 ± 15.2</td>
<td>102.8 ± 12.3</td>
<td>1578.3 ± 291.4</td>
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</table>

Tg(mpl) mice contain significantly fewer large (> 20 cells) CFU-Mks (Figure 2B; P < .005) and Tg(mpl) megakaryocyte progenitors grown in liquid culture showed a degree of maturation arrest, with almost no cells reaching a DNA content of 32N or greater (Figure S1, available on the Blood website; see the Supplemental Materials link at the top of the online article).

To see whether transgene rescue had also reconstituted hematopoietic stem cell–repopulating ability we performed competitive repopulation assays transplanting WT C57BL/6, mpl<sup>−/−</sup>, or Tg(mpl) marrow cells (all Ly5.2) mixed with an equal number of WT Ly5.1 competitor marrow cells into lethally irradiated WT Ly5.1 recipients. Irradiated WT Ly5.1 mice that received transplants of only WT Ly5.2 or Tg(mpl) marrow demonstrated endogenous reconstitution of less than 10%, indicating that nearly all contributing stem cells came from donor marrow (data not shown). As expected, the repopulating ability of the mpl<sup>−/−</sup> marrow was severely impaired at only 3.6% plus or minus 0.8% of WT marrow (Figure 3). In comparison, the Tg(mpl) mice demonstrated a nearly 10-fold increase in marrow-repopulating activity, though the recovery was still not complete, reaching 33.5% plus or minus 5.6% of the WT Ly5.1 level. Thus, the transgenic vector used to restore mpl expression in the mpl<sup>−/−</sup> mouse generates overcorrection of the platelet count but incomplete recovery of stem cell–repopulating activity.
9-fold lower level of Mpl protein expression in their platelets (Figure 4B). The reduced expression of Mpl in platelets accounts for the high plasma TPO levels seen in the Tg(mpl) mice. Even in the setting of marked thrombocytosis the total number of Mpl receptors available to bind TPO was diminished. However, high plasma TPO levels and low expression of Mpl do not exclude the possibility that megakaryocytopoiesis in Tg(mpl) marrow is less dependent on TPO stimulation than in WT marrow.

To compare the relative megakaryocytopoietic response of WT and Tg(mpl) marrow to an equivalent TPO stimulus, we tracked the stem cell sources of both peripheral blood platelets and leukocytes in chimeric mice. For these experiments, we used Tg(GFP) mice as WT donors because they exhibit normal hematopoiesis and express GFP in all lineages, including platelets. A determination of the relative donor contribution to megakaryocytopoiesis was not possible in the prior competitive repopulation assays because CD45 is not expressed on platelets. We used a 3:1 ratio of Tg(mpl)/Tg(GFP) marrow cells because of the lower repopulating activity shown in Tg(mpl) marrow. At 4 months after transplantation, peripheral blood was analyzed and 15 of the 19 mice that received transplants were chimeras, with each donor graft contributing 2% or more of peripheral blood leukocytes. In chimeras, the mean contribution of leukocytes coming from Tg(mpl) marrow was 48.4% plus or minus 3.2%; for platelets, it was 13.3% plus or minus 5.0%. Furthermore, in the peripheral blood of 14 of the 15 chimeras there was a higher percentage contribution of Tg(mpl) cells in circulating leukocytes than in platelets (Figures 5A, S2; P < .001). Because of the low number of marrow cells transplanted from the Tg(GFP) donors, 4 of the 19 recipient mice did not end up as stable chimeras and instead were repopulated with only Tg(mpl) marrow. We compared the platelet counts and TPO levels in the 15 chimeras to the 4 mice repopulated by only Tg(mpl) stem cells. The chimeric mice had normal platelet counts and lower TPO levels than the mice engrafted with only Tg(mpl) marrow cells (Figure 5B).

These results demonstrate that megakaryocytopoiesis from Tg(mpl) marrow is not only TPO dependent, but actually requires a greater stimulus for platelet production than WT marrow. Therefore, the thrombocytosis found in Tg(mpl) mice is solely due to an increased stimulus to megakaryocytopoiesis from high plasma TPO levels. Furthermore, the imbalance in TPO homeostasis in Tg(mpl) mice can be reversed by the engraftment of a small number of WT stem cells that generate megakaryocytes and platelets with normal expression of Mpl.

**TPO-mediated signaling in megakaryocytes**

We examined the phosphorylation of Jak2, Stat3, Stat5, and MAPK in megakaryocyte progenitors grown in liquid culture. The Tg(mpl) megakaryocytes stimulated by TPO showed significantly less
tyrosine phosphorylation of all 4 signaling proteins when compared with WT cells (Figure 6B). Phosphorylation of Jak2 was more robust in WT cells, with a 7.2- and 8.1-fold increase over Jak2 phosphorylation in Tg(mpl) cells in response to TPO stimulation at 50 and 500 ng/mL, respectively (Figure 6B). Similarly, phosphorylation of Stat3 and Stat5 was more pronounced in WT cells, with 6.8-fold (50 ng/mL) to 7.5-fold (500 ng/mL) greater Stat3 phosphorylation and 5.1-fold (50 ng/mL) to 6.3-fold (500 ng/mL) fold greater Stat5 phosphorylation than in Tg(mpl) cells. Phosphorylation of MAPK was 4.1-fold (50 ng/mL) to 4.3-fold (500 ng/mL) greater in WT megakaryocytes (Figure 6B). In contrast, the total protein levels of Jak2, Stat3/5, and MAPK were similar in megakaryocytes from both WT and Tg(mpl) mice. However, as in our earlier studies with platelets, Mpl expression was markedly reduced in the Tg(mpl) megakaryocytes (Figures 4B,6A). The reduced expression of Mpl in the Tg(mpl) mouse correlated with the observed lower levels of TPO-induced kinase phosphorylation in megakaryocytes and explains the lower contribution of platelet production from Tg(mpl) megakaryocytes in the Tg(mpl)/Tg(GFP) chimeras (Figure 5A,B).

**Mpl message levels in megakaryocytes and undifferentiated progenitors**

We performed qPCR to determine relative expression levels of Mpl mRNA in WT and Tg(mpl) liquid culture–generated megakaryocytes and LSK cells. LSK cells are a population of primitive marrow progenitors highly enriched for HSCs.34-36 Relative mpl message levels were significantly higher in both megakaryocytes and LSK cells from WT mice (Figure 7). However, the difference in mpl mRNA levels between WT and Tg(mpl) mice was more pronounced in megakaryocytes (27.8 ± 4.7-fold change) than in LSK cells (4.7 ± 1.1-fold change). Furthermore, the markedly reduced level of mpl message in Tg(mpl) megakaryocytes paralleled the Western blot results on megakaryocytes and platelets (Figures 4B,6A), indicating that the message coming from the ectopic transgene is efficiently translated. Moreover, the discrepancy in relative mpl mRNA expression patterns in Tg(mpl) megakaryocytes and LSK cells is consistent with the functional results that showed a greater contribution to the leukocyte than platelet compartment by Tg(mpl)–repopulating cells in chimeric mice (Figure 5A).

**Discussion**

TPO-induced proliferation and survival effects are limited to cells that express Mpl: immature multipotent progenitors and cells in the megakaryocyte lineage.7,37,38 Furthermore, plasma TPO levels are inversely proportional to the total number of Mpl receptors available for binding. Because most of Mpl is expressed on platelets and megakaryocytes, plasma TPO levels fluctuate in response to the physiologic demand for megakaryocytopoiesis.13-15,19 Studies that induced dyshematopoiesis from forced overexpression of Mpl in WT mice demonstrate that TPO effects are tightly regulated by the location and level of Mpl expression.21,24

At the present time, stem cell transplantation is the only modality available to cure CAMT, which is an otherwise lethal disease. However, not all patients with CAMT will have access to an HLA-compatible donor and, even in cases of closely matched transplants, there remains the risk for graft rejection, regimen-related toxicities, and acute and chronic graft-versus-host disease. HSC gene therapy offers a potential cure for CAMT but only if vector gene addition restores normal Mpl expression patterns. The 2-kb noncoding region just upstream from the Mpl start site has promoter activity with the ability to direct appropriate lineage-restricted transgene expression.25 Here, we have demonstrated that ectopic expression of an mpl transgene by this same promoter can correct platelet, progenitor, and stem cell defects found in the mpl−/− mouse without also inappropriately expanding erythroid or myeloid lineage-committed cells. However, in the Tg(mpl) mice there was a disparity in the degree of correction. The platelet counts were overcorrected, a finding also reported by another group that used an Mpl transgenic expression vector with the same 2-kb promoter to rescue the mpl−/− mouse.39 In contrast, the stem cell–repopulating ability of the marrow compartment in

![Figure 6. TPO-induced signaling in WT and Tg(mpl) megakaryocytes.](image-url)

![Figure 7. Expression levels of mpl in megakaryocytes and LSK cells.](image-url)
the Tg(mpl) mice was restored to only about 25% to 35% of the
level found in congenic WT controls.

There are 2 possible causes for the thrombocytosis seen in the
transgene-rescued mice: (1) an inherent defect in function of the
transgenic Mpl receptor leading to excessive megakaryocyte
lineage proliferation or (2) dysregulation of TPO homeostasis.
Excessive signal transduction from the transgenic Mpl receptor
would raise concerns about an increased risk for the development
of a myeloproliferative disorder. It was recently demonstrated
that expression of a constitutively active mutant Mpl in mice produces a
myeloproliferative disorder with myelofibrosis.23 In our study, the
Mpl transgene expressed the WT protein and therefore should not
be subject to activation without stimulation by TPO. Furthermore,
we looked at some of the common downstream targets of TPO-
induced phosphorylation to determine the activity of the Mpl
receptor in Tg(mpl) megakaryocytes and discovered a marked
reduction in signaling that paralleled the decreased Mpl protein
expression.

Paradoxically, the cause for thrombocytosis in the Tg(mpl)
mouse is low expression of Mpl on platelets and megakaryocytes.
Disruption of the normal receptor/ligand interaction that dynami-
cally regulates megakaryocytopenia results in higher plasma TPO
levels and excessive platelet production. Using circulating leuko-
cytes as a measure for repopulating cell engraftment in chimeric
mice (Figure 5A), we determined that under an equivalent TPO
stimulus, the relative degree of megakaryocytopenia derived from
Tg(mpl) marrow was actually much less than from WT marrow.
Furthermore, in vitro studies demonstrated both maturation and
signaling defects in Tg(mpl) megakaryocytes, even with stimula-
tion by saturating levels of TPO (Figures 2B, 6, and S1). Therefore,
the thrombocytosis seen in the Tg(mpl) mice is most likely the
result of persistent unchecked signaling in the megakaryocyte
compartment rather than the restoration of normal megakaryocyte
physiology that has been adjusted to a higher TPO set point.

Reduced Mpl expression in HSCs of the Tg(mpl) mouse probably
accounts for the failure of the transgene to fully restore
hematopoietic-repopulating ability. In fact, our studies found that
the relative restoration of mpl mRNA levels in the Tg(mpl) LSK
cells closely matched the degree to which stem cell–repopulating
ability was corrected. Because the mpl−/− mouse does not show
progression to bone marrow failure, it is hard to predict from this
model how much restoration of stem cell function would be needed
to durably correct the hematopoietic defects in patients with
CAMT. The difference in the hematopoietic phenotype of human
and mouse Mpl deficiency could be due to the relative excess in
stem cell residual capacity in mice,40 or because other cytokine
pathways are more able to compensate for the loss of Mpl signaling
in the mouse system. However, it was recently demonstrated that
Tpo−/− mice undergo progressive loss of both phenotypically
defined HSCs and functionally determined marrow-repopulating
cells as they age.41 We would expect the same to be true for mpl−/−
mice. Our competitive repopulation studies were performed at
4 months after transplantation. It would be interesting to serially
follow chimeric mice for up at least 1 year after transplantation to
see if the contribution of the graft from Tg(mpl) HSCs remains
stable over time.

Though we did not observe thrombotic or hemorrhagic compli-
cations in the Tg(mpl) mice, it is unclear whether this mechanism
for thrombocytosis could predispose to these pathologic outcomes.
In general, nonclonal causes of thrombocytosis are asymptomat-
ic.42 However, there have been 2 reports of autosomal dominant
familial essential thrombocythemia caused by high serum TPO
levels resulting from mutations in the 5′ untranslated region of the
TPO gene that produced alterations in posttranscriptional mRNA
processing.43,44 In one of these families, an increased incidence of
both thrombotic and hemorrhagic complications was described.44,45

Nonclonal thrombocytosis in the setting of reduced Mpl expres-
sion has been described in a couple of clinical settings. Mpl
Baltimore is a single-nucleotide polymorphism that results in a
defect in posttranslational receptor processing.46 Patients homozy-
gous for this polymorphism exhibit marked thrombocytosis and
degreased Mpl protein levels on their platelets. In their description
of Mpl Baltimore, Moliterno et al speculated that the thrombocyto-
sis was caused either by an hyperfunctional Mpl receptor or by a
 mechanism independent of TPO/Mpl signaling. However, plasma
TPO levels and an evaluation of Mpl signal transduction pathways
in megakaryocytes were not reported in their study. We hypothesize
that the decrease in platelet and megakaryocyte expression of Mpl
in individuals with Mpl Baltimore leads to high plasma TPO levels
that drives excessive megakaryocytopenia, like in the Tg(mpl)
mice. At birth, preterm infants exhibit both elevated serum TPO
levels and reduced expression of platelet Mpl mRNA. Over the first
month of life, these infants develop a relative thrombocytosis
which then resolves at ages 2 to 6 months in association with a
decrease in serum TPO levels and an increase in platelet Mpl
mRNA expression.47 Reduced expression of Mpl disrupts TPO
homeostasis and can induce states of thrombocytosis if enough
residual receptor function remains to respond to the resultant high
TPO levels. States of low Mpl expression may be an unrecognized
mechanism for explaining thrombocytosis in patients with idiopathic
nonclonal thrombocytosis.

We found a discrepancy in the degree of restoration of mpl
mRNA expression in LSK cells and megakaryocytes in the Tg(mpl)
mouse, a result that suggested the 2-kb promoter had greater
relative activity in HSCs than megakaryocytes. However, one
limitation of our study is that we only examined expression levels
in mature megakaryocyte progenitors that had been kept in culture
for 72 hours. Tiedt et al recently found that mpl mRNA expression
directed by the same 2-kb promoter decreased over the course of
megakaryocyte maturation.49 Thus, the apparent differences in the
restoration of mpl mRNA expression between the 2 hematopoietic
compartments may have been significantly influenced by the
maturation state of the megakaryocyte progenitors we studied.

To fully correct both the stem cell and platelet compartments of
the mpl−/− mice, transgenes that can accurately restore normal Mpl
expression patterns are needed. It is likely that some regulatory
elements important for the control of native mpl expression were
not included in the 2-kb promoter used in the Tg(mpl) mouse. As
shown in Figure S3, there are regions of significant homology
between the human and murine noncoding regions out to 9 kb
upstream of the mpl start site. By incorporating more of these
homologous sequences into new promoters, we hope to generate
vectors that will be capable of restoring physiologic Mpl signaling
and TPO regulation in the mpl−/− mouse, with the eventual goal of
generating human Mpl expressing retroviral vectors to treat pa-
tients with CAMT.

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Authorship
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References


Incomplete restoration of Mpl expression in the mplt−/− mouse produces partial correction of the stem cell–repopulating defect and paradoxical thrombocytosis

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