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RAPID COMMUNICATION

Deficiencies in Progenitor Cells of Multiple Hematopoietic Lineages and Defective Megakaryocytopoiesis in Mice Lacking the Thrombopoietin Receptor c-Mpl

By Warren S. Alexander, Andrew W. Roberts, Nicos A. Nicola, Ruili Li, and Donald Metcalf

Mice with a null mutation in the thrombopoietin (TPO) receptor c-Mpl were generated by gene targeting. c-*mpl*–deficient mice developed normally but were deficient in megakaryocytes and severely thrombocytopenic. The hematocrit and numbers of mature circulating leukocytes were normal in $mpl^{-/-}$ mice, as was the distribution of morphologically identifiable precursors in hematopoietic tissues. Bone marrow and spleen cells of adult $mpl^{-/-}$ mice lacked specific binding sites for TPO, were unresponsive to TPO in culture, and displayed a marked deficiency in progenitor cells with megakaryocytic potential. Significantly, total hematopoietic progenitor cell numbers were also reduced in $mpl^{-/-}$ mice, including multipotential, blast cell, and committed progeni- tors of multiple lineages. The megakaryocyte deficiency was evident as early as 14 days of gestation in *mpl*-deficient mice, although reductions in progenitor cell numbers arose only later in development. The data suggest that the critical function of c-Mpl signalling in megakaryocytopoiesis is in maintenance of mature megakaryocyte numbers through control of progenitor cell proliferation and maturation. Moreover, our results also imply an important role for TPO and c-Mpl in the production of primitive pluripotent progeni- tor cells as well as progenitor cells committed to nonmega- karyocytic lineages.
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CONSIDERABLE evidence supports an important role for the c-*mpl* gene in the regulation of hematopoiesis. c-*mpl* encodes a cell-surface protein that contains the distinctive amino acid sequence motifs common to the hematopoietin receptor superfamily, members of which transduce signals from a range of predominantly hematopoietic growth factors and cytokines.[1,2] A constitutively active form of the receptor, expressed by the myeloproliferative leukemia virus (MPLV), can function in a range of hematopoietic cells and induces a lethal myeloproliferative disease in mice.[3,4] Finally, tissues that normally support hematopoiesis express c-*mpl*. The receptor is found predominantly in primitive hematopoietic cells, megakaryocytes, and platelets.[4-8]

Consistent with its expression in platelets and their precursors, a specific role for c-Mpl in megakaryocytopoiesis was conclusively established with the recent discovery that thrombopoietin (TPO) is the c-Mpl ligand. TPO, which was originally defined as a thrombopoietic activity in thrombocytopenic serum, was isolated and cloned by virtue of its interaction with c-Mpl and its capacity to stimulate cells expressing the receptor.[9-11] Studies with recombinant or purified plasma-derived material have shown that TPO has potent effects on megakaryocyte proliferation and differentiation. In vitro, TPO stimulates proliferation of megakaryocyte pro-

genitor cells, and this activity may be augmented by the combination of TPO with other cytokines, in particular interleukin-3 (IL-3), stem cell factor (SCF), IL-11, or erythropoietin (EPO).[12-15] TPO also stimulates maturation; megakaryocytes treated with TPO increase in size, become polyploid, and display cytoplasmic reorganization and fragmentation typical of platelet release.[12,14-18] Similarly, TPO administration to mice[11,15,19] or rhesus monkeys[20] stimulates production of megakaryocyte progenitors, elevates megakaryocyte numbers in the bone marrow and spleen, and significantly increases the number of circulating platelets. The magnitude of the thrombocytosis induced by TPO is the largest for any cytokine studied to date.[11,15] Moreover, TPO has been suggested to mediate the megakaryocytopoietic effects of other cytokines, including IL-6, IL-11, and SCF, because their action can be blocked by a soluble form of the c-Mpl receptor.[21] Together, these data imply that TPO, acting through the c-Mpl receptor, is the major regulator of megakaryocytopoiesis.

To date, most in vitro studies have suggested that TPO is a lineage-restricted regulator, with little or no activity on nonmegakaryocytic lineages.[13-15] Nevertheless, in populations of mouse fetal liver enriched for primitive hematopoietic cells, a significant proportion of cells express c-*mpl*.[16] To explore in vivo whether c-Mpl plays a wider role in hematopoiesis as well as to more precisely define the action of TPO and c-Mpl in megakaryocytopoiesis, we have used homologous recombination in embryonic stem (ES) cells to generate mice carrying disrupted *mpl* alleles that prevent receptor expression. In this study, we describe reduced megakaryocyte production and thrombocytopenia in adult $mpl^{-/-}$ mice, consistent with a previous report of *mpl*-deficient animals.[22] To determine the physiologic basis of perturbed megakaryocytopoiesis in mice lacking c-*mpl*, we have extended these previous observations with a detailed analysis of precursor and progenitor cells in the hematopoietic organs of adult, neonatal, and fetal mice. Our results suggest that the indispensable role of c-Mpl in steady-state megakaryocytopoiesis is in the maintenance of normal levels of mature megakaryocytes through the control of progenitor cell prolif-

eration and differentiation. Moreover, we report a significant reduction in the number of hematopoietic progenitors of multiple cell lineages in homozygous mutant mice, suggesting that the role of TPO and c-Mpl is not confined solely to megakaryocytopoiesis.

MATERIALS AND METHODS

Disruption of c-mpl by homologous recombination in embryonic stem cells. A 5.5-kb *Hind*III-*Xba* I clone encompassing exons 1 to 6 of the murine c-*mpl* gene[7] was digested with *Sma* I; the 2.6-kb fragment bearing exons 1 to 5 was removed and replaced with a β-galactosidase–PGKneo cassette.[23] An additional 8.3-kb *Hind*III fragment of 3′ c-*mpl* genomic DNA was added such that in the final construct the β-gal-PGKneo genes were flanked by c-*mpl* sequences extending 1.8 kb 5′ and 9.2 kb in the 3′ direction. The targeting vector (Fig 1A) was excised from this construct as a 14.5-kb fragment, gel purified, and electroporated into the W9.5 ES cell line.[24] Clones surviving selection in 175 μg/mL of G418 were divided into pools of four and screened for homologous recombinants by the polymerase chain reaction (PCR) using primers within the β-galactosidase sequence and within the c-*mpl* gene immediately 5′ to the targeting vector. Homologous recombination in individual clones from positive pools was determined in subsequent PCR reactions and confirmed by Southern blot analysis of genomic DNA extracted from the candidate ES cell clones. Of 384 G418-resistant clones analyzed, 15 (3.9%) contained a targeted c-*mpl* allele.

Mice. Chimeric mice were generated by the implantation into pseudo-pregnant females of blastocysts injected with ES cells bearing a single disrupted c-*mpl* allele. Chimeric males were mated with C57B1/6 female mice and heterozygous offspring interbred to yield wild-type (*mpl*⁺/⁺), heterozygous (*mpl*⁺/⁻), and homozygous mutant (*mpl*⁻/⁻) mice for analysis. Each of three targeted ES cell clones tested transmitted the c-*mpl* mutation through the germline. Mice arbitrarily chosen from one line were used in all experiments reported. Key results were confirmed in animals from a second independent line. Unless otherwise indicated, mice of 8 to 12 weeks of age were used in all experiments. Mouse genotypes were routinely determined by Southern blot analyses of genomic DNA extracted from tail biopsies. DNA preparation, digestion with restriction endonucleases, and processing of blots was performed as described.[25] The probes used were a 0.6-kb *Xba* I fragment of genomic DNA situated 6 kb 5′ of the c-*mpl* gene (probe A) and a c-*mpl* cDNA fragment extending from the initiation ATG codon to the *Sph* I site 0.23 kb downstream (probe B, Fig 1A).

Flow cytometry. Single cell suspensions of bone marrow, spleen, and thymus from four mice of each genotype were incubated with saturating amounts of 2.4G2 anti-Fcγ receptor antibody[26] to reduce background staining, then with specific monoclonal antibodies to murine cell surface antigens: anti CD4 and CD8 (Becton Dickinson, Lincoln Park, NJ), anti-IgM (5.1),[27] anti-Ly5-B220 (RA3-6B2),[28] anti-Mac-1 (M1/70),[29] F4/80,[30] anti-Gr-1 (RB6-8C5),[31] Ter-119,[32] anti-Thy1.2 (30-H12),[33] anti-TCRα/β (H57-697.1),[34] and anti-TCRγ/δ (GL3-1A).[35] The antibodies were directly coupled to fluorescein isothiocyanate (FITC) or biotin, the latter being visualized with R-phycoerythrin-streptavidin. Analyses were performed on a FACScan cell sorter (Becton Dickinson) and dead cells and erythrocytes were excluded by propidium iodide (1 μg/mL) staining and gating of forward angle and side scatter of light.

Thrombopoietin and c-Mpl receptor binding studies. Recombinant murine TPO was produced in Chinese hamster ovary (CHO) cells using a pEF-BOS vector[36] containing the full-length mature protein coding region preceded by a FLAG epitope and the murine IL-3 leader sequence. The protein was purified using an M2 anti-FLAG affinity column (Eastman Kodak, New Haven, CT) and eluted
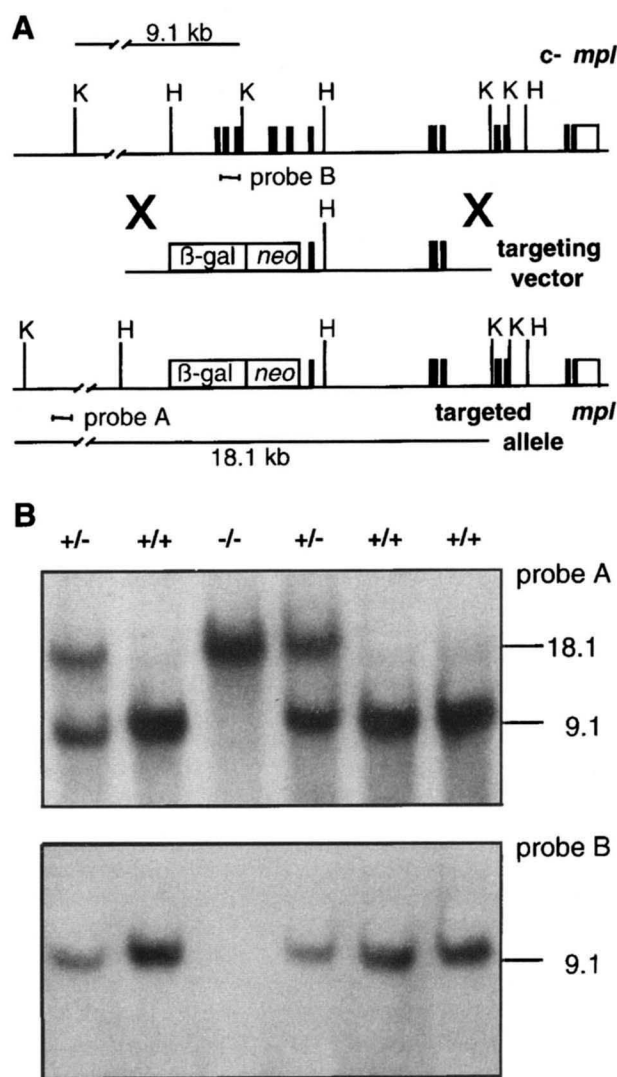
Fig 1. (A) Disruption of the c-*mpl* locus by homologous recombination. A map of the murine c-*mpl* gene is shown (H, *Hind*III; K, *Kpn* I) with exons as raised boxes and translated regions shaded. The targeting vector contains a β-gal-PGKneo cassette in place of the first five c-*mpl* exons (see Materials and Methods) and generates the targeted *mpl* allele after homologous recombination. The probe fragments used in Southern blots to verify gene targeting are shown. (B) Southern blot of *Kpn* I-digested genomic DNA extracted from the tails of mice derived from a cross between heterozygous (*mpl*⁺/⁻) mice. The blot was initially probed with probe A, which is situated in the c-*mpl* locus outside the targeting vector, to allow a distinction between endogenous (9.1 kb) and mutant *mpl* (18.1 kb) alleles, and then with probe B, which is part of c-*mpl* deleted by homologous recombination, to show the loss of genetic material in *mpl*⁻/⁻ mice.

with 0.06 mmol/L FLAG peptide (DYKDDDDK). The purified protein was exchanged into normal saline buffered with 10 mmol/L sodium phosphate (pH 7.4) containing 0.02% (wt/vol) sodium azide and 0.02% (vol/vol) Tween-20. TPO (2 to 3 μg in 10 μL) was radioiodinated on tyrosine using a modified iodine monochloride method[37] and separated from free iodine by gel filtration on Sephadex G-25M columns (Pharmacia, Uppsala, Sweden). ¹²⁵I-TPO yielded a specific radioactivity of 2×10^7 cpm/pmol determined by self displacement analysis[37] and showed no detectable loss of bioactivity

in a proliferation assay of Ba/F3 cells transfected with c-*mpl* (data not shown).

Binding studies were performed by incubating 0.5 to 1×10^7 bone marrow or spleen cells with ^{125}I-TPO (approximately 3×10^5 cpm) in the presence or absence of unlabeled TPO (2 μg/mL, approximately 1,000-fold excess) in 100 μL binding buffer (RPMI medium containing 10% fetal calf serum [FCS] and 10 mmol/L HEPES, pH 7.4) for 1 hour at room temperature (23°C). The mixture was then layered over 200 μL FCS and centrifuged at 13,000g for 10 seconds and the tube was cut just above the cell pellet. Cell pellets were counted in a gamma counter and the specific binding was determined as the difference in counts from duplicate tubes incubated in the absence or presence of unlabeled TPO. As a positive control, Ba/F3 cells expressing c-*mpl*,[25] which bound 4.1×10^4 specific cpm/10^6 cells, were used.

Hematologic analysis. The peripheral blood hematocrit, white blood cell count, and platelet counts were determined using either manual or automated (Sysmex NE8000; TOA, Kobe, Japan) counting techniques. Cell suspensions from femoral bone marrow, spleen, and liver were prepared by standard techniques and the cell numbers were enumerated in hemocytometers after eosin staining. Manual 100 to 400 cell leukocyte differential counts of peripheral blood, bone marrow, liver, and spleen were performed on smears or cytocentrifuge preparations stained with May-Grunwald-Giemsa.

Clonal culture of hematopoietic progenitors. Clonal cultures of hematopoietic cells were performed as previously described.[38] Cytokines were obtained from commercial sources or produced by expression of recombinant proteins in *Pichia pastoris* (SCF), *Saccharomyces cerevisiae* (macrophage colony-stimulating factor [M-CSF]), or CHO cells (TPO, see above) and purified before use. Briefly, cultures of 2.5×10^4 adult bone marrow cells or 10^5 spleen cells in 0.3% agar in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% newborn calf serum were stimulated with a final concentration of 100 ng/mL murine SCF and 10 ng/mL murine IL-3 (PeproTech, Rocky Hill, NJ) and incubated for 7 days at 37°C in a fully humidified atmosphere of 10% CO_2 in air. These conditions resulted in optimal stimulation of neutrophil (G), neutrophil-macrophage (GM), macrophage (M), eosinophil (Eo), megakaryocyte (Meg), and blast cell colony-forming cells (CFC). Similar cultures stimulated with single growth factors (10 ng/mL human G-CSF [Amgen, Thousand Oaks, CA], 10 ng/mL murine GM-CSF [Schering, Kenilworth, NJ], 10 ng/mL murine M-CSF, 200 ng/mL TPO and SCF) were established in parallel. To enumerate erythroid and mixed erythroid-myeloid CFC, 0.3% agar cultures of 10^4 fetal liver cells, 2×10^4 neonatal liver, spleen, and bone marrow cells and 2.5×10^4 adult bone marrow cells were established in Iscove's modified Dulbecco's medium (IMDM) supplemented with 25% FCS, SCF, IL-3, and 4 U/mL human EPO (Amgen) and incubated for 7 days at 37°C in a humidified atmosphere of 5% CO_2 in air. Agar cultures were fixed and sequentially stained for acetylcholinesterase, Luxol fast Blue, and hematoxylin, and the cellular composition of each colony determined at 100- to 400-fold magnification.

Methylcellulose cultures. Spleen cells (10^5) or fetal liver cells (10^4) were suspended in 1.5% methylcellulose (Fluka, Buchs, Switzerland) in IMDM supplemented with 20% FCS, IL-3, SCF, and EPO and incubated at 37°C for 7 days in an humidified atmosphere of 5% CO_2 in air. Colonies were scored as erythroid, myeloid, or mixed-erythroid at 35-fold magnification and colonies appearing to contain erythroid cells were verified by staining with benzidine dihydrochloride.

Histologic analysis. Sections of sternum, femur, liver, spleen, heart, lung, thymus, kidney, intestine, and reproductive organs were prepared by standard techniques from 5 adult $mpl^{+/+}$, $mpl^{+/-}$, and $mpl^{-/-}$ mice, respectively, as were sternum, liver, and spleen sections from neonatal mice killed within 54 hours of birth and liver sections

from fetuses at day 14 of gestation. All sections were stained with hematoxylin and eosin and examined by light microscopy.

Statistical analysis. Initial comparisons among $mpl^{+/+}$, $mpl^{+/-}$, and $mpl^{-/-}$ groups were made by analysis of variance. If the null hypothesis was rejected ($P < .05$), further pairwise comparisons were performed using the Student-Newman-Keuls test or Dunnett's test as appropriate. The Mann-Whitney rank-sum test was used for the comparison of the size distribution of megakaryocyte colonies.

RESULTS

*Generation of c-*mpl *mutant mice.* To disrupt the c-*mpl* gene by homologous recombination in ES cells, a targeting vector was designed to replace 2.6 kb of endogenous c-*mpl*, including the first five exons, with a selectable neomycin resistance cassette (Fig 1A). In addition to the loss of sequences encoding the c-Mpl signal peptide and first hematopoietin receptor domain, this strategy also deletes the protein initiation ATG codon and the site for RNA transcription initiation.[7] After electroporation of the targeting vector into W9.5 ES cells, several independent clones exhibiting the expected homologous disruption of c-*mpl* were used to generate chimeric mice by blastocyst injection (see Materials and Methods). Interbreeding of heterozygous offspring generated mice of the three expected genotypes (Fig 1B). A normal Mendelian ratio for segregation of the disrupted *mpl* allele was observed (65:152:71 for wild-type [$mpl^{+/+}$] :heterozygous [$mpl^{+/-}$]:homozygous mutant [$mpl^{-/-}$]), indicating that $mpl^{-/-}$ mice survived gestation and weaning normally. Homozygous mutant mice also remained clinically healthy as adults. No premature mortality has been observed in our colony, which includes animals more than 7 months of age. No evidence of hemorrhage was seen in mice killed for analysis and no histologic anomalies were observed in an extensive survey of nonhematopoietic tissues (see Materials and Methods).

To confirm that the targeted disruption of c-*mpl* prevented receptor expression, ligand binding experiments were performed with radioiodinated TPO. Whereas c-Mpl receptors on bone marrow and spleen cell suspensions from $mpl^{+/+}$ and $mpl^{+/-}$ mice bound ^{125}I-TPO specifically, no significant specific binding to $mpl^{-/-}$ cells was observed (Table 1). In addition, Northern blots of RNA extracted from bone marrow cells showed specific c-*mpl* transcripts in $mpl^{+/+}$ and $mpl^{+/-}$ cells that were absent in cells from $mpl^{-/-}$ mice (data not shown). Finally, the absence of c-Mpl expression in homozygous mutant mice was further confirmed by the complete inability of hematopoietic cells from these mice to respond to TPO in culture (see below).

*Mice lacking c-*mpl *are severely thrombocytopenic.* Consistent with the role of c-Mpl as the TPO receptor, examination of peripheral blood showed that *mpl*-deficient animals were severely thrombocytopenic: $mpl^{-/-}$ mice had only 6% the number of platelets observed in wild-type or heterozygous littermates (Table 2). Histologic sections of bone marrow and spleen further showed a marked deficit of megakaryocytes in $mpl^{-/-}$ animals (Table 2). The hematocrits, total white blood cell count, and numbers of neutrophils, lymphocytes, monocytes, and eosinophils in the blood were similar in mice of each genotype. The cellularity of the femoral bone marrow and spleen, the spleen weight, and the distribution of

Table 1. Binding of ^{125}I-TPO to Hematopoietic Cells of mpl Mutant Mice

| | Specific Binding of ^{125}I-TPO (cpm/10^7 cells) | | | | | |
| | Bone Marrow | | | Spleen | | |
Genotype	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
mpl$^{+/+}$	1,531 ± 260	1,372 ± 321	4,076 ± 239	2,650 ± 971	1,129 ± 235	1,768 ± 178
mpl$^{+/-}$	1,608 ± 482	2,089 ± 322	1,115 ± 473	557 ± 311	1,000 ± 203	1,086 ± 186
mpl$^{-/-}$	290 ± 580	494 ± 256	−126 ± 571	148 ± 842	−376 ± 62	−203 ± 133

Mean ± standard deviations of the difference between the amount of ^{125}I-TPO bound to cells in the absence and presence of excess unlabeled TPO. Each experiment represents results from a single mouse of each genotype.

morphologically identifiable precursor cells in cytocentrifuge preparations of bone marrow and spleen cells were also normal in mpl$^{-/-}$ mice (Table 2). Flow cytometric analysis of cells from bone marrow, spleen, and thymus, using monoclonal antibodies specific for a range of T-lymphoid, B-lymphoid, myeloid, and erythroid markers (see Materials and Methods), showed no perturbations in the mpl mutant mice (data not shown). Thus, among mature hematopoietic cells, the loss of the c-Mpl receptor appears to deplete specifically the megakaryocyte lineage.

Megakaryocyte progenitor cells in mpl$^{-/-}$ *mice.* To further characterise megakaryocytopoiesis in the absence of c-mpl, megakaryocyte progenitor cells were assayed in clonogenic culture. In response to TPO, 2.5 ± 1 small, purely megakaryocytic colonies developed from 2.5 × 10^4 wild-type bone marrow cells. No colonies of any type developed from similarly stimulated cultures of mpl$^{-/-}$ marrow and an intermediate number of TPO-responsive megakaryocyte colonies (0.5 ± 0.5 per 2.5 × 10^4 cells) were observed in cultures of heterozygous mpl$^{+/-}$ bone marrow. Cultures of spleen cells from mpl$^{-/-}$ mice also failed to yield colonies in response to TPO (data not shown). To determine megakaryocyte progenitor cell numbers and proliferative potential in normal and mpl-deficient mice, the combination of SCF and IL-3 was used. This factor combination stimulated the formation of similar numbers of megakaryocyte-containing colonies from wild-type marrow to the number stimulated by TPO plus IL-3 (data not shown). When compared with wild-type or heterozygous controls, mpl$^{-/-}$ mice displayed a six-fold reduction in progenitor cells with megakaryocytic potential (Table 3A). In addition to a deficiency in unilineage megakaryocyte progenitor cells, there was a significant reduction in the numbers of bipotential megakaryocyte progenitor cells in cultures that included EPO. Multilineage colonies containing megakaryocytes were also under-represented in cultures from mpl-deficient animals (Table 3B). The cell numbers in megakaryocyte colonies from mpl$^{-/-}$ bone marrow were skewed in distribution toward larger colonies (Fig 2), suggesting that the mpl$^{-/-}$ defect was most evident among more mature committed megakaryocyte progenitors with least proliferative potential. The numbers of large mature polyploid megakaryocytes with abundant cytoplasm were reduced within colonies cultured from mpl$^{-/-}$ mice.

mpl$^{-/-}$ *mice are deficient in progenitor cells of multiple hematopoietic lineages.* Although mature hematopoietic cells in mpl$^{-/-}$ mice were selectively deficient in megakaryocytes, the reduction in progenitor cell numbers was not restricted to cells of this lineage (Table 3). In clonal cultures of bone marrow cells, the total progenitor cell number in mpl$^{-/-}$ mice was found to be 38% to 44% of that observed in wild-type animals. Neutrophil-CFC, neutrophil-macrophage–CFC, and pure erythroid-CFC were present at approximately 50% of normal levels. The number of mixed colonies was similarly reduced, and strikingly blast cell-CFC, the most primitive progenitor population detectable in vitro, were reduced as dramatically as megakaryocyte progenitors. No significant differences were observed between cultures of mpl$^{+/+}$ and mpl$^{+/-}$ bone marrow cells.

Table 2. Hematologic Profile of mpl Mutant Mice

| | Genotype | | |
	mpl$^{+/+}$	mpl$^{+/-}$	mpl$^{-/-}$
Peripheral blood			
Platelets (×10^{-6}/mL)	938 ± 304	1027 ± 228	59 ± 13*
Hematocrit (%)	47 ± 2	48 ± 1	46 ± 2
White blood cell count (×10^{-6}/mL)	4.82 ± 2.23	5.28 ± 2.08	4.50 ± 1.63
Neutrophils	0.84 ± 1.01	0.70 ± 0.42	0.40 ± 0.26
Lymphocytes	3.66 ± 1.62	4.16 ± 1.80	3.82 ± 1.54
Monocytes	0.26 ± 0.18	0.30 ± 0.12	0.23 ± 0.11
Eosinophils	0.06 ± 0.05	0.12 ± 0.08	0.05 ± 0.04
Bone marrow			
Cellularity (×10^{-6}/femur)	23.2 ± 3.5	25.5 ± 3.7	23.7 ± 3.1
Blasts (%)	4 ± 2	4 ± 1	3 ± 0
Promyelocytes/myelocytes (%)	10 ± 4	8 ± 4	9 ± 2
Metamyelocytes/neutrophils (%)	27 ± 5	24 ± 5	24 ± 2
Lymphocytes (%)	27 ± 5	29 ± 4	28 ± 5
Monocytes (%)	5 ± 2	6 ± 2	5 ± 2
Eosinophils (%)	2 ± 2	4 ± 1	2 ± 1
Nucleated erythroid cells (%)	25 ± 8	25 ± 4	29 ± 7
Megakaryocytes (per 10 hpf)†	58 ± 8	54 ± 10	5.5 ± 2*
Spleen			
Weight (mg)	81 ± 18	74 ± 22	86 ± 16
Cellularity (×10^{-6})	109 ± 28	94 ± 36	163 ± 128
Blasts (%)	2 ± 1	3 ± 2	3 ± 2
Promyelocytes/myelocytes (%)	0 ± 0	0 ± 0	0 ± 1
Metamyelocytes/neutrophils (%)	2 ± 1	1 ± 1	2 ± 1
Lymphocytes (%)	89 ± 3	91 ± 2	88 ± 4
Monocytes (%)	2 ± 2	1 ± 0	3 ± 1
Eosinophils (%)	1 ± 1	1 ± 1	0 ± 1
Nucleated erythroid cells (%)	4 ± 2	3 ± 2	4 ± 3
Megakaryocytes (per 10 hpf)†	9.5 ± 1.5	7.5 ± 2	0.5 ± 1*

Mean ± standard deviations of data from 4 mice of each genotype, excepting peripheral white blood cell count and differential data (n = 10 per genotype).

* P < .0001 for comparison with mpl$^{+/+}$ and mpl$^{+/-}$ mice. There were no other statistically significant differences between genotypes.

† Determined from histologic sections at 400× magnification, with a minimum of 20 high power fields (hpf) counted per mouse.

Table 3. Bone Marrow Progenitor Cell Analysis of *mpl* Mutant Mice

Stimulus	Genotype	Progenitor Cells per 2.5 × 10⁴ Bone Marrow Cells						
		Total	Neutrophil	GM	Macrophage	Eosinophil	Megakaryocyte	Blast
A. SCF + IL-3	$mpl^{+/+}$	71 ± 15	21 ± 1	13 ± 6	8 ± 3	5 ± 2	18 ± 8	6 ± 2
	$mpl^{+/-}$	60 ± 18	19 ± 5	13 ± 8	6 ± 2	4 ± 1	13 ± 2	5 ± 1
	$mpl^{-/-}$	27 ± 5*	9 ± 2*	6 ± 2	6 ± 3	2 ± 1	3 ± 1*	1 ± 0.5*

Stimulus	Genotype	Total	Myeloid	Erythroid	Megakaryocyte	Blast	Meg/E	Meg/Myeloid	E/Myeloid	Multi
B. SCF + IL-3 + EPO	$mpl^{+/+}$	90 ± 6	50 ± 4	10 ± 2	8 ± 1	8 ± 2	7 ± 2	3 ± 1	2 ± 1	2 ± 1
	$mpl^{+/-}$	88 ± 2	48 ± 1	12 ± 1	7.5 ± 2	7 ± 2	8 ± 1	2 ± 1	1.5 ± 0	2 ± 0
	$mpl^{-/-}$	40 ± 5*	26.5 ± 3*	5.5 ± 2†	3 ± 1*	1 ± 1*	3.5 ± 1*	0 ± 1‡	0.5 ± 0.5‡	0 ± 0.5‡

Mean ± standard deviations of colony numbers in replicate cultures from 4 mice per genotype in (A) and 3 mice per genotype ($mpl^{+/-}$, n = 2) in (B). Cultures were incubated in either 10% CO_2 in air (A) or 5% CO_2 in air (B) for 7 days. Colony numbers and composition were determined by examination of stained cultures at 200× magnification. In (B), myeloid comprises all neutrophil, GM, macrophage, and eosinophil colonies. Statistical comparisons were made with data from $mpl^{+/+}$ mice.

Abbreviations: GM, granulocyte-macrophage; Meg/E, mixed megakaryocyte/erythroid; Multi, mixed granulocyte/erythroid/macrophage/megakaryocyte.
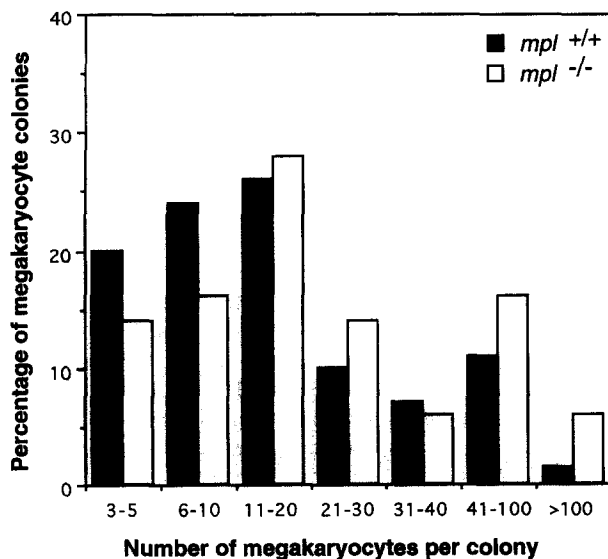
* P < .01.

† P = .07.

‡ P < .05.

The deficiency in progenitor cell numbers was also observed in the spleens of *mpl*-deficient mice. Methylcellulose cultures of 10⁵ spleen cells established to ensure optimal growth of erythroid progenitors showed reductions in pure erythroid ($mpl^{+/+}$, 17 ± 5, n = 2; $mpl^{-/-}$, 3 ± 1, n = 2), mixed erythroid ($mpl^{+/+}$, 17 ± 7; $mpl^{-/-}$, 3 ± 2), and myeloid ($mpl^{+/+}$, 23 ± 11; $mpl^{-/-}$, 4 ± 1) colonies in homozygous mutant mice.

Cultures stimulated by single growth factors confirmed

the deficiency in progenitor cells of multiple hematopoietic lineages in *mpl*-deficient mice (Table 4). Whereas similar numbers of colonies developed from $mpl^{+/+}$ and $mpl^{+/-}$ bone marrow cells in response to granulocyte-CSF (G-CSF), granulocyte-macrophage–CSF (GM-CSF), M-CSF, or SCF, significantly fewer colonies arose from cells of *mpl*-deficient mice.

Hematopoiesis during development in mpl-*deficient mice.* To explore the function of c-Mpl during ontogeny, clonal cultures of liver cells from $mpl^{-/-}$ fetuses at day 12 of gestation were compared with wild-type and heterozygous littermates (Table 5). The cellularity of fetal livers was similar in mice of each genotype and, in contrast to the pattern observed in adult bone marrow and spleen, there were no deficiencies in progenitor cells in $mpl^{-/-}$ animals. This included the total number of progenitor cells and the relative frequencies of unipotential, bipotential, multipotential, and blast cell colonies and pure megakaryocyte-CFC. These results were confirmed in methylcellulose cultures of pure erythroid ($mpl^{+/+}$, 13 ± 2 per 10⁴ cells, n = 2; $mpl^{-/-}$, 18 ± 6,

Fig 2. Size of megakaryocyte colonies cultured in parallel from wild-type (n = 4) and *mpl*-deficient (n = 4) mice using IL-3 and SCF as the stimulus. The histogram plots the distribution of the numbers of megakaryocytes (acetylcholinesterase-staining cells) per colony. The mean number ± standard deviations of megakaryocytes per colony was 20 ± 23 for $mpl^{+/+}$ (n = 71) and 33 ± 43 for $mpl^{-/-}$ (n = 50) colonies (P = .08, Mann-Whitney rank-sum test).

Table 4. Colony Formation Stimulated by Individual Growth Factors in Cultures of Bone Marrow From *mpl* Mutant Mice

Stimulus	Colonies per 2.5 × 10⁴ Bone Marrow Cells		
	$mpl^{+/-}$	$mpl^{+/-}$	$mpl^{-/-}$
G-CSF	7 ± 3	6 ± 3	3.5 ± 1*
GM-CSF	32 ± 6	28 ± 7	19 ± 8†
M-CSF	32 ± 11	27 ± 9	16 ± 7†
SCF	30 ± 6	27 ± 8	13 ± 2†

Mean ± standard deviations of colony numbers from 5 independent experiments ($mpl^{+/+}$, n = 6; $mpl^{+/-}$, n = 4; $mpl^{-/-}$, n = 5). Cultures were incubated in 10% CO_2 in air for 7 days. Statistical comparisons were made with data from $mpl^{+/+}$ mice.

* P < .1.

† P < .05.

Table 5. Fetal Liver Progenitor Cell Analysis of mpl Mutant Mice

Genotype	Cells per Liver ($\times 10^3$)	Progenitor Cells per 10^4 Fetal Liver Cells								
		Total	Myeloid	Erythroid	Megakaryocyte	Blast	Meg/E	Meg/Myeloid	E/Myeloid	Multi
$mpl^{+/+}$	36.0 ± 15	101 ± 4	32 ± 2	27 ± 0	16.5 ± 5	5 ± 0	9 ± 3	2.5 ± 1	3 ± 3	6 ± 0
$mpl^{+/-}$	25.5 ± 12	96 ± 13	31 ± 8	22 ± 5	16 ± 6	6 ± 3	10 ± 1	2 ± 1	5 ± 2	4 ± 1
$mpl^{-/-}$	33.8 ± 11	114 ± 24	42 ± 5	22 ± 9	20 ± 4	6 ± 1	14 ± 3	2 ± 2	2 ± 1	6 ± 1

Mean ± standard deviations of colony numbers grown from liver cells of fetal mice of 12 days gestation. Cultures were stimulated with SCF + IL-3 + EPO for 7 days in a humidified atmosphere of 5% CO_2 in air. There were no statistically significant differences among genotypes in either the number or lineage distribution of progenitor cells. Abbreviations are as in Table 3.

n = 4), mixed erythroid ($mpl^{+/+}$, 10 ± 2; $mpl^{-/-}$, 14 ± 6), and myeloid ($mpl^{+/+}$, 50 ± 5; $mpl^{-/-}$, 67 ± 13) colonies from fetal liver cells. The production of megakaryocytes was nevertheless compromised during fetal hematopoiesis. Histologic sections of fetal livers from $mpl^{-/-}$ mice of 14 days of gestation showed a marked deficiency of mature megakaryocytes (3 ± 2 per 10 high-power fields [hpf]) when compared with sections from littermate $mpl^{+/+}$ (26 ± 3 per 10 hpf) and $mpl^{+/-}$ (24 ± 4 per 10 hpf) mice.

To determine the developmental stage at which c-Mpl deficiency prevents normal hematopoietic progenitor production, we also examined neonatal mice. $mpl^{-/-}$ neonates were severely thrombocytopenic, with platelet counts (51 ± 19 × 10^6/mL, n = 4) approximately 12% of controls ($mpl^{+/+}$ 411 ± 37 × 10^6/mL, n = 2; $mpl^{+/-}$ 406 ± 59 × 10^6/mL, n = 7), although no evidence of hemorrhage associated with birth was noted at autopsy. Histologic examination of liver, spleen, and sternal bone marrow of neonatal $mpl^{-/-}$ mice confirmed the marked reduction in megakaryocyte number that was already evident during fetal development (data not shown). However, in contrast to fetal liver, the bone marrow, spleen, and liver of neonatal $mpl^{-/-}$ mice contained significantly fewer pure megakaryocyte-CFC than $mpl^{+/+}$ and $mpl^{+/-}$ animals (Table 6). The paucity of multilin-

eage colonies that developed precluded assessment of less mature mixed megakaryocytic and multipotential progenitors. However, similar to the pattern observed in adult mice, the numbers of myeloid and erythroid progenitors were also reduced in mpl-deficient animals (Table 6). Thus, although c-Mpl is dispensable for progenitor development during early fetal hematopoiesis, by birth its absence prevents the maintenance of normal progenitor cell numbers.

DISCUSSION

Biologic studies of TPO and its receptor c-Mpl provide important insights into the molecular regulation of megakaryocyte and platelet production. Our analysis of mice genetically manipulated to lack c-Mpl showed an indispensable role for this receptor in steady-state megakaryocytopoiesis. Consistent with initial descriptions of similar mice by Gurney et al,[22] we found dramatically reduced numbers of megakaryocytes in the bone marrow and spleen of adult $mpl^{-/-}$ mice accompanied by a severe thrombocytopenia. We have extended these observations to show that megakaryocyte progenitor cells are also deficient. Colony formation in response to TPO was abolished in cultures of $mpl^{-/-}$ bone marrow. However, the use of alternative stimuli, including IL-3 and SCF, showed that, although megakaryocyte progen-

Table 6. Progenitor Cell Analysis of Neonatal mpl Mutant Mice

Organ	Genotype	Progenitor Cells per 2 × 10^4 Cells								
		Total	Myeloid	Erythroid	Megakaryocyte	Blast	Meg/E	Meg/Myeloid	E/Myeloid	Multi
Bone marrow	$mpl^{+/+}$	47	26.5	9.5	4.5	1	2.5	0	2	1
	$mpl^{+/-}$	55 ± 10	29.5 ± 7	7 ± 1	7 ± 2	1.5 ± 0.5	4 ± 2	0.5 ± 0.5	2.5 ± 1	3 ± 1
	$mpl^{-/-}$	28 ± 4*	15 ± 2.5*	5 ± 1*	2 ± 1*	0 ± 0.5*	3 ± 0.5	0 ± 0.5	1 ± 1†	1 ± 0.5*
Spleen	$mpl^{+/+}$	42	21	11	2.5	1	2.5	0.5	2.5	1
	$mpl^{+/-}$	65.5 ± 23	33 ± 8	8 ± 2	6.5 ± 2	5 ± 2.5	7 ± 5	1 ± 0.5	2 ± 2	3 ± 2.5
	$mpl^{-/-}$	23.5 ± 7*	12.5 ± 4*	4.5 ± 2‡	2 ± 1*	0.5 ± 0.5*	2 ± 2	0.5 ± 0.5	1.5 ± 1	0 ± 0.5
Liver	$mpl^{+/+}$	39.5	21	7	5	2.5	1.5	0	1	1.5
	$mpl^{+/-}$	33 ± 4	17 ± 4	5 ± 1.5	4 ± 1.5	1.5 ± 0.5	2.5 ± 0.5	0.5 ± 0.5	1 ± 1	1.5 ± 0.5
	$mpl^{-/-}$	15 ± 1†	8 ± 2*	3.5 ± 1	1 ± 1*	0.5 ± 1	0.5 ± 0.5*	0.5 ± 0.5	0.5 ± 0.5	0.5 ± 0.5‡

Mean ± standard deviations of colony numbers from a litter of seven mice ($mpl^{+/+}$ n = 1, $mpl^{+/-}$ n = 3, $mpl^{-/-}$ n = 3). Replicate cultures from each mouse were stimulated with SCF + IL-3 + EPO and incubated for 7 days in a humidified atmosphere of 5% CO_2 in air. No statistical comparisons were possible with $mpl^{+/+}$ data; comparisons between $mpl^{+/-}$ and $mpl^{-/-}$ were performed using Student's t-test. Abbreviations are as in Table 3.

* $P < .05$.
† $P < .01$.
‡ $P < .1$.

itor cells were present in *mpl*-deficient animals and capable of yielding colonies in the absence of c-Mpl, their numbers were significantly reduced from those in normal mice. Although no differences in total megakaryocyte progenitor cell number or in the number of mature megakaryocytes or platelets were seen between wild-type and heterozygous $mpl^{+/-}$ mice, a reduction in megakaryocyte colony formation from $mpl^{+/-}$ bone marrow was observed in cultures stimulated solely with TPO. This suggests that cells from heterozygous $mpl^{+/-}$ mice may express reduced levels of c-Mpl, which limits proliferation under these culture conditions, but which appear not to have significant adverse effects in vivo.

Our results clearly support a critical role for c-Mpl in the maintenance of megakaryocyte numbers. Whereas defects both in the production of megakaryocyte progenitor cells and in the formation of mature megakaryocytes coexist in adult *mpl*-deficient mice, the sixfold reduction in progenitor cells is significantly less than the 10- to 20-fold deficiency in mature cells. This suggests that, although c-Mpl has a role in maintaining the progenitor cell pool, its function may be most critical during later stages of megakaryocyte production and maturation. Indeed, in $mpl^{-/-}$ animals, we observed the greatest deficiency among the most mature committed megakaryocyte-CFC of limited proliferative potential. This bias is also reflected in studies that show the greatest impact of TPO in culture is on the development of the most mature megakaryocyte progenitors.[13,15,18]

Despite the dramatic reduction in circulating platelets in *mpl*-deficient mice, c-Mpl signalling may not be directly involved in the late stages of platelet production and release. The magnitude of the platelet deficiency in $mpl^{-/-}$ mice is no greater than that of mature megakaryocytes. Based on these observations, the thrombocytopenia in these animals may be explained most simply by the shortfall in megakaryocytes without the need to invoke an additional defect in platelet production from individual mature cells. Previous studies of the actions of TPO support this contention. TPO does not increase platelet shedding in cultures of mature megakaryocytes[17] and in animals the thrombocytotic effect of TPO injection takes 3 or more days to develop in parallel with the increase in mature megakaryocytes[15] and megakaryocyte-CFC.[20] It is also noteworthy that mice lacking the transcription factor p45 NF-E2 have a near absolute thrombocytopenia without a reduction in megakaryocyte numbers.[39] These animals do not show a compensatory increase in TPO levels that might have been expected if TPO was a major regulator of platelet release.

Despite the defects in megakaryocytopoiesis, $mpl^{-/-}$ mice produce residual numbers of platelets that are sufficient to prevent bleeding and allow normal survival. Thus, although signalling through c-Mpl is a major factor in platelet production, a limited degree of megakaryocyte development and maturation can still proceed in its absence. As our studies with ^{125}I-TPO provided no evidence of specific binding to bone marrow or spleen cells from $mpl^{-/-}$ mice, the residual megakaryocytopoiesis in these animals is unlikely to be mediated by TPO acting through an alternative receptor. It seems likely that one or a combination of other cytokines with more modest effects on megakaryocytopoiesis maintain

the low platelet levels in *mpl*-deficient mice. Blocking the c-Mpl receptor has been reported to eliminate the megakaryocyte stimulatory activity of IL-6, IL-11, and SCF, whereas the activity of IL-3 is at least partially spared.[21] Thus, IL-3 may contribute to the residual megakaryocytopoiesis in $mpl^{-/-}$ mice, although it should be noted that production of this cytokine has not been shown in normal mice.[40] Genetic crosses between *mpl*-deficient mice and similar animals engineered to lack other receptors or cytokines implicated in megakaryocyte development may help dissect the contributions of these other factors to platelet production in vivo. Moreover, although residual steady-state thrombopoiesis is adequate for the health of $mpl^{-/-}$ mice, it is yet to be determined whether these mice are capable of responding to emergency situations requiring rapid platelet production.

The effects of c-Mpl deficiency on megakaryocytopoiesis were observed in neonatal mice and were already manifest during fetal development. By 14 days of gestation, fetal livers of $mpl^{-/-}$ mice showed a reduction in megakaryocytes of a similar magnitude to that observed in neonatal and adult tissues. Intriguingly, signalling through c-Mpl was dispensable for production of fetal liver megakaryocyte progenitor cells, the numbers of which were equivalent in $mpl^{-/-}$ and wild-type littermates. By birth, the deficiency in progenitor cell production that characterizes adult $mpl^{-/-}$ mice was evident. These observations further support the conclusion that the major role of c-Mpl signalling is in production of megakaryocytes and show that this function is required throughout the course of hematopoietic development. The reduction in progenitors of other hematopoietic lineages that typifies neonatal and adult $mpl^{-/-}$ mice (see below) was also not evident in fetal livers. Thus, the mechanisms for regulating the production of both megakaryocytic and other hematopoietic progenitor cells may differ between early and late fetal hematopoiesis, with only the latter developmental stages becoming strictly dependent on c-Mpl.

An important finding of this study is that the hematopoietic defect of $mpl^{-/-}$ mice is not confined to cells of the megakaryocyte lineage. The numbers of neutrophil-, granulocyte-macrophage-, erythroid-, multipotential, and mixed-CFC in *mpl*-deficient mice were at best only 50% of those in wild-type littermates and the deficit was even more substantial for less mature blast cell-CFC. The reduction was not reflected in mature cell populations. The peripheral blood hematocrit, white blood cell, and neutrophil counts, as well as spleen and marrow precursor cell numbers, were normal in $mpl^{-/-}$ mice, suggesting that compensatory mechanisms exist during the final stages of maturation. We have largely excluded the possibility that the reduction in progenitor cell number observed in $mpl^{-/-}$ mice was simply a function of lower production in vitro of ancillary megakaryocyte-derived growth factors. Single stimulus cultures using G-CSF, M-CSF, and SCF, which provided conditions under which megakaryocytes did not survive, confirmed the reduction in $mpl^{-/-}$ progenitor cells observed in cultures stimulated by combinations of IL-3, SCF, and EPO. The precise mechanism by which the absence of Mpl signalling results in a reduction in committed progenitor cells of nonmegakaryocytic potential remains unclear. The effects observed may

be contributed to by the indirect consequences of progenitor cell development in *mpl*$^{-/-}$ mice in the absence of sufficient hematopoietic growth factors normally produced in vivo by megakaryocytes and platelets. Alternatively, there may be a direct requirement for Mpl signalling in early hematopoietic cells. A direct role for c-Mpl in a wide variety of committed progenitor cells seems unlikely because the receptor appears to be expressed in only a small fraction of CD34$^+$ cells.[8] The reduction in progenitor cells of several hematopoietic lineages in *mpl*$^{-/-}$ mice may therefore reflect reduced input from the presence of fewer multipotent ancestors.

Indeed, the deficiency in multipotential progenitors and particularly blast-CFC in *mpl*$^{-/-}$ mice observed in this study suggests an important function for c-Mpl in maintenance of the most primitive hematopoietic cells. Such a role would be consistent with studies that show c-*mpl* expression in hematopoietic populations enriched for immature cells.[6,16] Moreover, the capacity of c-Mpl to signal in primitive cells is confirmed by analyses of mice infected with MPLV, which induces a true myeloproliferative disease involving cells of all lineages.[3,4] It remains to be determined whether *mpl*$^{-/-}$ mice have deficiencies in colony-forming units-spleen (CFU-S) or repopulating stem cells. Direct experiments examining the effects of TPO on the survival, proliferation, and differentiation of purified candidate stem cell populations[16] will also be required to define the role of TPO and c-Mpl in the earliest stages of hematopoiesis.

Although most studies of TPO have reported little if any activity on progenitor cells of nonmegakaryocytic lineages, recent data suggest that, in combination with EPO, TPO may augment growth of erythroid progenitors[41] and cells from several nonmegakaryocytic human leukemias can proliferate in response to TPO.[42] An elevation in the number of mixed granulocyte/erythroid/macrophage/megakaryocyte-CFC (GEMM-CFC) has also been observed in early studies of the effects of TPO upon administration in monkeys.[20] None of the known regulators of hematopoiesis is restricted entirely in its activity to cells of one lineage. The phenotype of *mpl*$^{-/-}$ mice strongly implies a broader role for TPO than its effects on megakaryocytopoiesis, particularly in the maintenance of multipotential progenitor cells as well as those committed to several individual hematopoietic lineages. Because this is a vital consideration in potential therapeutic strategies, it will be important to closely monitor effects on all hematopoietic cells when evaluating TPO administration in vivo.

ACKNOWLEDGMENT

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