Physiological Regulation of Early and Late Stages of Megakaryocytopoiesis by Thrombopoietin

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Summary

Thrombopoietin (TPO) has recently been cloned and shown to regulate megakaryocyte and platelet production by activating the cytokine receptor c-mpl. To determine whether TPO is the only ligand for c-mpl and the major regulator of megakaryocytopoiesis, TPO deficient mice were generated by gene targeting. $TPO^{-/-}$ mice have a >80% decrease in their platelets and megakaryocytes but have normal levels of all the other hematopoietic cell types. A gene dosage effect observed in heterozygous mice suggests that the TPO gene is constitutively expressed and that the circulating TPO level is directly regulated by the platelet mass. Bone marrow from $TPO^{-/-}$ mice have decreased numbers of megakaryocyte-committed progenitors as well as lower ploidy in the megakaryocytes that are present. These results demonstrate that TPO alone is the major physiological regulator of both proliferation and differentiation of hematopoietic progenitor cells into mature megakaryocytes but that TPO is not critical to the final step of platelet production.

latelets are small anucleated cells produced from mega-karyocytes. Normal hemostasis requires the maintenance of an adequate number of circulating platelets in the peripheral blood. Multiple cytokines (IL-1, IL-3, IL-6, IL-11, GM-CSF, and erythropoietin) stimulate megakaryocytopoiesis both in vitro and in vivo (1, 2), but their effect is only modest compared with the recently identified ligand for the protooncogene c-mpl (3–9). This ligand, called thrombopoietin (TPO), is a novel cytokine with a unique structure; the NH_2-terminal domain is homologous to erythropoietin, and the COOH-terminal glycosylated domain is unrelated to any known protein (6–8). In vitro and in vivo experiments with recombinant TPO indicate that it stimulates both megakaryocyte colony formation and megakaryocyte maturation (6, 8–10). TPO stimulates the formation of CFU-megakaryocytes (CFU-megs) both alone and in combination with early acting factors (9, 11). It stimulates the production of megakaryocytes and functional platelets from enriched murine or human stem cell populations (12, 13). Injection of TPO into mice increases platelet counts 4–6-fold and stimulates a 20-fold increase in bone marrow megakaryocytes (7, 9). The importance of c-mpl in the physiological regulation of platelet production in vivo was demonstrated by the generation of mice deficient in c-mpl (14). These mice exhibit an 85% reduction in peripheral platelet counts and in marrow and spleen megakaryocytes. Although these results demonstrate that c-mpl is critical in thrombopoiesis, they do not exclude the possi-

bility that another receptor could be activated by TPO and could therefore be responsible for the remaining platelets in the $c-mpl^{-/-}$ mice. To investigate this and the mechanism of control of megakaryocyte and platelet production by TPO, we have generated mice deficient in TPO by using homologous recombination.

Materials and Methods

Embryonic Stem (ES) Cell–Targeted Mutation. Genomic clones were isolated from a mouse 129 library using the human TPO cDNA as a probe (6). The targeting vector was constructed from a 10-kb XhoI–SacI subclone. The targeting vector (20 µg) was linearized and electroporated at 275 V, 200 µF, using a BTX 300 electroporator (BTX Inc., San Diego, CA) into ES.D3 C-12, a subclone of D3 ES cells (a gift from T. Doetschman, University of Cincinnati, Cincinnati, OH) (15). Cells were subjected to G418 selection at 400 µg/ml for 10 d. Single-colony wells were expanded for DNA isolation and Southern blot analysis, using a murine oligo probe 5′ to the XhoI site on the targeting vector. Five gene-targeted clones were selected to generate chimeric mice by microinjection into the blastocele cavity of 3.5-d C57Bl/6J blastocysts (16). Chimeric males were mated with C57Bl/6J females, and agouti color offspring were screened for germline transmission by PCR analysis for the *neo*ʳ gene and confirmed by Southern blot analysis of tail DNA for the mutant TPO allele. Heterozygous mice were interbred to obtain homozygous animals.

PCR-based Genotyping of TPO-deficient Mice. The presence of the mutated TPO allele was detected using PCR primers to the wild-type allele: p.TPO.SacI (5′-GGTGAATGTAACCTGGGA-

TAA-3′), an oligonucleotide from the fourth intron near the SacI site; and p.TPO.SalI (5′-GTCGACCCTTTGTCTATCCCT-3′), an oligonucleotide in exon 4 upstream of the SalI site. Homologous recombination places the 1.9-kb pgk-Neo gene between this oligo pair. The reaction conditions favor the smaller 330-bp wild-type product such that the predicted 2.1-kb band is not detected. An additional set of oligo primers 5′-CGGTTCTTTTTGTC-AAGAC-3′ and 5′-ATCCTCGCCGTCGGGCATGC-3′ specific for the neo' gene are used in each reaction to detemine heterozygous mice. Each reaction is performed with 1 μg of mouse genomic DNA prepared from tail biopsies using a PCR reaction kit (Perkin-Elmer, Norwalk, CT). Each reaction was analyzed on a 2% agarose TBE gel stained with ethidium bromide.

Northern Blot Analysis. Poly(A)$^+$ RNA was prepared from the livers and kidneys of mice as described (17). 2 μg of liver mRNA or 5 μg of kidney mRNA per lane was resolved on a 1.2% agarose–formaldehyde gel and transferred to a nylon membrane (Genescreen Plus; Du Pont, Wilmington, DE). The membrane was hybridized overnight with a random-primed ^{32}P-labeled cDNA probe containing the entire coding region of murine TPO. The membrane was washed at 42°C in 0.2× SSC/0.1% SDS and exposed overnight to a phosphor imaging plate (Fuji, Stamford, CT).

Ploidy Analysis of Megakaryocytes. A Percoll step gradient (20, 40, 60, and 80%) was used to enrich for megakaryocytes in bone marrow. Aproximately 120 × 10^6 bone marrow cells per 13-ml gradient were spun at 3,000 g for 30 min at 10°C. Cells from the 20/40% interface were collected, washed in HBSS, and stained with anti-CD61 (integrin-β$_3$ chain)-FITC (PharMingen, San Diego, CA) and the DNA intercalating fluorescent dye propidium iodide. The DNA content in CD61$^+$ cells was quantified by two-color flow cytometry (Epics Elite; Coulter Corp., Hialeah, FL).

CFU-meg Assay. Megakaryocyte colony formation was assayed in semisolid agar culture of bone marrow of 6–10-wk-old mice as described (11) in the presence of 100 ng/ml of the various cytokines. After 7 d, the plates were dried and stained for acetylcholinesterase (18). Megakaryocyte colonies were defined as stained colonies containing three or more cells.

Results and Discussion

A targeting vector containing a 10-kb TPO mouse genomic clone (Fig. 1 *A*) was electroporated into ES cells. The construct was designed to remove 23 amino acids of the third coding exon via insertion of a neo' cassette. Deletion of this fragment located in the erythropoietin (EPO) homology domain that is responsible for receptor binding and activation (6) should ensure that homologous recombination will lead to a nonfunctional TPO gene. Gene targeting was detected in 1 of 80 ES colonies screened, and 5 colonies were selected for microinjection into blastocysts (Fig. 1 *B*). All five clones gave germ line transmission, and three separate lines were interbred to generate homozygous gene-targeted mice (TPO$^{-/-}$). The litters generated had a normal Mendelian distribution of the three genotypes, indicating viable fetal development of the TPO$^{-/-}$ mice. The adult mice were healthy and displayed no overt abnormalities. Expression of TPO mRNA in the liver and kidney was assessed by Northern blot analysis (Fig. 1 *C*). A 1.8-kb band can be detected in both tissues in TPO$^{+/+}$ mice. The band intensity is reduced by half in the heterozygotes, as measured by quantification of the radioactivity present in each band using a PhosphoImager (model BAS2000; Fuji, Stamford, CT). TPO mRNA is undetectable in the liver and kidney of TPO$^{-/-}$ animals.
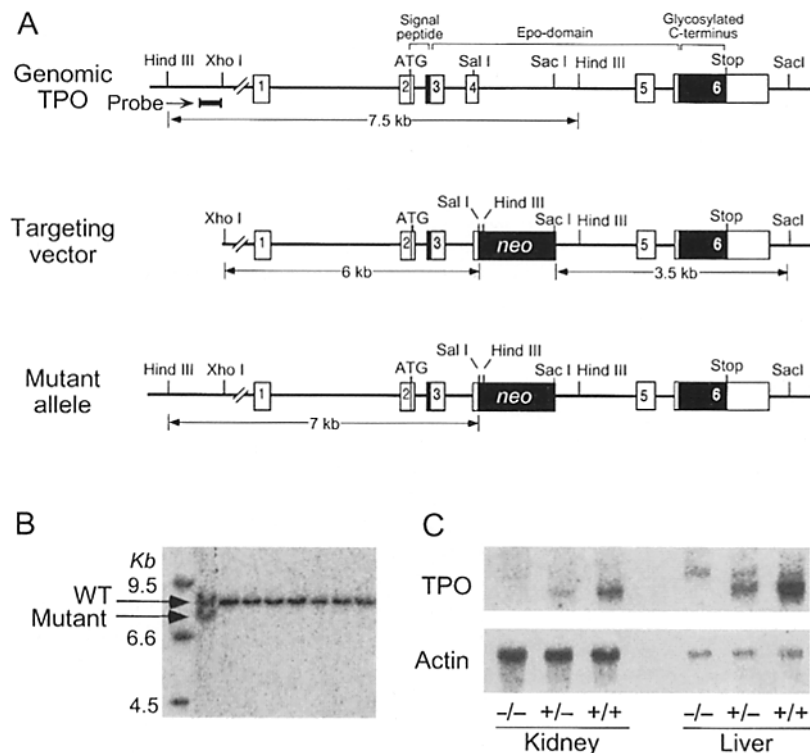

Figure 1. Targeting of the TPO gene by homologous recombination. (*A*) Structure of the TPO gene (19) and gene-targeting vector. The restriction enzyme sites are those from the mouse, whereas the intron exon organization is taken from the human. A neo' gene under control of the Pgk promoter (19) was inserted between the SalI site in the third exon of TPO and the XhoI site present in the following intron. An oligonucleotide probe was designed from sequences 5′ to those present on the targeting vector. After homologous recombination, the mutated allele was detected by the addition of a HindIII site from the neo' gene. (*B*) Targeting of the TPO gene in ES cells. (*C*) Northern blot analysis of TPO expression in c-mpl$^{-/-}$, TPO$^{-/-}$, TPO$^{-/+}$, and wild-type mice. RNA was prepared from the livers and kidney of the indicated mice, separated on agarose–formaldehyde gels, transferred to a nylon membrane, and hybridized with a TPO DNA probe.

Complete blood cell counts performed on TPO$^{-/-}$ and TPO$^{+/+}$ mice revealed an 88% drop in platelet counts in the gene-targeted animals, with 100% penetrance in mice derived from three independent ES cell clones (p <0.0001) (Fig. 2 A). There was also a significant increase in volume of the remaining platelets, with a mean of 8.7 cubic micrometers for TPO$^{-/-}$ mice compared with 4.7 cubic micrometers for TPO$^{+/+}$ mice (p <0.0001). These findings are similar to those observed in the c-$mpl^{-/-}$ mice (14). However, a significant alteration in platelet counts (67% of wild type) was observed in TPO heterozygous mice. These data suggest that the TPO$^{-/-}$ mice are unable to produce normal levels of megakaryocytes, and therefore platelets, because of the loss of a functional TPO gene, and that even the loss of one allele affects platelet levels. We previously detected an increased level of TPO activity in the serum of c-mpl$^{-/-}$ mice that was comparable to levels present in aplastic porcine plasma (14). No increase in IL-3, IL-6, or GM-CSF, which are other cytokines with a weak mega-karyocytopoietic ability, could be detected by ELISA-based assays. These data suggest that in response to thrombocytopenia, TPO levels are upregulated. However, the gene dosage effect in TPO$^{+/-}$ mice would argue against mechanisms involving regulation of TPO production. Northern blot analysis of TPO$^{-/-}$, TPO$^{+/-}$, and wild-type mice confirms that there is no regulation of TPO mRNA expression, as the heterozygous mice had reduced mRNA levels. It was unexpected that the regulator of platelet production, which shares many similarities with erythropoietin, appears to be constitutively expressed and is not regulated at the transcriptional level in response to a platelet sensing mechanism. This system would have been similar to the sensing mechanism by which the kidney controls erythrocyte production through transcription of the erythropoietin gene (20). Instead, our results support the concept of circulating levels of TPO being directly regulated by platelet mass (21). When platelet levels are normal, they will bind and remove from the circulation TPO released by the liver and/or the kidney. In thrombocytopenic situations, the circulating concentration of TPO will therefore increase and stimulate platelet production by bone marrow megakaryocytes. This regulatory mechanism whereby the ligand availability is controlled by the mature cell could be similar to the way the G-CSF level is regulated in neutropenic situations (22). A gene dosage effect is also observed in G-CSF$^{+/-}$ mice, where the neutrophil counts of the G-CSF$^{+/-}$ mice are 67% of the G-CSF$^{+/+}$ (23). The observed gene dosage effect suggests that mild congenital thrombocytopenias may be caused by mutation of a single allele of the TPO gene.

Interestingly, the TPO$^{-/-}$ animals are not completely devoid of platelets, which suggests that although c-mpl/TPO activation is the key regulator of megakaryocyte and platelet production, alternative mechanisms may be triggered in the c-mpl$^{-/-}$ or TPO$^{-/-}$ mice to promote mega-karyocytopoiesis. Histopathology of paraffin-embedded sternums also demonstrated a major loss of megakaryocytes in the bone marrow of TPO$^{-/-}$ mice (<10% of control)
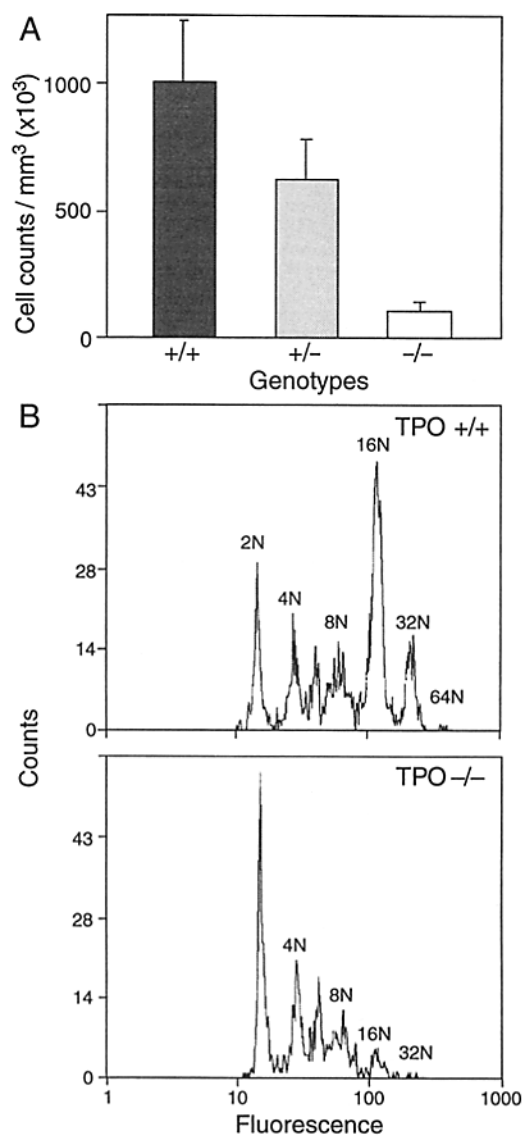

Figure 2. Platelet and megakaryocyte analysis in TPO$^{-/-}$ mice. (A) Blood was collected by retroorbital venous puncture and analyzed in a hematology analyzer (System 9000 Diff; Serono-Baker Diagnostics, Allentown, PA) to determine platelet counts. n = 5 mice per group. (B) Ploidy analysis of bone marrow megakaryocytes. A Percoll step gradient (20, 40, 60, and 80%) was used to enrich for megakaryocytes in bone marrow. Cells from the 20/40% interface were stained with anti-CD61 (integrin-β_3 chain)-FITC and the DNA intercalating fluorescent dye propidium iodide. The DNA content in CD61$^+$ cells was quantified by two-color flow cytometry.

and heterozygous mice (60% of control). Ploidy of bone marrow megakaryocytes was measured to identify qualitative differences between the megakaryocytes present in the TPO$^{-/-}$ compared with normal mice (Fig. 2 B). These data indicate a 10-fold drop in the 32N megakaryocytes and a 5-fold drop in the 16N megakaryocytes. Thus there is a reduction not only in the absolute number of megakaryocytes but also in their maturity as measured by their DNA content. This result demonstrates that TPO regulates the maturation of megakaryocytes at the physiological
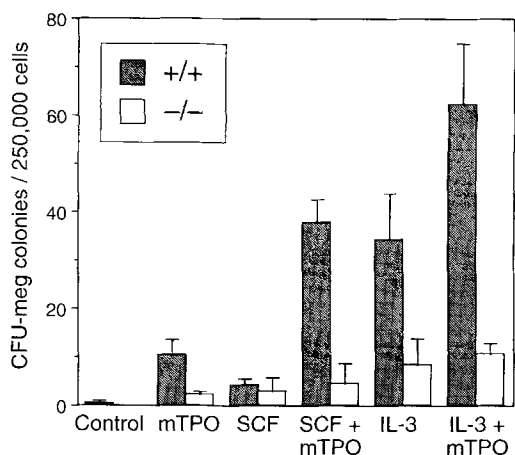
Figure 3. Effect of TPO, SCF, and IL-3 alone or in combinations on CFU-Meg colonies. Megakaryocyte colony formation was assayed in semisolid agar culture of bone marrow of 6–10-wk-old mice as described (8) in the presence of 100 ng/ml of the various cytokines. After 7 d, the plates were dried and stained for acetylcholinesterase (18). Megakaryocyte colonies were defined as stained colonies containing three or more cells.

level. There was no significant difference in red blood cells, total white blood cells, neutrophils, bands, and eosinophils in peripheral blood between $TPO^{+/+}$ and $TPO^{-/-}$ mice, as determined by differential cell counts. The size and cellularity of lymphoid organs were also unaffected. Thus, disruption of the TPO gene results in a highly specific loss of both megakaryocytes and platelets, leaving other cell lineages unaffected.

To determine whether TPO affects an early or late stage of megakaryocyte development, we tested the physiological influence of TPO on the number of bone marrow megakaryocyte progenitor cells by establishing CFU-meg cultures from $TPO^{+/+}$ and $TPO^{-/-}$ mice in serum containing semisolid media in the presence of TPO, stem cell factor (SCF), and IL-3 (Fig. 3). Analysis of the number of CFU-meg colonies after acetylcholinesterase staining indicates that murine TPO supports the growth of CFU-megs alone, synergizes with SCF, and is additive with IL-3 as described (11). However, the number of colonies present in the cultures from $TPO^{-/-}$ cells were greatly reduced com-

pared with the wild type (mTPO, 10 ± 3 vs. 2 ± 0.5; mTPO + SCF, 38 ± 5 vs. 5 ± 4). This effect was also observed in CFU-meg cultures stimulated by IL-3 (34 ± 10 vs. 8 ± 6), indicating that IL-3 does not stimulate a population of progenitor cells independent of TPO. The involvement of TPO and c-mpl at an early stage of megakaryocytopoiesis as indicated by the reduction in CFU-meg progenitors present in the bone marrow of $TPO^{-/-}$ mice is consistent with the detection of c-mpl expression in $AA4^{+}$ Sca^{+} murine stem cell population (12).

To determine whether it is possible to rescue the mutant phenotype, platelet production in response to TPO was compared in $c\text{-}mpl^{-/-}$ or $TPO^{-/-}$ and wild-type mice. The TPO effect is completely missing in the $c\text{-}mpl^{-/-}$ mice, confirming that this receptor has been properly disrupted on a functional level and that other receptors do not exist that respond to TPO (data not shown). The $TPO^{-/-}$ and $TPO^{-/+}$ mice do respond to TPO in a similar fashion to wild-type mice, giving a fivefold increase in platelets (Fig. 4). It takes 12 d of TPO treatment for $TPO^{-/-}$ mice to reach normal levels, which is probably due to the lower initial starting population of megakaryocyte progenitors. The platelet levels in $TPO^{-/-}$ mice eventually surpass untreated wild-type levels (Fig. 4), indicating that the protein replacement therapy using TPO can correct the engineered genetic defect.

These results, combined with those observed in the $c\text{-}mpl^{-/-}$ mice, demonstrate that there is no redundancy in the TPO/mpl system where a single ligand mediates its effect through a unique receptor. The thrombocytopenia observed in the $TPO^{-/-}$ mice indicates that TPO is the major physiological regulator of megakaryocytopoiesis. The phenotype of the $TPO^{-/-}$ mice provides direct evidence of the dual function of thrombopoietin at the physiological level. TPO can act both as an early acting factor and as a late maturation factor, as indicated by ploidy analysis of bone marrow megakaryocytes and CFU-meg assays. Furthermore, the 80–90% reduction in megakaryocyte numbers in the $TPO^{-/-}$ and the 40% reduction in the $TPO^{+/-}$ mice lead to a proportional reduction in the number of circulating platelets. These results suggest that TPO is predominantly involved in megakaryocytopoiesis and not in
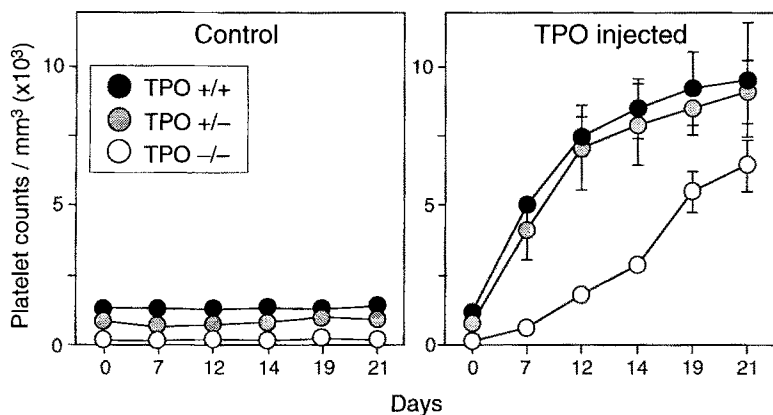

Figure 4. $TPO^{-/-}$, $TPO^{+/-}$, or wild-type mice were injected subcutaneously with either PBS or 1 µg TPO twice daily for 14 d. Blood samples were taken by retroorbital venous puncture on days 0, 7, 12, 14, 19, and 21 and were analyzed in a hematology analyzer. $n = 6$ mice per group. Data are indicated as platelet number.

the latter stages of platelet formation. Treatment of wild-type mice with pharmacological doses of TPO causes a 20-fold increase in megakaryocytes but only a 5-fold increase in platelets (7, 9), suggesting that other mechanisms or factor(s) may be required for platelet formation and are now limiting even in the presence of elevated TPO. Targeted disruption of the p45 NF-E2 gene (24) also indicates that

TPO may not be required for the terminal differentiation of megakaryocytes and platelet formation. These mice have a fully functional TPO/mpl system and have normal megakaryocyte levels. However, these megakaryocytes show no cytoplasmic platelet formation. Availability of these models constitutes a powerful tool to dissect the molecular mechanisms of megakaryocytopoiesis and platelet formation.

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