Studies of the c-Mpl Thrombopoietin Receptor through Gene Disruption and Activation

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Abstract. The c-*mpl* gene encodes a receptor for thrombopoietin (TPO), a cytokine that potently stimulates megakaryocytopoiesis. To study the mechanisms of c-Mpl activation, we generated constitutively active receptor mutants. Substitution of cysteine residues into a dimer interface homology domain of c-Mpl forced ligand-independent homodimerization and constitutive receptor activation. In factor-dependent cells, mutant receptors induced autonomous growth and tumorigenicity. The receptors were constitutively phosphorylated in these cells, as were signal transduction molecules implicated in Mpl function. These data suggest that the normal process of ligand-induced Mpl activation involves receptor homodimerization and that mutated forms of the cellular *mpl* gene can contribute to tumorigenicity. We have also examined the biological role of c-Mpl in *mpl*-deficient mice generated via homologous recombination in embryonic stem cells. Homozygous mutant animals were deficient in megakaryocytes and severely thrombocytopenic. Mature cells from all other hemopoietic lineages were unaffected. Bone marrow cells from *mpl*$^{-/-}$ mice were incapable of binding to TPO or responding to the cytokine in clonogenic assays, and further displayed a marked deficiency in progenitor cells capable of megakaryocyte colony formation in response to other stimuli. Moreover, total progenitor numbers were also deficient and included significant reductions in colony-forming cells of multiple hemopoietic lineages. Unexpectedly, the numbers of progenitor cells of all lineages were not perturbed in mid-gestation *mpl*$^{-/-}$ fetal liver. Our analyses suggest an indispensable role for c-Mpl in megakaryocyte development and reveal that the function of TPO and its receptor is not confined solely to activities in megakaryocytopoiesis.
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Introduction

The c-*mpl* gene encodes a cell surface receptor that belongs to the hemopoietin receptor superfamily, members of which transduce signals from a diverse range of predominantly hemopoietic growth factors and cytokines. Hemopoietin receptors are characterized by a conserved extracellular hemopoietin domain which is defined by the presence of two pairs of evenly spaced cysteine residues, a series of alternating hydrophobic and charged amino acids and the hallmark Trp-Ser-Xaa-Trp-Ser (WSXWS) motif [1, 2]. Hemopoietin receptors lack any known intrinsic catalytic activity, although their intracellular domains share regions of homology that are essential for normal receptor function and are likely to represent sites for interaction with components of the cellular signal transduction machinery [3].

Compelling evidence supports a pivotal role for the c-Mpl receptor in hemopoiesis. The receptor was first isolated as a fusion protein with retroviral *env* sequences, the product of the v-*mpl* oncogene of the myeloproliferative leukemia virus (MPLV) [4]. In mice, MPLV induces a lethal myeloproliferative disease involving the rapid emergence of factor-independent hemopoietic progenitor cells and an acute leukemia of multiple hemopoietic lineages [4-6]. c-*mpl* expression is restricted almost exclusively to hemopoietic tissues, where it is found predominantly in primitive cells, megakaryocytes and

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platelets [4, 7-9]. Consistent with this expression pattern, a role for c-Mpl in megakaryocyte development was first suggested by experiments demonstrating that antisense oligonucleotides to c-*mpl* that reduced receptor expression specifically inhibited megakaryocyte colony formation from $CD34^+$ cells [8]. This role was conclusively established with the discovery that the ligand for c-Mpl is thrombopoietin (TPO), also referred to as Mpl ligand or megakaryocyte growth and development factor. TPO was originally described as an activity in thrombocytopenic serum that was capable of elevating platelet numbers in recipient animals [10]. The molecular cloning of this factor relied upon its interaction with c-Mpl and its capacity to stimulate cells engineered to express the receptor [11-13]. Studies with purified or recombinant TPO have begun to define its potent megakaryocytopoietic activities. Acting alone or in combination with other cytokines, TPO is a colony-stimulating factor in vitro, promoting proliferation of megakaryocyte progenitor cells [14-17]. It can also stimulate maturation, inducing differentiation of large, polyploid megakaryocytes [14, 17, 18]. Similarly, in animals TPO induces megakaryocyte progenitor production, elevates megakaryocyte numbers in the hemopoietic organs and significantly increases the number of circulating platelets [13, 17, 19, 20]. To date, most studies have concentrated on the role of TPO in megakaryocytopoiesis with little activity reported on cells of other hemopoietic lineages.

To help define the biochemical mechanisms of c-Mpl activation and to study the biology of c-Mpl function, we have undertaken experiments to genetically manipulate receptor activity. This paper summarizes our studies of *mpl* mutants engineered to display constitutive receptor activity and describes the biological consequences of preventing c-Mpl expression in mice via gene knockout techniques.

Constitutive Activation of c-Mpl by Mutation of a Conserved Dimer Interface Domain

To study the biochemical mechanisms of c-Mpl activation, we have generated mutants targeting domains that may be involved in receptor aggregation [21]. Within the hemopoietin receptor family, subunit oligomerization is a common characteristic of ligand-induced receptor activation. While hetero-oligomerization of ligand-specific α chains with shared signal-transducing β chains characterizes the functional receptors for many hemopoietin receptors [22, 23], a distinct subset forms homodimers upon interaction with their cognate ligand. The receptors for G-CSF, prolactin and growth hormone (GH) typify this group [24-28]. The erythropoietin receptor (EPOR) appears also to be activated in this manner. Mutations which force EPOR dimerization constitutively activate receptor function with the mutant receptors no longer requiring ligand to stimulate cell growth [29-31]. Determination of the crystal structure of the GH-GH receptor complex identified a region of the hemopoietin domain of the receptor that contributes to the stabilization of receptor dimers through intermolecular hydrogen bonds and salt bridges [32]. Significantly, this domain aligns with the region of the EPOR sensitive to mutational activation [31]. Both regions fall within a loop connecting the predicted a′ and b′ β-strands [32] of the membrane proximal subdomain of the hemopoietin domain. Thus a structurally conserved dimer interface region appears to exist within these receptors.

To explore whether activation of c-Mpl involved an analogous mechanism, potential dimer interface regions in each of the two Mpl hemopoietin domains were identified by amino acid alignments between c-Mpl and the receptors for GH and EPO [21]. By analogy with the activating EPOR mutations, we reasoned that if Mpl activation involved aggregation mediated by these domains, the substitution of cysteine residues to permit disulfide-linked dimerization might confer constitutive receptor activity. Accordingly, two residues in each of the N-terminal (Arginine-117 and Serine-120) and membrane-proximal (Serine-368 and Serine-369) dimer interface homology domains of c-Mpl were individually changed by site-directed mutagenesis to encode cysteine residues [21].

To assess the activity of the mutant receptors, each mutant cDNA was incorporated into the LXSN retroviral vector [33] for expression in the factor-dependent hemopoietic cell lines FDC-P1 and Ba/F3. These cells are strictly dependent upon interleukin 3 (IL-3) for survival and growth but can be converted to responsiveness to TPO by exogenous expression of c-Mpl. Each of the Mpl dimer interface mutants was appropriately expressed and transported to

the cell surface because Ba/F3 or FDC-P1 cells infected with retroviruses containing the normal or mutant *mpl* cDNAs all proliferated in response to TPO. However, in the absence of exogenous factors, only cells expressing mutant Mpl receptors were capable of growth. Mutants of the membrane-proximal dimer interface homology domain most efficiently induced factor independence: 50% of Ba/F3-*mpl*S368C cells capable of responding to TPO were autonomous, as were 7% of TPO-responsive Ba/F3-*mpl*S369C cells. Mutations in the more N-terminal domain were less effective. Only sporadic cells expressing MplR117C were factor-independent and no autonomous cells arose from *mpl*S120C virus-infected populations [21].

Factor-Independent Growth and Tumorigenicity of Cells Expressing MplS368C

To examine the nature of factor-independent growth induced by the constitutively active c-Mpl receptors, autonomous colonies were selected from primary cultures of *mpl*S368C-infected cells for expansion in liquid medium. All colonies picked from such Ba/F3 and FDC-P1 cultures continued to proliferate in the absence of exogenous factors, and the presence and appropriate transcription of *mpl*S368C proviruses in these clones were confirmed in genomic DNA and Northern blot analyses [21].

In agar cultures lacking added factors, a cloning efficiency of 65%-85% for FDC-P1-*mpl*S368C clones and 30%-50% for their Ba/F3 counterparts was observed. The cloning efficiency for all clones proved independent of the number of cells plated and was not influenced by the addition of maximal concentrations of IL-3 or TPO to the cultures. Moreover, the medium conditioned by the growth of these clones contained no factors capable of stimulating proliferation of Ba/F3 cells expressing normal Mpl receptors [21]. Thus the factor-independent growth of cells expressing MplS368C is established in the absence of any apparent autocrine mechanism, consistent with a constitutive proliferative signal emanating directly from the mutated receptor.

The c-*mpl* gene was originally isolated as a potent retroviral oncogene [4-6]. It is therefore noteworthy that in addition to the induction of factor-independent growth, expression of the constitutive *mpl*S368C mutant also induced tumorigenicity. Unlike parental cells or cells expressing the normal Mpl receptor, the subcutaneous injection of FDC-P1- or Ba/F3-*mpl*S368C clones rapidly produced tumors in syngeneic mice. A proviral integration pattern identical to the injected cells was observed in Southern blots of DNA derived from tumor samples [21]. Although the c-*mpl* gene has not been found to be consistently overexpressed in human leukemias [34], the tumorigenicity of constitutively active mutants raises the prospect that more subtle genetic alterations to c-*mpl* may contribute to neoplasia.

Biochemistry of Constitutive MplS368C Activity

Biochemical examination of receptor structure in cells expressing the mutant receptors supported the premise that constitutive activity was induced by receptor homodimerization. Ba/F3 cells were derived that expressed wild-type or mutant MplS368C receptors containing an epitope tag at the N-terminus of the mature protein. Using an antibody to this epitope, receptor protein was purified from cell lysates and examined in Western blots using the same antibody. A protein consistent with the size of c-Mpl, and absent in parental Ba/F3 cells, was abundant in cells expressing either wild-type or MplS368C receptors. Moreover, significant levels of protein at a molecular weight twice that of c-Mpl were detected specifically in Ba/F3-*mpl*S368C cells under nonreducing conditions. This species reduced to monomeric size upon treatment with 2-mercaptoethanol (2-ME) consistent with it representing disulfide-linked MplS368C homodimers [21]. Moreover, while factor-independent proliferation of FDC-P1- or Ba/F3-*mpl*S368C cells was inhibited in culture by reducing agents such as 2-ME, α-monothioglycerol or glutathione, in the presence of TPO, when the cells no longer depend on constitutive receptor activity, these agents were ineffectual at the same concentrations [21]. These results confirm not only the formation of disulfide-bonded MplS368C dimers, but further emphasize that this dimerization is critical for constitutive MplS368C activity.

The autonomous growth induced by MplS368C receptors appeared to be mediated by constitutive activation of similar signal transduction pathways normally associated with Mpl activity. As with other members of the hemopoietin receptor family, proteins in the Janus kinase (Jak) and signal transducers and activators

of transcription (STAT) families have been strongly implicated in c-Mpl signal transduction [35]. Consistent with the results of others [36, 37], upon stimulation of Ba/F3 cells expressing the wild-type Mpl receptor with TPO, we observed rapid phosphorylation of c-Mpl, Jak2 and STAT-3, as well as association of Jak2 with the receptor. In contrast, in Ba/F3-*mpl*S368C cells, phosphorylation of the receptor, Jak2 and STAT-3, and the formation of the Jak2-receptor complex, were independent of TPO, being evident in unstimulated cells [*A.B. Maurer* and *W.S. Alexander*, unpublished results]. These results substantiate the involvement of these molecules in c-Mpl signal transduction and reveal that the constitutive activation of MplS368C by disulfide-bonded dimerization mimics normal ligand-stimulated receptor activation.

Our demonstration of the constitutive activity of disulfide-bonded MplS368C receptors provides compelling evidence that the normal process of TPO-mediated c-Mpl activation also involves receptor dimerization mediated by a dimer interface region that is structurally conserved within the hemopoietin domains of the EPO, GH and c-Mpl receptors (Fig. 1). Given that the hemopoietin domain is a feature of all members of this receptor family, the analogous regions of other receptors may also mediate the subunit interactions that characterize their activation. Moreover, since inactivating mutations that abolish receptor dimerization have been described within this domain of the GHR [38], the dimer interface region may also provide a target for strategies designed to prevent hemopoietin receptor function.

Mpl-Deficient Mice

In the analysis of gene function, a powerful complement to gain-of-function studies, such as our examination of constitutive *mpl* mutants, is to evaluate the consequences of gene ablation. To help define in vivo the role

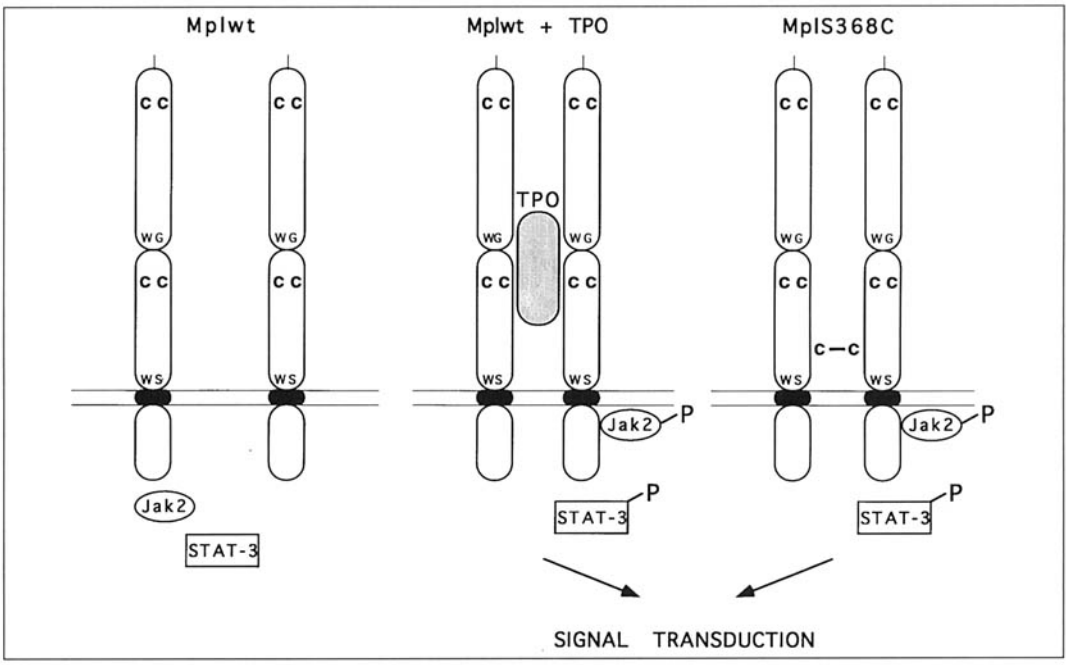

Figure 1. Model of c-Mpl activation. *The substitution of cysteine residues within a dimer interface homology region of c-Mpl induces ligand-independent, disulfide-linked homodimerization of* Mpl*S368C receptors allowing constitutive activation of signal transduction. This implies that wild-type* Mpl *receptors, which are inactive in the absence of ligand (TPO), are activated by TPO-induced dimerization which is also mediated by this dimer interface domain.*

of c-Mpl in hemopoiesis and development, we have exploited homologous recombination in embryonic stem (ES) cells to generate mice unable to express the receptor [39]. A targeting vector was constructed in which a 2600 nucleotide fragment containing exons 1 to 5 was excised from a c-*mpl* genomic clone and replaced with a cassette including the selectable neomycin gene under the control of the phosphoglycerate kinase promoter. The vector was designed such that its homologous recombination with the endogenous c-*mpl* gene yields a targeted allele lacking both the site for RNA transcription initiation and the protein initiation ATG codon, as well as sequence encoding the Mpl receptor signal peptide and first hemopoietin domain. Of 384 G418-resistant clones derived by electroporation of the targeting vector into W9.5 ES cells [40], Southern blot analysis confirmed that 15 (3.9%) had undergone homologous recombination at the c-*mpl* locus. Chimeric mice were generated from several of these clones by injection into blastocysts and animals were identified that transmitted the disrupted *mpl* allele through the germline. The absence of c-Mpl did not prevent normal survival during gestation and weaning as a normal Mendelian ratio was observed for segregation of the mutant *mpl* allele in offspring from mice heterozygous for the disrupted allele ($mpl^{+/-}$). Homozygous mutant ($mpl^{-/-}$) mice also remained clinically healthy as adults. No abnormal bleeding occurred and no abnormalities were observed in histological sections of liver, heart, lung, thymus, kidney, intestine and reproductive organs [39].

Ligand binding experiments with radiolabeled TPO confirmed the absence of Mpl expression in $mpl^{-/-}$ animals. Although Mpl receptors on bone marrow and spleen cells from wild-type ($mpl^{+/+}$) and heterozygous littermates were readily detected, no significant binding of ^{125}I-TPO was observed to cells from $mpl^{-/-}$ mice [39]. While stimulation of wild-type bone marrow cells yielded the expected number of small, purely megakaryocytic colonies in agar cultures, the absence of c-Mpl expression in $mpl^{-/-}$ mice was further confirmed by the complete failure of cells from homozygous mutant mice to respond to TPO in this assay [39].

Megakaryocytopoiesis in mpl$^{-/-}$ *Mice*

The phenotype of $mpl^{-/-}$ mice reflected the known function of c-Mpl as the TPO receptor and demonstrated that Mpl signaling is indispensable for normal megakaryocytopoiesis. Consistent with a previous report of mice lacking c-*mpl* [41], our *mpl*-deficient animals were severely thrombocytopenic. Examination of the peripheral blood revealed that only 6% the number of platelets observed in wild-type or heterozygous littermates were circulating in $mpl^{-/-}$ mice (Fig. 2). This shortfall paralleled a similar 10- to 20-fold reduction in mature megakaryocytes that was observed in histological sections of $mpl^{-/-}$ bone marrow and spleen [39]. Among mature hemopoietic cells, the absence of c-Mpl appeared to prevent specifically the development of the megakaryocyte lineage. The hematocrit, total white cell count (Fig. 2) and numbers of circulating lymphocytes, monocytes, neutrophils and eosinophils in the blood of $mpl^{-/-}$ mice were normal [39]. Similarly, the number and distribution of immature hemopoietic cells in preparations of $mpl^{-/-}$ bone marrow and spleen cells were not perturbed. The patterns of bone

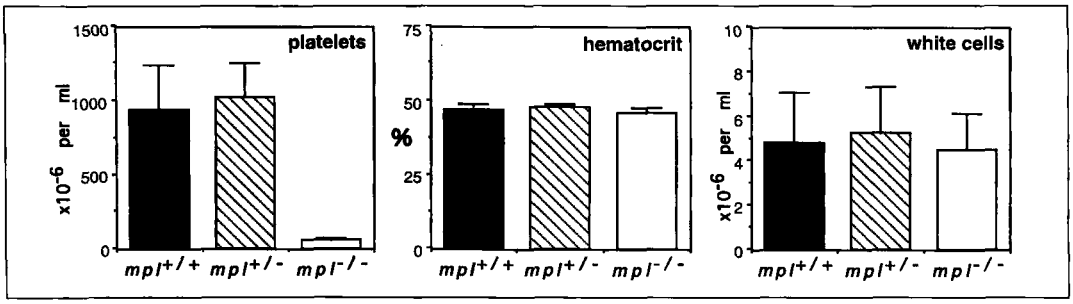

Figure 2. Thrombocytopenia in mice lacking c-Mpl. *Peripheral blood profile showing dramatically reduced platelet numbers in homozygous* mpl *mutant* (mpl$^{-/-}$) *animals in comparison to wild-type* (mpl$^{+/+}$) *or heterozygous* (mpl$^{+/-}$) *littermates. The hematocrit and white cell counts are normal.*

marrow cells and splenocytes expressing the Mac-1, Gr-1, or F4/80 myeloid, the B220 or IgM B-lymphoid, the CD4 or CD8 T-lymphoid, or the Ter-119 erythroid cell surface markers were also similar in flow cytometric analyses of wild-type and *mpl* mutant littermates. In similar experiments, thymocyte subpopulations expressing combinations of Thy-1, CD4, CD8 or products of the T cell receptor loci were also normal [39].

To determine the physiological basis of the megakaryocytopoietic defect in *mpl*$^{-/-}$ mice, clonogenic bone marrow cultures were used to analyze megakaryocyte progenitor cells. A reduction of sixfold in the total number of progenitor cells with megakaryocytic potential was revealed in *mpl*-deficient animals using factor combinations including IL-3, stem cell factor (SCF) and EPO. This included reductions in both unilineage and bipotential progenitor cells as well as cells capable of generating multilineage colonies containing megakaryocytes [39]. The reduction in megakaryocyte progenitor cells in *mpl*-deficient mice reveals a role for Mpl in progenitor cell development but was significantly less than the shortfall in mature cells. Thus the function of c-Mpl may be most critical during the later stages of megakaryocyte production. Indeed, cultures of *mpl*$^{-/-}$ marrow tended to yield larger megakaryocyte colonies than those from wild-type mice [39] consistent with a more profound defect among the mature *mpl*$^{-/-}$ progenitor cells of limited proliferative potential. Consistent with this observation, studies in vitro suggest the predominant target for TPO action are the most mature megakaryocyte progenitors [15, 17, 18].

Studies of megakaryocytes in culture suggest that TPO does not elevate platelet shedding [42] and in vivo the thrombopoiesis resulting from TPO injection is delayed to coincide with the elevation in megakaryocytes and their progenitors [17, 20]. The phenotype of *mpl*$^{-/-}$ mice supports the notion that Mpl signaling may not be directly involved in platelet release. As platelet levels are reduced in *mpl*-deficient animals to a similar degree observed for mature megakaryocytes, the thrombopoiesis in these animals can be accounted for by the shortfall in megakaryocytes without the need to invoke a concomitant defect in platelet release. It is also noteworthy that although signaling through c-Mpl makes a substantial contribution to platelet production, residual megakaryocyte development and maturation can still occur in its absence: *mpl*$^{-/-}$ mice generate sufficient platelets to prevent bleeding and maintain normal health. An attractive hypothesis is that alternative cytokines to TPO provide the residual megakaryocytopoietic stimulus required to maintain the platelet levels observed in *mpl*$^{-/-}$ mice. To address this issue, we are currently investigating genetic crosses between *mpl*$^{-/-}$ mice and mice lacking other receptors or cytokines implicated in megakaryocytopoiesis, as well as assaying the effects of administering such cytokines to *mpl*-deficient mice.

An unexpected finding was that although deficient megakaryocytopoiesis was already evident during fetal development in *mpl*$^{-/-}$ mice—megakaryocyte numbers in fetal liver were reduced to a similar extent as in adult tissues—the numbers of megakaryocyte progenitor cells were normal. By birth, however, the progenitor cell deficiency typical of adult *mpl*$^{-/-}$ mice was evident [39]. Thus, although the production of mature megakaryocytes requires c-Mpl throughout development, the mechanisms regulating the production of progenitor cells may alter during fetal hemopoiesis with only the latter stages becoming strictly dependent on c-Mpl.

Deficiencies in Progenitor Cells of Multiple Hemopoietic Lineages in mpl$^{-/-}$ *Mice*

Significantly, although the profile of mature hemopoietic cells in *mpl*$^{-/-}$ mice reflected a selective deficit in megakaryocytopoiesis, the deficiency in progenitor cells was not restricted to this lineage. Agar cultures stimulated with IL-3, SCF and EPO revealed that the total number of hemopoietic progenitors in the bone marrow of *mpl*$^{-/-}$ mice was only 40% that of normal animals. The numbers of neutrophil, granulocyte-macrophage, erythroid, bipotential and mixed myeloid progenitor cells were all reduced [39]. As the levels of mature cells in these lineages are normal in *mpl*$^{-/-}$ mice, compensatory mechanisms must exist during later stages of differentiation to overcome the progenitor cell deficiency. The pattern of progenitor cell development for these additional lineages during hemopoietic ontogeny in *mpl*$^{-/-}$ mice mirrored that of megakaryocyte progenitors. Deficiencies were not observed in early fetal hemopoiesis but became evident in analyses of newborn mice [39].

We have not formally distinguished whether the reduction in progenitor cells of multiple hemopoietic lineages in *mpl*$^{-/-}$ mice reflects direct or indirect consequences of the absence of c-Mpl. The deficiency was confirmed in single stimulus cultures using G-CSF, M-CSF or SCF [39], which do not support megakaryocyte development. It is therefore unlikely that the reduction in progenitor cell numbers observed in *mpl*$^{-/-}$ mice simply reflects lower production in vitro of ancillary megakaryocyte-derived growth factors. Nevertheless, we cannot formally exclude the possibility that reduced progenitor cell development is contributed to by the absence of growth factors normally provided in vivo by megakaryocytes and platelets.

Alternatively, our data raise the intriguing possibility of a direct requirement for c-Mpl signaling in early hemopoietic cells. As c-Mpl appears to be expressed by only a fraction of CD34$^+$ cells [43], a direct role in the wide range of committed progenitor cells affected in *mpl*$^{-/-}$ mice seems unlikely. The deficiency in progenitor cells of multiple hemopoietic lineages in *mpl*$^{-/-}$ mice may therefore reflect reduced input from fewer multipotent ancestors. The dramatic reduction in blast cell colony-forming cells in *mpl*$^{-/-}$ mice, which is of similar magnitude to that of megakaryocyte progenitors [39], strongly supports an important role for c-Mpl in maintaining primitive hemopoietic cells. Indeed, c-Mpl expression has been reported in early hemopoietic populations [8, 44] and the multilineage myeloproliferative disorder induced by v-*mpl* [5, 6] confirms that the receptor can signal in multipotent cells. It remains to be evaluated whether defects in colony-forming unit-spleen or repopulating stem cells are also a feature of *mpl*$^{-/-}$ mice.

In summary, the phenotype of mice lacking c-Mpl implies a broader hemopoietic role for TPO and c-Mpl signaling in addition to their activities in megakaryocytopoiesis. In particular, our data suggest an important function in the maintenance of normal numbers of progenitor cells of multiple hemopoietic lineages, possibly through a direct action on primitive pluripotential cells. As the precise role of TPO is an important consideration in its potential therapeutic utility, it will be necessary to closely monitor effects on all hemopoietic cells in the clinical evaluation of this cytokine.

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