RAPID COMMUNICATION

Thrombopoietin (c-mpl Ligand) Acts Synergistically With Erythropoietin, Stem Cell Factor, and Interleukin-11 to Enhance Murine Megakaryocyte Colony Growth and Increases Megakaryocyte Ploidy In Vitro

By Virginia C. Broudy, Nancy L. Lin, and Kenneth Kaushansky

Thrombopoietin (Tpo), the ligand for the c-mpl receptor, is a major regulator of platelet production in vivo. Treatment of mice with purified recombinant Tpo increases platelet count fourfold and expands colony-forming unit-megakaryocyte (CFU-Meg) numbers. Other cytokines including interleukin-3 (IL-3), IL-6, IL-11, erythropoietin (Epo), and stem cell factor (SCF) can stimulate megakaryopoiesis. Therefore, we examined the effects of recombinant murine Tpo in combination with these cytokines on megakaryopoiesis in vitro. Murine marrow cells were cultured in agar in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% horse serum and β-mercaptoethanol in the presence of recombinant growth factors, and CFU-Meg colonies were counted on day 5. Megakaryocyte ploidy was analyzed using murine marrow cells cultured for 5 days in IMDM supplemented with 1% nutridoma-SP and recombinant growth factors. Megakaryocytes were identified by labeling with the A45 antibody and ploidy was analyzed by flow cytometry. Tpo supported the growth of CFU-Meg in a dose-dependent manner. Although the addition of SCF (50 ng/mL), Epo (2 U/mL), or IL-11 (50 ng/mL) alone exerted only a modest effect on CFU-Meg growth, the combinations of SCF plus Tpo, Epo plus Tpo, or IL-11 plus Tpo resulted in a synergistic enhancement of the number of CFU-Meg colonies. IL-3 alone supported CFU-Meg colony growth, and the effects of IL-3 plus Tpo or IL-6 plus Tpo on colony growth appeared to be approximately additive. Fifty percent of megakaryocytes generated in cultures containing IL-3 or Epo displayed ≥16N ploidy. In contrast, cultures containing Tpo uniquely generated large numbers (30% to 35% of the total) of megakaryocytes with ≥64N ploidy. These results show that Tpo stimulates both proliferation of committed megakaryocytic progenitor cells and maturation of megakaryocytes, and that two multipotent cytokines, SCF and IL-11, as well as a late-acting erythroid cytokine, Epo, can synergize with Tpo to stimulate proliferation of CFU-Meg.

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regulation of platelet production predicts that Tpo would be a late-acting factor that primarily regulates terminal maturation of megakaryocytes (endoreduplication and cytoplasmic maturation). However, our results show that Tpo also stimulates proliferation of CFU-Meg as effectively as IL-3, and that Epo, SCF, and IL-11, three cytokines that display minimal megakaryocyte colony-simulating activity alone, synergize with Tpo to promote CFU-Meg growth. These data suggest that Tpo acts on both the mitotic and endomitotic pathways of megakaryopoiesis, in contrast with IL-3, which is primarily a proliferation factor.

**MATERIALS AND METHODS**

Megakaryocyte colony assay. B6D2F1 mice, 8 to 10 weeks old, were obtained from Jackson Laboratories (Bar Harbor, ME), and were killed by rapid cervical dislocation. Marrow cells were harvested by flushing the femur with Iscove’s modified Dulbecco’s medium (IMDM; Gibco, Grand Island, NY) supplemented with 2% fetal calf serum (HyClone, Logan, UT). The marrow cells were plated at a concentration of 2 to 2.5 × 10^5 cells/mL in IMDM supplemented with combinations of growth factors as described below. 10% horse serum (HyClone), 5 × 10^-3 M NaCl, and 1mM mercaptoethanol (Sigma Chemical Co, St Louis, MO), and penicillin-streptomycin (Sigma), and made semisolid with 0.275% agar (Difco, Detroit, MI) in triplicate plates. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂, and megakaryocytic colonies were counted on day 5 using an inverted microscope. Megakaryocyte colonies were defined as containing at least three large refractile cells. Identification of the megakaryocytic colonies was confirmed by staining with acetylcholinesterase. In some experiments, the addition of Tpo or IL-3 was delayed until day 3 of culture, to assess the effects of immediate versus delayed addition of these cytokines on CFU-Meg growth.

Hematopoietic growth factors. Recombinant murine Tpo was expressed in baby hamster kidney (BHK) cells. Fifty units of activity were defined as the quantity of Tpo that gave one-half maximal stimulation in the BaF3/mpl proliferation assay. Recombinant human erythropoietin (Epo), recombinant murine IL-3, and recombinant murine GM colony-stimulating factor were expressed in BHK cells as previously described. Purified recombinant murine stem cell factor was a gift from Amgen, Inc (Thousand Oaks, CA). Purified recombinant human IL-6 and IL-11 were obtained from Genzyme. Human IL-6 and human IL-11 are active on murine megakaryocytic cells.

Analysis of megakaryocyte ploidy. Murine marrow cells were cultured in suspension at a concentration of 1 × 10^6 cells/mL in IMDM supplemented with 1% murine serum (Sigma) plus hematopoietic growth factors at 37°C for 5 days. The growth factors used included Tpo (10 to 2,000 U/mL), Epo (12 U/mL), and IL-3 (20 ng/mL) alone or in combination. The DNA content of the megakaryocytes was quantitated by two-color flow cytometric analysis after labeling the cells with a megakaryocyte-specific antibody and with the DNA-intercalating fluorescent reagent propidium iodide. The cultured cells were washed once in phosphate-buffered saline containing 0.5% bovine serum albumin (Sigma), then incubated for 30 minutes at 4°C with purified rat 4A5 MoAb (15 μg/mL, a gift from Dr Sam Burstein, University of Oklahoma, Oklahoma City, OK) or with control rat anti-mouse IgG2a antibody (15 μg/mL, Pharmingen, San Diego, CA). The 4A5 antibody recognizes an epitope present on murine platelets and megakaryocytes. The cells were washed three times, then incubated for 1 hour at 4°C with fluorescein isothiocyanate (FITC)-conjugated goat antirat IgG (Jackson ImmunoResearch Labs, Inc, Westgrove, PA) at a concentration of 30 μg/mL. The cells were washed once, then incubated for 36 minutes at 22°C in hypotonic citrate solution consisting of 0.1% sodium citrate, 50 μg/mL propidium iodide (Molecular Probes, Inc, Eugene, OR), 30 μg/mL DNAase-free RNase (Boehringer Mannheim) and 0.1% Triton X-100, and analyzed in a Coulter Epics Elite flow cytometer with an argon ion laser (emission 488 nm). A 70-μm prefilter was used. An electronic gate was set on the green fluorescence channel that excluded 99% of the cells labeled with the control MoAb. The DNA content of the cells labeled with the 4A5 antibody was analyzed by setting markers at the nadirs between the propidium iodide peaks. The 2N and 4N peak positions were identical to those of ungated cells. Approximately 2,000 to 10,000 megakaryocytes from each individual liquid culture were analyzed. Geometric mean ploidy was calculated as described.

**RESULTS**

Megakaryocytic colony growth. We examined the ability of recombinant murine Tpo alone or in combination with other cytokines to support the growth of megakaryocytic colonies in serum-containing semisolid media. Tpo exerted a concentration-dependent effect on CFU-Meg proliferation that was maximal at ~2,000 U/mL of Tpo (Fig 1). Although Epo (2 U/mL) alone did not support CFU-Meg growth (0.3 ± 0.3, 0.7 ± 0.7, 1 ± 0, 2 ± 0.6 and 5 ± 0.6 colonies/2 × 10⁵ cells in five separate experiments), the combination of Epo plus Tpo dramatically increased CFU-Meg colony numbers at all concentrations of Tpo tested (Fig 2 and Table 1). Likewise, the use of SCF alone resulted in only a modest effect on CFU-Meg colony growth (0, 0.5, 2.7 ± 1.4, and 5.7 ± 0.7 colonies/2 × 10⁵ cells plated in four separate experiments). However, the combination of SCF plus Tpo also markedly increased the number of CFU-Meg colonies in a suprapaddiative fashion (Fig 2 and Table 1). Other than Tpo, IL-3 was the only cytokine tested that was able to reproducibly support growth of CFU-Meg when added as a single agent under our culture conditions (9 ± 0.6, 11 ± 2, 16 ± 1, 20.3 ± 0.3, and 23.3 ± 2.0 CFU-Meg colonies/2 × 10⁵ marrow cells plated in five separate experiments). The effects of Tpo plus IL-3 on the number of megakaryocytic colonies appear to be approximately additive, in contrast to results with Tpo plus Epo or Tpo plus SCF (Fig 2 and Table 1). Although IL-11 displayed little megakaryocyte colony-stimulating activity, the addition of IL-11 to low concentrations of Tpo (15 to 75 U/mL) increased the number of CFU-Meg colonies by at least twofold, in comparison with the number found in the presence of Tpo alone (Fig 3). IL-6, a cytokine that predominately affects megakaryocyte maturation, had an approximately additive effect on CFU-Meg growth in the presence of low concentrations of Tpo (Fig 3). At higher concentrations of Tpo, the presence of IL-6 did not augment the growth of CFU-Meg colonies (Fig 3). In two experiments, the addition of GM-CSF (10 ng/mL) to various concentrations of Tpo (15 to 2,000 U/mL) had no effect on CFU-Meg colony growth (data not shown).

The number of cells in each CFU-Meg colony reflects the activity of the mitotic pathway of megakaryopoiesis. Growth factors that act primarily on proliferation of megakaryocyte progenitor cells would be expected to generate colonies containing large numbers of megakaryocytes. The CFU-Meg
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Fig 1. Effect of recombinant murine Tpo on growth of CFU-Meg colonies. Murine marrow cells (2 x 10^5/mL) were cultured in semisolid media in the presence of various concentrations of Tpo for 5 days. The data represent the number of CFU-Meg colonies (mean ± SEM of triplicate plates) from one of six similar experiments.

colonies grown in the presence of IL-3 contained more megakaryocytes per colony, on average, than those grown in Tpo, Epo, or SCF (Table 1). IL-3 uniquely generated a few very large colonies containing more than 50 megakaryocytes (Table 1). Although the combination of Tpo plus IL-3 displayed an additive effect on the number of CFU-Meg colonies, there was an increase in the proportion of larger colonies, so that overall many more megakaryocytes were produced than in the presence of either cytokine alone. The few CFU-Meg colonies identified in the presence of Epo or SCF alone were small (Table 1). However, in addition to augmenting the number of CFU-Meg colonies, the combination of Tpo plus Epo or Tpo plus SCF also increased the size of the colonies, resulting in at least 50% of the colonies containing 6 to 19 megakaryocytes. In the presence of 2,000 U/mL of Tpo, the addition of IL-6 did not change the size distribution of the megakaryocyte colonies.

To determine whether delayed addition of Tpo or IL-3 would affect either the number or the size of CFU-Meg colonies, we performed experiments in which one of these
cytokines was added on day 3 of culture (Table 2). When the marrow cells were cultured in Tpo and IL-3 was added on day 3, both the number and the size of the CFU-Meg colonies were approximately the same as those generated in the presence of Tpo alone, suggesting that IL-3 does not stimulate the proliferation of the more mature megakaryocytic cells present after 3 days in culture. However, when the cells were cultured in the presence of IL-3 and Tpo was added on day 3, the number of CFU-Meg colonies was increased over that seen in the presence of IL-3 alone, indicating that Tpo can enhance the proliferation of more mature cells in the megakaryocytic lineage.

Analysis of megakaryocyte polyploidy. We cultured murine marrow cells in serum-free liquid cultures in the presence of hematopoietic growth factors to analyze the effects of Tpo on megakaryocyte endoreduplication. In the presence of Tpo (2,000 U/mL), large smooth cells developed by day 3 to 5 of culture (Fig 4). Cytoplasmic blebbing was observed in some of these cells. Acetylcholinesterase staining verified that the large cells are megakaryocytes. In accord with the
effects of IL-3 on CFU-Meg colony growth, and prior reports, IL-3 (20 ng/mL) also supported generation of large numbers of megakaryocytes in serum-free liquid cultures (data not shown). For analysis of megakaryocyte ploidy, the cells generated in liquid culture were labeled with purified 4A5 MoAb (or with rat IgG2a control antibody) and incubated with propidium iodide. Flow cytometric analysis showed that in cultures containing Tpo, cells of greater than 4N ploidy were frequent enough to be identified on a DNA histogram that included all of the cells (data not shown). The DNA content of the cells that were most intensely stained with the 4A5 antibody was analyzed as described.24 As the concentration of Tpo increased, megakaryocyte ploidy increased (Table 3). Geometric mean ploidy25 was maximal at a Tpo concentration of 500 U/mL (two independent experiments). In the presence of Tpo (≥150 U/mL), the modal megakaryocyte ploidy number was 64N, and 5% to 7% of the megakaryocytes had a ploidy of 128N (Table 3, Fig 3. Effect of Tpo in combination with IL-6 or IL-11 on growth of CFU-Meg colonies. Murine marrow cells were cultured as described in Fig 2 in the presence of Tpo with or without IL-6 (50 ng/mL) or IL-11 (50 ng/mL). The number of CFU-Meg colonies (mean ± SEM) from one of four similar experiments are presented. (●), Tpo; (○), Tpo + IL-6; (■), Tpo + IL-11.

### Table 1. Effect of Growth Factors on CFU-Meg Colony Size

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>No. of Colonies Containing the Following:</th>
<th>Total No. of Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3-5 Meg</td>
<td>6-19 Meg</td>
</tr>
<tr>
<td>Tpo (2,000 U/mL)</td>
<td>27.3 ± 1.8</td>
<td>13 ± 1.2</td>
</tr>
<tr>
<td>IL-3 (20 ng/mL)</td>
<td>6.7 ± 3.2</td>
<td>9 ± 0.5</td>
</tr>
<tr>
<td>Tpo + IL-3</td>
<td>18 ± 1.5</td>
<td>23.3 ± 2.6</td>
</tr>
<tr>
<td>Epo (2 U/mL)</td>
<td>5 ± 0.6</td>
<td>0</td>
</tr>
<tr>
<td>Tpo + Epo</td>
<td>33.7 ± 0.9</td>
<td>35 ± 1</td>
</tr>
<tr>
<td>SCF (50 ng/mL)</td>
<td>5.7 ± 0.7</td>
<td>0</td>
</tr>
<tr>
<td>Tpo + SCF</td>
<td>19.3 ± 2.4</td>
<td>26.7 ± 2.3</td>
</tr>
<tr>
<td>IL-11 (50 ng/mL)</td>
<td>1 ± 0</td>
<td>0</td>
</tr>
<tr>
<td>Tpo + IL-11</td>
<td>28.3 ± 0.3</td>
<td>18.7 ± 2.0</td>
</tr>
<tr>
<td>IL-6 (50 ng/mL)</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>Tpo + IL-6</td>
<td>28.7 ± 0.3</td>
<td>14 ± 1.0</td>
</tr>
</tbody>
</table>

Murine marrow cells were cultured at a concentration of 2 x 10^5 cells/mL in 0.275% agar in IMDM supplemented with 10% horse serum, 5 x 10^{-4} mol/L β-mercaptoethanol, and combinations of cytokines. The number of colonies containing 3 to 5, 6 to 19, 20 to 50, or more than 50 individual megakaryocytes was counted on day 5 using an inverted microscope. The data represent the mean ± SEM of triplicate plates. Two additional experiments gave similar results.

- Average of duplicate plates.
Table 2. Effect of Delayed Addition of Tpo or IL-3 on CFU-Meg Colony Growth

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>No. of CFU-Meg Colonies/2 × 10^5 Cells Plated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tpo (2,000 U/mL)</td>
<td>58.3 ± 5.4</td>
</tr>
<tr>
<td>IL-3 (20 ng/mL)</td>
<td>30.6 ± 4.7</td>
</tr>
<tr>
<td>Tpo + IL-3</td>
<td>78.7 ± 5.4</td>
</tr>
<tr>
<td>Tpo + day 3 IL-3</td>
<td>60.8 ± 3.4</td>
</tr>
<tr>
<td>IL-3 + day 3 Tpo</td>
<td>45.5 ± 5.3</td>
</tr>
</tbody>
</table>

Murine marrow cells were cultured as described in Table 1, and CFU-Meg colonies were counted on day 5. Where indicated, the addition of IL-3 or Tpo was delayed until day 3 of culture. The data represent the mean ± SEM of triplicate or quadruplicate plates from one experiment. A second experiment with delayed addition of Tpo or IL-3 gave similar results.

Fig 4. Appearance of cells generated in serum-free suspension cultures in the absence of presence of Tpo. Murine marrow cells were cultured in IMDM supplemented with 1% nutridoma-SP without (A) or with (B) Tpo (2,000 U/mL) for 5 days.

Fig 5. In contrast, more than 50% of the megakaryocytes in IL-3-containing cultures exhibited ≥8N ploidy, and relatively few displayed ≥64N ploidy (Fig 6). These data indicate that, although IL-3 is a potent stimulant of CFU-Meg proliferation, it is much less active than Tpo at promoting megakaryocyte endomitosis. No megakaryocytes of ≥64N ploidy were identified in liquid cultures that contained Epo alone (Fig 6). In the presence of Epo plus a high concentration of Tpo (2,000 U/mL), the distribution of megakaryocyte DNA content was very similar to that of cells cultured in Tpo alone (Table 3), showing that at the concentration tested, Epo did not augment the megakaryocyte endoduplication induced by Tpo.

DISCUSSION

The induction of acute thrombocytopenia by exchange transfusion or by treatment with antiplatelet antibodies re-
Table 3. Effect of Tpo on Megakaryocyte Ploidy

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>% of Megakaryocytes in Each Ploidy Class</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2N</td>
</tr>
<tr>
<td>Exp. 1</td>
<td></td>
</tr>
<tr>
<td>Tpo (10 U/mL)</td>
<td>17</td>
</tr>
<tr>
<td>Tpo (40 U/mL)</td>
<td>15</td>
</tr>
<tr>
<td>Tpo (150 U/mL)</td>
<td>16</td>
</tr>
<tr>
<td>Tpo (500 U/mL)</td>
<td>16</td>
</tr>
<tr>
<td>Tpo (2,000 U/mL)</td>
<td>20</td>
</tr>
<tr>
<td>Tpo (2,000 U/mL) + Epo (2 U/mL)</td>
<td>14</td>
</tr>
<tr>
<td>Exp. 2</td>
<td></td>
</tr>
<tr>
<td>Tpo (2,000 U/mL)</td>
<td>13</td>
</tr>
<tr>
<td>Tpo (2,000 U/mL) + Epo (2 U/mL)</td>
<td>14</td>
</tr>
</tbody>
</table>

Murine marrow cells were cultured in IMDM supplemented with 1% nutridoma-SP for 5 days. The cells were labeled with 4A5 MoAb and propidium iodide and analyzed by flow cytometry. The number of 4A5' cells analyzed ranged from 2,300 to 4,800. Two additional independent experiments gave similar results.

Results in a reproducible sequence of events. Megakaryocyte size and ploidy increase by 48 to 72 hours. In contrast to these effects on megakaryocyte maturation, modest to no changes in the number of marrow megakaryocyte precursor and progenitor cells are found 4 to 5 days after the development of thrombocytopenia. Conversely, hypertransfusion of platelets reduces megakaryocyte ploidy in normal rats. These findings have led to the concept of two distinct levels of regulation of thrombopoiesis in vivo: a megakaryocyte proliferation factor that stimulates CFU-Meg growth, and a megakaryocyte potentiation factor that stimulates megakaryocyte endoreduplication. In this model of the regulation of thrombopoiesis, megakaryocyte proliferation factor is proposed to be constitutively present, whereas megakaryocyte potentiation factor is exquisitely regulated by the platelet count.

IL-3, which is constitutively produced by T cells present in the marrow microenvironment, is a candidate for the megakaryocyte proliferation factor, but the cytokine that predominantly affects megakaryocyte maturation had remained elusive. Both IL-6 and IL-11 enhance megakaryocyte endoreduplication in vitro, but are pleiotropic cytokines whose levels do not fluctuate inversely with the platelet count. However, Tpo levels increase within 8 hours after thrombocytopenia develops and platelet transfusions within this period abolish the increase in Tpo. The present results show that Tpo has a profound effect on megakaryocyte endoreduplication, generating a large proportion of megakaryocytes with a ploidy of 32 to 128N, reinforcing the identification of Tpo as megakaryocyte potentiation factor. Using morphological criteria, Levine et al have suggested that the majority of 4N to 8N megakaryocytes are immature and that 95% of megakaryocytes that are capable of producing platelets are at least 16N to 32N. Thus the ability of Tpo to enhance megakaryocyte polyploidization may have a significant impact on total platelet production capacity.

Contrary to the prediction of the model of thrombopoiesis, the effects of Tpo are not limited to promotion of megakaryocyte maturation; Tpo is also a potent stimulant of CFU-Meg proliferation. Experiments with enriched populations of hematopoietic stem cells and progenitor cells indicate that the proliferative effects of Tpo are not mediated by accessory cells. Our data document the effects of Tpo on proliferation of the more differentiated megakaryocyte progenitor cell, CFU-Meg. Whether more primitive megakaryocytic progen-
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Fig 6. Relative number of 4A5+ cells in each ploidy class generated in suspension culture. Murine marrow cells were cultured in Epo (2 U/mL), IL-3 (20 ng/mL) or Tpo (1,250 U/mL) for 5 days and analyzed as described in Fig 5.

...itor cells (BFU-Meg, high proliferative potential [HPP]-CFU-Meg) will respond to Tpo requires further investigation. Although full maturation and platelet production is not achieved in vitro in the absence of Tpo, inhibition of the biologic effects of Tpo using a soluble form of the c-mpl receptor suggests that a subset of CFU-Meg proliferate in vitro in response to IL-3 alone. The present results show that optimal levels of IL-3 or Tpo can stimulate the growth of similar numbers of CFU-Meg, and that these growth factors exert an additive effect on megakaryopoiesis in vitro. Thus there may be two populations of CFU-Meg progenitor cells: one that is responsive to IL-3 and maintains steady state thrombopoiesis, and one that is responsive to Tpo and may contribute to megakaryocyte expansion in response to acute thrombocytopenia.

These data lead us to propose a modified model of regulation of thrombopoiesis. In this model, IL-3 maintains steady-state proliferation of CFU-Meg. Tpo present in the serum at a low concentration, IL-6, and IL-11 modestly augment the proliferative effects of IL-3, and predominantly induce megakaryocyte maturation. In response to acute thrombocytopenia, serum levels of Tpo increase abruptly, resulting in a prompt increase in megakaryocyte size and polyploidy, and an increase in platelet production per megakaryocyte. At high concentrations, Tpo is also a potent stimulant of CFU-Meg proliferation and recruits additional megakaryocyte progenitor cells to proliferate. SCF and Epo circulate at measurable levels in the blood under normal circumstances, and synergistically enhance the effect of moderate to high concentrations of Tpo on CFU-Meg proliferation. However, elevated levels or the administration of SCF or Epo fail to raise the platelet counts above normal because of the Tpo dependence of platelet production mediated by SCF or Epo.

Mice that do not express the c-mpl receptor have peripheral platelet counts that are 15% of normal, and normal red blood cell and white blood cell numbers. These results support the concept that Tpo plays a major role in the control of thrombopoiesis in vivo. However, some platelets are produced even in the absence of c-mpl activity, suggesting that Tpo may not be absolutely essential for terminal megakaryocyte maturation in vivo. Determining whether the number of CFU-Meg in the marrows of c-mpl-deficient mice is increased, normal, or reduced could provide information about the contribution of Tpo to proliferation of CFU-Meg in vivo. However, it would be difficult to accurately quantitate the number of CFU-Meg in these mice by colony assays in vitro because of the lack of c-mpl receptor expression. Studies in Tpo knock-out mice could potentially assess the role of Tpo in the generation of CFU-Meg in vivo.

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REFERENCES


Thrombopoietin (c-mpl ligand) acts synergistically with erythropoietin, stem cell factor, and interleukin-11 to enhance murine megakaryocyte colony growth and increases megakaryocyte ploidy in vitro.

VC Broudy, NL Lin and K Kaushansky