THROMBOPOIETIN PROMOTES MIXED LINEAGE AND MEGAKARYOCYTIC COLONY FORMING CELL GROWTH BUT INHIBITS PRIMITIVE AND DEFINITIVE ERYTHROPOIESIS IN CELLS ISOLATED FROM EARLY MURINE YOLK SACS

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ABSTRACT

The role of thrombopoietin (Tpo) in promoting hematopoiesis has been extensively studied in late fetal, neonatal, and adult mice. However, the effects of Tpo on early yolk sac hematopoiesis have been largely unexplored. We examined whole embryos or the cells isolated from embryo proper and yolk sacs and identified both Tpo and c-mpl (Tpo receptor) mRNA transcripts in tissues as early as embryonic day 6.5 (E6.5). Pre-somite whole embryos and somite staged yolk sac and embryo proper cells were plated in methylcellulose cultures and treated with selected hematopoietic growth factors in the presence or absence of Tpo. Tpo alone failed to promote CFU formation. However, in the presence of other growth factors, Tpo caused a significant dose dependent reduction in primitive and definitive erythroid CFU growth in cultures containing E7.5 and E8.0 whole embryos and E8.25-9.5 yolk sac-derived cells. Meanwhile, Tpo treatment resulted in a significant dose dependent increase in CFU-mixed lineage (CFU-Mix) and CFU-megakaryocyte (CFU-Meg) formation in cultures containing cells from similar staged tissues. Addition of Tpo to cultures of sorted E9.5 yolk sac c-Kit+CD34+ hematopoietic progenitors also inhibited erythroid CFU growth but augmented CFU-Mix and CFU-Meg colony forming activity. Tpo effects on CFU growth were blocked in the presence of a monoclonal antibody with Tpo neutralizing activity but not with control antibody. Thus, under certain growth factor conditions, Tpo directly inhibits early yolk sac erythroid CFU growth, but facilitates megakaryocyte and mixed lineage colony formation.
INTRODUCTION

Thrombopoietin (Tpo) is a hematopoietic growth factor that stimulates the proliferation and differentiation of hematopoietic stem cells, primitive progenitors, megakaryocytes, and platelets. The cellular receptor for Tpo, c-mpl, belongs to the cytokine receptor super family. Early studies employing antisense oligonucleotides to disrupt c-mpl function suggested that the effects of Tpo may be restricted to megakaryocytic progenitor cells. A platelet lineage-restricted action of Tpo on c-mpl expressing cells was also observed in animals treated with Tpo. Mutant mice in which the genes for Tpo or c-mpl have been disrupted display a similar phenotype with deficient megakaryopoiesis. Both mutant strains suffer from thrombocytopenia with 100% penetrance. No significant difference in the number of other blood cells, including red blood cells, neutrophils, lymphocytes, monocytes and eosinophils has been observed in either mutant strain.

Expression of c-mpl has subsequently been demonstrated on hematopoietic stem cells. In vivo administration of Tpo to normal and myelosuppressed mice has also been reported to cause multi-lineage effects. Transplantation of c-mpl/- mutant mice marrow into lethally irradiated recipients results in poor repopulating ability by these donor cells. Tpo has recently been found to expand HSC in vitro as evidenced by higher levels of donor cell engraftment post-transplantation into lethally irradiated mice compared with freshly transplanted marrow cells. These results have demonstrated that the effects of Tpo on hematopoiesis are more diverse than originally reported.

Although much has been learned about the role of Tpo in fetal liver and adult marrow hematopoiesis, little is known of the effect of Tpo on early yolk sac hematopoiesis. Tpo has been reported to enhance the proliferation and differentiation of erythroid cells in the presence or
absence of erythropoietin in cultured day 10.5 post coitus (pc) yolk sac cells\textsuperscript{13}. In other studies, the addition of Tpo to embryonic stem cell (ES) cultures during the growth of embryoid bodies (EB) resulted in a significant increase in the total number of hematopoietic progenitors generated by day 6 EBs. However, the presence of Tpo in EB cultures resulted in a dramatic decrease in erythroid CFU activity\textsuperscript{14}. Because the pattern and kinetics of erythropoiesis in the murine ES differentiation system largely mirror those of the murine yolk sac, the results of the two studies above appear to be contradictory\textsuperscript{15}.

We have examined the effects of Tpo on hematopoietic progenitor cell growth in vitro in cells isolated from the early murine embryo and from isolated yolk sac and embryo proper cells. We report that Tpo causes dose dependent inhibition of primitive and definitive erythroid CFU growth but enhances mixed lineage and megakaryocytic CFU growth in vitro in cells derived from early embryos and extraembryonic yolk sacs.

**MATERIALS AND METHOD**

**Mice maintenance**

C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Gpi/BoyJ mice were derived from B.6Gpi-1A (gift of Dr. David Harrison, Jackson Labs, Bar Harbor, ME) × B.6SJL-Pep3B/Boy/J (purchased from Jackson Labs) breeding. The F1 progeny were screened for expression of glucose phosphate isomerase 1A (Gpi-1A) enzyme activity as previously described\textsuperscript{16} and CD45.1 expression on peripheral blood leukocytes by FACS. To obtain timed pregnant mice, two females were caged with a male overnight, and then examined for the evidence of a copulation plug the next morning. The care and use of animals in these studies
was approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

**Yolk sac cell preparation**

Timed-pregnant mice were killed by CO₂ inhalation and the uteri were removed from the peritoneum and washed with phosphate buffered saline or Hank’s balanced salt solution (GibcoBRL, Gaithersburg, MD) several times. Embryos were obtained and embryo proper and yolk sac were isolated as described previously. Presomite embryos were staged by morphological criteria. Embryos were staged by somite counting. Dissected embryo proper or yolk sac were incubated with 0.1% collagenase/dispsase (Sigma, St. Louis, MO) in 20% fetal bovine serum (FBS, Hyclone, Logan, UT) for 1-1.5 hour at 37°C, and dispersed yolk sac or embryo proper were drawn through a 70μm strainer, deposited into a polystyrene tube, and spun at 500g for 10 minutes. The pellet was washed with Iscove’s modified Dulbecco’s medium (IMDM) with 10% FBS, 2% penicillin and streptomycin (pen/strep, GibcoBRL), 2mM glutamine and 450μM monothioglycerol (MTG, GibcoBRL) and centrifuged again at 500g for 10 minutes. Cells in the suspension were counted and viability tested via Trypan blue (GibcoBRL) exclusion criteria.

**RNA preparation and reverse transcriptase - polymerase chain reaction (RT-PCR)**

RNA was isolated from yolk sac and embryo proper using TRIzol Reagent (GibcoBRL) according to the manufacturer’s instructions. Approximately, 1ml of TRIzol was used for 10-20 embryos. RNA (1-5μg) was treated with 1μl of RNase-free DNaseI (Promega, Madison, WI) at 37°C for 15 minutes followed by heat inactivation of DNaseI at 70°C for 15 minutes to deplete DNA. The total RNA was reverse transcribed into cDNA (SuperScription for Reamplification System, GibcoBRL) according to the manufacturer’s instructions. The specific primers were
used in nested PCR to amplify cDNA. Primers 1, 2 and primers 3, 4 were used for first and second round PCR, respectively. The final products are 220bp for thrombopoietin (GeneBank accession number L34169) and 368bp for c-mpl (GeneBank number X73677).

Tpo primer1: CGGGGAAAGGTGCCTCTCCTG,
Tpo primer2: GTTTCCTGAGACAAATTCCT,
Tpo primer3: TTCAGAGTCAAGATTACTCC,
Tpo primer4: GGAGAAGGAGGAGTCCACCC,
c-mpl primer1: CGGGAGAAGGCCGTGAGGACT,
c-mpl primer2: CTTCAGGGCTGCTGCCAATAG,
c-mpl primer3: CCTACTGCTGCTAAAGTGGCAAT,
c-mpl primer4: CAATAGCTTAGTGGTAGGAG.

PCR amplification was 45 seconds denaturation at 94°C, 45 seconds annealing at 45°C for Tpo or 52°C for c-mpl and 45 seconds for extension at 72°C for 35 cycles. Samples with DEPC water (instead of RNA) as template were run as negative controls. The PCR fragments were visualized by ethidium bromide staining of 10-20µl PCR product after electrophoresis in a 2% agarose gel. The size of PCR fragment was determined by comparing to a 100 bp DNA ladder (Promega)

**Fluorescence activated cell sorting**

The antibodies used for flow cytometry analysis were purchased from PharMingen (San Diego, CA). All antibodies were conjugated with fluorescein isothiocyanate (FITC) or allophycocyanin (APC). Rat monoclonal antibodies used in the studies included: FITC conjugated anti-mouse CD34 and APC conjugated anti-mouse c-kit. At the same time, FITC conjugated purified rat IgG2a and APC conjugated purified rat IgG2b were used as isotype
control antibodies. A single cell suspension was prepared as described in yolk sac cell preparation. After centrifugation, the cell pellet was suspended in 100µl Fc blocking antibody conditioned medium produced by the 2.4G2 cell line (ATCC) prior to the addition of 1µg of each specific antibody per 10⁶ cells²¹-²³. After 45-60 minutes incubation, cells were washed with IMDM containing 10% FBS, 2% pen/strep, 2mM glutamine and 450µM MTG. Cells were spun down and resuspended in the same medium for sorting, which was performed on a FACStarPLUS instrument (Beckton Dickinson). C-kit+CD34+ and c-kit-CD34- cells were collected for hematopoietic progenitor assay. In some studies, single c-kit+CD34+ cells were sorted and deposited into 96-well plates for hematopoietic progenitor assay.

**Primitive erythroid colony assay**

Cells were plated in duplicates or triplicates at 1-2.5×10⁵ cells/ml in 0.9% methylcellulose-based media (StemCell Technologies, Vancouver, Canada) which included IMDM, 2mM glutamine, 1% pen/strep, 5% protein free hybridoma medium-II (PFHM-II, GibcoBRL), 50µg/ml ascorbic acid, 450µM MTG, 200 µg/ml iron-saturated holo-transferrin (Sigma), 15% plasma derived serum (Animal Technology, Antech, TX), 4U/ml recombinant human erythropoietin (Epo, Amgen, Thousand Oaks, CA), and ± 50ng/ml recombinant human Tpo (Peprotech, Rocky Hill, NJ). Cultures were incubated in a humidified incubator at 37°C in 5% CO₂ and colonies were counted on day 7²⁴.

**Definitive committed progenitor assay**

Cells were plated in duplicates or triplicates at 1-2.5×10⁵ cells/ml in 0.9% methylcellulose-based media which included IMDM, 2mM glutamine, 1% pen/strep, 10⁻⁵M β-mercaptoethanol, 30% fetal bovine serum, 4U/ml recombinant human Epo (Amgen, Thousand Oaks, CA), 100U/ml recombinant murine interleukin-3 (IL-3, Peprotech), 100ng/ml recombinant
murine stem cell factor (SCF, Peprotech), and ± 50ng/ml recombinant human Tpo (Peprotech). Cultures were incubated in a humidified incubator at 37°C in 5% CO2 in air and colonies were counted on day 7 

**CFU-Meg and BFU-Meg assay and Immunohistochemical staining**

Cells were plated in duplicates or triplicates at 1-2.5×10^5 cells/ml in 0.3% agar-based McCoy’s 5A medium (GibcoBRL) which included 10% fetal bovine serum, 100U/ml recombinant IL-3, and ± 50ng/ml recombinant human Tpo. Cultures were incubated in a humidified incubator at 37°C in 5% CO2 in air. After 7 days (for CFU-Meg) or 14 days (for BFU-Meg) incubation, these 35mm grid culture dishes were air dried over night and fixed with 1:3 methanol: acetone (Fisher, Fair Lawn, NJ). Fixed cultures were rehydrated in pH7.6, 0.05M Tris/0.15M NaCl buffer for 20 minutes, followed by additional 0.5ml 5% mouse serum for 20 minutes. Primary antibody (10 µg/ml rat anti-mouse CD41, PharMingen, San Diego, CA) or rat IgG κ isotype control antibody (5 µg/ml rat IgG2a, PharMingen) in Tris/NaCl buffer with 5% mouse serum was added to the cultures and incubated for 30 minutes. After washing, secondary antibody (10 µg/ml biotinylated anti-rat IgG, Vector, Burlingame, CA) was added and incubated for 30 minutes, followed by 18 µg/ml alkaline phosphatase streptavidin (Vector) for 30 minutes. Plates were washed and subjected to alkaline phosphatase substrate (Vector) according to the manufacturer’s instruction. Gently rinsed plates and colonies were scored after drying. CFU-Meg and BFU-Meg were scored based on their colony morphology and CD41 expression.

**Neutralizing antibody blocking studies**

Anti-murine Tpo neutralizing antibody or anti-human Tpo neutralizing antibody or isotype control antibody (Peprotech) were incubated with murine Tpo or human Tpo (Peprotech) at 37°C for 1 hour, respectively. Then, the mixture of antibody and cytokine were added to the
regular culture medium for primitive erythroid, definitive committed progenitor, and megakaryocyte assays and plated as described above.

**Colony morphology analysis**

CFU-Mix from definitive committed progenitor assay were plucked and digested in 50µl 0.25% trypsin (StemCell Technologies, Vancouver, Canada) for 2 minutes and diluted in 100µl IMDM with 10% serum. The single cell suspension was added to 100 µl 10% BSA (Ortho Clinical Diagnostics, Paritan, NJ) and centrifuged onto glass microscope slides at 500 rpm in a Cytospin (Shandon, England) device for 5 minutes. After air-drying, slides were fixed and stained with Diff-Quik Stain Set (Dade Behring, Dudingen, Switzerland). Cells were scored morphologically under oil-immersion light microscopy.

**G1E-ER2 erythroid cell line**

G1E-ER2 cells have been previously described 27-29. These cells were grown in Iscove’s Modified Dulbecco’s Medium (IMDM) (Invitrogen) with 15% heat inactivated fetal bovine serum (Fisher), recombinant Epo (2u/ml) (Amgen, Thousand Oaks, CA), recombinant mouse (rm) SCF (50 ng/ml) (Amgen, Thousand Oaks, CA).

**Apoptosis analysis on G1E-ER2 cells**

G1E-ER2 cells were starved for 5 hours of growth factors and serum and cultured for 48 hours in the presence of 50 ng/ml of SCF or TPO or 2 U/ml of Epo. Apoptosis was measured by staining the cells with Annexin and analyzed by flow cytometry. Briefly, cells were resuspended in 100 µl of 1X binding buffer and 5 µl of Annexin V. Cells were then vortexed and incubated for 15 minutes at room temperature. After which an additional 400 µl of 1X binding buffer was added, and cells were analyzed by flow cytometry as described before 28.

**Immunoprecipitation**
G1E-ER2 cells were starved for 6 hours at 37°C and stimulated with 500 ng/ml of TPO for indicated times or left unstimulated. Cells were lysed and equal amount of protein was subjected to immunoprecipitation using an anti-murine Mpl antibody (kindly provided by Frederic J. de Sauvage, Genetech Inc, South San Francisco, CA) and western blot analysis was performed with an anti-phosphotyrosine antibody as described previously 28.

Statistical analysis

Data are expressed as the mean ± SEM where applicable. Differences between groups were analyzed by means of a non-parametric Mann-Whitney test. A probability value of less than 0.05 was considered significant. All experiments were performed in triplicate on two to four experiments.

RESULTS

Tpo and c-mpl are expressed in early embryonic development

We examined early whole embryos for Tpo and c-mpl mRNA expression. For E6.5 and E7.0 embryos, the whole embryos were used for RNA extraction. For E8.0 and later embryos, we dissected yolk sac and embryo proper tissues for RNA extraction and nested RT-PCR analysis. A 220 base pair (bp) nucleotide PCR product representing Tpo and a 368 bp nucleotide product representing c-mpl were observed in E6.5 and E7.0 whole embryo tissue (Fig. 1). Similar products were present in both yolk sac and embryo proper cells isolated on E8.0, E9.0, E10.0, E11.0, E13.0 and E14.0. These results indicated that both Tpo and c-mpl mRNA are expressed throughout the hematopoietic phase of yolk sac development and in the early embryo during the period of initiation of definitive hematopoiesis.
Figure 1. RT-PCR analysis of thrombopoietin and c-mpl mRNA expression. Whole embryos or embryo proper (ep) and yolk sac (ys) were dissected from timed pregnant female mice. Total RNA was isolated and reverse transcribed into cDNA followed by PCR amplification using specific primers for Tpo (220bp) + c-mpl (368bp). Products were run on 2% agarose gel and the size was determined by a 100bp DNA standard ladder.

The presence of mRNA for these molecules in the gastrulating embryo caused us to question whether Tpo and c-mpl were expressed prior to gastrulation. We isolated mRNA from murine blastocysts and an embryonic stem cell line (R1) for RT-PCR analysis and identified both Tpo and c-mpl mRNA transcripts (Xie and Chen, accepted for publication of FEBS). These results indicate that Tpo and c-mpl are expressed in pluripotent cells prior to and during gastrulation. This early appearance of Tpo and c-mpl message predates the emergence of not only megakaryocytes but of all hematopoietic elements in both the yolk sac and embryo proper.

Tpo diminishes primitive and definitive erythroid CFU formation but enhances mixed lineage and megakaryocyte CFU enumeration
To determine the effect of Tpo on early hematopoietic progenitor formation in vitro, we examined primitive and definitive erythroid CFU growth. In E7.5 whole embryos, only primitive erythroid CFU (EryP) can be detected (Fig. 2a). In E8.25 whole embryo and E8.5 yolk sac, both primitive and definitive CFU cells can be detected (Fig. 2b and 2c). In the E9.5 yolk sac, primitive erythroid CFU had disappeared and only definitive CFU were enumerated in culture (Fig. 2d). The presence of Tpo in the culture system resulted in a decreased number of EryP in cultured E7.5 and E8.25 whole embryo and E8.5 yolk sac and embryo proper cells (Fig. 2a, 2b and 2c). Tpo also decreased the output of definitive erythroid CFU cells (BFU-E) in E8.25 whole embryo and E8.5 and E9.5 yolk sac cell cultures (Fig. 2b, 2c and 2d).

Figure 2. Effect of Tpo on embryo or yolk sac primitive erythroid (EryP), burst forming unit-erythroid (BFU-E), colony forming unit-mixed (MIX), colony forming unit-granulocyte-macrophage (GM), colony forming unit-megakaryocyte (CFU-Meg), and burst forming unit-megakaryocyte (BFU-Meg) CFC formation. E7.5 whole embryo (a), E8.25
whole embryo (b), E8.5 yolk sac (c) and E9.5 yolk sac (d) cells were plated in the primitive erythroid colony assay or progenitor assay or megakaryocyte assays (as described in the materials and methods) in the presence or absence of 50ng/ml Tpo. * P<0.05, –Tpo compared to +Tpo.

However, the presence of Tpo in the same cultures augmented the number of CFU-Mix enumerated in E8.25 whole embryo and E8.5 and E9.5 yolk sac cell cultures (Fig. 2b, 2c and 2d). As anticipated, Tpo increased the production of both CFU-Meg and BFU-Meg in E8.25 whole embryo and E8.5 and E9.5 yolk sac cell cultures (Fig. 2b, 2c and 2d). We also identified colonies in cultures containing E8.5 and E9.5 embryo proper cells. Although the number of CFU enumerated was much lower than that of yolk sac (Table 1), Tpo diminished BFU-E output by 50%, had no effect on CFU-Mix, and increased CFU- and BFU-Meg by 2-fold.

Table 1. Progenitor number per E8.5 or 9.5 embryo proper cultured in the presence or absence of 50ng/ml Tpo.

<table>
<thead>
<tr>
<th></th>
<th>E8.5 embryo proper</th>
<th>E9.5 embryo proper</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Tpo</td>
<td>+Tpo</td>
</tr>
<tr>
<td>EryP</td>
<td>46.0 ± 2.1</td>
<td>31.7 ± 2.2</td>
</tr>
<tr>
<td>BFU-E</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Mix</td>
<td>0.63 ± 0.32</td>
<td>0.63 ± 0.32</td>
</tr>
<tr>
<td>GM</td>
<td>0.32 ± 0.32</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>CFU-Meg</td>
<td>0 ± 0</td>
<td>0.48 ± 0.48</td>
</tr>
<tr>
<td>BFU-Meg</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
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</table>

Dose dependent effects of Tpo on hematopoietic progenitor formation in vitro

To further confirm the above results, we examined the dose dependent effects of Tpo on different progenitor compartments, including EryP, BFU-E, CFU-Mix, CFU-GM and CFU-Meg in cells
isolated from these early embryos. Except for CFU-GM, all CFU responded to added Tpo in a dose dependent fashion. BFU-E and EryP CFU decreased in a reciprocal fashion as the concentration of Tpo increased in the cultures containing E8.5 or E9.5 yolk sac cells (Fig. 3a and 3b). On the contrary, CFU-Mix and CFU-Meg frequencies linearly increased with increasing concentrations of added Tpo (Fig. 3b). Therefore, colony formation of EryP, BFU-E, CFU-Mix and CFU-Meg are responsive to Tpo over a wide range of concentrations, while CFU-GM appeared unresponsive (data not shown).

![Graph](image1)

**Figure 3.** Tpo treatment modulates formation of EryP, BFU-E, CFU-MIX, and CFU-Meg in a dose dependent fashion. E8.5 yolk sac cells were plated at 10,000 cells/ml in primitive erythroid colony assay containing various concentration of Tpo (a). E9.5 yolk sac cells were plated 10,000 cells/ml in the progenitor assay or megakaryocyte assay containing various concentrations of Tpo. * P<0.05, various concentrations of Tpo compared to no Tpo.

**Effect of anti-Tpo neutralizing antibody on Tpo modulation of CFU formation**

To further substantiate that the effects of Tpo on hematopoietic progenitor formation are a direct effect of Tpo, we examined the consequences of the addition of an antibody that binds to
and neutralizes Tpo action by blocking binding of Tpo to c-mpl. Because of the endogenous expression of Tpo and c-mpl, we first confirmed that addition of the anti-murine Tpo neutralizing antibody (anti-mTpo) to cultures had no effect on baseline CFU formation (Table 2).

Table 2. Addition of anti-murine Tpo neutralizing antibody to cultures has no effect on baseline CFU formation.

<table>
<thead>
<tr>
<th></th>
<th>EryP</th>
<th>BFU-E</th>
<th>CFU-Mix</th>
<th>CFU-Meg</th>
</tr>
</thead>
<tbody>
<tr>
<td>G.F. only</td>
<td>474.5 ± 40.0</td>
<td>10.3 ± 0.5</td>
<td>19.8 ± 0.8</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>G.F. + hTpo</td>
<td>235.0 ± 22.3</td>
<td>5.3 ± 0.5</td>
<td>33.3 ± 2.5</td>
<td>6.0 ± 0.4</td>
</tr>
<tr>
<td>G.F. + anti-m Tpo</td>
<td>474.5 ± 35.6</td>
<td>12.0 ± 0.9</td>
<td>20.8 ± 1.0</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>G.F. + control</td>
<td>455.5 ± 30.6</td>
<td>10.0 ± 0.9</td>
<td>19.0 ± 0.9</td>
<td>1.0 ± 0.0</td>
</tr>
</tbody>
</table>

E8.5 yolk sac for EryP assay and 10,000 cells/ml of E9.5 yolk sac for other progenitor assays. Anti-murine neutralizing antibody (anti-mTpo) or control antibody was 0.3 µg/ml.

We noted no change in the number of hematopoietic progenitors cultured in the presence or absence of the anti-mTpo neutralizing antibody suggesting that endogenous murine Tpo present in the early embryo is not playing a significant role in the methycellulose cultures upon plating of yolk sac or embryo proper cells. Adding anti-human Tpo (anti-hTpo) neutralizing antibody to the culture system in the presence of human (hTpo) blocked the previously observed hTpo-induced inhibition of EryP colony formation. This resulted in an observed increase in the number of EryP CFU compared to cultures with hTpo but no added neutralizing antibody. Increasing concentrations of neutralizing antibody completely rescued EryP colony numbers in the presence of hTpo, while a control antibody did not show any effect (Fig. 4a). Similarly, BFU-E colony numbers were significantly increased by adding anti-hTpo neutralizing antibody in hTpo-supplemented cultures to the level approaching those cultures containing no hTpo (Fig. 4b). However, anti-hTpo neutralizing antibody decreased the number of CFU-Mix and CFU-Meg in cultures with added hTpo (Fig. 4a and 4b). CFU-GM formation was the same in cultures with or without addition of anti-hTpo neutralizing antibody or control antibody (data not shown).
These results strongly suggest that the effects of hTpo on EryP, BFU-E, CFU-Mix and CFU-Meg colony formation in cells from E8.5 and E9.5 yolk sac cells are mediated through c-mpl signaling upon Tpo binding.

**Figure 4.** The inhibitory effect of hTpo on erythroid and the positive effect of hTpo on CFU-MIX and CFU-Meg are blocked by adding neutralizing anti-hTpo antibody in the cultures. E8.5 yolk sac cells were plated at 2,500 cells/250µl in 24-well plates in the primitive erythroid colony assay containing various concentrations of neutralizing anti-hTpo antibody or control antibody (a). E9.5 yolk sac cells were plated at 10,000 cells/ml in the progenitor assay or megakaryocyte assay containing 0.5µg/ml neutralizing anti-hTpo antibody or control antibody (b). * The statistical significance when various conditions are compared to the condition of no Tpo, P<0.05. ** The statistical significance when hTpo+anti-hTpo group is compared to that of hTpo and hTpo+control antibody, P<0.05.
Phenotype of Mixed lineage colonies in the presence and absence of Tpo

As previously described, the presence of Tpo in the culture medium can increase the number of CFU-Mix colonies. In order to investigate whether Tpo changes the composition of these colonies, we compared the cellular composition of CFU-Mix colonies grown in the presence or absence of Tpo. CFU-Mix are heterogeneous colonies of multiple lineages that we have classified according to composition including: I, red blood cell and macrophage; II, red blood cell, macrophage and neutrophil; III, red blood cell, macrophage, neutrophil and megakaryocyte. The percentages of different cell types were not different in the presence or absence of Tpo. In 24 CFU-Mix colonies grown in the absence of Tpo, 32% were type I composition, 36% type II, and 32% type III. In CFU-Mix colonies grown in the presence of Tpo, 30% were type I composition, 35% type II, and 35% type III. Thus, while Tpo significantly increased CFU-Mix formation, Tpo had no effect on changing the composition of cell lineages within CFU-Mix colonies.

Effect of Tpo on c-kit⁺CD34⁺ population

To document that Tpo acts directly on hematopoietic progenitor cells and not indirectly via nonhematopoietic cells present in the yolk sac or embryo proper tissue preparations, we isolated a E9.5 yolk sac c-kit⁺CD34⁺ population that has been shown to be enriched for hematopoietic progenitor and long term repopulating HSC. Sorted cells were plated in definitive committed CFU assays as above with or without addition of Tpo. In the presence of Tpo and other hematopoietic growth factors, c-kit⁺CD34⁺ cells gave rise to more CFU-Mix and CFU-Meg colonies, but less BFU-E colonies than in the absence of added Tpo. As expected, the presence of Tpo did not change the frequency of CFU-GM colonies (Table 3). As a negative
control, we also plated E9.5 pc yolk sac c-kit−CD34− cells. These antigen negative cells failed to give rise to any CFU cells with or without the addition of Tpo to the cultures. These data suggest that enriched hematopoietic progenitor cells behave similar to unfractionated yolk sac cells in response to Tpo in clonogenic assays. We interpret these results to indicate that the effects of Tpo on hematopoietic progenitors is not dependent on the presence of a Tpo responsive accessory cell type that is secreting unknown factors to inhibit erythroid and augment mixed lineage and megakaryocyte CFU formation.

Table 3. Effect of Tpo on c-kit+CD34+ cells.

<table>
<thead>
<tr>
<th></th>
<th>BFU-E</th>
<th>CFU-Mix</th>
<th>CFU-GM</th>
<th>CFU-Meg</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-kit+CD34+</td>
<td>-Tpo</td>
<td>11.5 ± 1.7</td>
<td>24.5 ± 2.1</td>
<td>12.2 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>+Tpo</td>
<td>5.0 ± 1.0</td>
<td>39.5 ± 1.6</td>
<td>12.8 ± 0.5</td>
</tr>
<tr>
<td>c-kit-CD34-</td>
<td>-Typo</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+Tpo</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

Sorted cells from E9.5 yolk sac were plated at 500 cells/ml.

To confirm the effect of Tpo on sorted cells, were performed a single cell analysis of progenitor cell formation in vitro. In three experiments, single c-kit+CD34+ cells from E9.5 yolk sac were sorted into 96-well plates in the presence or absence of Tpo. This assay is more informative since it compares all lineage readouts under identical in vitro conditions. Representative data from one of three experiments (CFU plating efficiency is 145% with 15.4% of single cell deposition and 10.6% of standard CFU assay) revealed that the number of BFU-E cloned was 5/373 cells plated in the presence of Tpo and 13/374 cells plated in the absence of Tpo while the number of CFU-Mix cloned was 34/373 and 33/374, respectively. Tpo had no apparent effect on the number of CFU-GM, as the number of CFC cloned was 15/373 cells plated in the presence of Tpo and 15/374 in the absence of Tpo. The number of megakaryocyte containing CFC was 10/373 cells plated in the presence of Tpo and 4/374 in the absence of Tpo.
While the effect of Tpo was not apparent on CFU-Mix formation in these experiments, the effects of Tpo on BFU-E and megakaryocyte colonies was similar to the results obtained when c-kit+CD34+ yolk sac-derived cells were plated in a bulk progenitor assays and supports a direct effect of Tpo on these progenitor cells.

**Effect of Tpo on late yolk sac and adult marrow CFU**

Given the evidence that Tpo has a positive effect on E10.5 yolk sac and adult marrow erythroid CFU formation, we also examined the effect of Tpo on late yolk sac and adult marrow progenitors under our culture conditions. In E10.5 embryo proper (Fig 5a), few colonies were identified; a finding consistent with previous studies. Significantly, more colonies were found in plated E10.5 yolk sac cell cultures (Fig 5b). Tpo did not alter BFU-E and CFU-Mix formation, but CFU-Meg significantly increased (Fig. 5b).
Figure 5. Effect of Tpo on E10.5 embryo, yolk sac and adult bone marrow BFU-E, CFU-Mix, CFU-GM, and CFU-Meg. E10.5 embryo (a) and yolk sac (b) cells at 10,000 cells/ml and adult bone marrow cells (c) at 50,000 cells/ml were plated in the progenitor assay (as described in the materials and methods) in the presence or absence of 50ng/ml Tpo. * P<0.05, –Tpo compared to +Tpo.

To further characterize the lack of effect of Tpo on BFU-E formation at the functional and biochemical level, we utilized an ES cell derived erythroid progenitor cell line, G1E-ER2 27-29. These cells were derived from in vitro differentiated GATA-1/- ES cells and represent primary GATA-1/- erythroblasts as determined by the expression of erythroid, but not myeloid genes 27. Remarkably, restoration of GATA-1 function in these cells restores erythroid maturation 27,28. Utilizing these cells, we examined the effect of Tpo in augmenting or inhibiting cell survival and proliferation, alone, or in the presence of SCF and Epo. As seen in Figure 6b, in spite of significant phosphorylation of c-Mpl in response to Tpo stimulation, Tpo alone or in the presence of SCF or Epo did not significantly modulate erythroid cell survival (Figure 6a) or proliferation (data not shown). These results demonstrate that in spite of the presence of a functional c-Mpl receptor, SCF or Epo fail to augment or inhibit the effects of Tpo in erythroid cells. These results are consistent with our results in primary late yolk sac cultures, and suggest that Tpo might differentially regulate erythropoiesis at different stages of erythroid cell development. Tpo also increased BFU-E formation in plated adult bone marrow cells (Fig 5c). Consistent with the lack of a Tpo effect on CFU-GM in early murine cells, Tpo did not change CFU-GM formation in plated E10.5 yolk sac or adult marrow cells. Thus the effect of Tpo on
early yolk sac hematopoietic cells varies from the effect of Tpo on late yolk sac, adult marrow or cultured erythroblast cells.

Figure 6. Biochemical and functional effects of Tpo on erythroid cell line G1E-ER2. a. G1E-ER2 cells were starved for 6 hours and stimulated with TPO for indicated time or left unstimulated. Immunoprecipitation was performed using an anti-c-Mpl antibody, followed by western blot analysis using an anti-phosphotyrosine antibody. Top panel shows the phosphorylation of c-Mpl, and the bottom panel shows the expression of total c-Mpl protein in each lane. b. G1E-ER2 cells were starved for 5 hours and cultured for 48 hours in the presence of 50 ng/ml of SCF or TPO or 2 u/ml of Epo. Apoptosis was measured by staining the cells with annexin and analyzed by flow cytometry as described in methods.

DISCUSSION

We have demonstrated the presence of Tpo and c-mpl mRNA transcripts in pregastrulating embryonic cells and in yolk sac and embryo proper cells during early embryogenesis. We have also provided evidence that Tpo inhibits erythroid colony formation in
vitro in cells isolated from pre-somite staged embryos and yolk sacs but augments megakaryocyte lineage (CFU-Meg and BFU-Meg) and mixed lineage CFU cell formation from these same tissues. These data support a growing body of literature suggesting that the effects of Tpo on hematopoiesis are multi-lineage and highlights differences in the response of hematopoietic progenitors to growth factors at different stages of ontogeny.

Murine hematopoiesis arises in yolk sac blood islands and becomes morphologically identifiable at E7.0-7.5\textsuperscript{31,32}. At first, primitive erythroid progenitors comprise nearly all blood island progenitors, however, definitive progenitors begin to emerge at E8.25\textsuperscript{33}. We have investigated the temporal emergence of Tpo and c-mpl mRNA transcripts and examined the biological responsiveness of early yolk sac and embryo proper hematopoietic progenitors to pharmacological doses of Tpo in vitro. Based on our RT-PCR analysis, the transcripts of Tpo and c-mpl were present prior to the appearance of the first yolk sac blood islands and throughout the hematopoietic phase of embryo development. Since blood cells start to emerge on E7.0–7.5, we isolated embryos at this stage and later to determine if hematopoietic progenitors were Tpo responsive. In E7.5 embryos and older, we found two waves of detectable progenitors: primitive erythroid and definitive progenitors. The first wave of primitive erythroid CFU emerged on E7.5 but were undetectable at E9.5 in either the yolk sac or embryo proper. The second wave of definitive progenitors was first identifiable on E8.25. These findings are consistent with those published by Palis et al.\textsuperscript{19} who used an 11 growth factor combination to identify primitive erythroid progenitors on E7.0 of gestation and emergence of definitive progenitor cells in the yolk sac on E8.25. From E8.5 and later in the present studies, isolated embryos were dissected into embryo proper and yolk sac and we identified definitive CFU cells in both yolk sac and embryo proper. At all stages, the frequency of each CFU lineage in the yolk sac exceeded those
found in the embryo proper. Our data support the results of Palis et al. 19 who speculated that the definitive hematopoietic progenitors emerge predominantly in the yolk sac and subsequently enter the circulation for colonization of the fetal liver.

We identified a dose dependent inhibitory effect of Tpo on both primitive and definitive erythroid CFU formation. In contrast, Tpo augmented megakaryocyte lineage (CFU-Meg & BFU-Meg) and CFU-Mix formation in a dose dependent manner. These apparent direct effects of hTpo on hematopoietic progenitors were confirmed by anti-hTpo neutralizing antibody studies. When cultures containing hTpo were supplemented with anti-hTpo neutralizing antibody, the biologic effects of the growth factor were abrogated in every experimental group. Interestingly, the inhibitory effect of hTpo on BFU-E formation was totally rescued by adding 0.5 µg/ml anti-hTpo neutralizing antibody in the hTpo supplemented culture system (Fig. 4b). However, the inhibitory effect of hTpo on EryP formation was not totally rescued using the same concentration of neutralizing antibody (Fig. 4a). At the same time, the positive effect of hTpo on CFU-Mix and CFU-Meg formation was also not totally abolished by 0.5 µg/ml anti-hTpo neutralizing antibody (Fig. 4b). These observations may be explained by varying sensitivities of different progenitors to Tpo.

We did not observe an effect of Tpo on E10.5 yolk sac erythroid progenitor cells, as previously noted by Era et al. 13. This may be explained by the different serum and cytokines that we used in our study. We also failed to see any effect of Tpo on our erythroblast cell line despite the evidence of c-Mpl receptor activation. We did observe a positive effect of Tpo on BFU-E formation in plated adult marrow cells. This effect is similar to that observed when mice deficient in Tpo expression (Tpo/- mice) were treated with exogenous Tpo. While the lack of Tpo (Tpo/-) resulted in diminished BFU-E, CFU-Mix, CFU- and BFU-Meg progenitor cell
numbers, all of these progenitor compartments were rescued to greater than wild-type control levels when exogenous Tpo was administrated in vivo. These results indicate that Tpo differentially affects erythroid CFU formation depending on the stage and site of hematopoietic development in the mouse whereas the effects of Tpo on CFU-Mix and CFU- and BFU-Meg are similar throughout murine ontogeny.

There are several possibilities to explain the mechanism through which Tpo simultaneously inhibits erythroid but augments mixed lineage and megakaryocyte CFU formation in the early embryo. First, Tpo might send an inhibitory signal to erythroid precursors but a growth progression signal to mixed lineage and megakaryocyte progenitors directly or indirectly, although whether this is true or not need to be further studied. As noted above, we did not observe a Tpo induced apoptotic response in cultured erythroid cells. Alternatively, similar to the in vitro study of ES cell-derived hematopoiesis, the idea of bi-potent clonogenic precursor for both erythroid and megakaryocyte may explain what we observed. According to this theory, the cell may respond to Tpo by preferentially differentiating into mixed lineage and megakaryocyte progenitors at the expense of erythroid progenitors. Because of the different kinetics of erythropoiesis and megakaryocytopenesis (a common precursor may give rise to a number of erythroid progenitors, but just a few megakaryocyte progenitors), comparing CFU cell generation on a 1:1 basis from the putative bi-potent precursor may be inadequate if not inaccurate. However, it may still be interesting to determine if such bi-potent burst progenitors may give rise to definitive erythropoiesis and megakaryopoiesis in the early yolk sac and if the pattern of differentiation is altered in the presence of Tpo.

Taken together, our data indicate that Tpo and c-mpl are expressed in early embryonic development. Tpo negatively regulates the formation of erythroid but up-regulates mixed
lineage and megakaryocyte CFU growth in early yolk sac and embryo proper cells in vitro. It remains unclear if Tpo plays a significant role in steady state hematopoiesis in the yolk sac in vivo. Detailed analysis of yolk sac hematopoiesis in existing mutant mice in which Tpo or c-mpl have been disrupted may be informative. Such variation in the response of early and late yolk sac and adult marrow cells to Tpo is similar to previously reported differences in growth factor responsiveness of embryonic versus fetal and adult mouse progenitor cells treated with Epo or SCF and highlights how differences in the hematopoietic microenvironment influence progenitor cell behavior\textsuperscript{39,40}.

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REFERENCE


Thrombopoietin promotes mixed lineage and megakaryocytic colony forming cell growth but inhibits primitive and definitive erythropoiesis in cells isolated from early murine yolk sacs

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