Thrombopoietin acts synergistically with LIF to maintain an undifferentiated state of embryonic stem cells homozygous for a Shp-2 deletion mutation

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Abstract Thrombopoietin (Tpo) and its receptor, c-mpl, are expressed in murine embryonic stem (ES) cells. ES cells are maintained in a pluripotent state by leukemia inhibitory factor (LIF) via activation of the Janus kinase (Jak)–STAT3 signaling pathway. Tpo, like LIF, activates STAT3. We report that Tpo increases the number of undifferentiated colonies derived from wild type or Shp-2 mutant (Shp-2$^{\Delta 46-110}$) ES cells. Tpo plus LIF acted synergistically on the Shp-2$^{\Delta 46-110}$ ES cells to maintain undifferentiated colonies but no evidence of synergism via Jak–STAT3 activation was detected. Collectively, these data suggest that Tpo can play a role in preventing ES cell differentiation via Jak–STAT3 activation and perhaps via novel pathways that are enhanced in the absence of functional Shp-2.

Key words: Tpo; Shp-2; ES cell; Differentiation; LIF; STAT3

1. Introduction

Thrombopoietin (Tpo) and its receptor (c-mpl) were identified as critical for normal megakaryocyte and platelet growth [1–3]. However, Tpo has also been shown to be important for the maintenance and growth of hematopoietic stem cells (HSCs) [4,5]. The first clue regarding Tpo's involvement in HSC homeostasis was the observation that a truncated, constitutively active form of the Tpo receptor encoded by *v-mpl*, induced a myeloproliferative disorder in mice infected with this oncogene [6]. HSCs from mice in which either the *Tpo* or *c-mpl* locus is genetically disrupted do not undergo normal self-renewal and differentiation [4,5]. Additionally, Tpo has been shown to mediate expansion of HSC ex vivo [7]. Given the expanded function of Tpo from megakaryopoiesis to HSC homeostasis, we were interested in examining its role in the early murine embryo.

Embryonic stem (ES) cells are derived from the inner cell mass of the developing blastocyst and are able to differentiate into multiple tissues in vitro [8]. Originally, ES cells were cultured in the presence of mitotically inactivated murine fibroblasts that produced 'differentiation inhibitory activity' (DIA) which allowed for propagation with the maintenance of ES cell pluripotency in culture. Subsequently, DIA was found to be substitutable by leukemia inhibitory factor (LIF) [9,10]. However, there is evidence that cytokines in addition to LIF have the capacity to maintain ES cells in an undifferentiated state [11].

Shp-2 is a cytoplasmic tyrosine phosphatase with two SH2 domains at the N-terminus. ES cells homozygous for an exon 3 deletion from the *Shp-2* locus causing an in-frame deletion of amino acids 46–110 (Shp-2$^{\Delta 46-110}$), were found to be hypersensitive to the differentiation-inhibitory effect of LIF [12] and to have increased LIF-stimulated phospho-STAT3 (P-STAT3) levels compared to wild type (WT) ES cells (Chan et al., manuscript in preparation). Several studies have demonstrated that both Shp-2 and STAT3 are recruited and activated, respectively, upon c-mpl stimulation [13–15]. Therefore, we have examined the effect of Tpo and the combination of Tpo plus LIF on maintaining an undifferentiated state of WT and Shp-2$^{\Delta 46-110}$ ES cells as well as on P-STAT3 levels in Shp-2$^{\Delta 46-110}$ ES cells.

Collectively, these studies show that Tpo-stimulated pathways are able to contribute to maintaining ES cells in an undifferentiated state, although the effect is more pronounced in the Shp-2$^{\Delta 46-110}$ ES cells. Additionally, Tpo plus low concentrations of LIF act synergistically to maintain Shp-2$^{\Delta 46-110}$ in an undifferentiated state; however, the Tpo plus LIF-induced P-STAT3 level in Shp-2$^{\Delta 46-110}$ ES cells was not increased synergistically, suggesting that pathways in addition to LIF- and Tpo-stimulated P-STAT3 are necessary to maintain ES cells in an undifferentiated state.

2. Materials and methods

2.1. ES cell culture and cell lines

ES cells were maintained on gelatinized plates in Dulbecco's modified Eagle's medium with 15% ES cell-qualified fetal calf serum (Hyclone, Logan, UT, USA), 55 μM β-mercaptoethanol, and 1000 U/ml LIF (ESGRO, Gibco BRL). The Shp-2$^{\Delta 46-110}$ ES cell line, IC3, has been described previously [12,18].

2.2. Reverse-transcribed polymerase chain reaction (RT-PCR)

Total cellular RNA was prepared using TRIzol Reagent (Gibco

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Abbreviations: Tpo, thrombopoietin; LIF, leukemia inhibitory factor; STAT3, signal transducer and activator of transcription; HSC, hematopoietic stem cell; ES cell, embryonic stem cell; DIA, differentiation inhibitory activity; Jak, Janus kinase; WT, wild type

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BRL) according to the manufacturer's instructions. First strand synthesis of cDNA was performed using poly-dT primer and reverse transcriptase (SuperScript™, Invitrogen, Carlsbad, CA, USA). Specific primers were used in nested PCR to amplify cDNA. Fragments were subcloned into a TA Cloning Vector (Invitrogen) and sequenced to verify fragment identity.

2.3. Colony differentiation assay

WT and Shp-$2^{\Delta 46-110}$ ES cells were cultured at a colony dilution on gelatinized plates for 5–7 days in ES cell LIF-containing media. The resulting colonies were cultured for 96 h in media containing various concentrations of LIF, recombinant human Tpo (rhTpo), and in some assays, various concentrations of anti-rhTpo neutralizing antibody (R&D, Minneapolis, MN, USA). The colonies were stained with Giemsa and scored as differentiated when surrounded by flattened, fibroblast-like outgrowths. Additionally, colonies were stained with alkaline phosphatase diagnostic kit R-86 (Sigma, St. Louis, MO, USA). Colonies were scored as undifferentiated when the entire colony was stained red and were scored as differentiated when surrounded by flattened, alkaline phosphatase-negative cells.

2.4. Immunoblot analysis and antibodies

Control and LIF-stimulated cell lysates were prepared as previously described [19]. ES cells were cultured in serum-free, LIF-free maintenance media containing 0.5% bovine serum albumin (Sigma) for 6 h, followed by stimulation for various times with LIF, Tpo or the combination. Total cell lysates were electrophoresed on a 10% polyacrylamide gel followed by transfer to a nitrocellulose membrane. Anti-P-STAT3 and anti-STAT3 were from New England Biolabs (Beverly, MA, USA). Signals were detected by enhanced chemiluminescence.

2.5. Statistical analysis

Groups were compared using non-paired, two-sided, Student's *t*-test.

3. Results

3.1. Tpo and c-mpl are expressed in WT and Shp-$2^{\Delta 46-110}$ ES cells

Previous studies have indicated the presence of Tpo and c-mpl messages in the murine embryo as early as 6.5 days post coitum (Xie et al., manuscript submitted). Using RT-PCR, we found both the Tpo and c-mpl messages expressed in the murine blastocyst, WT ES cells, and Shp-$2^{\Delta 46-110}$ ES cells (Fig. 1). We have sequenced the corresponding bands, and found the expected sequences of Tpo and c-mpl (data not shown). Negative controls utilizing water in lieu of template yielded no Tpo or c-mpl bands (data not shown).

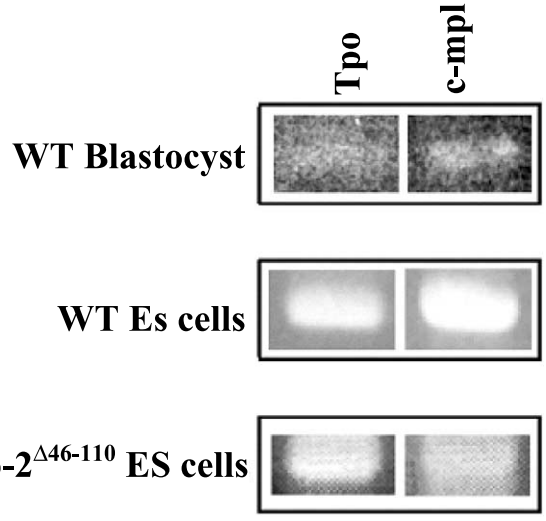

Fig. 1. RT-PCR products of Tpo and c-mpl from WT blastocysts, WT ES cells, and Shp-$2^{\Delta 46-110}$ ES cells.

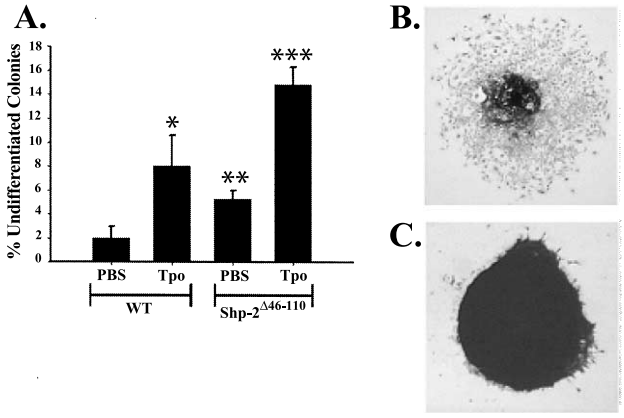

Fig. 2. A: Percentage of undifferentiated ES cell colonies following culture +/− 10 ng/ml Tpo. $*P = 0.07$ comparing WT ES cells treated with PBS vs. Tpo; $**P = 0.03$ comparing WT to Shp-$2^{\Delta 46-110}$ ES cells, both treated with PBS; $***P < 0.0001$ comparing Shp-$2^{\Delta 46-110}$ ES cells treated with PBS vs. Tpo. B: Representative differentiated colony surrounded by flattened, fibroblast-like cells. C: Representative undifferentiated, well-circumscribed colony.

3.2. Tpo mediates an undifferentiated phenotype of WT and Shp-$2^{\Delta 46-110}$ ES cells

The presence of Tpo and c-mpl in ES cells prompted us to examine their roles in ES cell function. WT and Shp-$2^{\Delta 46-110}$ ES cell colonies were cultured without LIF but in the presence of 10 ng/ml Tpo or vehicle (phosphate-buffered saline, PBS). Nearly all of the WT colonies became differentiated in the presence of PBS (2% undifferentiated colonies, Fig. 2). Upon the addition of Tpo, however, the number of undifferentiated colonies increased to 8%. The Shp-$2^{\Delta 46-110}$ cells had more undifferentiated colonies compared to the WT cells when cultured in the presence of PBS (2% vs. 5%, Fig. 2), as described previously [12]. The addition of Tpo, however, increased the number of undifferentiated colonies from 5% to 15% (Fig. 2). These data demonstrate that Tpo has the capacity to maintain ES cells in an undifferentiated state and that the effect appears to be stronger in the absence of normal Shp-2 function.

3.3. Tpo acts synergistically with LIF to maintain an undifferentiated phenotype of Shp-$2^{\Delta 46-110}$ ES cells

Previous work demonstrated that the Shp-$2^{\Delta 46-110}$ cells are hypersensitive to LIF signaling [12]; therefore, we examined the effect of LIF and of Tpo plus LIF on maintaining an undifferentiated ES cell phenotype. WT and Shp-$2^{\Delta 46-110}$ ES cell colonies were cultured with vehicle (PBS), 10 ng/ml Tpo, 10 U/ml LIF, 10 ng/ml Tpo plus 10 U/ml LIF, 100 U/ml LIF, or 1000 U/ml LIF. LIF at 100 or 1000 U/ml resulted in an increased number of undifferentiated colonies for Shp-$2^{\Delta 46-110}$ compared to WT ES cells, consistent with previous studies (Fig. 3A). The addition of Tpo to low concentrations of LIF (10 U/ml) had no effect on maintaining WT ES cells in an undifferentiated state compared to 10 U/ml LIF alone (Fig. 3A). However, the combination of Tpo and LIF (10 U/ml) caused a dramatic increase in the amount of undifferentiated Shp-$2^{\Delta 46-110}$ colonies (Fig. 3A). The observation that this synergistic effect is limited to the Shp-$2^{\Delta 46-110}$ cells implies that

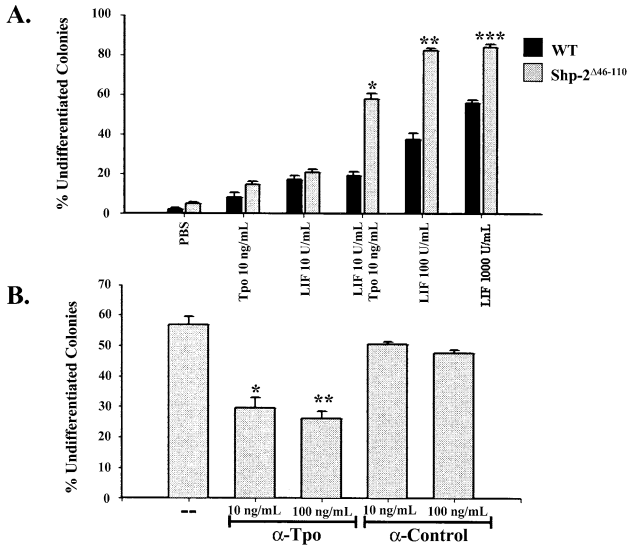

Fig. 3. A: Percentage of undifferentiated colonies after culture with PBS, 10 ng/ml Tpo, 10 U/ml LIF, 10 U/ml LIF plus 10 ng/ml Tpo, 100 U/ml LIF, or 1000 U/ml LIF. $*P<0.0001$ comparing Shp-$2^{\Delta 46-110}$ ES cells treated with 10 U/ml LIF vs. 10 U/ml LIF plus 10 ng/ml Tpo; ** and $***P<0.0001$ comparing WT to Shp-$2^{\Delta 46-110}$ ES cells treated with 100 U/ml or 1000 U/ml LIF, respectively. B: Percentage of undifferentiated Shp-$2^{\Delta 46-110}$ ES cell colonies after culture with 10 U/ml LIF plus 10 ng/ml Tpo +/− varying concentrations of anti-Tpo neutralizing antibody or control antibody. $*P=0.0016$ and $**P<0.0001$ comparing addition of anti-Tpo at 10 ng/ml and 100 ng/ml, respectively, to 10 U/ml LIF plus 10 ng/ml Tpo alone.

Shp-2 functions to down-regulate Tpo-stimulated signaling pathways, similar to LIF-stimulated pathways (Chan et al., manuscript in preparation).

To determine specifically the contribution of Tpo signaling to this synergistic effect, we examined the consequences of adding Tpo-neutralizing antibody to these cultures. The Tpo-neutralizing antibody blocks binding of Tpo to its receptor, c-mpl. Addition of the Tpo-neutralizing antibody (either at 10 or 100 ng/ml) decreased the undifferentiated colony number (Fig. 3B), in contrast to what is observed when control antibody was added. These data demonstrate that Tpo signaling via c-mpl strongly contributes to the undifferentiated Shp-$2^{\Delta 46-110}$ ES cell phenotype induced by the Tpo plus LIF combination, and that this synergistic effect is not due solely to hypersensitivity of the Shp-$2^{\Delta 46-110}$ ES cells to LIF.

To examine further the effect of Tpo and LIF on maintaining an undifferentiated state of Shp-$2^{\Delta 46-110}$ cells, colonies were also subjected to alkaline phosphatase activity examination, as alkaline phosphatase levels are known to decrease upon ES cell differentiation [20]. As observed in the morphology assay, the addition of 10 ng/ml Tpo alone resulted in an increased number of undifferentiated Shp-$2^{\Delta 46-110}$ colonies compared to PBS (Fig. 4A). Additionally, the combination of 10 U/ml LIF plus 10 ng/ml Tpo caused a synergistic increase in the number of undifferentiated Shp-$2^{\Delta 46-110}$ colonies.

3.4. *Tpo induces activation of STAT3 in ES cells, but does not act synergistically with LIF to activate STAT3*

P-STAT3 has been shown to be crucial for the maintenance of ES cells in an undifferentiated state [16,17] and for self-renewal of ES cells [21,22]. We sought to determine if Tpo

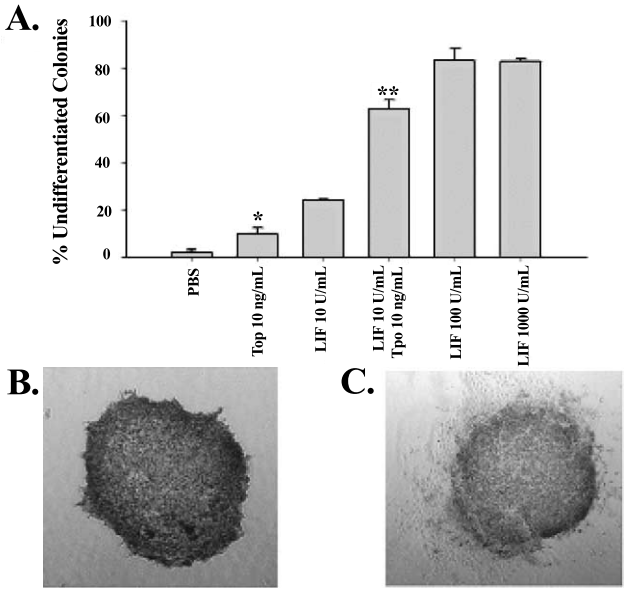

Fig. 4. A: Alkaline phosphatase staining of Shp-$2^{\Delta 46-110}$ ES cell colonies after culture in the presence of PBS, 10 ng/ml Tpo, 10 U/ml LIF, 10 U/ml LIF plus 10 ng/ml Tpo, 100 U/ml LIF, or 1000 U/ml LIF. $*P=0.02$ comparing treatment with 10 ng/ml Tpo vs. PBS; $**P<0.0001$ comparing treatment with 10 U/ml LIF plus 10 ng/ml Tpo vs. 10 ng/ml Tpo. B: Representative undifferentiated colony strongly stained for alkaline phosphatase. C: Representative differentiated colony surrounded by alkaline phosphatase-negative cells.

induced activation of STAT3 in Shp-$2^{\Delta 46-110}$ ES cells. We found that Tpo stimulation causes activation of STAT3 in a dose-dependent manner (Fig. 5A). We next examined the effect of 10 ng/ml Tpo plus 10 U/ml LIF stimulation on P-STAT3 levels, as these doses produced the synergistic increase in the number of undifferentiated colonies. However, the level of P-STAT3 was not increased upon treatment with 10 ng/ml Tpo plus 10 u/ml LIF compared to treatment with 10 u/ml LIF alone (Fig. 5B). We did, however, observe an increase in P-STAT3 upon treatment with 100 or 1000 U/ml LIF, correlating well with LIF doses at which the Shp-$2^{\Delta 46-110}$

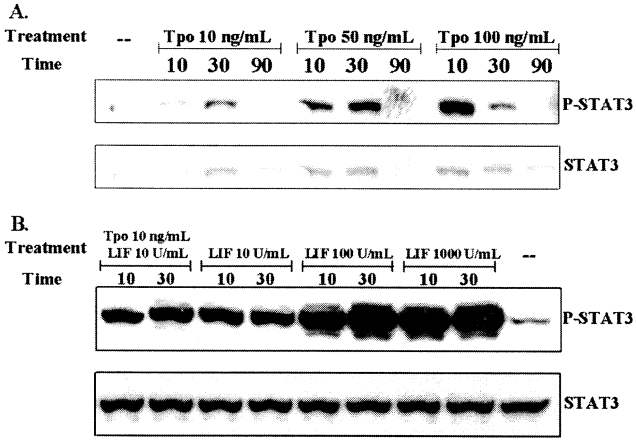

Fig. 5. A: Shp-$2^{\Delta 46-110}$ ES cells were unstimulated (dashed line) or stimulated with 10 ng/ml, 50 ng/ml, or 100 ng/ml Tpo, for 10, 30, or 90 min followed by immunoblot analysis for P-STAT3 and STAT3. B: Shp-$2^{\Delta 46-110}$ ES cells were unstimulated (dashed line) or stimulated with 10 ng/ml Tpo plus 10 U/ml LIF, 10 U/ml LIF, 100 U/ml LIF, or 1000 U/ml LIF for 10 and 30 min followed by immunoblot analysis for P-STAT3 and STAT3.

cells demonstrated LIF hypersensitivity in the colony studies above, suggesting that a synergistic effect of 10 ng/ml Tpo plus 10 U/ml LIF on P-STAT3 levels could be detected if present.

4. Discussion

Tpo was originally found to be a megakaryocytic growth factor [1–3] and subsequently was found to be important for HSC homeostasis [4,5]. We have demonstrated that Tpo can mediate ES cell function as well, namely, by inhibiting ES cell differentiation in vitro. Previously, Filippi et al. reported Tpo-induced effects on ES cell-derived hematopoiesis [23]. In this study, we have demonstrated Tpo-mediated functional effects on ES cells rather that ES cell-derived hematopoietic cells.

The presence of Tpo and c-mpl in the early murine embryo and blastocyst suggested a potential role in ES cell function. We examined the effect of Tpo on WT and Shp-$2^{\Delta 46-110}$ ES cells, as Shp-2 has been shown to be critical in mediating normal LIF-regulated ES cell function [12]. We found that both Tpo and c-mpl are expressed in WT and Shp-$2^{\Delta 46-110}$ ES cells. Although we evaluated RNA levels rather than protein levels, we believe the response of ES cells to Tpo stimulation in the form of P-STAT3 is strong evidence that c-mpl protein is expressed and is functional on ES cells.

As Tpo sustains HSC in an undifferentiated and self-renewing state in vitro [7], we sought to determine the capacity of Tpo to maintain ES cells in an undifferentiated state compared to LIF, the cytokine used routinely to maintain ES cell pluripotency in vitro. Compared to PBS only (vehicle), Tpo increased the number of ES cell colonies that remained undifferentiated. This effect was more pronounced in the Shp-$2^{\Delta 46-110}$ ES cells compared to the WT cells, and the effect was not nearly as great as the effect of LIF (1000 U/ml) for either cell line. These data demonstrate that Tpo is able to participate in maintaining an undifferentiated state of ES cells, but is not sufficient to do so.

We next examined the combination of Tpo plus LIF on ES cell function and found that Tpo worked synergistically with LIF to maintain Shp-$2^{\Delta 46-110}$ ES cell colonies in an undifferentiated state as determined both morphologically and by alkaline phosphatase staining. The synergistic effect of Tpo was blocked by neutralizing antibody, indicating that Tpo stimulation contributes significantly to this synergism. That the dose-dependent effect of the neutralizing antibody was not stronger is likely because 10 ng/ml antibody is adequate to block the low concentration (10 ng/ml) of added Tpo. The fact that we observed a more pronounced effect of Tpo and the Tpo plus LIF combination on the Shp-$2^{\Delta 46-110}$ ES cells implies that the absence of normal Shp-2 function confers increased sensitivity of the Shp-$2^{\Delta 46-110}$ ES cells to Tpo signaling and suggests that Shp-2 acts to down-regulate Tpo signaling in WT ES cells.

As LIF-stimulated P-STAT3 is necessary to maintain ES cells in an undifferentiated state [16] and promotes ES cell self-renewal [21,22], and as Tpo is known to signal to STAT3, we investigated STAT3 as a potential molecule downstream of Tpo and LIF that may mediate the synergistic effect observed in the Shp-$2^{\Delta 46-110}$ ES cells. Consistent with Tpo activation of STAT3 as reported [13,14], phosphorylation of STAT3 was observed in a dose-dependent manner upon stimulation of the Shp-$2^{\Delta 46-110}$ cells. However, surprisingly, we did not observe a synergistic increase in the level of P-STAT3 upon stimulation with LIF plus Tpo, in contrast to the dramatic functional changes observed upon treatment of the Shp-$2^{\Delta 46-110}$ ES cell colonies. These data indicate that signaling molecules downstream of LIF and Tpo in addition to STAT3 are necessary for maintenance of ES cells in an undifferentiated state and suggest that ES cell pluripotency is maintained through the effect of multiple cytokines, working through parallel and potentially overlapping pathways.

The in vitro data presented above demonstrate that exogenously added Tpo can contribute to the maintenance of ES cells in an undifferentiated state. However, the relevance of the observed Tpo effect in vivo on the early murine embryo remains to be examined. The Tpo−/− and c-mpl−/− mice provide excellent models to study these novel roles of Tpo and c-mpl function in early embryogenesis.

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