Brief report
Thrombospondin-1 is not the major activator of TGF-β1 in thrombopoietin-induced myelofibrosis

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Transforming growth factor-β1 (TGF-β1) is the most important cytokine involved in the promotion of myelofibrosis. Mechanisms leading to its local activation in the bone marrow environment remain unclear. As a recent study has highlighted the role of thrombospondin-1 (TSP-1) in platelet-derived TGF-β1 activation, we investigated the role of TSP-1 in the TPOhigh murine model of myelofibrosis. Two groups of engrafted mice, WT TPOhigh and Tsp-1–null TPOhigh, were constituted. All mice developed a similar myeloproliferative syndrome and an increase in total TGF-β1 levels in the plasma and in extracellular fluids of marrow and spleen. Surprisingly, we were able to detect the active form of TGF-β1 in Tsp-1–null TPOhigh mice. Accordingly, these mice developed marrow and spleen fibrosis, with intriguingly a higher grade than in WT TPOhigh mice. Our results show that TSP-1 is not the major activator of TGF-β1 in TPO-induced myelofibrosis, suggesting the contribution of another mechanism in the megakaryocyte/platelet compartment.

Introduction
Transforming growth factor-β1 (TGF-β1) is a pleiotropic cytokine involved in normal tissue repair, and its sustained production induces fibrosis in numerous organs, including myelofibrosis. Mechanisms leading to its activation or in vivo TGF-β1 activity (from inactive proforms) remain unclear, involving interactions with integrins,4-6 thrombospondin-1 (TSP-1),7 or shear forces.8 In a murine model of myelofibrosis obtained by thrombopoietin (TPO) overexpression,9 enhanced release of TGF-β1 occurs mainly from megakaryocytes and, to a lesser extent, from monocytes.10 Because both TGF-β1 and TSP-1 are synthesized and stored in megakaryocyte α-granules,11 abnormal and concomitant release of both molecules may hypothetically lead to pathologic local TGF-β1 activation. As a recent study has highlighted the role of TSP-1 in platelet-derived TGF-β1 activation,12 we investigated the role of TSP-1 in TPO-induced myelofibrosis.

Methods
We used the TPOhigh murine model. All procedures were approved by the Institut Gustave Roussy ethics committee. Bone marrow (BM) cells from C57BL/6 wild-type (WT) or Tsp-1–null13 male littermates were infected with a retrovirus encoding murine TPOβ and engrafted into lethally irradiated WT or Tsp-1–null female hosts, respectively, leading to the following 2 engraftment combinations: WT/WT (ie, WT TPOhigh mice, n = 22) and Tsp-1–null/Tsp-1–null (ie, Tsp-1–null TPOhigh mice, n = 15). Lethally irradiated hosts were engrafted with 4 × 106 cells in 2 independent experiments. Peripheral blood was analyzed every 4 weeks during 3 months using an automated blood counter calibrated for mouse blood (MS9, Melet Schloessing). Control mice included unmanipulated WT and Tsp-1–null mice. At weeks 8 and 12 after engraftment, mice were killed for histologic analysis. Reticulin fibers revealed by silver staining were quantified using ImageJ Version 1.42q software (National Institutes of Health). Microvascular density (MVD) was determined by CD34 staining.14 TPO and TGF-β1 levels in plasma or extracellular fluids were determined with the appropriate Quantikine kits from R&D Systems.

Results and discussion
To assess the role of TSP-1 in the TGF-β1-induced BM fibrosis observed in TPOhigh mice, 2 groups of engrafted mice with BM TPO-transduced cells. WT TPOhigh and Tsp-1–null TPOhigh, were constituted. The transduction efficiency of the progenitor cells, evaluated with a colony-forming assay, was similar in the 2 mouse genotypes (77% ± 5%). Four weeks after engraftment, the TPO concentrations in plasma of WT and Tsp-1–null TPOhigh mice ranged from 1000- to 10 000-fold higher than in WT and Tsp-1–null control mice and remained elevated during the 3 months of follow-up (Figure 1A). The magnitude of this increase was comparable regardless of the TPOhigh mice groups. Chimerism, analyzed by fluorescent in situ hybridization on the Y chromosome (BM donors were male, recipients were female) on whole nucleated BM cells at weeks 8 and 12, when mice were killed for histologic analysis, showed levels more than 90% and similar in both TPOhigh mice groups (data not shown).
WT and TPOhigh mice versus WT TPOhigh mice: *TGFβ levels in extracellular fluids of BM as well as plasma levels (D) are shown in WT and Tsp−1−null control mice and in WT and Tsp−1−null TPOhigh mice during the 3 months of follow-up after engraftment. Results are the mean of 6 to 15 animals per experimental group. Results of statistical analysis with the Wilcoxon test are as follows: Tsp−1−null control mice versus WT control mice and Tsp−1−null TPOhigh mice versus WT TPOhigh mice: *P < .05. Total TGFβ−1 (active + latent forms) levels were quantified in plasma (D) and extracellular fluids (E,G) using an enzyme-linked immunosorbent assay after acidification of samples. Active TGFβ−1 levels in extracellular fluids (F,H) were determined without acidification. Total TGFβ−1 in plasma and extracellular fluids from WT and Tsp−1−null TPOhigh mice (5.7 ± 2.9) did not rise above the one observed in Tsp−1−null TPOhigh mice, suggesting that alternative mechanisms are responsible for local TGFβ−1 activation in this model of myelofibrosis.

As predicted by the detection of the active form of TGFβ−1, Tsp−1−null TPOhigh mice developed BM and spleen fibrosis (Figure 2A), with, intriguingly, a greater grade than in WT TPOhigh mice (Figure 2B). Our results show that local activation of TGFβ−1 in the hematopoietic environment leading to BM and spleen fibrosis occurs in the absence of TSP−1, according to a previous study reporting that TSP−1 was not a major activator of TGFβ−1 in platelets.15 However, Ahamed et al recently demonstrated that TSP−1 contributes to TGFβ−1 activation in both serum and platelet releasates under conditions of stirring and/or shear.12 Such mechanisms are probably not involved within the BM environment. TSP−1 has been clearly demonstrated to be the major activator of TGFβ−1 in lung and renal fibrosis.19,20 However, one can speculate that TGFβ−1 could be regulated in a tissue-specific way, particularly in the megakaryocyte/platelet compartment. Numerous studies have shed light on the major role of integrins in TGFβ−1 activation.4,6 We hypothesize that β3 integrins expressed by platelets and megakaryocytes21 may compensate for the absence of TSP−1 in this model.

Unexpectedly, the fibrosis observed in Tsp−1−null TPOhigh mice was more enhanced than the one observed in WT TPOhigh mice (Figure 2B). To explain this surprising finding, we first hypothesized that this increased fibrosis was related to an augmentation of neoangiogenesis14 mediated by the antiangiogenic function of TSP−1.16 We therefore analyzed the MVD in BM. MVD in control Tsp−1−null was higher than in control WT (10 ± 4.7 vs 3.4 ± 1.6; P < .001), as expected. However, MVD observed in Tsp−1−null TPOhigh mice (8.3 ± 4.4) did not rise above the one observed in control Tsp−1−null mice and was similar to MVD observed in WT TPOhigh mice (5.7 ± 2.9). Thus, the increase of myelofibrosis in Tsp−1−null TPOhigh mice cannot be explained by an augmentation
healing with reepithelialization. To this purpose, we compared the expression of several TGF-β1-signaling pathway members in Tsp-1−/− and WT BM stromal cells. Unfortunately, we did not clearly demonstrate a sustained TGF-β1 activation nor an increase in TGF-β1 receptors or collagen expression in Tsp-1−/− stromal cells (data not shown). Thus, mechanisms leading to the increased myelofibrosis in Tsp-1−/− mice remain unclear. However, similar greater amounts of fibrosis have been recently reported in lung of Tsp-1−/− mice after bleomycin treatment.

Together, these results show that TSP-1 is not the major activator of TGF-β1 in TPO-induced BM fibrosis, suggesting the contribution of another mechanism in the megakaryocyte/platelet compartment.

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Authorship

Contribution: S.E. and O.W.-B. performed animal studies, enzyme-linked immunosorbent assay, fluorescence in situ hybridization, and Western blotting; O.B. performed mRNA analysis; M.T. performed histologic analysis; P.R. performed reticulin fiber quantification; P.G. performed statistical analysis; E.Z. and J.P. performed angiogenesis analysis; A.B. provided TSP-1−null mice; J.-L.V. provided MPZenTPO virus-producing GP + E86 cells; W.V. and S.G. designed studies and analyzed data; and O.W.-B. analyzed data, generated figures, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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