

Thrombopoietin/TGF- β 1 Loop Regulates Megakaryocyte Extracellular Matrix Component Synthesis

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ABSTRACT

Extracellular matrix (ECM) components initiate crucial biochemical and biomechanical cues that are required for bone marrow homeostasis. In our research, we prove that a peri-cellular matrix composed primarily of type III and type IV collagens, and fibronectin surrounds human megakaryocytes in the bone marrow. The data we collected support the hypothesis that bone marrow megakaryocytes possess a complete mechanism to synthesize the ECM components, and that thrombopoietin is a pivotal regulator of this new function inducing transforming growth factor- β 1 (TGF- β 1) release and consequent activation of the downstream pathways, both in vitro and in vivo. This activation results in a dose dependent increase of ECM component synthesis by megakaryocytes, which is reverted upon incubation with JAK and TGF- β 1 receptor specific inhibitors. These data are pivotal for understanding the central role of megakaryocytes in creating their own regulatory niche within the bone marrow environment. *STEM CELLS* 2016;34:1123–1133

SIGNIFICANCE STATEMENT

Bone marrow is a finely organized organ formed by extracellular matrix (ECM) components and various cell types. Increasing evidences are highlighting the importance of the ECM components in the regulation of different aspects of cell behavior. In this regard, we recently demonstrated that mouse megakaryocytes, the bone marrow resident platelet progenitors, are able to produce ECM components which in turn regulate their function. Here we extend this observation by describing the mechanism by which human and mouse megakaryocytes produce those ECM components in vitro and in vivo.

INTRODUCTION

The hematopoietic niche is perivascular, composed by mesenchymal and endothelial cells, extracellular matrix (ECM) components, and soluble factors and is normally located near the trabecular bone [1, 2]. In this niche, hematopoietic stem cells (HSCs) differentiate into megakaryocytes (Mks) under the control of thrombopoietin (TPO), which promotes HSC commitment [3, 4]. A growing body of evidence indicates that the characteristics of the extracellular matrix (ECM) components, in the bone marrow (BM), play an important role in the regulation of megakaryopoiesis [5–8]. It is generally accepted that ECM components are mainly produced by resident stromal cells

[9–11]. We recently shed new light on this concept by showing how mouse Mks can actively synthesize collagens and fibronectin, contributing to their own developmental regulation and to the whole BM homeostasis [12]. ECM component synthesis is modulated by the effect of various profibrotic cytokines, particularly by transforming growth factor- β 1 (TGF- β 1) [10, 11, 13–15], whose principal source, in the BM, are Mks [16–18]. The signals that regulate the production of ECM components by Mks in the BM have never been investigated.

In our research we demonstrate that TPO promotes active TGF- β 1 synthesis and release by Mks, both in vitro and in vivo. Furthermore, we show that acting through an autocrine loop on Mks, TGF- β 1 can trigger the synthesis

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of type III and type IV collagens and of fibronectin. Moreover, we demonstrate that an increase of ECM production occurs in patients affected by primary myelofibrosis (PMF), a well-known pathologic situation caused by altered JAK/STAT signaling [19]. Finally, we report that inhibition of TPO downstream intracellular STAT-coupled kinase JAK prevented the increase of ECM components synthesis in both control and patient derived Mks.

MATERIALS AND METHODS

Antibodies and Reagents

The following antibodies were used: anti-human CD41 (clone HIP8), anti-mouse CD41 (clone MWReg30) (eBioscience, Milan, Italy), mouse anti-CD3 (clone UCHT1), mouse anti-CD4 (clone 13B8.2), mouse anti-CD8 (clone B9.11), mouse anti-CD11b (clone Bear1), mouse anti-CD19 (clone J3-119), mouse anti-CD33 (clone D3HL60.251) (Beckman Coulter, Milan, Italy, <http://www.beckmancoulter.com>), mouse anti- β -actin (clone AC-15), rabbit polyclonal anti-laminin, mouse monoclonal anti-fibronectin (clone IST-4) (Sigma-Aldrich, Milan, Italy, <http://www.sigmaaldrich.com>), goat polyclonal anti-CD61 (clone C-20), and anti-goat horseradish peroxidase (HRP) conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, <http://www.scbt.com>), anti-rabbit and anti-mouse HRP conjugated secondary antibody (BioRad, Milan, Italy, <http://www.bio-rad.com>), rabbit monoclonal anti-pERK1/2 (Thr185/Tyr187) (clone AW39), rabbit polyclonal anti-fibronectin (Millipore, Milan, Italy, <http://www.millipore.com>), rabbit monoclonal anti-pAkt (Ser473), rabbit polyclonal anti-Akt, rabbit monoclonal anti-SAPK/JNK (clone 56G8), rabbit monoclonal anti-ERK1/2 (clone 137F5), rabbit monoclonal anti-STAT5 (clone RH7), rabbit monoclonal anti-pSmad2/3 (Ser465/467) (clone 138D4), rabbit monoclonal anti-pSAPK/JNK (Thr183/Tyr185) (clone 8111), rabbit monoclonal anti-pSTAT5 (Tyr694) (clone C11C5) (Cell Signaling Technology, MA, <http://www.cellsignal.com>), mouse monoclonal anti-p38 (Ab89454), rabbit monoclonal anti-pp38 (Thr180/Tyr182) (clone E229), rabbit monoclonal anti-N WASP (clone EPR6959), rabbit polyclonal anti-pN WASP (Tyr256), mouse monoclonal anti-human CD42b (clone HIP1), rabbit polyclonal anti-type IV collagen, rabbit polyclonal anti-type III collagen (Abcam, London, U.K., <http://www.abcam.com>); Alexa Fluor-conjugated antibodies (Life Technologies, Monza, Italy, <http://www.lifetech.com>). Precision Plus protein standard was from BioRad. Immunomagnetic separation system and Stemolecule ALK5 inhibitor were from Miltenyi Biotec (Bologna, Italy, <http://www.miltenyibiotec.com>). Recombinant human TPO (rhTPO), recombinant mouse TPO (rmTPO), interleukin 6 (IL-6), interleukin 11 (IL-11), and recombinant human TGF- β 1 (rhTGF- β 1) were from Peprotech (U.K., <http://www.peprotech.com>). Ruxolitinib was from Selleckchem (Munich, Germany, <http://www.selleckchem.com>). Enhanced chemiluminescence reagents were from Millipore. The prolyl 4-hydroxylase inhibitor, ethyl 3,4-dihydroxybenzoate (EDHB), was from Sigma-Aldrich.

Patient Privacy and Informed Consent

Human umbilical cord blood was collected following normal pregnancies and deliveries upon informed consent of the parents; BM samples were collected from healthy volunteers

who gave their informed consent; peripheral blood samples were obtained from healthy subjects and patients with primary myelofibrosis, who gave their informed consent. All human samples were collected in accordance with the ethical committee of the IRCCS Policlinico San Matteo Foundation and the principles of the Declaration of Helsinki.

In Vitro Megakaryocyte Culture

CD34⁺ cells were separated and cultured, as described previously [20, 21]. In order to evaluate Mk differentiation and the purity of in vitro cultures, Western blot and flow cytometry analysis were performed, as subsequently described. For in vitro testing of the effects of rhTPO on ECM synthesis and production, mature Mks at day 10 of culture were treated for 3 days with increasing concentration of rhTPO alone (0, 10, 50, and 100 ng/mL), without the addition of any other cytokines. At the end of the treatment Mks were lysed for Western blotting analysis, or RNA was extracted for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis. For ALK5 inhibition experiments, Mks at day 10 of culture were treated with 100 ng/mL of rhTPO in combination or not with 10 μ M Stemolecule ALK5 inhibitor (Miltenyi Biotec) for 3 days. For treatment with the JAK inhibitor Mks at day 10 of culture were incubated or not with 100 nM of JAK inhibitor (ruxolitinib, Selleckchem, Munich, Germany) for 3 days and then lysed. For inhibition of prolyl 4-hydroxylase Mks at day 10 of culture were treated with 100 ng/mL of rhTPO in combination or not with 50 μ M EDHB for 3 days and then lysed. For treatment with rhTGF- β 1 Mks at day 10 of culture were with 10 ng/mL rhTPO in the presence or not of 50 ng/mL rhTGF- β 1, for 3 days and then lysed.

Tissue Collection and Immunohistochemistry

Femurs from treated and control animals were removed and fixed for 24 hours in paraformaldehyde (PFA) 3%. Bones were decalcified in a solution of EDTA 10% in phosphate buffered saline (PBS) (w/o calcium and magnesium) pH 7.2, for 2 weeks at 4°C. Bones were embedded in OCT cryosectioning medium and snap frozen in a chilling bath. 8 μ m tissue sections were taken using a Microm Microtome HM 250 (Bio Optica S.P.A., Milan, Italy, <http://www.bio-optica.it>) and stained with anti-CD41 for fluorescence microscopy.

Formalin-fixed, paraffin-embedded human BM biopsy specimens from normal controls were obtained from the posterior superior iliac spine and decalcified using an EDTA based solution (33.27 g of EDTA and 10 mL of hydrochloric acid diluted in 1 L of distilled water) for 4 hours. Immunohistochemistry was performed using an automated staining system (BenchMark ULTRA, Ventana, Roche, Tucson, <http://www.ventana.com>). Heat-induced antigen retrieval was automatically obtained using a 0.05 mol/L EDTA solution, pH 8.0 at 95°C for 30 minutes. Reactions were revealed using ultraView Universal DAB Detection Kit (Ventana, Roche, Tucson) in accordance with the manufacturer's instructions; the negative control slides were incubated with normal goat serum instead of the primary antibody. Normal control samples consisted of BM biopsy specimens obtained for staging purposes, which were determined to be free of neoplasia and other abnormalities upon histologic and immunohistochemical examinations.

Quantitative Real-Time PCR

In vitro differentiated Mks at day 13 of culture were purified using immunomagnetic beads technique [6]. Total RNA was extracted using the Mammalian GeneElute total RNA kit (Sigma-Aldrich). Retrotranscription (RT) was performed using the iScript™ cDNA Synthesis Kit according to the manufacturer's instructions (BioRad). For quantitative real-time PCR, RT samples were diluted up to three times with ddH₂O, and the resulting cDNA was amplified in triplicate with 200 nM of primers and SsoFast Evagreen Supermix (BioRad). The amplification was performed in a CFX Real-time system (BioRad) as follows 95°C for 5 minutes, 35 cycles at 95°C for 10 minutes, 60°C for 15 minutes, 72°C for 20 minutes. Predesigned KiCq-Start primers were purchased from Sigma-Aldrich. The BioRad CFX Manager software 3.0 was used for the normalization of the samples (BioRad). β -2 Microglobulin gene expression was used for the quantitative analysis.

Western Blotting

For western blotting analysis, cultured Mks and BM sorted Mks were collected, washed twice at 4°C and lysed in HEPES-glycerol lysis buffer (HEPES 50 mM, 10% glycerol, 1% Triton X-100, MgCl₂ 1.5 mM, EGTA 1 mM) containing aprotinin 1 µg/mL and leupeptin 1 µg/mL, for 30 minutes at 4°C, as previously described [22]. After the clarification, centrifugation at 15,700 × *g* for 15 minutes at 4°C, Laemmli sample buffer was added to supernatants. Then samples were heated at 95°C for 3 minutes and loaded on 8% or 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and then transferred to polyvinylidene fluoride membranes. Membranes were incubated with 5% bovine serum albumin (BSA), 0.1% Tween in PBS, to avoid unspecific antibodies binding and then probed with primary antibodies and the appropriate peroxidase conjugated secondary antibodies. Western blots were developed with enhanced chemiluminescence reagents and Chemidoc XRS Imaging System (BioRad).

Immunofluorescence

For cell immunofluorescence staining 12 mm glass coverslips were coated with polylysine. At day 13 of culture 1×10^5 Mks were harvested and cytopun. Adhering cells were washed with PBS, fixed in 4% PFA, permeabilized with 0.1% Triton X-100, and stained for immunofluorescence evaluation with the indicated antibodies, as previously described [23]. The coverslips were mounted onto glass slides with ProLong Gold antifade reagent (Invitrogen, Milan, Italy, <http://www.invitrogen.com>). For immunofluorescence staining of BM samples, sections were fixed for 20 minutes in 4% PFA, washed with PBS, and blocked with 2% BSA (Sigma-Aldrich) in PBS for 30 minutes. Nonspecific binding sites were saturated with a solution of 5% goat serum, 2% BSA, and 0.1% glycine in PBS for 1 hour. Specimens were incubated with primary antibodies in washing buffer (0.2% BSA, 0.1% Tween in PBS) overnight at 4°C. After three washes, sections were incubated with appropriate fluorescently conjugated secondary antibodies in washing buffer for 1 hour at room temperature (RT). Nuclei were counterstained using Hoechst 33258 (100 ng/mL in PBS) at RT for 3 minutes. Sections were then mounted with microcover glass slips using Fluoro-mount (Bio-Optica). Negative controls were routinely performed by omitting the primary antibodies.

All images were acquired using the Olympus BX51 fluorescence microscopy (Olympus Deutschland GmbH, Hamburg, Germany, <http://www.olympus.com>).

ELISA

The ELISA assays were performed following manufacturer instructions (Sigma-Aldrich). For TGF- β 1 dosage, supernatants from Mks culture treated with increasing concentration of rhTPO were collected, clarified by centrifugation at 15,700 *g* and stored at -80°C. TGF- β 1 dosages were normalized on cell number. Each sample was used in triplicate.

Animals and In Vivo Treatment

C57/BL6 wild type mice were from Charles River Laboratories, Italy. Mice were housed at the animal facility of the Department of Physiology, section of General Physiology, University of Pavia (approval #1/2010, 24/06/2010; #3, 19/11/2013). All mice were sacrificed according to the current European legal Animal Practice requirements. Mice were injected with 100 µg/kg per day of rmTPO. At day 7 and 14 after the first injection, mice were sacrificed and blood collected for peripheral blood count. Femurs were fixed in 3% PFA or alternatively flushed and used for flow cytometry analysis and cell sorting experiments. Age paired mice were always injected with PBS as control. Cell count and differential cell count in blood samples were performed on an ADVIA 120 hematology analyzer (Siemens, Erlangen, Germany, <http://www.healthcare.siemens.com>).

Flow Cytometry and Cell Sorting

For the analysis of BM stem cells, femurs were flushed, and red blood cells lysed with ammonium chloride and stained with fluorescein isothiocyanate (FITC) lineage cocktail (Miltenyi Biotec), phycoerythrin (PE)-Cy7 anti-mouse Ly-6A/E (Sca-1, clone D7), allophycocyanin (APC)-eFluor 780 anti-mouse CD117 (c-Kit, clone 2B8), rat anti-mouse CD16/32-PE (clone 93), rat anti-mouse CD34-PE-Cy5 (clone MEC14.7), and rat anti-mouse CD127-APC (IL-7R α , clone A7R34) (Biolegend, London, U.K., <http://www.biolegend.com>) antibodies for 30 minutes on ice and analyzed. HSC were identified as lineage negative/Sca-1⁺/CD117⁺ cells. LK (Lin⁻, c-Kit⁺, Sca-1⁻) cells were gated and analyzed for the expression of CD16/32 and CD34 markers. Granulocyte macrophage progenitors (GMP) were considered as CD16/32⁺/CD34⁺ cells, common myeloid progenitors (CMP) cells were CD16/32^{low}/CD34⁺, while megakaryocyte erythroid progenitor (MEP) cells were CD16/32^{neg}/CD34^{neg}. For analysis of Mk ploidy, cells were fixed in ice cold 70% ethanol overnight at -20°C and resuspended in PBS with 100 mg/mL of RNase and propidium iodide solution and stained with FITC anti-CD41. All Mk samples, from the different tested sources, were routinely characterized as CD41⁺CD42b⁺ and CD3⁻CD4⁻CD8⁻CD11b⁻CD19⁻CD33⁻ cells (Beckman Coulter). All samples were acquired with a Beckman Coulter FACS Diva flow cytometer (Beckman Coulter). The analytical gating were set using unstained samples and relative isotype controls. Offline data were analyzed using Beckman Coulter Kaluza version software package (Beckman Coulter). Mks were sorted from BM cells as previously described [12]. Mk purity was routinely performed and assessed to be >95% \pm 3%. Cell sorting experiments were performed using a FACS Aria IIu (three lasers; BD Bioscience, www.bdbiosciences.com). Diva software (BD

Pharmingen, San Diego, CA, <http://www.bdbiosciences.com>) was used for data acquisition and analysis.

Fibronectin Purification and Dot Blot Assays

For the analysis of fibronectin secretion, the protein was purified from conditioned medium of human Mks cultured in the presence of 0, 10, 50, or 100 ng/mL rhTPO, or of control and PMF Mks cultured in the presence of 10 ng/mL rhTPO, in the presence or not of 100 nM of JAK inhibitor, according to previously published methods [24, 25]. Briefly, precleared medium was applied to a column of gelatin-sepharose in agitation overnight at 4°C and after a washing with the equilibrating buffer (1 M NaCl and 0.2 M Arg), the fibronectin bound fraction was eluted with 1 M arginine. Prior to dot blot analysis, 500 μ L of each pooled sample was concentrated in TBS-Na₃ buffer (50 mM Tris/HCl, pH 7.4 containing 0.15 M NaCl, 0.02% azide) using an Amicon Ultra-0.5 centrifugal filter unit (Millipore). For dot blot analysis, a nitrocellulose membrane (Amersham Hybond ECL Nitrocellulose Membrane, GE Healthcare Life Sciences, Pittsburgh, PA <http://www.gehealthcare.com>) was prewet in distilled water followed by equilibration in TBS and air dried before sample application. Then, 2 μ L of each sample was spotted in triplicate on the membrane and air dried. The nonspecific sites were blocked by soaking the membrane in 5% BSA in TBS containing 0.05% Tween 20 for 1 hour at room temperature. The membrane was then incubated with polyclonal rabbit anti-human fibronectin antibody (10 μ g/mL in 2% BSA in TBST) overnight at 4°C [26]. The membrane was extensively washed with TBST and incubated with secondary goat anti-rabbit HRP-conjugated antibody (GenScript, Piscataway <http://www.genscript.com>) for 1 hour at RT. Bound antibody was detected by chemiluminescence using an ECL kit (GE Healthcare). After the membrane exposure to ImageQuant LAS4000 (GE Healthcare), the spots were analyzed with ImageQuant TL software (GE Healthcare). The results were normalized to a calibration curve performed using increasing concentration of purified human plasma fibronectin and the protein concentration was expressed as μ g/mL.

For the analysis of type III and type IV collagens secretion, conditioned medium of human Mks, cultured in the presence of 0, 10, 50, or 100 ng/mL rhTPO, or of human Mks from PMF patients and healthy controls cultured in presence of 0 or 10 ng/mL rhTPO, in the presence or not of 50 μ M EDHB or 100 nM JAK inhibitor, was spotted in triplicate on nitrocellulose membrane and air dried. The nonspecific sites were blocked by soaking the membrane in 5% BSA in PBS containing 0.1% Tween 20 for 1 hour at room temperature. The membrane was then incubated with antibodies against human type III or human type IV collagen overnight at 4°C. After extensive washing and incubation with secondary HRP-conjugated antibody, the membranes were developed with enhanced chemiluminescence reagents and Chemidoc XRS Imaging System (BioRad). The spots were analyzed with Quantity One software (BioRad) and results were normalized with respect to conditioned medium derived from control condition.

Statistics

Values are expressed as mean \pm SD or median. *t* test or one-way ANOVA followed by Bonferroni post-test were used to analyze experiments. A value of $p < .05$ was considered statis-

tically significant. All experiments were independently repeated at least three times.

RESULTS

Human Megakaryocytes Express BM ECM Components

Human BM biopsy samples were stained with different ECM component antibodies. As shown in Figure 1A, type III and type IV collagens and fibronectin were diffusely distributed in the BM with a clear peri-cellular deposition around Mks. In order to verify whether human Mks were able to actively produce ECM components, BM derived CD34⁺ hematopoietic progenitor cells were differentiated with rhTPO in a serum-free medium. At day 13 of culture the purity of in vitro cultures was assessed by flow cytometry and Western blot analysis (Supporting Information Figs. 1A, 1B). Thereafter, expression of collagen synthesis enzymes [lysyl oxidase, prolyl 4-hydroxylase, and procollagen type III N-endopeptidase] (Fig. 1B), fibronectin, collagen type III and type IV (Fig. 1C) was demonstrated in Mks by qRT-PCR analysis relative to β 2-microglobulin. Finally, as shown in Figure 1D, the Western blot analysis of Mk lysates confirmed the presence of bands for all the tested ECM components. Data were further supported by immunofluorescence analysis of the ECM components in mature human Mks (Fig. 1D).

Thrombopoietin Drives In Vitro ECM Synthesis by Human Megakaryocytes Dose-Dependently

To get further insight into the driving mechanisms of ECM component synthesis, we used cord blood-derived CD34⁺ cells, a well-characterized source for differentiating Mks [22, 27]. We differentiated the cells under the same conditions of BM progenitors and verified that Mks had a similar pattern of production of all tested ECM components independently from the source of origin (Supporting Information Fig. 2). Starting from day 10 of differentiation, Mks were cultured with 10, 50, and 100 ng/mL of rhTPO for 3 days. The phosphorylation of STAT5, Akt, and ERK1/2, three of the major downstream effectors of the TPO receptor (c-Mpl), increased according to rhTPO doses (Fig. 2A). Importantly, this phosphorylation was paralleled by a significant dose-dependent increase of ECM component expression at both mRNA (Fig. 2B) and protein levels (Fig. 2C). Of note, increased levels of fibronectin and collagens were also detected in the extracellular milieu (Fig. 2D, 2E). A significant upregulation of the expression of the collagen synthesis enzymes, lysyl oxidase and prolyl 4-hydroxylase and P4H, was also observed upon rhTPO treatment, confirming the active ECM synthesis in treated Mks (Fig. 2F). Consistently, the selective inhibition of prolyl 4-hydroxylase activity, with 50 μ M of the specific inhibitor EDHB [28], caused an important reduction of both type III and type IV collagen synthesis (Fig. 2G). Finally, concomitant treatment of Mks with 100 ng/mL of rhTPO and 100 nM of a specific inhibitor of the intracellular STAT-coupled kinase JAK was sufficient to prevent the increase of ECM component synthesis and secretion (Fig. 2H–2L). To prove the specificity of the rhTPO stimulation on ECM component synthesis, we treated Mks with increasing doses of the basic fibroblast growth factor (10, 50, 100 ng/mL) [29], but we could not appreciate any evident differences (Supporting Information Fig. 3). Furthermore, Mks deprived of

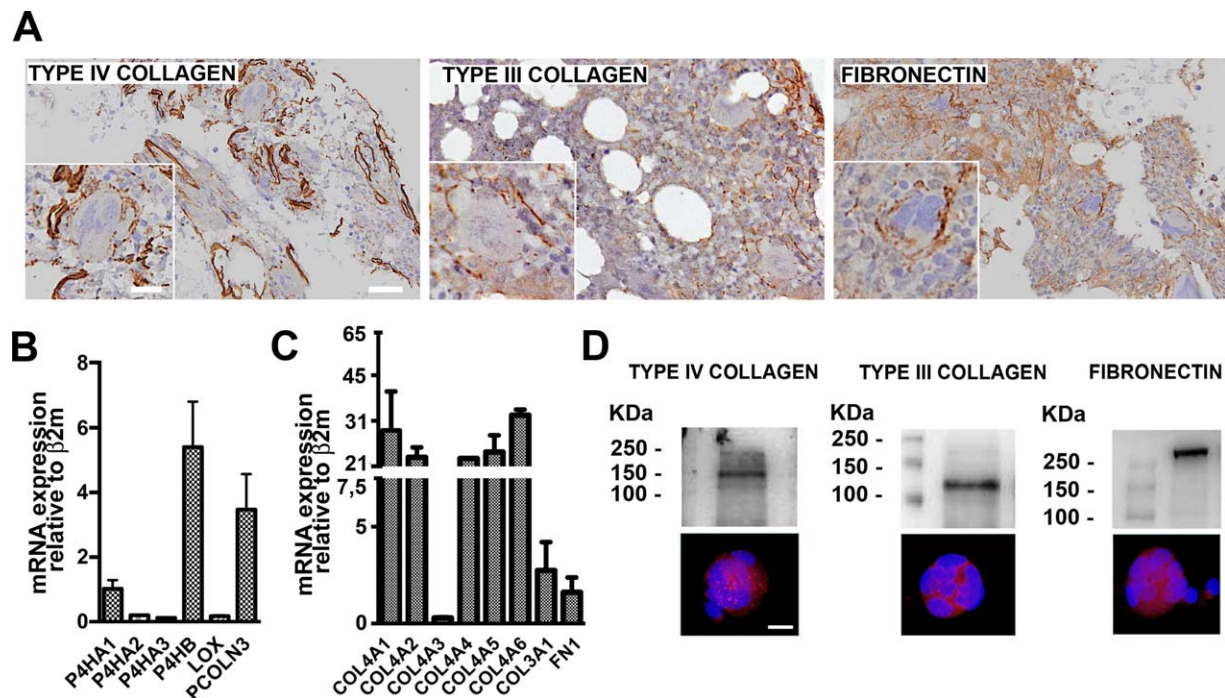


Figure 1. Human megakaryocytes (Mks) express bone marrow extracellular matrix (ECM) components. **(A):** Human bone marrow biopsies were stained for type IV and type III collagen and for fibronectin (scale bar = 50 μm ; box scale bar = 20 μm). **(B):** Mks were differentiated from bone marrow hematopoietic stem cells. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) products showing expression of collagen synthesis enzymes in CD61⁺ Mks. **(C):** qRT-PCR analysis of the indicated ECM component genes in Mks. Histograms show gene expression relative to $\beta 2$ -microglobulin. Data are shown as mean \pm SD. Reactions were repeated five times with independent samples. **(D):** Protein expression of the indicated extracellular matrix components in Mks. Images show Western blots and immunofluorescence analysis. In Western blot analysis molecular weight standard (left columns) indicates that ECM components are of the expected mass. Scale bar = 20 μm . Abbreviations: COL, collagen, FN, fibronectin; LOX, lysil oxidase; P4HB, prolyl 4-hydroxylase; PCOLN3, procollagen type III endopeptidase; $\beta 2m$, $\beta 2$ -microglobulin.

rhTPO (rhTPO 0), starting from day 10 of culture, showed a decreased expression of all the tested ECM components at both mRNA and protein levels (Supporting Information Fig. 4A, 4B). As a consequence, secretion of both fibronectin and collagens was reduced by about 60% in rhTPO 0 with respect to 10 ng/mL rhTPO (data not shown). Accordingly, TPO downstream signaling pathway activation was decreased (Supporting Information Fig. 4C). Finally, both ploidy and CD41/CD42b expression were significantly decreased in rhTPO 0 samples with respect to rhTPO treated samples, while no differences were observed among 10, 50, and 100 ng/mL rhTPO (Supporting Information Fig. 5).

Thrombopoietin Stimulates ECM Component Synthesis via a TGF- $\beta 1$ -Dependent Mechanism

TGF- $\beta 1$ is synthesized and secreted by numerous cell types in the body and is one of the most abundant cytokine found in Mks and platelets [30, 31]. Interestingly, TGF- $\beta 1$ has been described to stimulate ECM component production in different cell types [11, 13, 32]. The analysis of released TGF- $\beta 1$ in Mk culture supernatants revealed an increased concentration of the molecule in the presence of higher concentrations of rhTPO (Fig. 3A). TGF- $\beta 1$ has been demonstrated to be contained in Mk α -granules [30], and P-selectin is a protein contained in the same granules commonly used to study the release of their content [33]. Consistently, the treatment of human Mks with increasing rhTPO concentrations resulted in increased exposure of P-Selectin on Mk surface (Fig. 3B). Fur-

thermore, the phosphorylation of Wiskott-Aldrich syndrome protein, which has been previously correlated with TGF- $\beta 1$ secretion [34], was significantly augmented in the same experimental conditions (Fig. 3C). Following the binding of TGF- $\beta 1$ to its receptors (T β R), several signaling pathways are activated involving both the canonical SMAD pathway as well as other alternative signaling molecules [35, 36]. Western blot analysis demonstrated that increasing concentration of released TGF- $\beta 1$ determined a concomitant increase of SMAD2/3, p38, and SAPK/JNK phosphorylation (Fig. 3D). Of note, Mks cultured in the absence of rhTPO showed a decreased activation of the same pathways, with respect to 10 ng/mL rhTPO (Supporting Information Fig. 4D). To definitely demonstrate that, in the presence of high concentration of rhTPO, TGF- $\beta 1$ acts through the engagement of its specific receptors on Mks, we treated samples with 100 ng/mL rhTPO with or without 10 μM ALK5 inhibitor [36], a selective T β RI inhibitor. ALK5 inhibitor efficiently prevented the activation of SMAD2/3, p38, and SAPK/JNK (Fig. 3E). In the same experimental conditions, we also analyzed the activation of Akt and ERK1/2, which are signaling molecules downstream of both TGF- $\beta 1$ and TPO receptors, observing a decrease of Akt phosphorylation [37]. Additionally, the inhibition of ALK5 (T β RI) determined a significant decrease of ECM component synthesis (Fig. 3F). The effect of TGF- $\beta 1$ was further demonstrated by treating Mks at day 10 of culture with 10 ng/mL rhTPO in the presence or not of 50 ng/mL rhTGF- $\beta 1$ for 3 days. Western blot analysis confirmed the activation of the specific

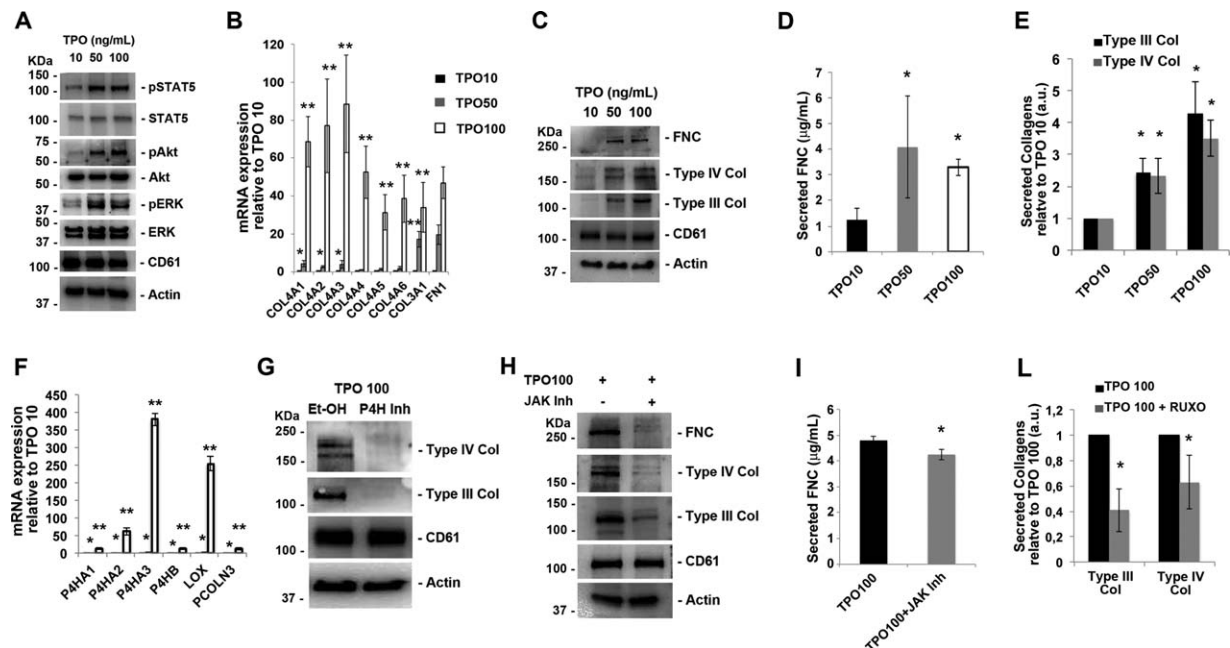


Figure 2. TPO drives in vitro extracellular matrix (ECM) synthesis by human megakaryocytes (Mks). **(A):** Western blot analysis of TPO signaling pathway activation after recombinant human TPO (rhTPO) treatment. **(B):** Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis of the relative expression of the indicated genes. Mks were treated with the indicated rhTPO concentrations. After 3 days of culture Mk RNA was extracted, and qRT-PCR analysis performed. Results refer to six independent experiments, and data are shown as mean \pm SD relative to 10 ng/mL rhTPO-treated cultures. **(C):** Western blot analysis showing the increase in the synthesis of the indicated ECM components after rhTPO treatment. **(D):** Quantification of purified secreted fibronectin in Mk culture supernatants after treatment with rhTPO. **(E):** Dot blot analysis of secreted collagens in the culture medium of Mks treated with increasing doses of rhTPO. **(F):** qRT-PCR analysis of collagen synthesis enzymes in rhTPO treated Mks. **(G):** Western blot analysis of collagen synthesis after treatment with the highest tested rhTPO concentration (100 ng/mL) in the presence or not of 50 μ M P4H inhibitor. **(H):** Western blot analysis of the indicated ECM component synthesis after treatment with 100 ng/mL rhTPO in the presence or not of 100 nM of a JAK inhibitor. Western blot data were normalized for actin and CD61 and the respective total protein. **(I):** Quantification of purified secreted fibronectin in Mk culture supernatants after treatment with rhTPO and 100 nM JAK inhibitor. **(L):** Dot blot analysis of secreted collagens in the culture medium of Mks treated with 100 ng/mL of rhTPO in presence or not of 100 nM of JAK inhibitor. ECM components are indicated as follows: FNC, Type IV Col, and Type III Col. *, $p < .05$; **, $p < .01$. Abbreviations: COL, collagen; FNC, fibronectin; LOX, lysyl oxidase; P4H, prolyl 4-hydroxylase; P4H inh, P4H inhibitor; PCOLN3, procollagen type III endopeptidase; TPO, thrombopoietin; type IV Col, type IV collagen; type III Col, type III collagen.

pathways concomitant with increased ECM component synthesis (Supporting Information Fig. 6).

BM Sorted Megakaryocytes from Thrombopoietin Treated Mice Show Increased Expression of ECM Components

To ascertain if the mechanisms we observed in vitro could be replicated in the in vivo context, we treated C57BL/6 mice with 100 μ g/kg per day of rmTPO for 5 days [38] and then characterized in vivo the BM Mks (Supporting Information Fig. 7A). Peripheral blood analysis showed increased platelet count after 7 days in treated animals, which returned to normal count after 14 days (Supporting Information Fig. 7B). The analysis of marrow HSCs showed a peak at day 7, which remained significantly higher than basal level after 14 days (Supporting Information Fig. 7C). Interestingly, the analysis of BM progenitors revealed an increase of MEP progenitor cell only at day 7, while no significant differences were observed in GMP and CMP at both day 7 and 14 (Supporting Information Fig. 7D). Consistently, the number of marrow CD41⁺ Mks per mm² was significantly increased (Supporting Information Fig. 7E). BM Mks were sorted by fluorescence-activated cell sorting, as previously described [12], after 7 and 14 days of rmTPO treatment (Fig. 4A). TGF- β 1 expression in rmTPO-

treated marrow Mks peaked at day 7 and was maintained slightly higher than untreated marrow Mks after 14 days (Fig. 4B). Importantly, fibronectin and type III collagen mRNA expression and protein levels were significantly increased in sorted Mks at day 7, up to day 14 of mouse treatment. Furthermore, we found a significant increase of type IV collagen at day 7 that returned normal at day 14 (Fig. 4C, 4D).

Altered JAK2 Signaling in a Patient with Primary Myelofibrosis Results in Increased ECM Component Production

As prove of concept of our results obtained in a physiologic setting, we analyzed the production of type III and type IV collagens and of fibronectin by in vitro differentiated Mks from two PMF patients with mutated JAK2 and healthy controls. Both patients were analyzed three independent times. Clinical data are reported in Supporting Information Table 1. Both patient and control Mks were grown with 10 ng/mL rhTPO. Results demonstrated higher phosphorylation of STAT5 in PMF Mks with respect to controls with a concomitant increased phosphorylation of Akt and ERK1/2 (Fig. 5A). The dosage of culture supernatants revealed a significant increase of released TGF- β 1 with respect to controls (3,655 \pm 402 pg/mL vs. 1,679 \pm 421 pg/mL, $p < .05$), consistent with our

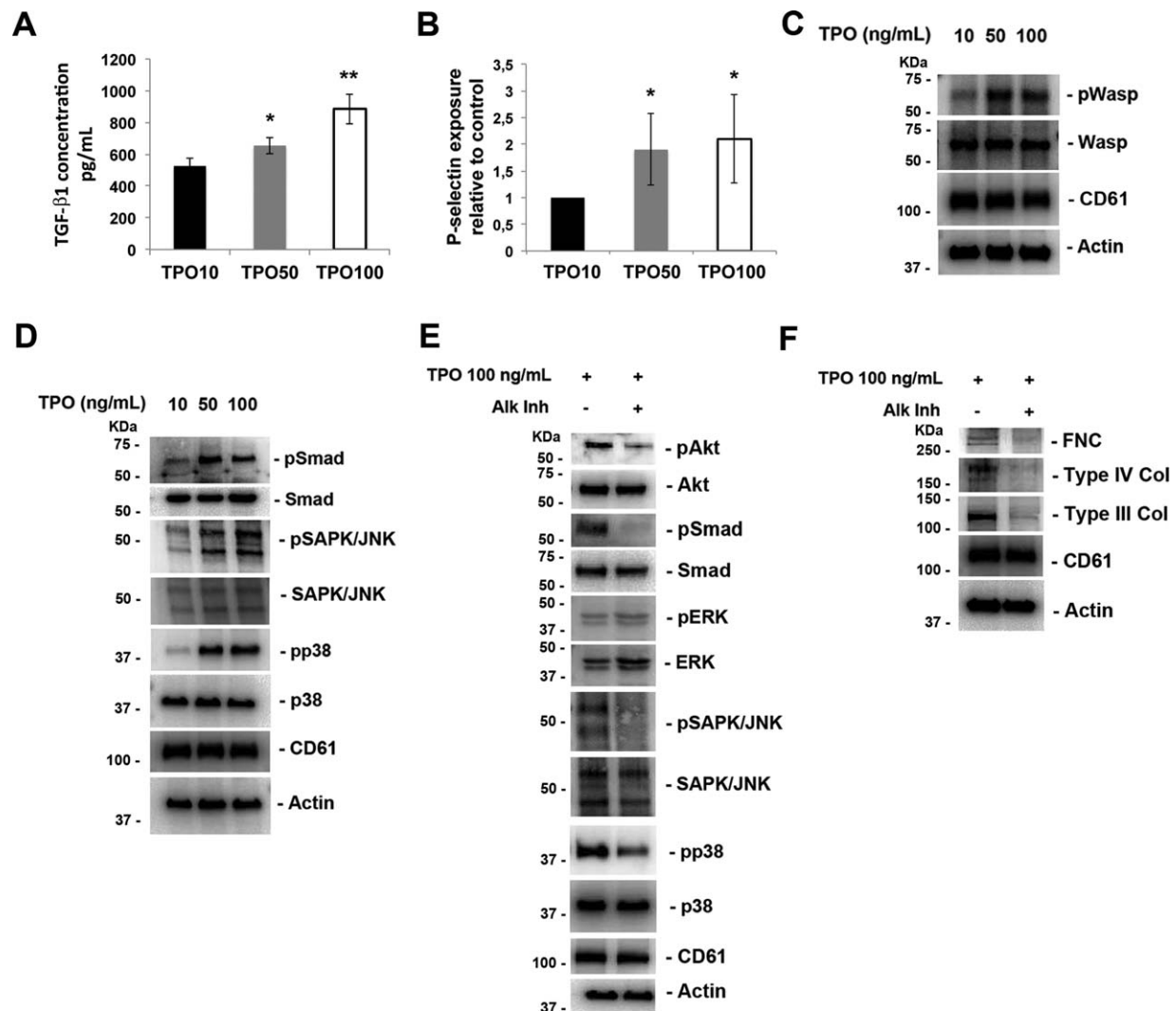


Figure 3. TPO stimulates extracellular matrix (ECM) component synthesis via a TGF- β 1-dependent mechanism. **(A):** Total TGF- β 1 dosage in recombinant human TPO (rhTPO) treated megakaryocyte (Mk) supernatants. Data are shown as mean \pm SD of six independent experiments. **(B):** Flow cytometry analysis of cell membrane P-selectin (CD62) exposure in rhTPO treated Mks. Results are reported as mean \pm SD relative to 10 ng/mL rhTPO-treated cells used as control. Results refer to five independent experiments. **(C):** Western blot analysis of WASP phosphorylation in rhTPO treated Mks. **(D):** Western blot analysis of TGF- β 1 signaling in Mks treated with 100 ng/mL of rhTPO in the presence or not of 10 μ M ALK5 (T β RI) inhibitor. **(E):** Western blot analysis of TGF- β 1 signaling in Mks treated with 100 ng/mL of rhTPO in the presence or not of 10 μ M ALK5 (T β RI) inhibitor. Western blot data were normalized to actin, CD61, and to the respective total protein. ECM components are indicated as follows: FNC, Type IV Col, and Type III Col. *, $p < .05$; **, $p < .01$. Abbreviations: Alk inh, Alk inhibitor; FNC, fibronectin; Type IV Col, Type IV collagen; Type III Col, Type III collagen; TGF, transforming growth factor; TPO, thrombopoietin; WASP, Wiskott Aldrich syndrome protein.

previous findings [36]. This was paralleled by increased phosphorylation of the TGF- β 1 signaling molecules SMAD2/3, p38, and SAPK/JNK. Consistently, the qRT-PCR analysis demonstrated increased expression of type III collagen and fibronectin compared to controls, while the expression of type IV collagen was not affected (Fig. 5B). Furthermore, Western blot analysis showed a clear increase of type III collagen and fibronectin production and to a lesser extent also of type IV collagen with respect to controls (Fig. 5C). In support of the importance of the JAK/STAT hyperactivation as a trigger for ECM component production by PMF Mks, we treated in vitro differentiated Mks with 100 nM JAK inhibitor and observed a reduced production of all tested ECM components (Fig. 5D). Interestingly, the increment of secreted ECM components

observed in PMF Mk cultures with respect to controls was reverted to control level by treatment with the JAK inhibitor (Fig. 5E, 5F). Finally, both PMF and control Mks at day 10 of differentiation, cultured for 3 days in complete absence of rhTPO demonstrated a reduced production of all ECM components, similarly to that observed in presence of 100 nm JAK inhibitor (Supporting Information Fig. 8).

DISCUSSION

In the BM environment, Mks interact with ECM components, that play the double function of being a regulator of platelet formation and a barrier that Mks need to rearrange and traverse in order to extend proplatelets [8, 39]. It has been

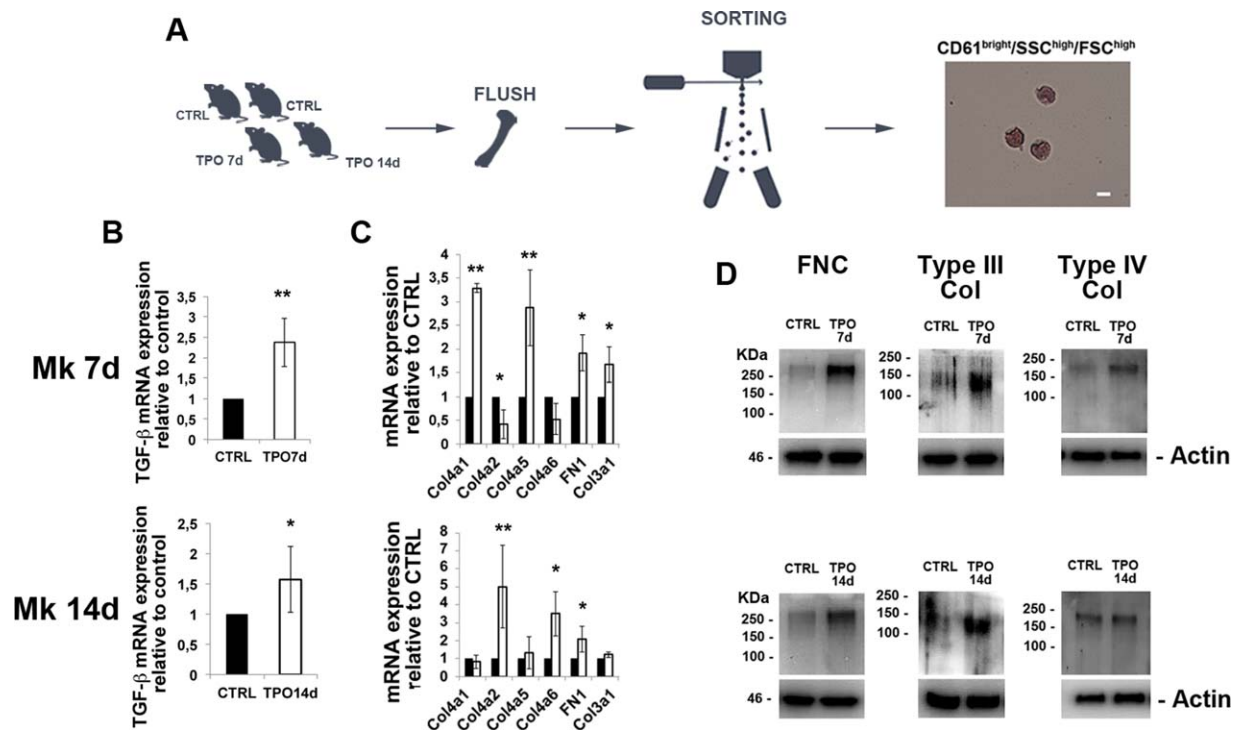


Figure 4. Isolated bone marrow megakaryocytes (Mks) from TPO treated mice produce more extracellular matrix (ECM) components than untreated mice. **(A):** Schematic flow of sorting experiments. Scale bar = 20 μ m. **(B):** Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis of TGF- β 1 expression at day 7 and 14 of recombinant mouse TPO (rmTPO) treatment in sorted bone marrow mouse Mks relative to untreated mice. **(C):** qRT-PCR analysis of the expression of the indicated genes at day 7 and 14 of rmTPO treatment in sorted bone marrow mouse Mks relative to untreated mice. **(D):** Western blot analysis of the production of the indicated ECM protein at day 7 and 14 of rmTPO treatment in sorted bone marrow mouse Mks relative to untreated mice. Results refer to eight independent experiments and are shown as mean \pm SD relative to control. *, $p < .05$; **, $p < .01$. Abbreviations: FNC, fibronectin; Mk, megakaryocyte; TGF β , transforming growth factor β ; TPO, thrombopoietin; Type IV Col, Type IV collagen; Type III Col, Type III collagen.

proven that Mks possess collagenase activity, as they express several Matrix MetalloProteinase (MMP), such as MMP-2, MMP-9, MMP-14, MMP-24, MMP-25 and assemble podosomes that rearrange the ECM through proteolytic activity [40]. In vivo, in the BM, Mks are surrounded by a peri-cellular matrix mainly composed of collagens and fibronectin [12]. BM ECM components are mainly synthesized by resident stromal cells [10], however, as we recently demonstrated, mouse Mks are able to synthesize fibronectin, type IV collagen and laminin both in vitro and in vivo [12]. In this research endeavor we extended these observations demonstrating that human Mks possesses the collagen synthesis mechanisms and synthesize and release fibronectin, type III and type IV collagens. The exact function of the self-produced ECM components is still unclear, however it is accepted that different autocrine loops are fundamental modulators of Mk development and function [6, 23, 41–43].

In vivo, in mice, Mk expression of fibronectin and type IV collagen is upregulated during BM reconstitution upon induced myelosuppression, while only type IV collagen is upregulated upon induced thrombocytopenia [12]. All these data are clear evidence that the peri-cellular matrix that we observed around Mks in mouse and human BM biopsies may constitute an “autocrine niche” that contribute to the regulation of Mk functions. This leads us to highlight how the composition of this niche appears to vary according to external stimuli [12, 44, 45].

TPO is the main cytokine that regulate megakaryopoiesis [3, 4]. Our studies demonstrate that, in vitro, increasing rhTPO concentrations determined a significant increase of ECM component expression by human Mks (Fig. 6). The importance of JAK signaling in regulating this process was proven by the cotreatment of Mks with high doses of rhTPO and a specific inhibitor of the intracellular STAT-coupled kinase JAK which prevented the increase of ECM component synthesis. It has been ascertained that TGF- β 1 regulates the synthesis of collagens and fibronectin by different cell types including osteoblasts, fibroblasts, and mesenchymal stromal cells [9, 11, 13, 32]. In a previous work, we had determined that maturing Mks release TGF- β 1 that upon binding to its receptors and activating the downstream signaling, promotes proplatelet formation by terminal differentiated Mks [36]. Our data extends this observation and proves that treatment of human maturing Mks with a selective T β RI inhibitor, in the presence of high doses of rhTPO, determines a significant reduction in the downstream signaling accompanied by reduced ECM component production. Upon activation of the specific intracellular pathways, direct treatment with TGF- β 1 promoted ECM production by human Mks. Finally, these findings were confirmed in vivo by treating mice with supra pharmacological doses of rmTPO [38]. This treatment has been extensively described to induce a transient BM fibrotic phenotype associated with increased number of BM Mks and thrombocytosis [38, 46]. In this condition Mks, sorted from the BM of treated mice,

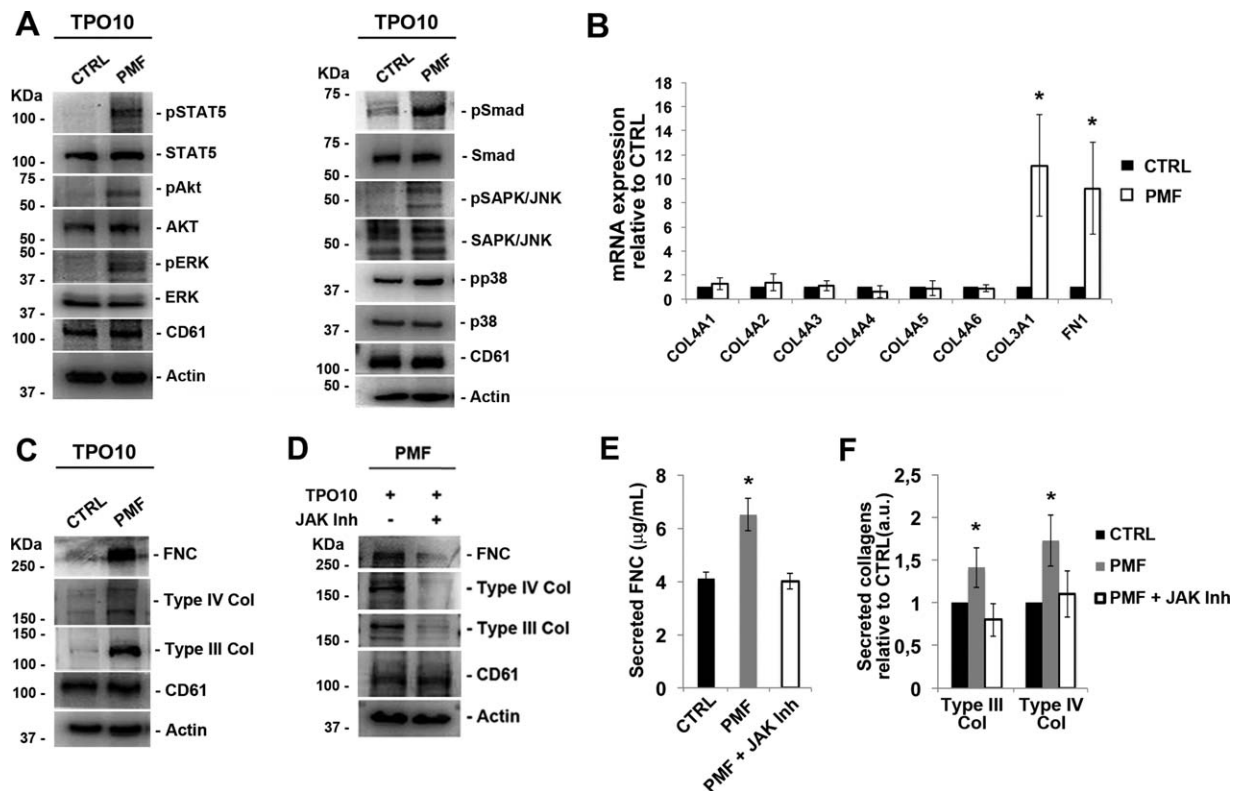


Figure 5. Megakaryocytes (Mks) from patients with primary myelofibrosis produce more extracellular matrix (ECM) components than healthy subjects. **(A):** Representative Western blot analysis of TPO and transforming growth factor β (TGF- β 1) signaling pathways in Mks from PMF patients and controls. **(B):** Quantitative reverse transcriptase polymerase chain reaction analysis of the expression of the indicated ECM component genes in Mks from PMF patients and controls. Data are shown as mean \pm SD relative to control; results refer to three independent experiments for the two PMF patients and six different controls. **(C):** Representative Western blot analysis of the expression of the indicated ECM components in Mks from PMF patients and controls. **(D):** Representative Western blot analysis of the expression of the indicated ECM proteins in Mks from PMF patients in the presence or not of 100 nM of a JAK inhibitor. Western blot data were normalized to actin, CD61, and to the respective total protein. **(E):** Quantification of purified secreted fibronectin in control and PMF Mk supernatants in the presence or not of the JAK inhibitor. **(F):** Dot blot analysis of secreted collagens in the Mk culture supernatants of controls and PMF patients treated with 10 ng/mL rhTPO in presence or not of 100 nM JAK inhibitor. ECM components are indicated as follows: FNC, fibronectin; PMF, JAK Inh, JAK inhibitor; TPO, thrombopoietin; Type IV Col, Type IV collagen; Type III Col, Type III collagen. *, $p < .05$. Abbreviations: FNC, fibronectin; PMF, JAK Inh, JAK inhibitor; TPO, thrombopoietin; Type IV Col, Type IV collagen; Type III Col, Type III collagen.

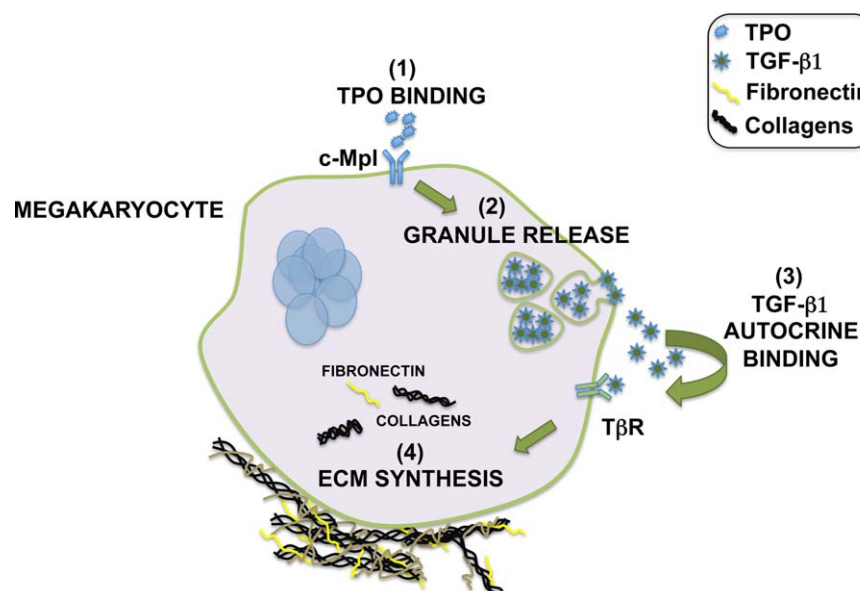


Figure 6. Outline of the TPO/TGF- β 1 autocrine loop regulating megakaryocyte ECM component synthesis. Abbreviations: ECM, extracellular matrix; TGF- β 1, transforming growth factor- β 1, transforming growth factor β ; T β R, TGF- β receptor; TPO, thrombopoietin.

presented higher expression of TGF- β 1, type III and type IV collagens, and of fibronectin at both mRNA and protein levels as compared to untreated controls.

The JAK/STAT system is one of the main signaling pathways from the membrane to the nucleus of hematopoietic cells, and the BCR-ABL-negative myeloproliferative neoplasms (MPNs) are malignant diseases driven by alterations of the JAK/STAT pathway [47–49]. Among MPNs, PMF is a pathological condition characterized by a profound alteration of BM structure and matrix composition. Patients affected by this pathology display high number of atypical Mks within the BM and progressive accumulation of reticulin and collagen which compromises patient prognosis [50–52]. An increased release of TGF- β 1 by PMF patient derived Mks has been demonstrated [18, 36]. On these premises, we investigated Mks derived, *in vitro*, from peripheral blood CD34⁺ cells of two well-characterized JAK-mutated PMF patients with high grade of BM fibrosis. Mks presented increased activation of both c-Mpl and T β R downstream signaling pathways. Accordingly, Mks exhibited increased expression of type III and type IV collagens and fibronectin at both mRNA and protein levels as compared to Mks derived from healthy controls. Finally, as reported in our basal experiments, *in vitro* treatment with a JAK inhibitor reduced the ECM component production by PMF patient Mks. Whether the increased ECM component expression by Mks could contribute to BM fibrosis in PMF is still to be established, but it looks like that alterations in composition and concentration of the Mk autocrine niche components might participate into the aberrant megakaryopoiesis found in PMF.

CONCLUSION

Mks are complex cells residing within the BM where they mature in order to release platelets under the control of a plethora of signals from the surrounding microenvironment. Increasing evidences highlight an important role for the autocrine regulation of Mk maturation and function [6, 23, 41–43]. In this work we demonstrated that human Mks produce and release fibronectin, type III and type IV collagens. ECM component synthesis by Mks is promoted by self-produced TGF- β 1, which is actively secreted by Mks under

the control of TPO. Importantly, these observations were replicated in the *in vivo* context by treating mice with supra-pharmacological doses of rmTPO. Finally, alterations of the proposed mechanism in Mks derived from two patients affected by primary myelofibrosis were shown. Although the exact role of the self-produced ECM components *in vivo* still need to be elucidated, overall, these results open fundamental perspectives in the study of megakaryopoiesis, by illustrating the physiological and pathological autocrine mechanisms that regulate the production of ECM components by Mks *in vitro* and *in vivo*.

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AUTHOR CONTRIBUTIONS

A.B.: supervised the project, conceived the idea, and wrote the manuscript; V.A. and C.A.D.B.: designed and performed the experiments, analyzed the data, and wrote the manuscript; C.G., A.M., U.G., G.C., A.A., and M.V.: performed the experiments and edited the manuscript; L.L., L.V., A.I., R.M., G.B., and V.R.: analyzed the data and edited the manuscript. V.A. and C.A.D.B. contributed equally to this work.

POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interests.

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