

# Involvement of TGF $\beta$ 1 in autocrine regulation of proplatelet formation in healthy subjects and patients with primary myelofibrosis

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## Design and Methods

### Materials

Lympholyte was from Accurate Chemical and Scientific Corporation (New York, NY, USA). Immunomagnetic separation system and Stemolecule ALK5 inhibitor were from Miltenyi Biotec (Bologna, Italy). Stem Span medium was from Stem-Cell Technologies (Vancouver, Canada). Recombinant human TPO, interleukin IL-6, IL-11 and recombinant human TGF $\beta$ 1 (mammalian derived) were from PeproTech (Rocky Hill, NJ, USA). The following anti-human antibodies have been used: rabbit polyclonal IgG T $\beta$ R2 were from Millipore (Temecula, CA, USA), mouse mAb T $\beta$ R1 was from Abcam (Cambridge, UK), goat polyclonal IgG Smad 2/3 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), rabbit mAb pSmad2/3 (Ser465/467), rabbit mAb pAkt (Ser473), rabbit mAb Akt, and rabbit mAb PTEN were from Cell Signaling Technology (Danvers, MA, USA), mouse mAb  $\beta$ -actin was from Sigma (Milan, Italy), mouse mAb  $\alpha$ -tubulin was from Sigma (Milan, Italy). AKT inhibitor VIII, isozyme-Selectine, AKTI-1/2 was from Calbiochem (La Jolla, CA, USA). TGF- $\beta$  receptor kinase inhibitor, SB431542, was from Sigma (Milan, Italy). BCA was from Pierce (Rockford, IL, USA). Enhanced chemiluminescence reagents (ECL) were from Millipore (Milan, Italy).

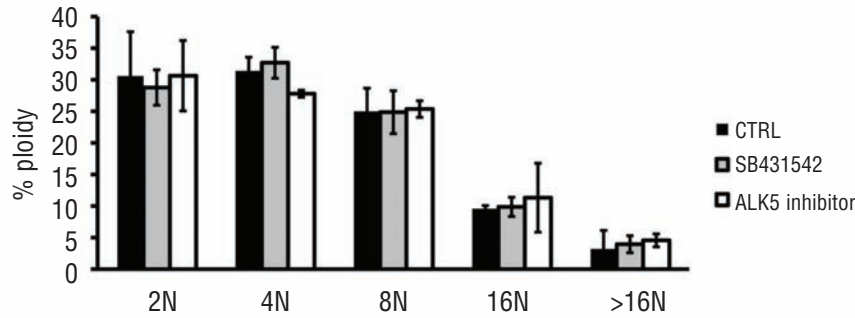
### Differentiation of megakaryocytes from human cord blood and peripheral blood

CD34<sup>+</sup> cells from human cord blood and peripheral blood were separated as previously described<sup>1</sup> and cultured for 13 days in Stem Span medium supplemented with 10 ng/mL TPO, IL-6, IL-11 at 37°C in a 5% CO<sub>2</sub> fully humidified atmosphere. Medium was changed at Days 3, 7 and 10 of culture. Cells at Day 13 of culture were harvested, plated onto coverslips coated with fibrinogen 100  $\mu$ g/mL in 24-well plates (1x10<sup>5</sup> cells per well) and allowed to adhere for 16 h at 37°C and 5% CO<sub>2</sub>. In some experiments, before being seeded, megakaryocytes were pre-incubated with TGF $\beta$  receptor inhibitors, SB431542 (10  $\mu$ M) and Stemolecule ALK5 inhibitor (10  $\mu$ M), or with AKT inhibitor VIII, AKTI-1/2 (10  $\mu$ M). Negative controls were prepared with cells incubated with identical concentrations of DMSO. The percentage of megakaryocytes extending proplatelets was then evaluated

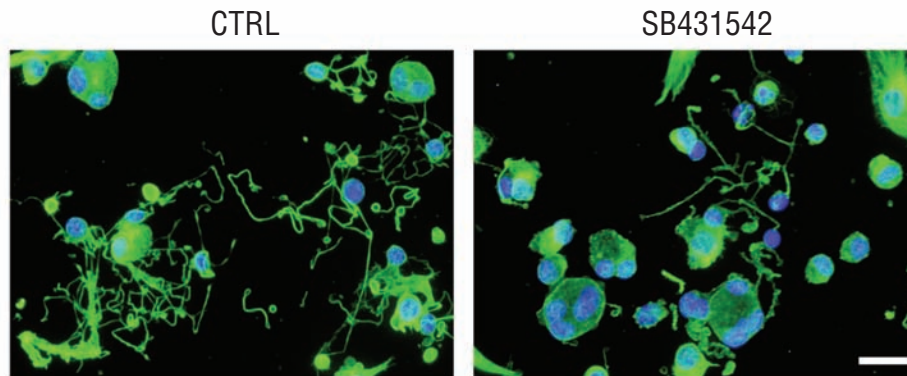
by phase-contrast microscopy (Nikon TMS-F, Tokyo, Japan) using a 20X objective, or by immunofluorescence microscopy. Briefly, for immunofluorescence analysis cells were fixed in 4% paraformaldehyde for 20 min at room temperature (RT). Upon washing with a phosphate buffer saline (PBS), cells were blocked with 3% bovine serum albumin (BSA) for 1 h at RT. Cells were then double-stained with anti- $\alpha$ -tubulin antibody (clone DM1A) (Sigma, Milan, Italy) and goat polyclonal CD61 antibody (St. Cruz Biotechnology, Heidelberg, Germany). Nuclear counterstaining was performed with Hoechst 33258 (100 ng/mL) in PBS. Specimens were mounted onto glass slides with ProLong Gold Antifade Reagent (Invitrogen, Milan, Italy) and images acquired by Olympus BX51 microscope (Olympus, Deutschland GmbH, Hamburg, Germany) using a 20X/0.5 objective. Megakaryocytes extending proplatelets were identified as CD61<sup>+</sup> cells extending  $\alpha$ -tubulin positive long filamentous structures ending with platelet-sized tips. The percentage of megakaryocytes bearing proplatelets was determined by analyzing the entire area of the coverslips.

### Bioassay for measurement of TGF $\beta$ 1 levels

TGF $\beta$ 1 levels in culture supernatants of cord blood and peripheral blood from control (CTRL) and primary myelofibrosis (PMF) megakaryocytes were determined by the CCL64 mink lung epithelial cell line biological assay, based on the lack of proliferation of these cells in presence of bioactive TGF $\beta$ <sup>2</sup> and measurements determined as previously described.<sup>3</sup> Briefly, CCL64 cells were collected during their logarithmic growth phase and suspended at 5x10<sup>4</sup> cells/mL in EMEM (LGC Promochem, Middlesex, UK) containing 10% fetal calf serum (Invitrogen, Milano, Italy). Hundred microliters of the suspension was added to flat bottom 96-well plates and incubated at 37°C for 24 h. Samples were then added to the CCL64 cells and after 28 h the cells were pulsed with 1 Ci [<sup>3</sup>H] thymidine (Amersham, Amsterdam, Holland) for 17 h. Cells were harvested onto glass-fiber filters and radioactivity was determined by liquid scintillation counting. All the conditions were carried out in triplicate. We quantified the amount of TGF $\beta$ 1 referring the CCL64 cell proliferation inhibition obtained with plasma from patients and CTRLs to that obtained with a standard curve of recombinant TGF $\beta$ 1 ranging from 0.001 to 6 ng/mL. To determine cell proliferation

**A**

**Online Supplementary Figure S1.** Effects of 16 h incubation with SB431542 (10  $\mu$ M) or Stemocleule ALK5 inhibitor (10  $\mu$ M) on megakaryocyte ploidy. Data are expressed as mean $\pm$ SD of 3 different experiments ( $P$ =NS, ANOVA and Bonferroni's t-test as *post-hoc* test) (A). Representative immunofluorescence image of proplatelet forming megakaryocytes, after incubation with control (CTRL) or T $\beta$ RI inhibitor (SB431542, 10  $\mu$ M) for 16 hours in culture ( $\beta$ -tubulin in green, Hoechst 33258 in blue, scale bars=40  $\mu$ m) (images were acquired by an Olympus BX51, magnification 20X) (B).

**B**

inhibition induced by total TGF $\beta$ 1, we acidified the plasma samples by 1 N HCl for 15 min at RT and neutralized them by 1.2 N NaOH/0.5 M HEPES.

#### Total RNA extraction and real-time PCR analysis

Quantitative real-time polymerase chain reaction (PCR) was performed to examine TGF $\beta$ 1 expression. Briefly, cells were collected at Days 7, 10 and 13 of culture and CD61<sup>+</sup> megakaryocytes separated using the immunomagnetic beads technique (Miltenyi Biotec, Bologna, Italy), as previously described.<sup>4</sup> Total cellular RNA was extracted with the GenElute Mammalian Total RNA Miniprep kit (Sigma, Milan, Italy), according to the manufacturer's instructions. Nucleic acids were quantified with a Nanodrop 1000 spectrophotometer (Thermo Scientific). cDNA synthesis was carried out using the iScript Kit (Bio-Rad, Milan, Italy). In brief, 100 ng of each total RNA sample was reverse transcribed using a blend of oligo-dT and random primers, subsequently diluted with nuclease-free water to a final cDNA concentration of 2.5 ng/ $\mu$ L and stored at -80°C. Primers for EvaGreen assays were designed using the Beacon Designer 7.9 software (Premier Biosoft International). For TGF $\beta$ 1, GAPDH and HPRT expression studies, the following primers were used: TGF $\beta$ 1 5' TGCTGAGGCTCAAGTTAA 3' and 5' AGGTATCGCCAGGAATTG 3'; GAPDH 5' CGGATTTGGTCGTATTGG 3' and 5' GGTGGAATCATATTGGAACA 3'; HPRT 5' TGACACTGGCAAAA-CAATGCA 3' and 5' GGTCTTTTCACCAGCAAGCT 3'. Quantitative real-time quantification of transcripts was carried

out in a 15  $\mu$ L reaction mix containing 1X SsoFast EvaGreen Supermix (BioRad, Milan, Italy) and 400 nM of each primer. The PCR conditions were the following: 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 60°C for 5 s. Melting curves were generated after amplification in the range 65-95°C with increments of 0.5°C every 5 s. The PCR data were collected using the CFX96 Real-Time System (Bio-Rad). Each sample was tested in duplicate.

#### Western blot analysis

*In vitro* differentiated megakaryocytes at Day 7, Day 10 and Day 13 of differentiation or cord blood and peripheral blood derived megakaryocytes at Day 13 of differentiation, pre-incubated or not with TGF $\beta$  receptor inhibitors, SB431542 or Stemocleule ALK5 inhibitor, as described above, were lysed with HEPES-glycerol lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5% MgCl<sub>2</sub>, 1 mM EGTA) containing 1 mM phenylmethylsulphonyl fluoride (PMSF), 1  $\mu$ g/mL leupeptin, 1  $\mu$ g/mL aprotinin, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 1 mM NaF, on ice for 30 min. Samples were clarified at 15,700  $\times$  g at 4°C for 15 min. Protein content was determined using the BCA assay. Proteins were separated using the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane. Membranes were blocked with 5% BSA containing 0.01% Tween-20 at room temperature for 1 h and then incubated overnight at 4°C with primary antibodies against human T $\beta$ RI, T $\beta$ RII, pSMAD2/3, SMAD2/3, pAkt, Akt, PTEN or  $\beta$ -actin, diluted 1:1000 in 0.5% BSA containing 0.01% Tween-20.

Immunoreactive bands were detected by horseradish peroxidase (HRP)-labeled secondary antibodies using enhanced chemiluminescence reagents.

### **Flow cytometry analysis of megakaryocyte ploidy**

For DNA content analysis, megakaryocytes treated or not with SB431542 or Stemocule ALK5 inhibitor were harvested and fixed with cold ethanol 70% and frozen at -20°C overnight. Subsequently, cells were stained with an antibody against CD41 in the dark with 50 µg/mL propidium iodide supplemented with 100 µg/mL RNase, at room temperature for 30 min. After incubation, cells were analyzed by 2-color flow cytometry (FACSCalibur flow cytometer; BD Biosciences). A minimum of 20,000 events were collected. Offline data analysis was performed using FCS Express Version 3.0 software (DeNovo Software).

### **Statistics**

Values were expressed as mean ± SD (standard deviation). Student's t-test was performed for paired observations.  $P < 0.05$  was statistically significant. Statistical analysis was carried out using SigmaStat 3.0 software. ANOVA, followed by the *post hoc*

Bonferroni's t-test was performed for grouped observations.  $P < 0.05$  was considered statistically significant. All experiments were independently replicated at least 3 times, unless specified otherwise.

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