SUPPLEMENTARY APPENDIX

Revealing eltrombopag's promotion of human megakaryopoiesis through AKT/ERK-dependent pathway activation

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 doi:10.3324/haematol.2016.146746

Received: March 25, 2016.

Accepted: August 4, 2016.

Pre-published: August 11, 2016.

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Running head: *Ex vivo* inquiry on Eltrombopag mechanism of action

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Acknowledgements

The authors would like to thank Dr. Gianluca Viarengo and Prof. Federica Meloni for technical assistance with the flow cytometry analysis; Dr. Cesare Perotti for supplying human cord blood; Dr. Lorenzo Tozzi and Daniel Smoot for technical assistance for the preparation of gel-spun silk microtubes; Prof. Joseph Italiano for providing β1-tubulin antibody. This paper was supported by Cariplo Foundation (2012-0529, 2013-0717), ERA-Net for Research Programmes on Rare Diseases (EUPLANE), the Italian Ministry of Health (RF-2010-2310098) and US National Institutes of Health (R01 EB016041-01). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Supplemental Methods

Silk solution preparation

Bombyx mori silkworm cocoons were supplied by Tajima Shoji Co., Ltd. (Yokohama, Japan). Silk fibroin protein aqueous solution was obtained from the cocoons according to previously published literature.[1-3] Briefly, cocoons were de-wormed, chopped and boiled for 10 minutes in 0.02 M Na_2CO_3 solution at a weight to volume ratio of 5 g to 2 L. The fibers were rinsed for 20 minutes for three times in distilled water and dried overnight. The dried fibers were solubilized for 4 hours at 60°C in 9.3 M LiBr at a weight to volume ratio of 3 g to 12 ml. The solubilized silk solution was dialyzed against distilled water using a Slide-A-Lyzer cassette (Thermo Scientific, Waltham, MA, USA) with a 3,500 MW cutoff for three days and changing the water a total of eight times. The silk solution was centrifuged at 3220xg for 10 minutes to remove large particulates. Finally, the concentration of the silk solution was determined by drying a known volume and massing the remaining solids.

Preparation of gel-spun microtubes and bioreactor assembly

Porous silk tubes were fabricated by gel spinning aqueous silk solutions around small diameter wires, as previously reported.[4,5] Briefly, 15% aqueous silk solution was mixed with the extracellular matrix proteins, fibronectin, type IV collagen and laminin to a final concentration of 25 µg/ml (all from Sigma Aldrich, St.Louis, MO), and with 300 ng/ml SDF-1α (PeproTech, London, UK). Pores in the tubes were obtained by adding 6% w/v polyethylene oxide (PEO) to the silk solution to a volume ratio of 10:1 silk:PEO. The solution was loaded into a syringe capped with a 23 G needle and then extruded onto a polytetrafluoroethylene (PTFE)-coated stainless steel wire that was rotating at a rate of 200 RPM. Axial slew rate and rotations per minute were optimized to generate evenly distributed and continuous scaffolds. The microtubes were stored at -20°C for 24 hours and then lyophilized on a semi-automatic cycle. The microtubes were subjected to a 60 minute methanol wash in order to transform the amorphous structure into its β-form conformation characterized by anti-parallel β-sheets, resulting in insolubility in aqueous conditions. The scaffolds were then soaked in deionized water on a shaker plate for approximately 24 hours to leach out the PEO porogen and stored in deionized water at 4°C. Resulting silk tubes presented 50±20 µm wall thickness with defined pore sizes of 22±4 µm. Before any cell-based experiments, the microtubes were sterilized in 70% ethanol for at least 2 hours. Finally, functionalized silk tubes were trimmed to approximately 1.5 cm in length and secured over the blunt end needles within a bioreactor chamber consisting of a polydimethylsiloxane (PDMS) block plasma bonded to cover glass (Goldseal, No. 1, 24X60 mm, Ted Pella, Redding, CA).[5] Stainless steel needles (23 G, blunt-ended) were positioned on either side of the bioreactor chambers, 50 µm from the bottom edge of the bioreactor.

Silk sponge preparation for mimicking bone marrow environment

A porous silk sponge was assembled around the silk micro-tube using a salt-leaching process, as previously described.[5] Specifically, an 8% aqueous silk solution was dispensed around the tube and then NaCl particles (approximately 500 µm in diameter) were sifted into the silk solution in a ratio of 1 ml of 8% silk solution to 2 g of NaCl particles. The tubes and scaffolds were placed at room temperature (RT) for 48 hours and then soaked in distilled water for 48 hours to leach out the salt particles. Scaffolds were trimmed to 5 mm in width and sterilized in 70% ethanol for 24 hours. Scaffolds were rinsed five times in PBS over 24 hours at 4°C.

For 3D cultures, $CD34^+$ cells from cord blood, were seeded within the silk sponge and cultured in the same conditions described for classic liquid culture, as described in the manuscript. Megakaryocyte (Mk) differentiation was assessed by immunofluorescence analysis and flow cytometry, as described in the manuscript. At day 13 of Mk differentiation the bioreactor chambers were sealed with a glass slide and the outlet ports were connected to the outlet needles. Culture media-filled syringes and tubing were connected to the inlet needles, while transfer bags for platelet collection containing acid-citrate-dextrose (ACD) were secured to the outlet ports. The syringes were placed into a syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA) and media was pumped for 6 hours at a physiologic shear rate of 60 s^{-1}.[5-7]

Western blotting analysis

In order to evaluate the expression of Mk transcription factors and/or the basal activation status of intracellular pathways, hematopoietic progenitor cells, cultured for 1 day in presence of 10 ng/ml rHuTPO or 2000 ng/ml Eltrombopag, or mature Mks, cultured for 13 days in presence of 10 ng/ml rHuTPO or 200, 500 and 2000 ng/ml Eltrombopag, were lysed with Hepes-glycerol lysis buffer (Hepes 50 mM, NaCl 150 mM, 10% glycerol, 1% Triton X-100, $MgCl_2$ 1.5 mM, EGTA 1 mM, NaF 10 mM, PMSF 1 mM, Na_3VO_4 1 mM, 1 µg/ml leupeptin, 1 µg/ml aprotinin) on ice for 30 minutes and lysates clarified by centrifugation at 15700xg, for 15 minutes at 4°C.[8,9]

In order to evaluate the kinetic of activation of the tested biochemical pathways upon stimulation, hematopoietic progenitor cells (day 1), immature (day 10) and mature (day 13) Mks were cyotkines-starved for 6 hours in culture media. Then, cells were stimulated with 10 ng/ml rHuTPO or 2000 ng/ml Eltrombopag only, at 37°C in a 5% CO_2 fully-humidified atmosphere, for 5, 20 or 60 minutes and then lysed, as described above.

Protein lysates were subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (BioRad, Milan, Italy). Membranes were probed with the following affinity-purified antibodies: mouse monoclonal (clone 2C10) anti-RUNX1 (1:1000, Sigma, Milan, Italy), rabbit polyclonal anti-NF-E2 (1:1000, Gene Tex, California, USA), mouse monoclonal (clone 9E12) anti phospho-STAT3 (1:1000, Merk-Millipore, Milan, Italy); rabbit

polyclonal anti STAT3 (Santa Cruz Biotechnology, California, USA); rabbit monoclonal (clone C11C5) anti phospho-STAT5 (1:1000, Cell Signaling Technology, Massachusetts, USA); rabbit monoclonal (clone 3H7) anti STAT5 (1:1000, Cell Signaling Technology); rabbit polyclonal anti phospho-AKT (1:1000, Cell Signaling Technology), rabbit polyclonal anti AKT (1:1000, Cell Signaling Technology), rabbit monoclonal (clone AW39) anti phospho-ERK1/2 (1:1000, Merk-Millipore), mouse monoclonal (clone 137F5) anti ERK1/2 (1:2000, Cell Signaling Technology), mouse monoclonal (clone DM1) anti-β-actin (1:5000, Sigma) following the conditions recommended by the manufacturers. Immunoreactive bands were detected by horseradish peroxidase-labeled secondary antibodies (BioRad), using enhanced chemiluminescence reagent (Merck-Millipore). Pre-stained protein ladders (BioRad) were used to estimate the molecular weights.

Flow cytometry analysis

For Mk differentiation analysis, 10^5 cells were collected at day 13 of culture and centrifuged at 250xg for 7 minutes. Cells suspended in phosphate buffered saline (PBS) were stained with a fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal (clone PM6/13) anti-human CD61 antibody (Abcam, Cambridge, UK) and phycoerythrin (PE)-conjugated mouse monoclonal (clone HIP1) anti-human CD42b antibody (Abcam), at RT, in the dark for 30 minutes. Fully differentiated Mks were gated as $CD61^+CD42b^+$ events. Mk proliferation was quantified by measuring the Mk output, which was defined as the absolute number of $CD61^+CD42b^+$ cells at day 13 of culture with respect to the absolute number of $CD34^+$ cells plated at the beginning of culture.

For ploidy analysis, cells at the end of differentiation were fixed with 70% ethanol at -20°C overnight, centrifuged at 2000xg for 10 minutes at RT and then suspended in PBS containing 1µg/ml Propidium Iodide (Sigma), 1 µg/ml RNAse (Sigma) and FITC-conjugated mouse monoclonal (clone HIP8) antibody against CD41a (eBioscience, Milan, Italy), at RT, in the dark for 30 minutes.

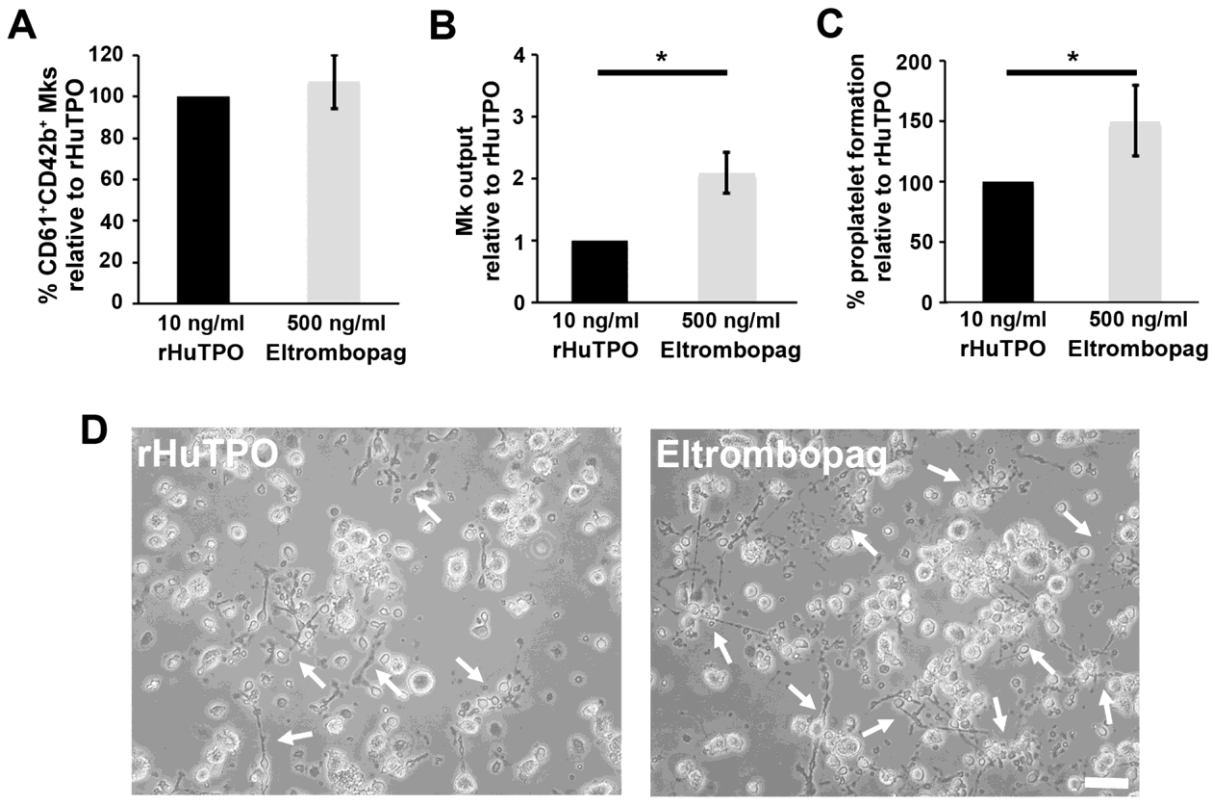
Physical parameters and functionality of *ex vivo* generated platelets were analyzed using the same settings we have previously described.[5] Briefly, collected platelets produced *ex vivo* after perfusion of silk microtubes were stained with a FITC-conjugated mouse monoclonal (clone PM6/13) anti-human CD61 antibody (Abcam) and a PE-conjugated mouse monoclonal (clone HIP1) anti-human CD42b antibody (Abcam) and their number was calculated using a TruCount bead standard (Becton Dickinson, S. Jose, CA). For the analysis of platelet functionality, platelets produced *ex vivo* by perfusing functionalized silk microtubes were suspended in Tyrode's buffer and allowed to rest for 1 hour at RT. Samples were then activated with 3 U/ml thrombin or 25 µM ADP (Sigma), for 15 minutes at 37°C, in presence of 2.5 mM Ca^{2+} and 2.5 mM Mg^{2+}. Unstimulated platelets were used to gate for non-activated platelets. All samples were probed with FITC-conjugated PAC-1 (Becton Dickinson), which specifically binds the activated conformation of CD41. $CD42b^+$ events

exhibiting PAC-1 binding were considered functional activated platelets. All samples were acquired with a Beckman Coulter Navios flow cytometer. Off-line data analysis was performed using Beckman Coulter Navios software package.

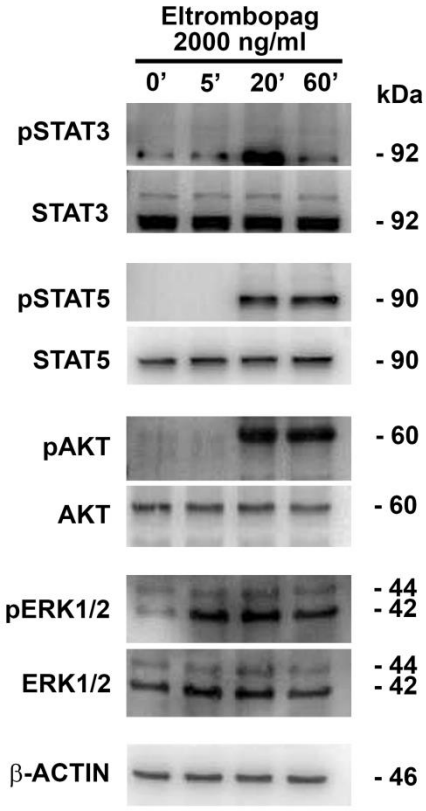
References

1. Kim UJ, Park J, Li C, Jin HJ, Valluzzi R, Kaplan DL. Structure and properties of silk hydrogels. Biomacromolecules. 2004;5(3):786-792.
2. Li C, Vepari C, Jin HJ, Kim HJ, Kaplan DL. Electrospun silk-BMP-2 scaffolds for bone tissue engineering. Biomaterials. 2006;27(16):3115-3124.
3. Lovett M, Cannizzaro C, Daheron L, Messmer B, Vunjak-Novakovic G, Kaplan DL. Silk fibroin microtubes for blood vessel engineering. Biomaterials. 2007;28(35):5271-5279.
4. Lovett ML, Cannizzaro CM, Vunjak-Novakovic G, Kaplan DL. Gel spinning of silk tubes for tissue engineering. Biomaterials. 2008;29(35):4650-4657.
5. Di Buduo CA, Wray LS, Tozzi L, et al. Programmable 3D silk bone marrow niche for platelet generation ex vivo and modeling of megakaryopoiesis pathologies. Blood. 2015;125(14):2254-64.
6. Mazo IB, von Andrian UH. Adhesion and homing of blood-borne cells in bone marrow microvessels. J Leukoc Biol. 1999;66(1):25-32.
7. Jiang J, Woulfe DS, Papoutsakis ET. Shear enhances thrombopoiesis and formation of microparticles that induce megakaryocytic differentiation of stem cells. Blood. 2014;124(13):2094-2103.
8. Di Buduo CA, Moccia F, Battiston M, et al. The importance of calcium in the regulation of megakaryocyte function. Haematologica. 2014;99(4):769-778.
9. Abbonante V, Di Buduo CA, Gruppi C, et al. Thrombopoietin/TGF-β1 Loop Regulates Megakaryocyte Extracellular Matrix Component Synthesis. Stem Cells. 2016;34(4):1123-1133.

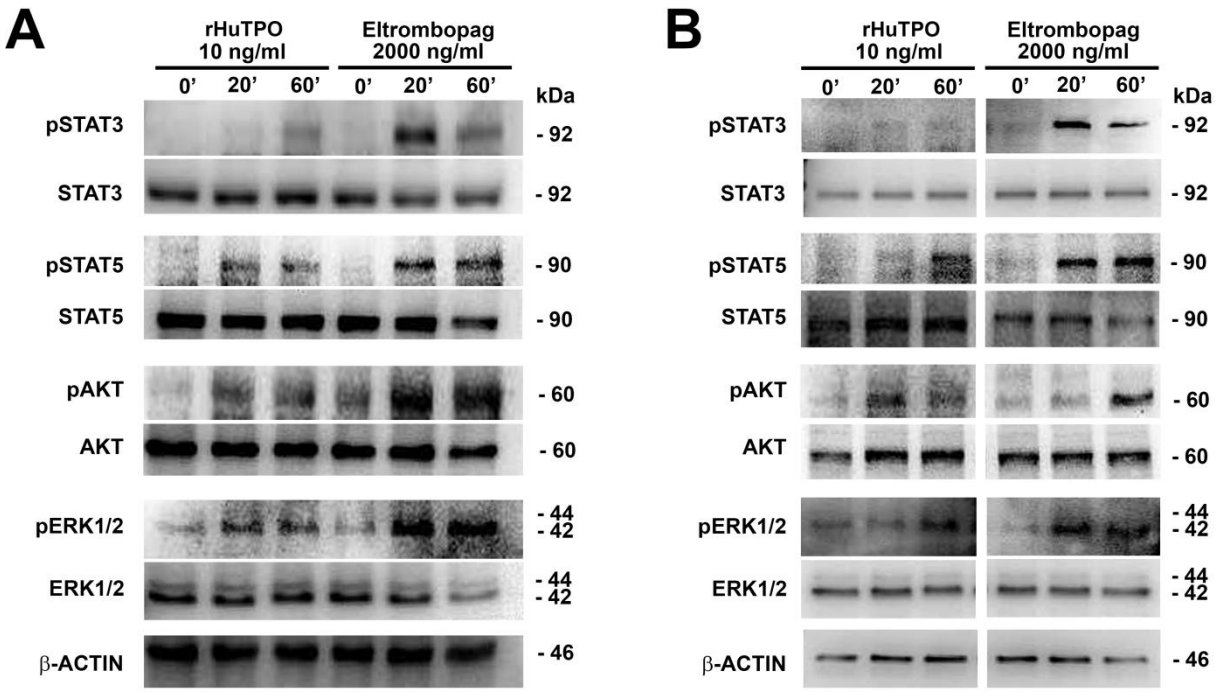
Supplemental Figures



Supplemental Figure 1. Eltrombopag promotes human adult hematopoietic progenitor differentiation into megakaryocyte. Megakaryocytes were differentiated from human adult peripheral blood progenitors and cultured in presence of 500 ng/ml Eltrombopag or 10 ng/ml recombinant human TPO (rHuTPO), as positive control. (A) Analysis of $CD61^{+}CD42b^{+}$ megakaryocytes (Mks) at the end of the culture in presence of 500 ng/ml Eltrombopag with respect to 10 ng/ml rHuTPO. Data are presented as mean±SD (p=NS). (B) Output was calculated as the number of $CD61^{+}CD42b^{+}$ cells at the end of the culture with respect to the total starting number of hematopoietic progenitors. Histograms show the fold increases of megakaryocyte (Mk) output in presence of 500 ng/ml Eltrombopag with respect to 10 ng/ml rHuTPO. Data are expressed as mean±SD (*$p<0.05$). (C) The percentage of proplatelet forming megakaryocytes was calculated as the number of cells displaying long filamentous pseudopods with respect to total number of round megakaryocytes per analyzed field. Histograms show the fold increase of proplatelet formation in presence of 500 ng/ml Eltrombopag with respect to 10 ng/ml rHuTPO, used as optimum standard condition to test proplatelet formation. Data are expressed as mean±SD (*$p<0.05$). (D) Representative light microscopy images of proplatelet formation in the presence of rHuTPO or Eltrombopag (scale bar=100 µm). Arrows indicate proplatelet-forming megakaryocytes.



Supplemental Figure 2. Eltrombopag activates c-Mpl downstream signaling molecules in *in vitro* differentiated megakaryocytes. Megakaryocyte at day 10 of culture were cytokine-starved and then stimulated with 2000 ng/ml Eltrombopag for 5, 20 or 60 minutes and then lysed. Samples were probed for phosphorylated-STAT3 (pSTAT3), phosphorylated-STAT5 (pSTA5), phosphorylated-AKT (pAKT) and phosphorylated-ERK1/2 (pERK1/2). Total STAT3, STAT5, AKT, ERK1/2 and β-actin were revealed to ensure equal loading.



Supplemental Figure 3. Early activation of biochemical pathways downstream of c-Mpl by Eltrombopag in *in vitro* differentiated megakaryocytes and hematopoietic progenitors. (A) Megakaryocyte at day 13 of culture were cytokine-starved and then stimulated with 10 ng/ml or 2000 ng/ml Eltrombopag for 20 and 60 minutes, and then lysed. Samples were probed for phosphorylated-STAT3 (pSTAT3), phosphorylated-STAT5 (pSTA5), phosphorylated-AKT (pAKT) and phosphorylated-ERK1/2 (pERK1/2). Total STAT3, STAT5, AKT, ERK1/2 and β-actin were revealed to ensure equal loading. (B) Hematopoietic progenitors at day 1 of culture were cytokine-starved and then stimulated with 10 ng/ml or 2000 ng/ml Eltrombopag for 20 and 60 minutes, and then lysed. Samples were probed for pSTAT3, pSTA5, pAKT and pERK1/2. Total STAT3, STAT5, AKT, ERK1/2 and β-actin were revealed to ensure equal loading.