SUPPLEMENTARY APPENDIX

A new path to platelet production through matrix sensing

Vittorio Abbonante,[1,2] Christian Andrea Di Buduo,[1,2] Cristian Gruppi,[1,2] Carmelo De Maria,[3] Elise Spedden,[4] Aurora De Acutis,[3] Cristian Staii,[4] Mario Raspanti,[5] Giovanni Vozzi,[3] David L. Kaplan,[6] Francesco Moccia,[7] Katya Ravid[8] and Alessandra Balduini[1,2,6]

[1]Department of Molecular Medicine, University of Pavia, Italy; [2]Laboratory of Biotechnology, IRCCS San Matteo Foundation, Pavia, Italy; [3]Interdepartmental Research Center "E. Piaggio", University of Pisa, Italy; [4]Department of Physics and Astronomy, Tufts University, Medford, MA, USA; [5]Department of Surgical and Morphological Sciences, University of Insubria, Varese, Italy; [6]Department of Biomedical Engineering, Tufts University, Medford, MA, USA; [7]Department of Biology and Biotechnology "Lazzaro Spallanzani", University of Pavia, Italy and [8]Department of Medicine and Whitaker Cardiovascular Institute, Boston University School of Medicine, MA, USA

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Correspondence: alessandra.balduini@unipv.it

SUPPLEMENTAL MATERIALS AND METHODS

Antibodies

The following antibodies were used: anti- β1 tubulin (kind gift of Prof. J. Italiano Jr); clone HUTS-4, anti-active β1 integrin (Millipore); anti- β1 integrin (Abcam); anti-βActin (Sigma Aldrich); anti-pAKT (ser473; Cell Signaling); anti-Akt (Cell Signaling); anti-pERK (Thr185/Tyr187; Millipore); anti-ERK (Cell Signaling); anti-TRPV4 (Abcam); anti-PE-pAKT (BD Pharmingen); anti-PKC substrates (pSer/pThr; Cell Signaling).

Evaluation of megakaryocyte spreading and proplatelet formation

To analyze Mk spreading and proplatelet formation (PPF) onto collagens, 12 mm glass coverslips or silk films were coated with 25 µg/mL type I (gift of Prof. Maria Enrica Tira; University of Pavia) or type IV collagen (acid soluble) (Sigma Aldrich) overnight at 4°C. At day 13 of differentiation, mature Mks were harvested and allowed to adhere at 37°C and 5% CO_2. To evaluate both the number of spread Mks and PPF, samples were fixed and stained at three well-defined time points, namely at 3, 8 and 16 hours post cell layering on coated surface [1]. These points have been previously shown to be representative of active adhesion (3 hours), starting of PPF (8 hours), and the zenith of PPF and platelet release (16 hours) [2]. The number of spread Mks was assessed as follows: β1 tubulin positive Mks exhibiting stress fibers (stained with TRITC-Phalloidin) were counted and expressed as percentage of spread Mks. PPF were counted as percentage of total Mks.

Immunoprecipitation and Western blotting

Cultured Mks and primary BM immunomagnetically-sorted Mks (CD41+; Biolegend) were collected, washed twice at 4°C and lysed in hepes-glycerol lysis buffer (Hepes 50 mM, 10% glycerol, 1% Triton x-100, $MgCl_2$ 1.5 mM, EGTA 1mM) containing aprotinin 1 µg/mL and leupeptin 1 µg/mL, for 30 min at 4°C, as previously described [3]. After centrifugation at 15700xg for 15' at 4°C, Laemmli sample buffer was added to supernatants. For active β1 integrin staining (clone HUTS-4) samples were not reduced. Samples were then heated at 95 °C for 3' and loaded and run on 8% or 12% sodium dodecyl

sulfate polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidine fluoride membranes (PVDF). Membranes were incubated with 5% BSA, 0.1% Tween in PBS to avoid non specific antibody 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). For immunoprecipitation, cellular lysates were precleared by incubation with protein A-Sepharose. Precleared lysates were incubated with 2 µg of anti-TRPV4 antibody (4 µg/mL; Abcam) for 4 h at 4°C on a rotatory shaker, followed by adding 100 µL of 50 mg/ml protein A-Sepharose and incubation overnight at 4°C on a rotatory shaker. Beads were washed three times in lysis buffer.

Immunofluorescence microscopy

The cover-slips were mounted onto glass slides with ProLong Gold antifade reagent (Invitrogen). For immunofluorescence staining of BM samples, sections were fixed for 20 minutes in 4% PFA, washed with PBS, and blocked with 2% bovine serum albumin (BSA) (Sigma-Aldrich) in PBS for 30 minutes. Non-specific 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 micro-cover glass slips using Fluoro-mount (Bio-Optica). Negative controls were routinely included by omitting the primary antibodies.

Internalization assays

For immunofluorescence internalization assay, Mks were washed in RPMI medium (Euroclone) containing 0.1% BSA and pre-cooled on ice before treatment with 15 µg/ml of anti-β1 integrin antibody (Abcam) ($1x10^5$ cells in 100 µl RPMI, 0.1% BSA with mAb) for 30 minutes on ice with gentle agitation. After washing with ice-cold RPMI, 0.1% BSA to remove unbound antibody,

cells were seeded on type I or type IV collagen-coated coverslips, or on type I collagen coated soft and stiff silk films for 3 hour at 37°C. Before fixation, cells were placed on ice and acid washed (three washes with ice-cold 50 mM glycine in Ca2+/Mg2+ HBSS, pH 2.5, and two washes with Ca2+/Mg2+ HBSS, pH 7.5) to remove the antibody from the cell surface. Mks were then fixed, permeabilized, and stained with the appropriate secondary antibody as described in the "immunofluorescence microscopy" section [4].

For western blotting internalization assay, Mks at day 13 of culture ($1.5x10^6$) were cooled on ice and washed in pre-chilled PBS before incubation with PBS 0.5 mg/mL thiol-cleavable Sulfo-NHS-S-S-Biotin (Pierce Chemical Co.) for 1 hour on ice. After washing with ice-cold PBS, labeled Mks were resuspended in serum-free RPMI medium and plated on the indicated substrates for the indicated time points at 37°C to allow β1 integrin internalization. After plating Mks for indicated times, samples were returned to ice, washed three times with ice-cold PBS, and treated with two successive reductions of 20 minutes with a reducing solution containing the non-membrane permeable reducing agent glutathione (GSH; 42 mM), 75 mM NaCl, 1 mM EDTA, 1% bovine serum albumin and 75 mM NaOH. To evaluate total labeling, a sample was not reduced with GSH.

Silk solution preparation

Silk fibroin aqueous solution was obtained from Bombyx mori silkworm cocoons according to previously published literature [5-7]. Briefly, Bombyx mori cocoons were de-wormed and chopped. 5 g of chopped cocoons were boiled for 10 minutes in 2 L of 0.02 M Na_2CO_3 solution. Resulting fibers were rinsed 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/12 mL. The solubilized silk solution was dialyzed against distilled water using a Slide-A-Lyzer cassette (Thermo Scientific) with a 3,500 MW cutoff for three days. Water was changed a total of eight times. The silk solution was centrifuged at 3220xg for 10 minutes to remove large particulates and stored at 4°C.

Silk film fabrication and assembly of the transwell chamber system

Silk solution (1% w/v), was cast on 6 well plates (45 μL/cm^2 of surface area) and dried at 22°C for 16 hours. Silk films were water annealed in a vacuum chamber, containing 100 mL of water at the bottom of chamber, for stiffness tuning. The water annealing chamber was maintained at either 60°C for 16 hours or 4°C for 6 hours to achieve stiff or soft silk film mechanical properties, respectively. Before Mk plating, silk films were exposed to ultraviolet light for 30 minutes per side inside of a sterile biological hood. For immunofluorescence experiments, films were cast on polydimethylsiloxane (PDMS) mold and dried at 22°C for 16 hours. Silk films were lift off the PDMS mold and water annealed as described in the text. Successively, silk films were secured between two rings of scotch tape (6 mm inner diameter, 12 mm outer diameter) and secured to the bottom of a 24-well plates using silicon rings (10 mm inner diameter, 15.5 mm outer diameter, McMaster Carr). All samples were sterilely washed three times in PBS over the course of 24 hours. The transwell chamber system for the analysis of platelet production was assembled as previously described [8]. Briefly, silk solution (1% w/v) was mixed with polyethylene oxide (PEO) porogen (0.05% w/v; Sigma) before casting on the PDMS mold, dried and water annealed in order to obtain porous silk films of different stiffness. The membrane from transwell inserts (Corning) was then removed under sterile conditions using a biopsy punch. Porous silk films were trimmed using an 8 mm diameter biopsy punch and secured to the transwell insert using a sterile, medical-grade silicon glue (Dow Corning). The films were rinsed three times in PBS over the course of 24 hours to remove the PEO porogen. In all experiments, silk films were soaked in cell culture media for one hour, prior to cell seeding.

Elastic modulus determination via Atomic Force Microscope

Elastic modulus maps were taken on an Asylum Research MFP-3D Atomic Force Microscope (AFM) (Asylum Research) using AC240TS-R3 cantilevers (Asylum Research) with a nominal spring constant of 2 N/m. Films were hydrated with PBS and a minimum of 300 AFM force vs. indentation curves were taken in the fluid solution on each film. Cantilevers were calibrated in air and in the buffer solution prior to measurement to determine accurate spring constant values. Elastic modulus values were determined using the inbuilt

Hertz Model fitting function of the Asylum Research MFP3D software [9]. To analyze collagen structures in different conditions, type I and type IV were coated on silk films cast on glass coverslips as described in “Silk film fabrication” and then observed by tapping-mode atomic force microscopy on a Digital Instruments multimode Nano- Scope III/a SPM (Digital Instruments) with Olympus OTR 8 oxide-sharpened silicon nitride probes [3].

Reverse transcription (RT)-PCR and quantitative Real Time PCR

In vitro differentiated Mks at day 13 of culture were purified using immunomagnetic beads technique [9]. Total RNA was extracted using the Mammalian GeneElute total RNA kit (Sigma-Aldrich). Retrotrascription (RT) was performed using the iScriptTM cDNA Synthesis Kit according to the manufacturer instructions (BioRad). RT-PCR was performed as previously described [3]. For quantitative Real Time PCR, RT samples were diluted up to three times with ddH_2O 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’, 35 cycles at 95°C for 10’’, 60°C for 15’’, 72°C for 20’’. Pre-designed KiCqStart primers were purchased from Sigma Aldrich (Milan, Italy). The BioRad CFX Manager software 3.0 was used for the normalization of the samples (BioRad). β-2 microglobulin gene expression was used for comparative quantitative analysis.

Elastic modulus determination via Atomic Force Microscope

Elastic modulus maps were taken on an Asylum Research MFP-3D Atomic Force Microscope (AFM) (Asylum Research) using AC240TS-R3 cantilevers (Asylum Research) with a nominal spring constant of 2 N/m. Films were hydrated with PBS and a minimum of 300 AFM force vs. indentation curves were taken in the fluid solution on each film. Cantilevers were calibrated in air and in the buffer solution prior to measurement to determine accurate spring constant values. Elastic modulus values were determined using the inbuilt Hertz Model fitting function of the Asylum Research MFP3D software [9].

[Ca^{2+}]i measurements

PSS consists of: NaCl 150 mM, KCl 6 mM, CaCl2 1.5 mM, MgCl2 1 mM, glucose 10 mM, Hepes 10 mM. In Ca2+-free solution (0Ca2+), Ca2+ was substituted with NaCl 2 mM and EGTA 0.5 mM was added. Solutions were titrated to pH 7.4 with NaOH. After washing in PSS, the coverslip was fixed to the bottom of a Petri dish and the cells were observed using an upright epifluorescence Axiolab microscope (Carl Zeiss), usually equipped with a Zeiss X63 Achroplan objective (water-immersion, 2.0mm working distance, 0.9 numerical aperture). Mks were excited alternately at 340 and 380 nm, and the emitted light was detected at 510 nm. See Supplemental methods. A first neutral density filter (1 or 0.3 optical density) reduced the overall intensity of the excitation light and a second neutral density filter (0.3 optical density) was coupled to the 380 nm filter to approach the intensity of the 340 nm light. A round diaphragm was used to increase the contrast. The excitation filters were mounted on a filter wheel (Lambda 10; Sutter Instrument). Custom software, working in the LINUX environment, was used to drive the camera (Extended-ISIS Camera; Photonic Science) and the filter wheel and to measure and plot on-line the fluorescence from 10 to 15 rectangular regions of interest (ROI) enclosing 10-15 single cells. [Ca^{2+}]i was monitored by measuring, for each ROI, the ratio of the mean fluorescence emitted at 510 nm when exciting alternatively at 340 and 380nm (shortly termed ‘‘ratio’’). An increase in [Ca^{2+}]i causes an increase in the ratio [2].

Animals and *in vivo* treatment

For in vivo BAPN treatment, mice were injected with 350 mg/Kg/day of BAPN (Sigma Aldrich) and were given drinking water containing 0.2% (w/v) BAPN for 14 days. At day 14 after the first injection, mice were sacrificed and blood was collected for peripheral blood count. Femurs were fixed in 3% PFA or alternatively flushed and used for flow cytometry analysis, BM explants and Mk cell sorting experiments. Age- and sex-paired mice (10-12 weeks-old males) were injected with PBS as control. Cell count and differential cell count in blood samples were performed on an ADVIA 120 hematology analyzer (Siemens).

In vivo bone marrow stiffness

Samples were subjected to cyclic uniaxial compression test with a strain rate of 0.01 s^{-1}, up to 4% deformation, at room temperature and in wet condition. Before testing, samples were preserved in PBS, and their length and cross sectional area were measured with a caliper (10 μm resolution). The compressive elastic modulus of each sample was evaluated from the slope of the first linear portion of the stress–strain curve [10].

Flow cytometry

All samples were acquired with a Beckman Coulter FacsDiva flow cytometer (Beckman Coulter Inc.). The analytical gating were set using unstained samples and relative isotype controls. Off line data were analyzed using Beckman Coulter Kaluza version software package (Beckman Coulter Inc.).

Bone marrow explants

Intact bone marrows were obtained by flushing mouse femurs with PBS buffer. Ten 0.5 mm thick transversal sections from one femur from the same mouse were placed at 37°C in an incubation chamber containing DMEM medium supplemented with 5% mouse serum. Living tissue sections were examined by phase contrast under an inverted microscope (Olympus IX53). Images for video were acquired sequentially at 8-minutes intervals with Olympus FluoView FV10i and processed with FV10 ASW 4.0 software (Olympus). Mks were stained as living cells with anti-CD41-FITC antibody (0.001mg/mL; Biolegend) making them visible and recognizable within the explants. Mks were classified as proplatelet forming cells when at least one thin extension presenting a proplatelet bud was observed. To perform TRPV4 immunoprecipitation in Mks from BM explants, Mks were sorted by immunostaining with a phycoerythrin (PE)-conjugated anti-mouse CD41 (0.1 mg/mL; Biolegend) followed by incubation with anti-PE immunomagnetic beads (Miltenyi biotech). The purity of isolated Mks, by means of CD42b and CD61 staining, were routinely analyzed.

Tissue collection and immunohistochemistry

Femurs from treated and control animals were removed and fixed for 24 hours in 3% paraformaldehyde (PFA). Bones were decalcified in a solution of 10% EDTA in 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 µm tissue sections were taken using a Microm Microtome HM 250 (Bio Optica S.P.A.) and stained with anti-CD41 (0.1 mg/mL in PBS/1%BSA/0.3% Triton X-100; Biolegend) and anti-pAkt (diluted 1:25 in PBS/1%BSA/0.3% Triton X-100; Cell signaling) and the appropriate secondary antibodies for fluorescence microscopy analysis [7].

Reticulated platelet analysis

To assess platelet production *in vivo*, a small sample of blood was collected from the tail vein and placed in anticoagulant. The blood was then diluted 20x in 2 mM EDTA in PBS before the addition of 100 ng/mL thiazole orange (Sigma Aldrich) for 60 minutes at room temperature to label reticulated platelets. The samples were then fixed in 1% PFA for 15 minutes and analyzed by flow cytometry. Thiazole orange-positive/$CD41^+$ platelets were considered reticulated [11].

Lox-mediated collagen crosslinking

Recombinant LOXL2 was purchased by R&D systems (2369-AO). Acid soluble type IV collagen (25 µg/mL; Sigma Aldich) was coated on 96-well plates alone or in combination with 4µg/mL of LOXL2 recombinant protein, or in combination with 4µg/mL of LOXL2 recombinant protein plus 200 µM BAPN, overnight at 4°C, following by incubation at 37°C for 24 hours and by three washes with PBS, prior to cell plating [12,13]. After 16 hours, non-adherent cells were removed and adhering Mks were fixed with 4% PFA. Proplatelet forming Mks were counted and expressed as percentage of proplatelet bearing Mks, using protocols we previously employed [1].

Statistics

Values are expressed as mean ± SD. *t test* was used to analyze experiments. A value of $p<0.05$ was considered statistically significant. All experiments were independently repeated at least three times.

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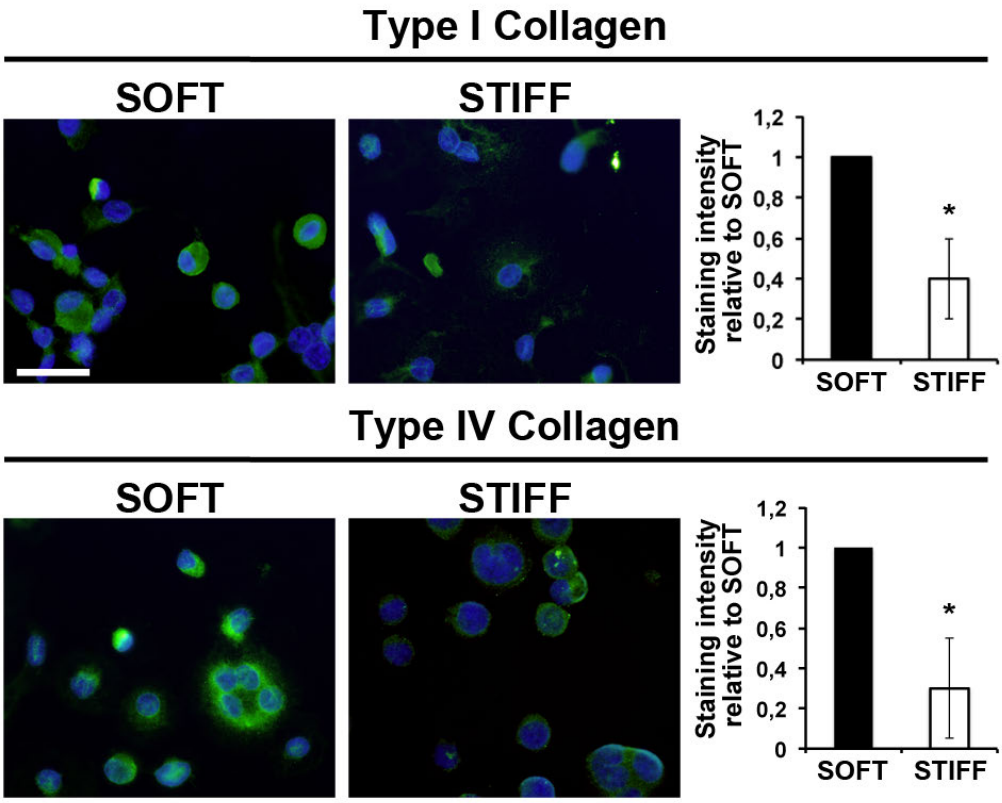
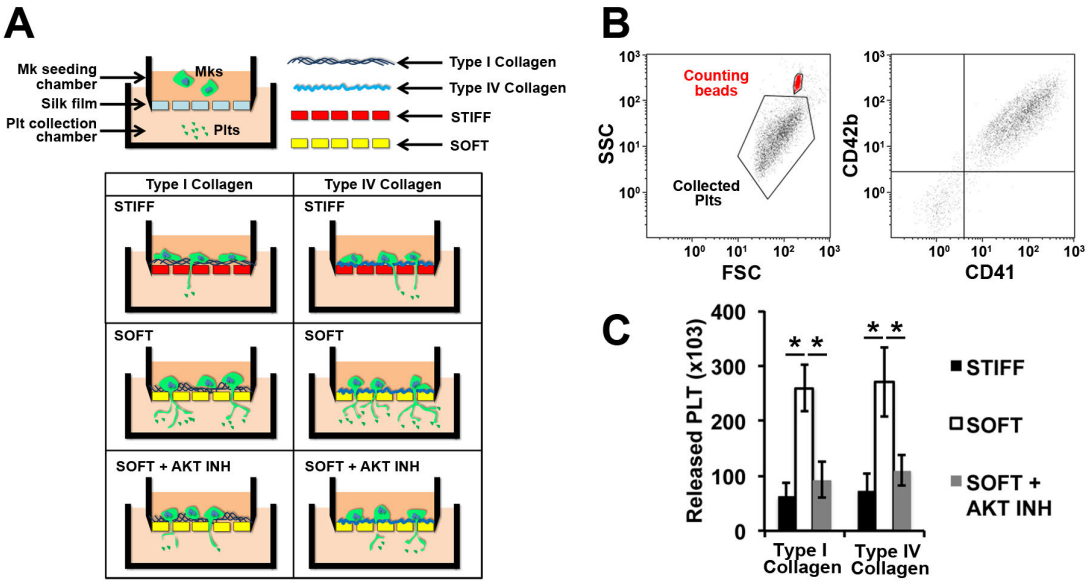
SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

SUPPLEMENTAL FIGURE 1



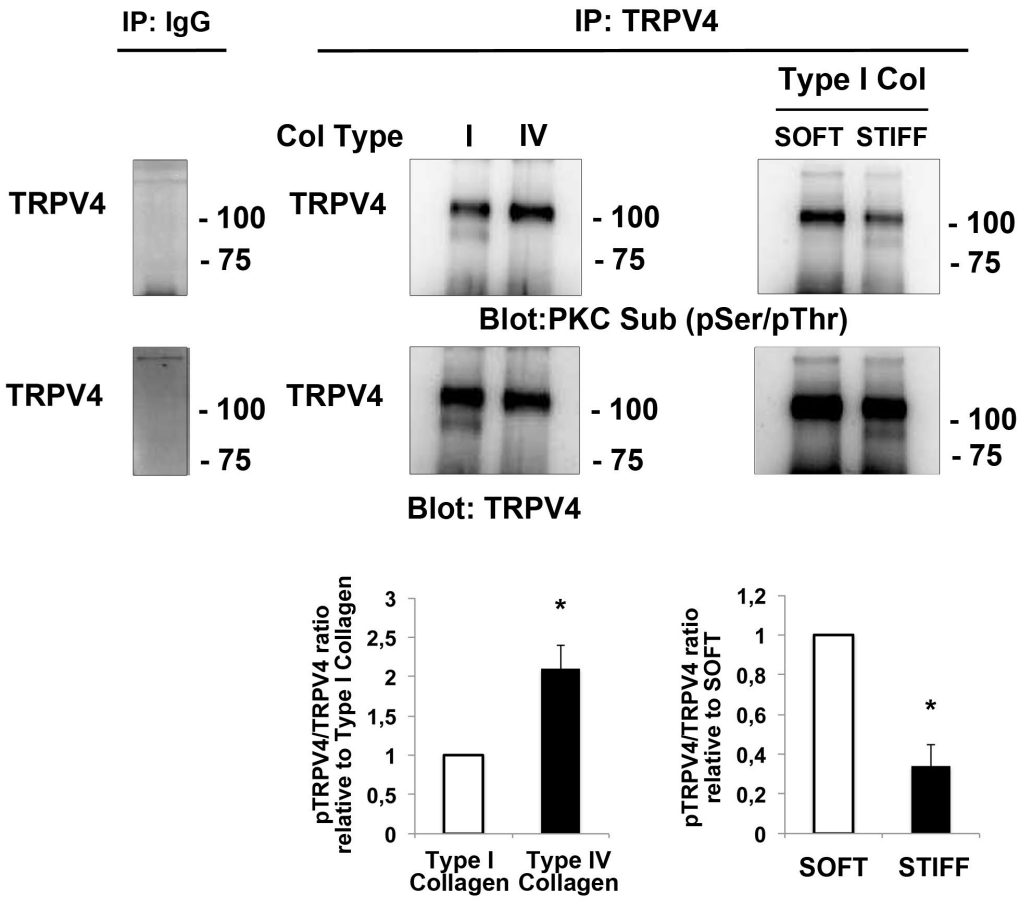
SUPPLEMENTAL FIGURE 1. A) Representative images of active beta 1 integrin immunofluorescence staining in human mature Mks plated, for 3 hours, on type I collagen or type IV collagen coated soft or stiff silk fibroin films. Staining intensities were quantified by ImageJ software. n = 100 Mks per experimental condition in at least three independent experiments. Staining intensities are expressed relative to soft. * $p < 0.05$

SUPPLEMENTAL FIGURE 2



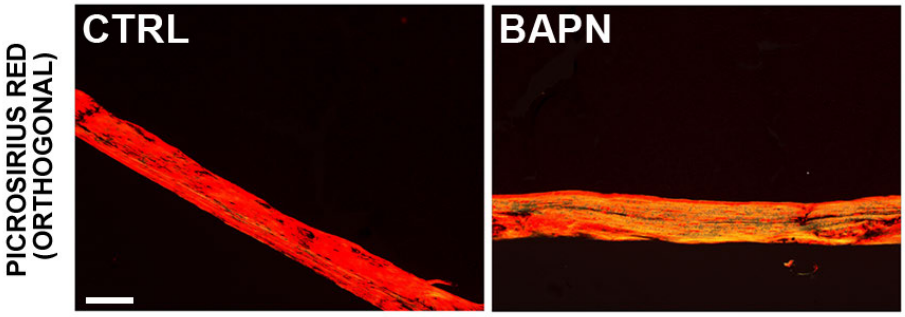
SUPPLEMENTAL FIGURE 2. A) Schematic representation of the transwell system for platelet (Plts) production. B) Representative flow cytometry dot plot of platelets collected in the lower chamber of the system. Platelets were stained with anti-CD41 and anti-CD42b antibodies and counted with bead standard. C) Number of $CD41^{+}/CD42b^{+}$ platelets in the different experimental conditions. n = 3 per experimental condition.

SUPPLEMENTAL FIGURE 3



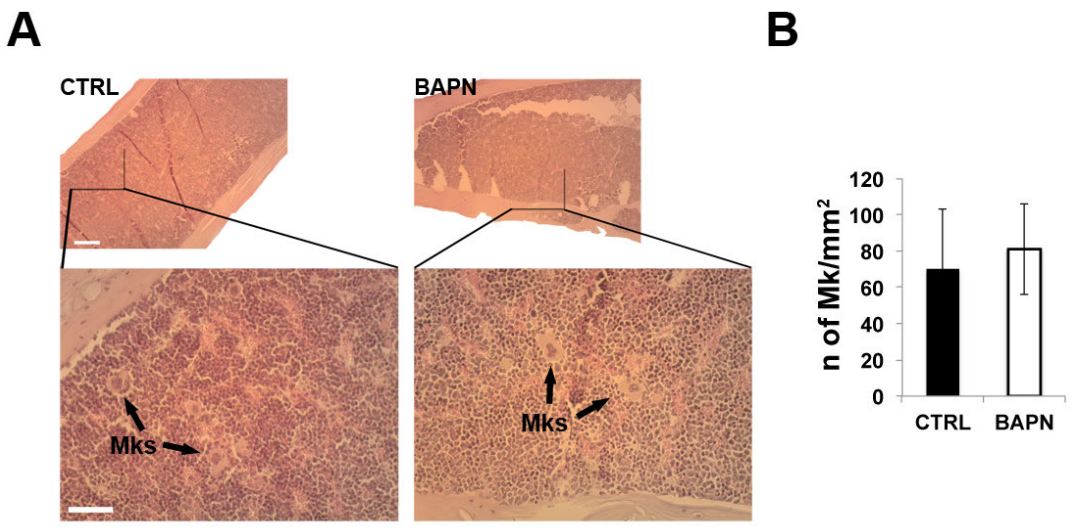
SUPPLEMENTAL FIGURE 3. A) Western blot analysis of phosphorylated TRPV4 in Mks plated for 3 hours on type I and type IV collagen. Mk were lysed and TRPV4 immunoprecipitated with anti TRPV4 antibody. Membranes were probed with anti-PKC substrates antibody and re-probed with anti-TRPV4 antibody to show the equal loading. B) Western blot analysis of phosphorylated TRPV4 in Mks plated for 3 hours on type I collagen coated SOFT or STIFF silk films. Mk were lysed and TRPV4 immunoprecipitated with anti TRPV4 antibody. Membranes were probed with anti-PKC substrates antibody and re-probed with anti-TRPV4 antibody to show the equal loading. A control sample was immunoprecipitated with an unrelated antibody (IgG). C) Densitometry analysis of the western blot in A and B. Data are expressed as mean ± SD for three independent experiments per each class of experiments. * $p<0.05$.

SUPPLEMENTAL FIGURE 4



SUPPLEMENTAL FIGURE 4. A) Photomicrographs of CTRL and BAPN treated mouse femurs stained with picrosirius red, viewed under an orthogonal polarizing filters. Scale bar 100 μm.

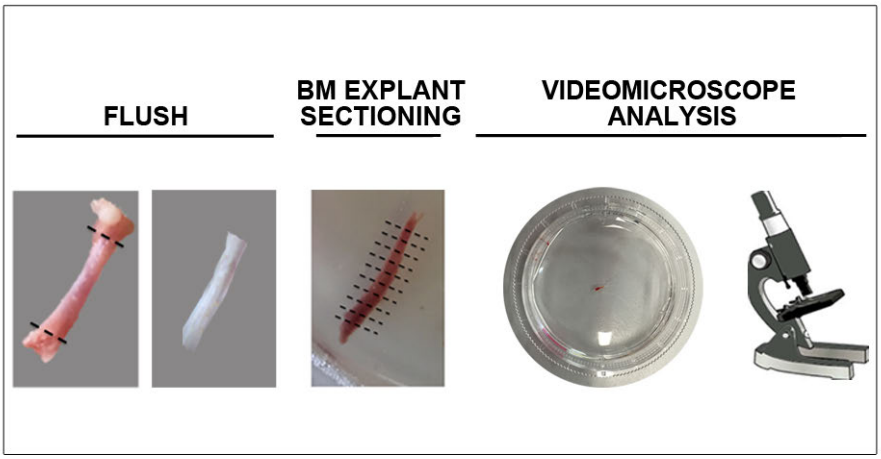
SUPPLEMENTAL FIGURE 5



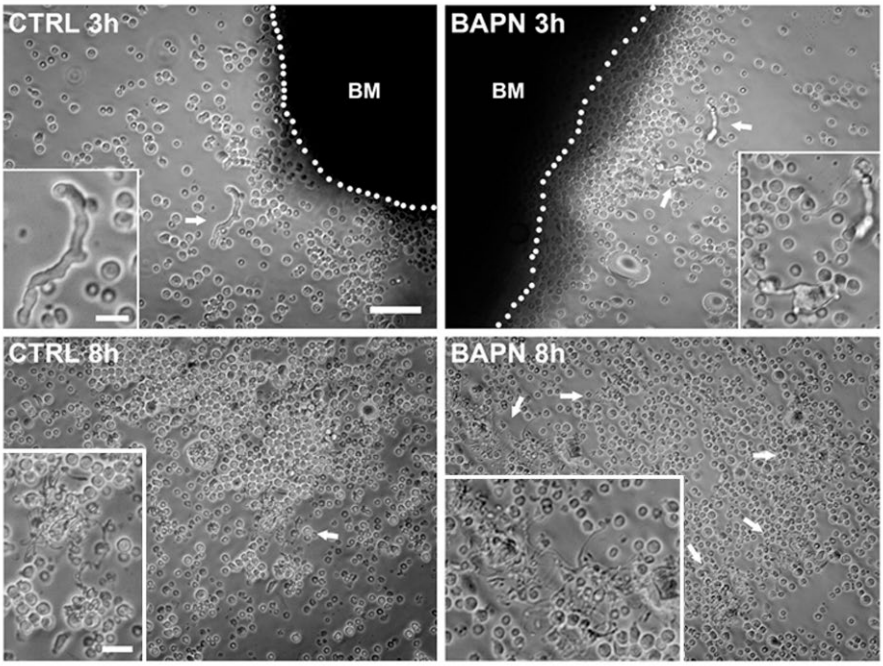
SUPPLEMENTAL FIGURE 5. A) Photomicrographs of CTRL and BAPN treated mouse femurs stained with Hematoxylin and Eosin. Bone marrow Mks are clearly visible (arrows). B) Bone marrow Mks were counted and expressed as number of Mks per mm^2. Data are expressed as mean ± SD (n=4).

SUPPLEMENTAL FIGURE 6

A

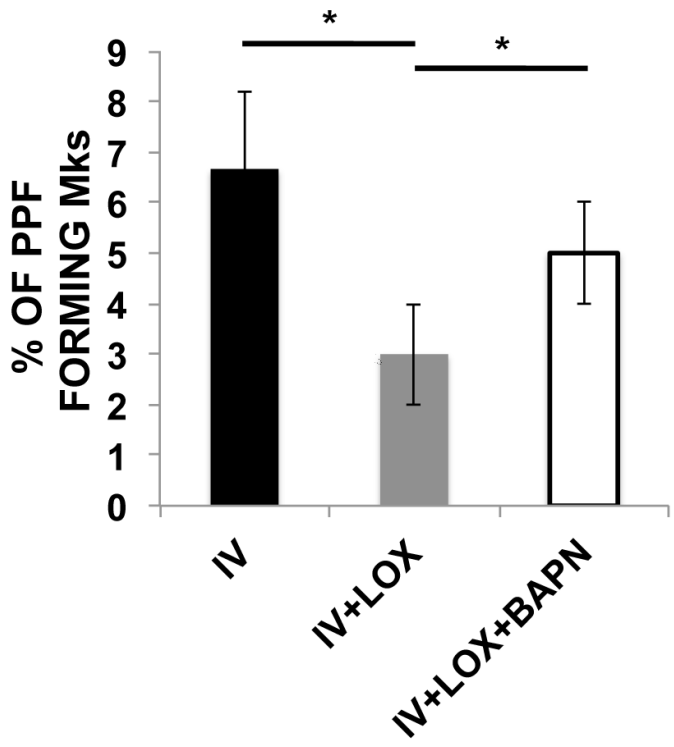


B



SUPPLEMENTAL FIGURE 6. A) Flow chart of the bone marrow explant experiments. B) Phase contrast photomicrographs of CTRL and BAPN treated bone marrow explants after 3 and 8 hours from the beginning of the experiments. Proplatelet extending Mks are visible (arrows). Scale bar 50 μm (box scale bar 10 μm). BM (bone marrow).

SUPPLEMENTAL FIGURE 7



SUPPLEMENTAL FIGURE 7. Mature Mks were plated for 16 hours on 96-well plates coated with 25 μg/mL type IV collagen alone, type IV collagen pre-treated overnight with 4μg/mL LOXL2, or type IV collagen pre-treated with 4μg/mL LOXL2 + 200 μM BAPN overnight, as detailed under Methods. After 16 hours of adherence cells were fixed and counted for proplatelet formation. Data are expressed as mean ± SD (n=5) * $p<0.05$.

SUPPLEMENTAL TABLE 1

TEST	CTRL (mean±SD)	BAPN (mean±SD)	P VALUE
WBC	3,03±1,12	2,76±1,25	n.s.
RBC	10,26±1,76	8,01±1,72	n.s.
PDW	51,58±8,26	81,18±4,63	p < .01
MPV	7,17±0,82	10,1±1,77	p < .01

SUPPLEMENTAL TABLE 1.

Blood cell count in CTRL and BAPN treated mice. Data refer to ten mice per group.

Supplemental Video 1. Videoclip of Mks extending proplatelets in bone marrow explants from CTRL mice. After bone marrow explant, Mks were stained as living cells with anti-CD41-FITC antibody. Images were acquired sequentially (1 frame/8 minutes) and the movie was accelerated to 1 frame/400 milliseconds. Total real duration: 160 min. An average of one Mk extending proplatelets per field is visible.

Supplemental Video 2. Videoclip of Mks extending proplatelets in bone marrow explants from BAPN treated mice. After bone marrow explant, Mks were stained as living cells with anti-CD41-FITC antibody. Images were acquired sequentially (1 frame/8 minutes) and the movie was accelerated to 1 frame/400 milliseconds. Total real duration: 160 min. An average of three Mks extending proplatelets per field is visible.

Supplemental Video 3. Videoclip of Mks extending proplatelets in bone marrow explants from BAPN treated mice treated with an Akt inhibitor. After bone marrow explant, Mks were stained as living cells with anti-CD41-FITC antibody. Images were acquired sequentially (1 frame/8 minutes) and the movie was accelerated to 1 frame/400 milliseconds. Total real duration: 160 min. Mks present a significantly decreased ability to extend proplatelets.