A new path to platelet production through matrix sensing

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ABSTRACT

Megakaryocytes (MK) in the bone marrow (BM) are immersed in a network of extracellular matrix components that regulates platelet release into the circulation. Combining biological and bioengineering approaches, we found that the activation of transient receptor potential cation channel subfamily V member 4 (TRPV4), a mechano-sensitive ion channel, is induced upon MK adhesion on softer matrices. This response promoted platelet production by triggering a cascade of events that lead to calcium influx, β1 integrin activation and internalization, and Akt phosphorylation, responses not found on stiffer matrices. Lysyl oxidase (LOX) is a physiological modulator of BM matrix stiffness *via* collagen crosslinking. *In vivo* inhibition of LOX and consequent matrix softening lead to TRPV4 activation cascade and increased platelet levels. At the same time, *in vitro* proplatelet formation was reduced on a recombinant enzyme-mediated stiffer collagen. These results suggest a novel mechanism by which MKs, through TRPV4, sense extracellular matrix environmental rigidity and release platelets accordingly.

Introduction

Megakaryocytes (MKs) reside within the bone marrow (BM), where they mature to extend proplatelets, at the end of which newly formed platelets are assembled and released into the bloodstream.[1-4] Different extracellular matrix components (ECM) in the BM actively regulate megakaryopoiesis.[5-7] In earlier studies, it was demonstrated that type IV collagen sustains proplatelet formation (PPF), opposite to type I collagen, which is a fundamental negative regulator of PPF through integrin α2β1/Rho/ROCK axis engagement.[8-12] In addition, recent studies highlighted the direct involvement of PI3K/Akt and MAPK/ERK pathways in PPF.[13-17] Interestingly, ECM component stiffness was shown to be inversely correlated to MK maturation and PPF.[8,10,18]

Cell contact with ECM components occurs through integrins which transduce the signals from the ECM to the cell cytoskeleton.[19-21] However, to sense the extracellular environment, integrins act in concert with different mechano-sensitive ion channels.[22,23] Among these, the transient receptor potential cation channel subfamily V member 4 (TRPV4) is a membrane mechano-sensitive ion channel whose activation has been linked to activation of β1 integrin a major collagen-binding integrin receptor subunit.[24-26] Interestingly, TRPV4 activity has been demonstrated to modulate PI3K/Akt and MAPK/ERK pathways in endothelial cells upon physical stimuli applied to the cell membrane.[24] In the current study, we demonstrate a new mechanism by which MKs sense the environmental mechanics to regulate their

maturation and platelet production. On soft collagenous substrates, TRPV4 expressed on the MK surface was activated, inducing calcium influx, β1 integrin activation and internalization, with consequent Akt phosphorylation and proplatelet formation.

Methods

In vitro megakaryocyte cultures

Human CD34$^+$ cells were isolated, separated and cultured, as described previously.[27,28] All human samples were collected in accordance with the ethical committee of the IRCCS Policlinico San Matteo Foundation, Pavia, Italy, and the principles of the Declaration of Helsinki. For collagen receptor inhibition, MKs at day 13 of culture were incubated with 10 μg/mL anti-β1 integrin blocking antibody (Millipore, clone P5D2), 10 μg/mL anti-GPVI blocking antibody (a kind gift of Prof. Jandrot Perrus) or with 200 nM (125 ng/mL) Discoidin Domain Receptor 1 (DDR1)-IN-1 (Tocris), a selective DDR1 tyrosine kinase inhibitor, for one hour prior to being plated on type I or type IV collagen for three hours (h). For Akt inhibition experiments, MKs at day 13 of culture were treated with 10 μM Akt1/2 inhibitor (Sigma Aldrich) for 30 minutes (min) and then plated on collagens for 16 h for PPT evaluation. For treatment with the TRPV4 inhibitors (RN-1734, HC067047, Sigma Aldrich), MKs at day 13 of culture were incubated with vehicle or 10 μM of the indicated TRPV4 inhibitor for 30 min prior to being plated on collagens for 3 or 16 h. For treatment with the TRPV4 agonist (GSK1016790A, Sigma Aldrich), MKs at day 13 of culture were incubated or not with 10 μM GSK1016790A for 10 min prior to being plated on collagens for 3 h.

Evaluation of megakaryocyte spreading and proplatelet formation

Evaluation of MK spreading and PPF onto collagens was performed as previously described.[29] Please refer to the *Online Supplementary Methods* for technical details.

Immunoprecipitation and Western blotting

For immunoprecipitation and Western blotting analysis, cultured MKs and primary BM immunomagnetically-sorted MKs (CD41$^+$; Biolegend) were collected, washed twice at 4°C, and lysed as previously described.[30] For active β1 integrin staining, samples were not reduced. Please refer to the *Online Supplementary Methods* for technical details.

Immunofluorescence microscopy

For cell immunofluorescence staining, 1x10^5 MKs at day 13 of culture were harvested and plated on collagen-coated coverslips. Adhering cells were washed, fixed, permeabilzed, and stained as previously described.[29] Please refer to the *Online Supplementary Methods* for technical details.

Internalization assays

For immunofluorescence internalization assay, MKs were treated as living cells with 15 μg/mL of the anti-β1 integrin mAb (Abcam) and acid washed before fixation as previously described.[31,32] MKs were then fixed, permeabilized, and stained with the appropriate secondary antibody. Western blot internalization was evaluated as previously described.[33] Please refer to the *Online Supplementary Methods* for technical details.

Silk film fabrication

Silk films were produced as previously described.[34] Type I or type IV collagen (25 μg/mL) were coated to the film surface overnight at 4°C. Please refer to the *Online Supplementary Methods* for technical details.

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. Please refer to the *Online Supplementary Methods* for technical details.

[Ca^{2+}]i measurements

Megakaryocytes at day 13 of culture were harvested and plated onto substrate-coated coverslips in 24-well plates (1x10^5 cells/well). After 60 min at 37°C and 5% CO_2, MKs were loaded with 4 μM fura-2 AM in physiological salt solution (PSS) for an additional 30 min and analyzed as previously described.[17] Please refer to the *Online Supplementary Methods* for technical details.

Animals and *in vivo* treatment

C57/BL6 wild-type mice were obtained from the Charles River Laboratories. Mice were housed at the animal facility of the Department of Physiology, section of General Physiology, University of Pavia (approval ref. n. 1302/2015). All mice were sacrificed according to the current European legal animal practice requirements. *In vivo* lysyl oxidase (LOX) inhibition by β-aminopropionitrile (BAPN) treatment was carried out using a protocol modified from previous studies.[35] Please refer to the *Online Supplementary Methods* for technical details.

In vivo bone marrow stiffness

The mechanical properties of femoral BM from mouse treated with BAPN and controls were measured with a Zwick/Roell Z005 testing device (Zwick GmbH & Co.) equipped with a 10 N load cell as previously described.[36] Please refer to the *Online Supplementary Methods* for technical details.

Flow cytometry

For the analysis of pAkt in BM MKs, femurs were flushed, red blood cells lysed with a 0.8% ammonium chloride solution, the remaining cells were washed by centrifugation with PBS and stained with anti-pAkt antibody (20 μL per test; BD Pharmingen) and anti-CD41 antibody (0.1 mg/mL; Biolegend) following the manufacturer's instructions. All MK samples, from the different tested sources, were routinely characterized as CD41$^+$CD42b$^+$ and CD3$^-$CD4$^-$CD8$^-$CD11b$^-$CD19$^-$CD33$^-$ cells, using appropriate antibodies (Beckman Coulter Inc.). Please refer to the *Online Supplementary Methods* for technical details.

Bone marrow explant analysis

Bone marrow explants were isolated and analyzed based on a previous protocol.[37] Please refer to the *Online Supplementary Methods* for technical details.

Statistical analysis

Values are expressed as mean±standard deviation (SD). *t*-test was used to analyze experiments. $P<0.05$ was considered statistically significant. All experiments were independently repeated at least three times.

For RT-PCR and quantitative real-time PCR, tissue collection and immunohistochemistry, reticulated platelet analysis and LOX-mediated collagen crosslinking please refer to the *Online Supplementary Appendix*.

Results

Type I and type IV collagen differently impact megakaryocyte adhesion

To evaluate the impact of collagen stiffness on MK behavior, coverslips were coated with type I or type IV collagens and Young's modulus was evaluated by AFM-based nano-indentation.[10,29] As we have previously demonstrated, type I collagen fibrils presented an elasticity more than 150 megapascal units (MPa), whereas material in early stage of self-aggregation had values in the order of 5-75 Mpa.[10] On the contrary, more homogeneous values in the order of less than 10 MPa were found during the analysis of type IV collagen Young's moduli. Consistently, nano-indentation studies on live cells plated on the two different collagens after 3 h of adhesion revealed values ranging from 290 to 3600 Pa (weighted average 1036 Pa) in MKs plated on type I collagen, while values ranging from 300 to 1400 Pa (weighted average 667 Pa) were obtained with MKs plated on type IV collagen (Figure 1A). As a result of the different stiffness, MKs remained spread over a 16-h incubation on type I collagen, while they rearranged their cytoskeleton and extended proplatelets on type IV collagen (Figure 1B and C).

Megakaryocyte β1 integrin activation and internalization vary depending on the type of collagen

We hypothesized that specific collagen receptors may be responsible for the MK behavior observed on type I and type IV collagens. qRT-PCR analysis revealed that the most expressed collagen-interacting integrin domains were β1>α2, with DDR1 and GPVI expressed at a similar level as α2 (Figure 2A). The most important reduction in Mk adhesion was obtained inhibiting β1 integrin, while DDR1 and GPVI inhibition did not significantly affect MK adhesion relative to untreated controls (Figure 2A).[10,30] β1 integrin activation was studied in MKs plated on type I and type IV collagens at the 3 time points used for functional studies (3, 8 and 16 h), by employing a monoclonal antibody directed against epitopes in the 355-425 region (hybrid domain), whose expression reflects the activity of β1 integrin.[38] An increase of β1 integrin activation was observed on type IV collagen, compared to type I collagen, after 3 and 8 h of adhesion, reaching a similar value under both conditions after 16 h (Figure 2B). Consistently, internalization assays showed that membrane distributed β1 integrin was reduced on MKs plated on type IV collagen as compared to type I collagen (Figure 2C).

Substrate stiffness modulates β1 integrin dynamics

To prove that β1 integrin activation and internalization were determined by collagen substrate stiffness, silk fibroin films were fabricated with imposed elasticity and were later coated with 25 μg/mL type I or type IV collagens.[34] Silk films with two different ranges of elasticity were chosen on the basis of previous results: a softer silk film (≤10 MPa) was chosen as the optimum condition to maximize MK function, while a stiffer silk film (≥90 MPa) was chosen as a condition proven to decrease PPF.[34] Importantly, it has been demonstrated that there are no specific cell-binding epitopes on the silk that would bias the outcomes.[39] MKs extended proplatelets after 16 h of adhesion on both collagens coated on soft films, while they maintained the spread form, independently of the

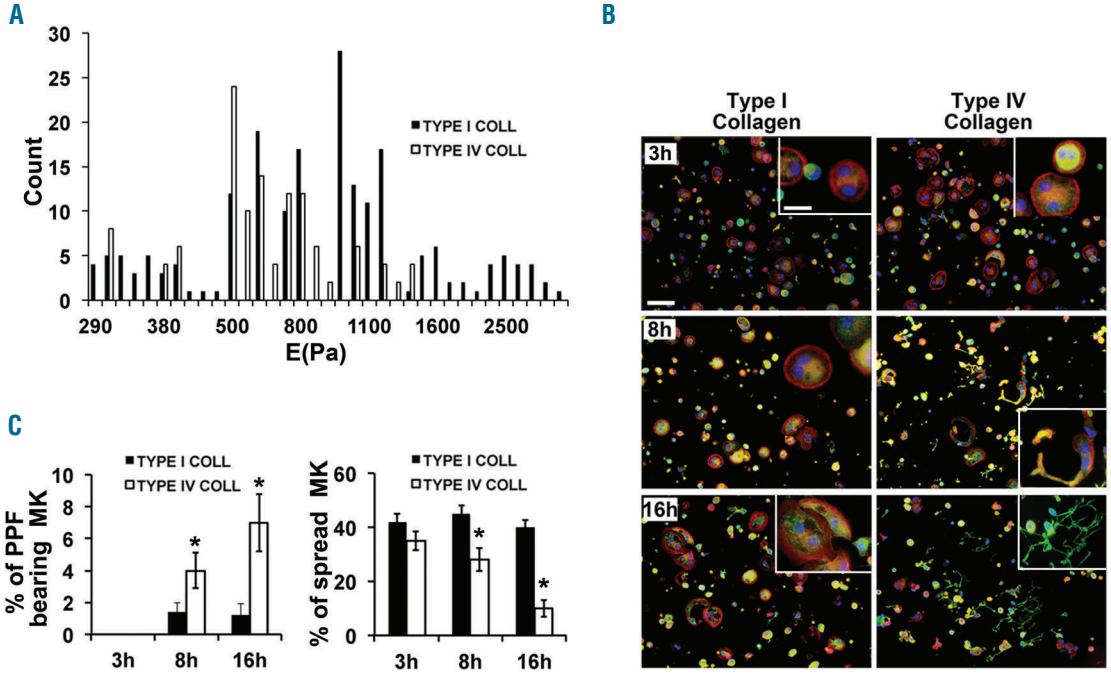

Figure 1. Type I and type IV collagens differently regulate megakaryocyte (MK) adhesion. (A) Distribution of Young's modulus values of live MKs plated on type I and type IV collagens (COLL). Three independent experiments were performed. (B) Human mature MKs, 1x10^5 per well in 24-well plates, were plated on type I and type IV collagen-coated coverslips for three different times. Cells were fixed and stained with anti-β1 tubulin (green) and tetramethylrhodamine (TRITC)-Phalloidin (red). Nuclei were counterstained with Hoechst (blue). Scale bar 50 μm; box scale bar 20 μm. (C) MKs exhibiting stress fibers were counted and presented as percentage of spread MK, while MKs extending proplatelets were counted and shown as percentage of proplatelet bearing MKs. Results shown in (C) are mean±standard deviation (SD) of six independent experiments. *P<0.05. Pa: Pascal units; h: hours.

collagen type, if plated on stiffer films (Figure 3A). Lower stiffness promoted higher activation of the β1 integrin in MKs plated on both collagen-type coated silk films, as demonstrated by western blot and immunofluorescence staining (Figure 3B and *Online Supplementary Figure S1*), with increased β1 integrin internalization (Figure 3C).

PI3K/Akt-dependent proplatelet formation on soft collagen substrate

To understand the involvement of PI3K/Akt and MAPK/ERK signaling pathways in mediating the effect of soft collagen, MKs plated on type I and type IV collagen were lysed after 3, 8 and 16 h. Western blot analysis demonstrated an important difference in Akt and ERK activation (Figure 4A). Specifically, ERK phosphorylation peaked after 3 h on type I collagen, remaining higher up to 16 h compared to type IV collagen, while Akt phosphorylation appeared constantly higher in MKs plated on type IV compared to type I collagen. Considering favorable PPF on type IV collagen, these results suggested a role for Akt phosphorylation as a positive mediator of PPF. Consistently, treatment of MKs plated on type IV collagen with a specific Akt inhibitor resulted in a significant reduction of PPF (Figure 4A). The role of substrate elasticity in Akt and ERK phosphorylation was further explored by using the silk fibroin film approach. Akt phosphorylation increased on soft collagen substrates, while ERK phosphorylation was unaffected (Figure 4B). Finally, inhibition of Akt in MKs plated on type I collagen-coated soft films led to a reduction in PPF by approximately 80%, compared to untreated cells (Figure 4B). Accordingly, taking advantage of our system for platelet production through porous silk films,[34] we showed that Akt inhibition significantly reduced the increase of platelet production obtained on soft collagen substrates (*Online Supplementary Figure S2*).

Megakaryocytes express functional mechano-sensitive TRPV4 ion channel

It has been demonstrated that, in endothelial cells, upon mechanical stress, β1 integrin activation determines the

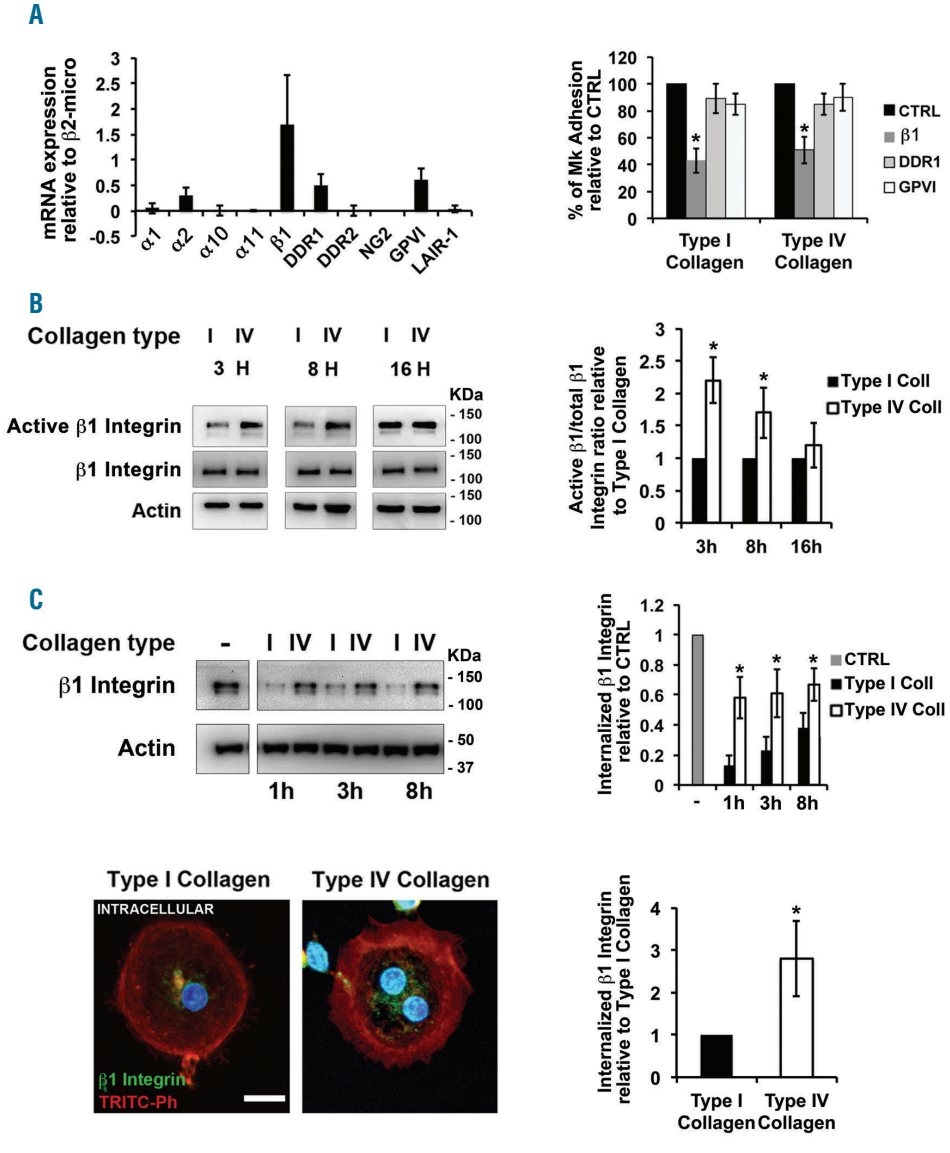

Figure 2. β1 integrin is strongly involved in mediating megakaryocyte (MK) adhesion on type I and type IV collagens. (A) Analysis of collagen (Coll) receptors. qRT-PCR analysis of different collagen receptors in human mature MKs [left; data are shown as mean±standard deviation (SD) of four independent experiments, relative to the housekeeping gene β2-microglobulin]. MK adhesion on type I and IV collagens was evaluated upon treatment with different collagen receptor blocking antibodies or blocking molecules (10 μg/mL anti-β1 integrin, 200 nM DDR1-IN-1, 10 μg/mL anti-GPVI) (right; data are shown as mean percentage of adhering MKs±SD of five independent experiments, relative to untreated cells). (B) Western blot analysis of active β1 integrin in MKs plated on type I and type IV collagens for different times. Total β1 integrin and actin were determined to show equal loading. The densitometry analysis of active β1 integrin level derived from three independent experiments is shown. (C) β1 integrin internalization assays. Western blot analysis of internalized β1 integrin after 1, 3 and 8 hours (h) of adhesion on type I and type IV collagens (top). To show equal loading actin was determined on total cell lysates. The first lane on the left represents the positive control of untreated MKs. The densitometry analysis of internalized β1 integrin level derived from four independent experiments is shown. Immunofluorescence analysis of internalized β1 integrin in MKs plated on type I and type IV collagens for 3 h (bottom). MKs were stained with anti-β1 integrin (green) and TRITC-Phalloidin (red). Nuclei were counterstained with Hoechst (blue). Scale bar 10 μm. The densitometry analysis of staining intensities derived from four independent experiments is shown. A minimum of 20 cells per experiment were evaluated. Data of densitometry analysis are all expressed as mean±SD. *P<0.05. CTRL: untreated.

TRPV4 ion channel opening, which allows calcium influx inside the cell cytoplasm, supporting further β1 integrin engagement. Here, we show that human MKs expressed functional TRPV4 ion channels on their membrane (Figure 5A and B) and that, upon adhesion onto type IV collagen, human MKs stained with FURA2-AM display calcium oscillations of significant higher frequency and amplitude than those observed during adhesion on type I collagen. Inhibition of TRPV4 channels with a selective antagonist (RN1734) led to a significant decrease in the amplitude of calcium spikes only on type IV collagen, but not on type I collagen, thus suggesting that calcium flux through TRPV4 differs in MKs plated on the two collagen types (Figure 5C). The increased activation of TRPV4 on type IV collagen and on soft collagen substrates was also confirmed by immunoprecipitation of phosphorylated TRPV4 followed by western blot analysis (*Online Supplementary Figure S3*).[40]

β1 integrin activation, Akt phosphorylation and proplatelet formation on type IV collagen were sustained by TRPV4

To test whether TRPV4 channels mediate β1 integrin activation and Akt phosphorylation, both proteins were analyzed upon MK treatment with a specific TRPV4 inhibitor (RN-1734) on type IV collagen and with a specific TRPV4 agonist (GSK1016790A) on type I collagen. Blocking TRPV4 activity with RN-1734 on type IV collagen resulted in a significant decrease of β1 integrin activation and Akt phosphorylation (Figure 6A). Consistently, the inhibition of TRPV4 activity resulted in diminished PPF on type IV collagen (Figure 6B), thus confirming the active role of the mechano-sensitive TRPV4/β1 integrin/Akt axis in PPF. On the contrary, forcing TRPV4 activation with GSK1016790A resulted in increased β1 integrin activation, Akt phosphorylation (Figure 6C) and β1 integrin internalization in MKs plated on type I collagen (Figure 6D).

Lysyl oxidase-mediated collagen cross-linking inhibition increases PPF and platelet production *in vivo*

A key regulator of collagen stiffness *in vivo* is the secreted enzyme LOX, which by oxidative deamination of lysine residues on collagen, leads to cross-linked aldehydes, contributing to a tight and stiffer ECM.[41] To support the impact of collagen substrate elasticity on platelet production *in vivo*, mice were treated with a specific LOX inhibitor, which has been demonstrated to reduce tissue stiffness.[42,43] Inhibition of LOX, by β-aminopropionitrile (BAPN) treatment, an irreversible inhibitor of LOX, diminished the bone marrow elastic modulus (Figure 7A) and reduced collagen fiber dimension in cortical bone (*Online*

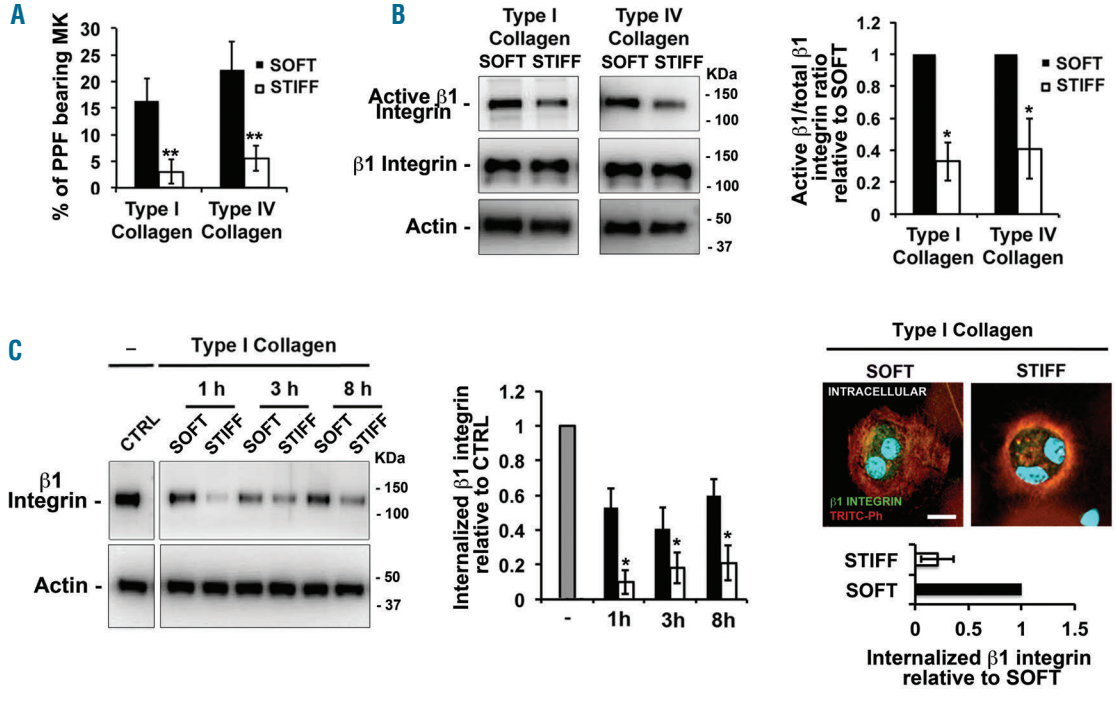

Figure 3. Collagen substrate elasticity regulates β1 integrin dynamics and proplatelet formation. (A) Megakaryocyte (MKs) extending proplatelets were counted and shown as percentage of proplatelet bearing MKs. Data are expressed as mean percentage±standard deviation (SD) of eight independent experiments. (B) Western blot analysis of active β1 integrin in MKs plated on type I and type IV collagen coated soft and stiff silk fibroin films. Total β1 integrin and actin were determined to show equal protein loading. The densitometry analysis of active β1 integrin level derived from four independent experiments is shown. (C) β1 integrin internalization assays. Western blot analysis of internalized β1 integrin after 1, 3 and 8 hours (h) of adhesion on type I collagen-coated soft and stiff silk fibroin films (left). Actin, in total cell lysates, was determined to show equal loading. The first lane on the left represents the positive control of untreated MKs. The densitometry analysis of internalized β1 integrin level derived from four independent experiments is shown. Immunofluorescence analysis of internalized β1 integrin in MKs plated on type I collagen-coated soft and stiff silk fibroin films for 3 h (right). Cells were stained with anti-β1 integrin (green) and TRITC-Phalloidin (red). Nuclei were counterstained with Hoechst (blue). Scale bar 10 μm. The densitometry analysis of staining intensities, derived from four independent experiments is shown. A minimum of 20 cells per experiment was evaluated. Data of densitometry analysis are all expressed as mean±SD. *P<0.05, **P<0.01. CTRL: untreated.

Supplementary Figure S4). The reduction of bone marrow stiffness led to an increase in peripheral platelet count, while the count of the other blood cells was unchanged (Figure 7B and *Online Supplementary Table S1*). The percentage of newly released reticulated platelets was significantly increased in treated mice, confirming that the boost in platelet numbers was mainly due to newly formed platelets (Figure 7B). Of note, the number of BM MKs was not significantly affected by BAPN treatment, thus demonstrating that the increase in peripheral blood platelet counts was not due to a higher MK number (*Online Supplementary Figure S5*). Interestingly, Akt phosphorylation was augmented in BAPN-treated bone marrow MKs as compared to untreated control (CTRL) by means of immunofluorescence imaging of bone marrow sections and flow cytometry analysis of flushed bone marrow (Figure 7C). To study PPF within bone marrow in LOX-inhibited and control mice, fresh BM explants were examined by videomicroscopy (*Online Supplementary Figure S6*). Through this approach, MKs, recognized by their morphology, became visible at the periphery of the explant as round cells or as cells extending thick protrusions or proplatelets. Importantly, TRPV4 activation was analyzed in MKs explanted from these scenarios by evaluating the extent of TRPV4 phosphorylation, which enhances TRPV4-mediated calcium inflow and may be employed as surrogate to monitor TRPV4 activation when the direct measurement of calcium signals is not feasible.[40] Diminishing the bone marrow stiffness resulted in an increase of TRPV4 activation in MKs as demonstrated by western blot analysis of immunoprecipitated TRPV4 (Figure 8A). Proplatelet forming MKs at 3 and 8 h from the beginning of the experiment were quantified. The number of visible MKs per experiment was comparable in both conditions (CTRL 51+9 *vs.* BAPN 58+13 at 3 h; CTRL 63+14 *vs.* BAPN 72+17 at 6 h), while the percentage of proplatelet-forming MKs was significantly higher in LOX-inhibited mice (Figure 8B and *Online Supplementary Videos 1* and *2*). Importantly, Akt inhibition in BM explants, from BAPN-treated mice, reverted the increase in proplatelet formation to a level lower than CTRL mice (Figure 8B and *Online Supplementary Video 3*). Finally, to further explore the direct dependence of PPF on collagen substrate crosslinking, and thus rigidity, PPF in MKs plated on type IV collagen pre-treated with recombinant LOXL2 protein was tested in the presence or not of BAPN. The incubation of type IV collagen with LOXL2 significantly reduced the percentage of proplatelet-forming MKs, while inhibition of LOXL2 with BAPN rescued PPF to a level comparable to type IV collagen alone (*Online Supplementary Figure S7*).

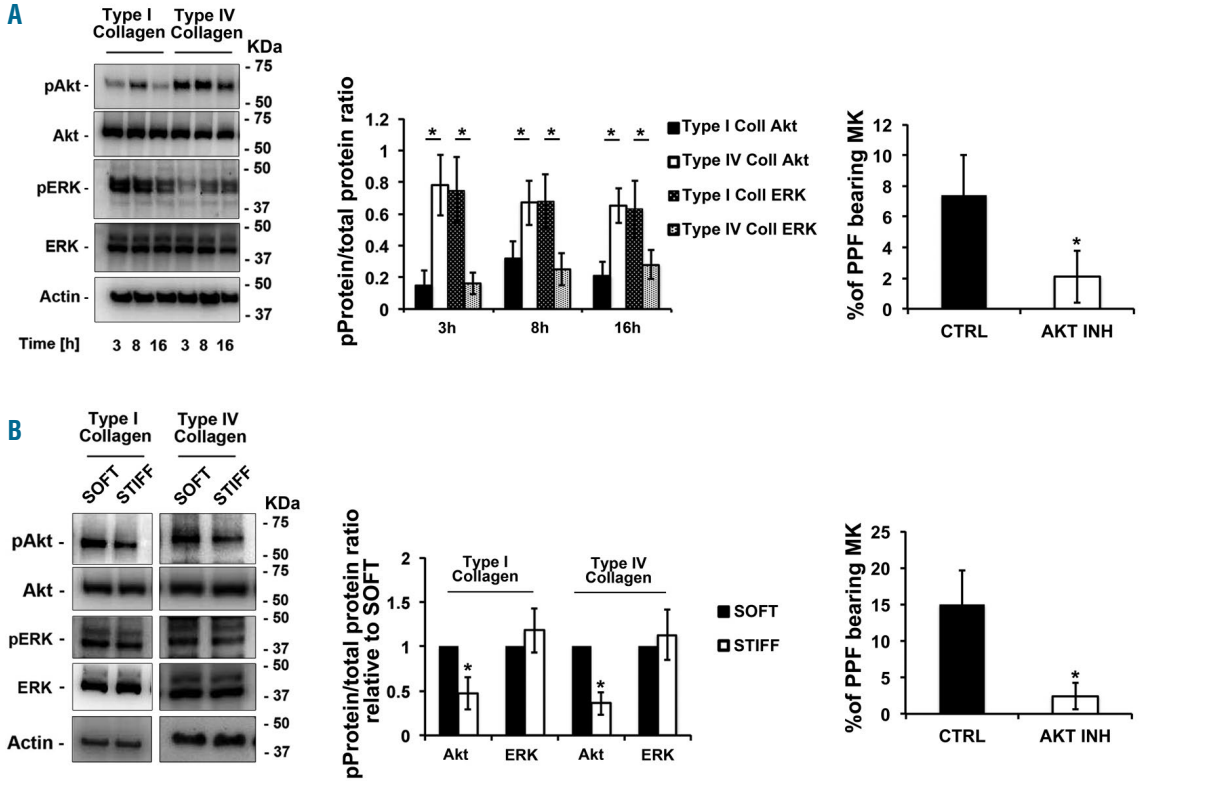

Figure 4. PI3K/Akt signaling mediates proplatelet formation on soft collagen (Coll) substrates. (A) Western blot analysis of Akt and ERK phosphorylation (pAkt and pERK) in MKs plated for 3, 8 and 16 hours (h) on type I and type IV collagens (left). Total Akt, ERK and actin were determined to show the equal loading. The densitometry analysis of pAkt and pERK level derived from four independent experiments is shown. Proplatelet formation assay in MKs plated for 16 h on type IV collagen in the presence or not of 10 μM Akt inhibitor (right). MKs extending proplatelet were counted and shown as percentage of proplatelet-bearing MKs. Data are expressed as mean percentage±standard deviation (SD) of five independent experiments. (B) Western blot analysis of pAkt and pERK in MKs plated for 3 h on type I and type IV collagen-coated soft and stiff silk fibroin films (left). Total Akt, ERK and actin were determined to show equal loading. The densitometry analysis of pAkt and pERK level derived from five independent experiments is shown. Proplatelet formation assay in MKs plated for 16 h on type I collagen-coated soft silk fibroin films in presence or not of 10 μM Akt inhibitor (right). MKs extending proplatelets were counted and expressed as percentage of proplatelet bearing MKs. Data are expressed as mean percentage±SD of five independent experiments. Data of densitometry analysis are all expressed as mean±SD. *P<0.05. CTRL: untreated.

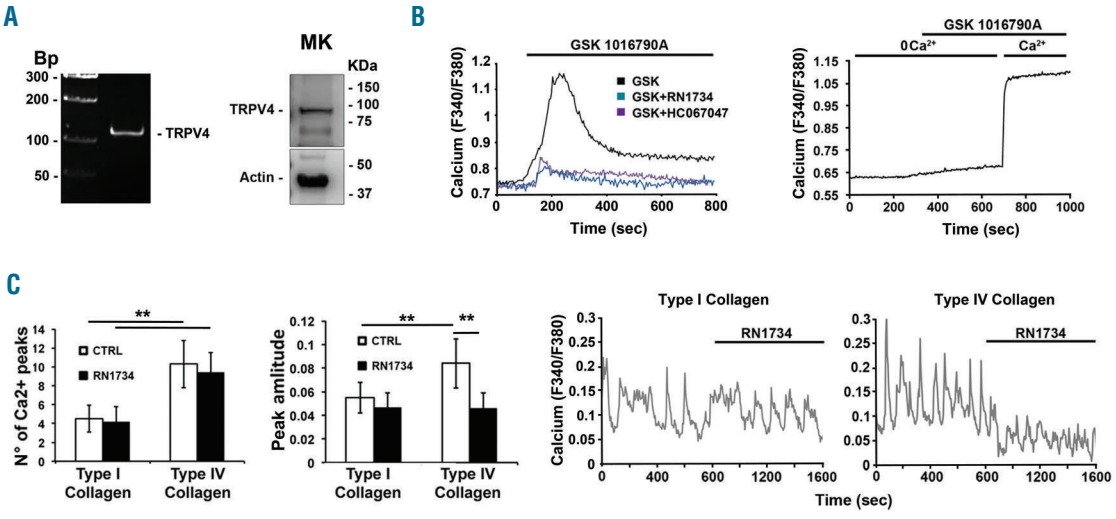

Figure 5. Megakaryocytes (MKs) express a functional TRPV4 ion channel. (A) TRPV4 expression in mature MKs. RT-PCR analysis of TRPV4 (left). Western blot analysis of TRPV4 (right). (B) Functional characterization of TRPV4. Analysis of calcium signaling in response to 10 nM GSK1016790A (TRPV4 agonist) in FURA2-AM loaded MKs, pre-treated or not, for 60 minutes (min), with 10 μM of the indicated TRPV4 inhibitor (RN-1734 or HC067047) (left). GSK1016790A was added at 100 seconds (sec) from the beginning of the analysis. Data are representative of three independent experiments. A minimum of 40 MKs per experiment were evaluated. In absence of extracellular calcium ($0Ca^{2+}$), treatment with 10 nM GSK1016790A did not elicit significant changes in calcium signaling in FURA2-AM-loaded MKs (right). After extracellular calcium restoration, in the presence of GSK1016790A, the increase in MKs fluorescence was indicative of extracellular calcium entry in MKs cytosol. GSK1016790A was added after 300 sec from the beginning of the experiment. Data are representative of three independent experiments. A minimum of 40 MKs per experiment were evaluated. (C) TRPV4 activity on type I and IV collagens. Number of calcium peaks per MKs plated on type I and type IV collagens in presence or not of 10 μM RN-1734. Time of analysis was 800 sec. Calcium peak amplitude in megakaryocytes plated on type I and type IV collagens in presence or not of 10 μM RN-1734 (left). Representative calcium oscillations in MKs plated on type I and type IV collagens (right). RN-1734 was added at 600 sec from the beginning of the analysis. Data in (C) refer to eight independent experiments. Data are expressed as mean±standard deviation (SD). *P<0.05, **P<0.01.

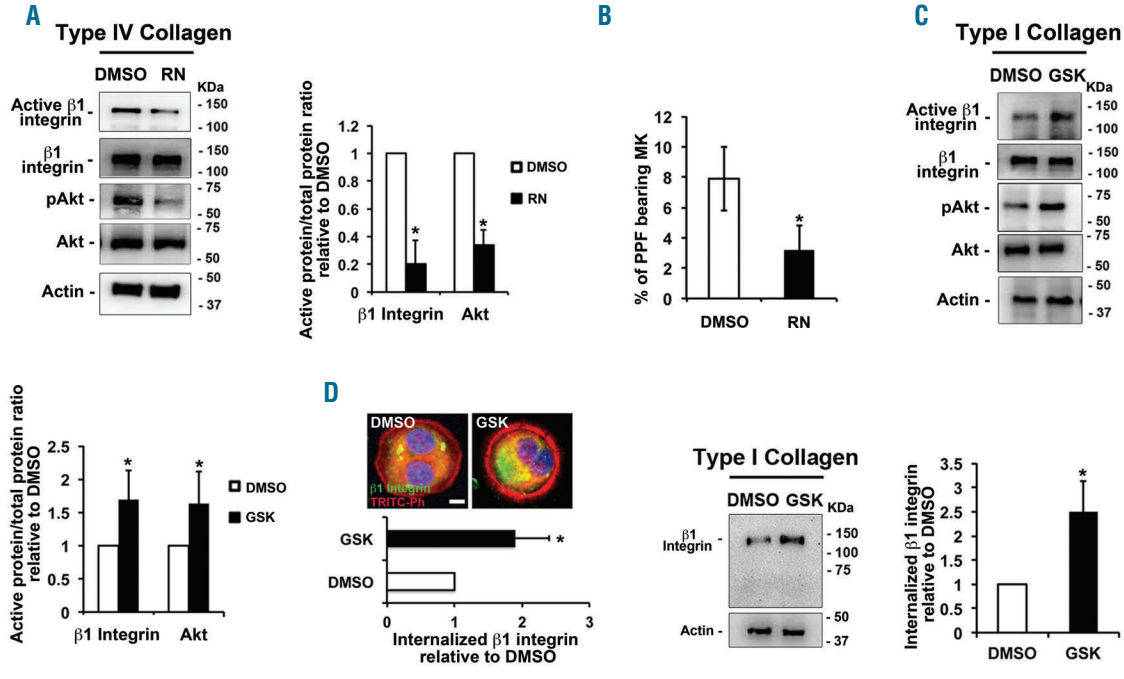

Figure 6. TRPV4 activity regulates β1 integrin dynamics and Akt phosphorylation. (A) Western blot analysis of active β1 integrin and phosphorylated Akt (pAkt) in megakaryocytes (MKs) plated on type IV collagen in the presence or not of 10 μM RN-1734 (RN). Total β1 integrin, Akt and actin were determined to show equal loading. The densitometry analysis of active β1 integrin level and pAkt derived from four independent experiments is shown. (B) Proplatelet formation assay in MKs plated on type IV collagen in presence or not of 10 μM RN-1734. Proplatelet bearing MKs were counted and shown as percentages of proplatelet-bearing MKs. Data are shown as mean percentage±standard deviation (SD) of six independent experiments. (C) Western blot analysis of active β1 integrin and pAkt in MKs plated on type I collagen in the presence or not of 10 nM GSK1016790A (GSK). Total β1 integrin, Akt and actin were determined to show equal loading. The densitometry analysis of active β1 integrin level and pAkt, derived from four independent experiments, is shown. β1 integrin internalization assays. (D) Immunofluorescence analysis of internalized β1 integrin in MKs plated on type I collagen in presence or not of 10 nM GSK1016790A (left). Cells were stained with anti-β1 integrin (green) and TRITC-Phalloidin (red). Nuclei were counterstained with Hoechst (blue). Scale bar 5 μm. The densitometry analysis of staining intensities derived from three independent experiments is shown. A minimum of 20 cells per experiment were evaluated. Western blot analysis of internalized β1 integrin in megakaryocytes plated on type I collagen in presence or not of 10 nM GSK1016790A (right). To show equal loading, actin was determined on total cell lysates. The densitometry analysis of internalized β1 integrin level derived from three independent experiments is shown. Data of densitometry analysis are all expressed as mean±standard deviation (SD). *P<0.05.

Discussion

The signaling pathways that control platelet formation in the bone marrow matrix environment have remained quite elusive. This study demonstrates that calcium influx through TRPV4 channels stimulates integrin β1 activation upon MK adhesion on soft collagen substrate, promoting Akt phosphorylation and platelet formation. This previously unknown mechanism for platelet production adds new insights into the role of the ECM in regulating MK function,[44-46] and determines the importance of ECM-dependent calcium influx in regulating MK spreading and PPF.[17]

The BM microenvironment consists of various ECM components that interact with each other to form a structural framework that supports tissue organization and positional cues regulating megakaryopoiesis. In this structure, type I and IV are the most abundant collagens. Notably, type IV can support PPF,[29] while type I collagen is the only ECM component known to inhibit this process.[9,10,12] This inhibition is triggered by the tensile strength of fibrils in type I collagen that regulates cytoskeleton contractility of MKs through activation of the Rho-ROCK pathway and MLC-2 phosphorylation.[8,10] Consistently, culturing MKs in soft methylcellulose (MC) hydrogels determines an increase of PPF through activation of the myosin IIA and MKL1 pathways.[18]

The current data showed that type I collagen displayed a significant higher stiffness than type IV collagen, as revealed by AFM analysis. Consistently, only MKs plated on the softer type IV collagen extended long and branched proplatelets. We hypothesized that specific collagen receptors may regulate the different MK behavior on type I and type IV collagens. Among the collagen receptors, integrins have been extensively described to be major mechano-receptors, due to their ability to modulate the signals transmitted inside the cell according to the physical properties of the ligand they bind.[47] In support of this hypothesis, the adhesion of MKs on both collagens was found to be mostly mediated by β1 integrin. However, β1 integrin was significantly more active and more internalized in MKs plated on type IV than on type I collagen during an 8-h incubation time. These data were in line with the notion that β1 integrin dynamics and internalization-re-cycling circle are regulated by matrix stiffness.[48,49] Importantly, integrin detachment and internalization on soft substrate was previously shown in mesenchymal stromal cells, and was ascribed to the instability of the binding, and thus to the low stress level necessary for the binding rupture.[50] In contrast, when the substrate stiffness increases, the relative number of nascent and retracting β1 integrin adhesions are reduced, and highly stable adhesions are promoted.[51] In accordance, by using passive silk films with different rigidities, substrate stiffness and β1 integrin activation in MKs were shown to be inversely proportional, as softer substrates promoted β1 integrin activation and internalization.

The increase in β1 integrin signaling that was observed on soft matrices, despite the higher rate of integrin internalization, is in line with recent work which demonstrated that endocytosis was necessary for full ECM-induced integrin-mediated signaling.[52] PI3K/Akt and MAPK/ERK signaling pathways are known to be activated downstream β1 integrin and responsible for PPF regulation.[13-17]

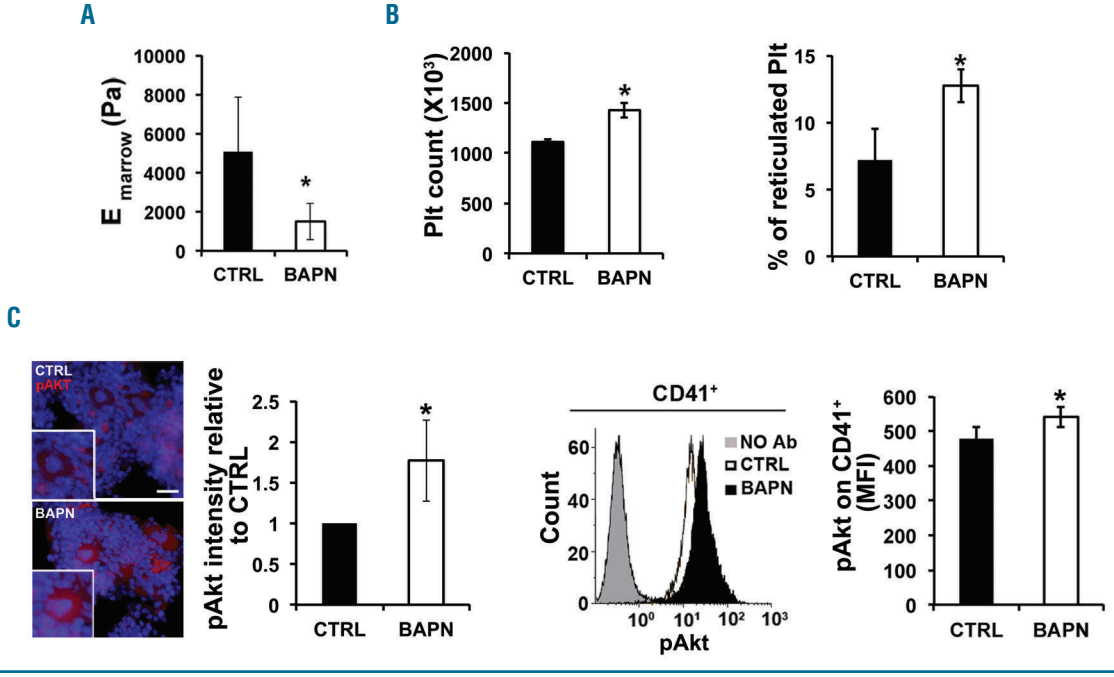

Figure 7. Lysyl oxidase-mediated collagen cross-linking inhibition increases platelet (Plt) production *in vivo*. (A) Elastic modulus of untreated (CTRL) and β-aminopropionitrile (BAPN)-treated mice bone marrows (BM). Data refer to 4 different mice per group. BM from both femurs per mouse was analyzed. (B) Peripheral blood platelet count of untreated (CTRL) and BAPN-treated mice. Data refer to 10 mice per group. Reticulated platelet measurements in PB of CTRL and BAPN-treated mice. Data refer to 8 mice per group. (C) pAkt analysis in CTRL and BAPN-treated mouse MKs was performed by BM section immunofluorescence (left) and by flow cytometry (right). The densitometry analysis of pAkt immunofluorescence staining, of BM MKs, is derived from 4 mice per group. At least 30 MKs per section were analyzed. The flow cytometry analysis of pAkt phosphorylation was performed in CD41⁺ BM MKs. The mean fluorescence intensity (MFI) is shown. Data are expressed as mean±standard deviation (SD) of five independent experiments. Data of densitometry analysis are all expressed as mean±SD. **P*<0.05.

Interestingly, thrombocytopenia is one of the most frequent hematologic adverse events in patients treated with perifosine, an oral Akt inhibitor used in cancer treatment.[53] Consistently, in our experiments, Akt and not ERK phosphorylation promoted MK maturation and PPF upon β1 integrin activation by soft substrates. These data prompted us to hypothesize that other mechano-sensitive molecules may be involved in the regulation of these signaling processes. The mechano-sensitive ion channels, a member of which is TRPV4, seemed to be the best candidates to explain the data. First, these ion channels detect and transduce external mechanical forces into electrical and/or chemical intracellular signals.[22,54] Second, it was demonstrated that stretch-activated endothelial cells regulate Akt and ERK activation through a TRPV4- β1 integrin-dependent mechanism.[24] Consistently, in the experiments MKs were shown to express functional TRPV4 ion channels that were selectively activated only upon MK adhesion on soft substrates.

Accordingly, TRPV4 channels present the unique ability to mediate an integrin-to-integrin signaling which is activated by mechanical forces transmitted by ECM components to β1 integrins. This results in the ultra-rapid (4 ms) alteration of the molecular conformation of TRPV4, which gates the channels and enables extracellular Ca^{2+} entry, thereby causing additional β1 activation.[24,25,55]

Overall, these *in vitro* studies lead us to posit a model by which a softer environment in the BM promotes the activation of the TRPV4 ion channel that, in turn, leads to further β1 integrin stimulation and Akt phosphorylation, culminating with PPF. In line with this contention, adult mice treated with a specific LOX inhibitor that reduces tissue stiffness[42,43] promoted TRPV4, β1 integrin and Akt activation, with a consequent increase of platelet count in the

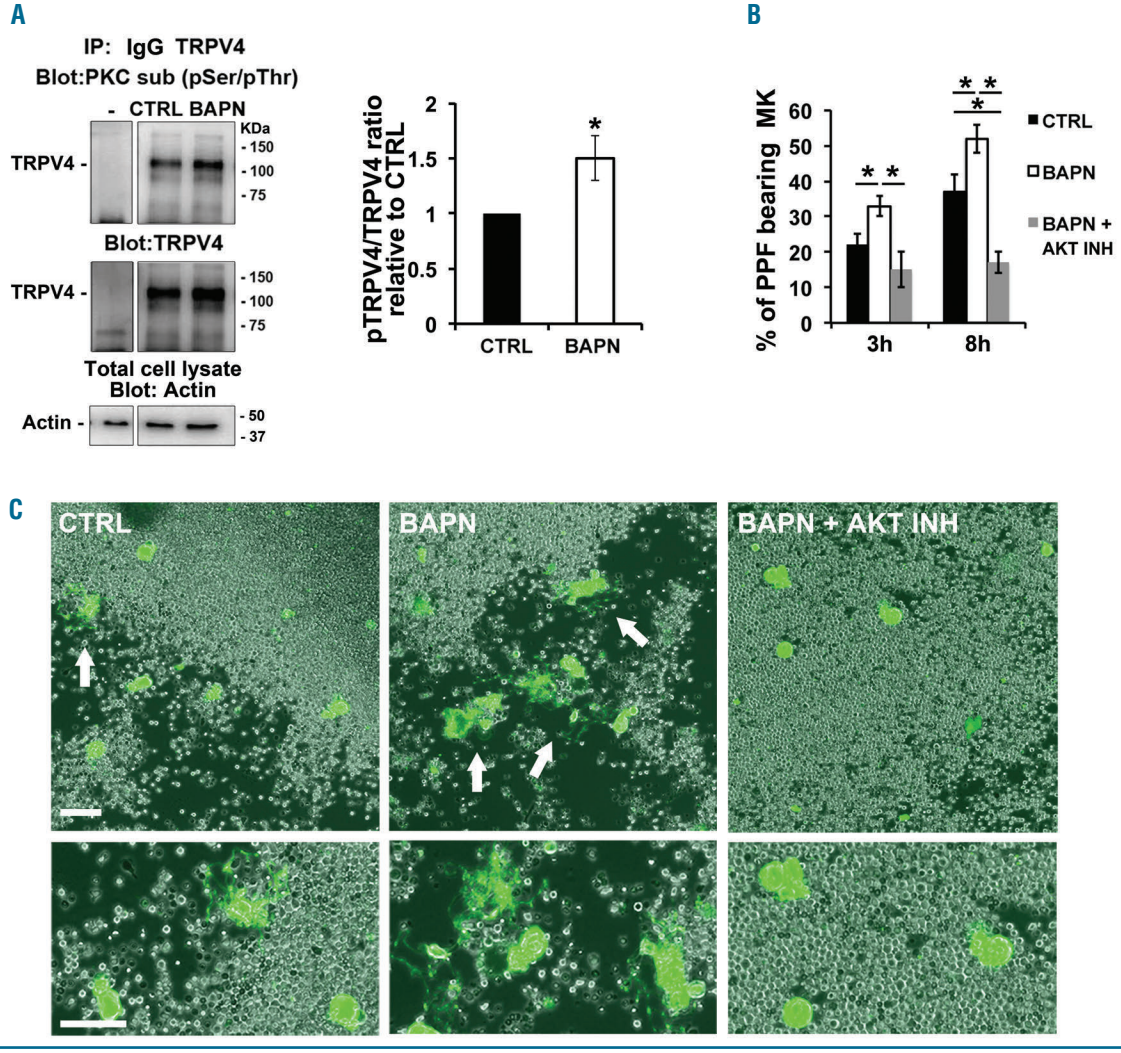

Figure 8. Lysyl oxidase-mediated collagen cross-linking inhibition increases proplatelet formation and TRPV4 activation *ex vivo*. (A) Western blot analysis of TRPV4 immunoprecipitated from bone marrow (BM) immunomagnetically-sorted megakaryocytes (MKs). A control sample (CTRL) was immunoprecipitated with an unrelated antibody (IgG). Membranes were probed with anti-PKC-substrates antibody (recognizing phosphorylated serine) and with anti-TRPV4 antibody. Actin staining on total cell lysates is shown to ensure equal loading. The densitometry analysis of the phosphorylated TRPV4/total TRPV4 ratio is shown. Data refer to three independent experiments. (B) Proplatelet formation assay in BM explants. MKs extending proplatelet were counted after 3 and 8 hours (h) from the beginning of the experiment, and the percentage of proplatelet-bearing MKs is shown. Data are expressed as mean percentage±standard deviation (SD). n=10 CTRL, 10 BAPN, 3 BAPN + AKT inhibitor (AKT INH). (C) Representative pictures of proplatelet-bearing MKs in BM explants. MKs were stained as living cells with anti-CD41-FITC antibody. Data of densitometry analysis are expressed as mean±SD. Scale bar = 50 μm upper pictures; 100 μm lower pictures. *P<0.05. PPF: proplatelet formation.

peripheral blood. This occurred while MK numbers in the BM were not affected. Of note, an unexplained trend in increased platelet count was also reported earlier upon LOX inhibitor administration,[35] although the dosage of inhibitor used in that study was low, and was tested in newborn to young mice. A recent study showed that up-regulated LOX, at a level similar to that found in pathological BM fibrosis, led to increased platelet adhesion to monomeric collagen mediated *via* α2β1 collagen receptors.[56] A similar increase in adhesion was observed with regard to LOX over-expressing MKs. Together with our current studies, we propose that LOX is capable of augmenting MK adhesion *via* collagen receptor activation, but whether these MKs effectively produce proplatelets or not also depends on LOX-regulated ECM stiffness and changes in TRPV4 activation.

During life, ECM components are continuously rearranged to permit cell-tissue function. However, some patho-physiological conditions, such as aging or augmented bone marrow fibrosis determine an increase of ECM component rigidity, causing tissue function alterations.[57,58] Interestingly, it is known that platelet count is inversely correlated with age and that in the late stages of primary myelofibrosis, when the BM is full of thick reticular fibers, thrombocytopenia occurs.[59,60] Moreover, in these conditions, other organs, such as the lung[61] or the spleen,[62] with a more favorable matrix environment, may become the site of platelet production. Based on these observations, and on the mechanism described in the current work, we propose that, beside the extensively described humoral-dependent mechanisms of platelet production, the physical properties of the ECM components that fill the BM are crucial regulators of MK function and platelet production.

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