The importance of calcium in the regulation of megakaryocyte function

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Manuscript received on August 21, 2013. Manuscript accepted on January 20, 2014.
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Running heads: SOCE regulates megakaryocyte function.

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ACKNOWLEDGMENTS

The authors would like to thank Marco Cattaneo for critical review of the manuscript. Cesare Perotti for human cord blood supply. Valentina Boz and Maria Rita Cozzi for helping during calcium measurements under flow. Gianluca Viarengo for flow cytometry analysis. This paper was supported by Cariplo Foundation (grant 2010.0807), Regione Lombardia - Project SAL-45, Almamater Foundation (Pavia) and Regione Lombardia - Progetto di cooperazione scientifica e tecnologica internazionale, National Institutes of Health (grant EB016041-01) and Italian Ministry of Health (grant RF-2009-1550218). The founders had no role in study design, data collection and analysis, or preparation of the manuscript.

SUPPLEMENTAL METHODS

Materials

Apyrase from potato (Grade VII), adenosine 5′-diphosphate (ADP), Hoechst 33258, Poly-L-lysine solution, paraformaldehyde, Triton X-100, 2-Aminoethyl diphenylborinate (2-APB), 1-[6-[((17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione (U-73122), 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM), phalloidin-tetramethylrhodamine B isothiocyanate, cyclopiazonic acid from *Penicillium cyclopium* (CPA), and Protein A-Sepharose from *Staphylococcus aureus* were from Sigma-Aldrich (Milan, Italy). MRS 2179 and MRS 2211 were from Tocris Bioscience (Missouri, USA). N-(4-[3,5-Bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide (BTP-2) was from Calbiochem (Merck Millipore, Milan, Italy). Fura-2 acetoxymethyl ester (Fura-2 AM) and FLUO 3-acetoxymethyl ester-AM (FLUO-3 AM) were from Molecular Probes Europe BV (Leiden, The Netherlands). Precision Plus protein standard was from Bio-Rad (Milan, Italy). The following antibodies were used: anti–human CD41 (FITC) (clone HIP8) (eBioscience, Milan, Italy); monoclonal anti–CD61 (clone SZ21) (Immunotech, Marseille, France); mouse monoclonal anti–tubulin (clone DM1A) and mouse anti–β-actin (clone AC-15) (Sigma-Aldrich, Milan, Italy); mouse monoclonal anti–STIM1 (Abcam, Cambridge, UK); goat polyclonal anti–CD61 (clone C-20), rabbit polyclonal anti–Orai1 (clone H-46), mouse monoclonal anti–TRPC1 (clone E-6) and anti–goat HRP conjugated secondary antibody (Santa Cruz Biotechnology, California, USA); rabbit monoclonal anti–phospho-ERK1/2 (Thr185/Tyr187) (clone AW39) (Millopre, Milan, Italy); rabbit monoclonal anti–phospho-Akt (Ser473), rabbit polyclonal anti–phospho-FAK (Tyr397), rabbit monoclonal anti–phospho-Syk (Tyr525/526) (clone C87C1), rabbit monoclonal anti–phospho-Src Family (Tyr416) (clone D49G4), and rabbit polyclonal anti-phospho-MLC (Ser19) (Cell Signaling Technology, Massachusetts, USA); anti–mouse and anti–rabbit HRP conjugated secondary antibodies (Bio-Rad, Milan, Italy);

Alexa Fluor–conjugated antibodies (Invitrogen, Milan, Italy). Fibronectin was a kind gift of Dr.ssa Livia Visai. Fibrinogen, was purified by chromatography from human plasma as previously reported[1] and diluted in phosphate buffered saline (PBS; 20 mM Na_2HPO_4, 20 mM NaH_2PO_4, 2.7 mM KCl, 0.15 M NaCl, pH 7.4) at 200 µg/mL. Type I collagen was purified as described previously.[2]

Solutions

Physiological salt solution (PSS) had the following composition: NaCl 150 mM, KCl 6 mM, $CaCl_2$ 1.5 mM, $MgCl_2$ 1 mM, glucose 10 mM, Hepes 10 mM. In Ca^{2+}-free solution ($0Ca^{2+}$), Ca^{2+} was substituted with NaCl 2 mM and EGTA 0.5 mM was added. Solutions were titrated to pH 7.4 with NaOH.

Megakaryocytes differentiation from human cord blood hematopoietic progenitor cells

Human cord blood was collected from the local blood bank following normal pregnancies and deliveries with informed consent of the parents, in accordance with the ethical committee of the IRCCS Policlinico San Matteo Foundation and the principles of the Declaration of Helsinki. $CD34^+$ cells from cord blood samples were separated by immunomagnetic bead selection (Miltenyi Biotec, Bologna, Italy) and differentiated, as previously described.[3-5] At the end of the culture (13^{th} days), $1x10^5$ cells were collected, cytospun on glass cover-slips, fixed in 4% paraformaldehyde (PFA) and stained with a primary antibody against CD61 (1:100) to evaluate megakaryocyte output. The cover-slips were mounted onto glass slides with ProLong Gold antifade reagent (Invitrogen, Milan, Italy) and images acquired using a Olympus BX51 (Olympus, Deutschland GmbH, Hamburg, Germany).

Flow cytometry analysis of *in vitro* differentiated megakaryocytes

For megakaryocyte analysis by flow cytometry, $2x10^5$ cells at day 13 of culture or the whole cells that had migrated in the trans-well migration chamber system were collected and centrifuged at 250xg for 7 minutes. Cells were then incubated in PBS and stained with a FITC-conjugated antibody against human CD41, at room temperature, in the dark for 30 minutes. After incubation, samples were acquired with a Beckman Coulter Navios flow cytometer. Non-stained samples were used to set the correct analytical gating. Off-line data analysis was performed using Beckman Coulter Navios software package. At least 3 independent experiments were performed.

Evaluation of cell adhesion and proplatelet formation on extracellular matrix components

In order to analyze megakaryocyte adhesion and proplatelet formation onto different extracellular matrix components, 12 mm glass cover-slips were coated with 100 µg/ml fibrinogen, 25 µg/ml fibronectin or 25 µg/ml type I collagen, overnight at 4°C. Polylysine was used as neutral control compound. At day 13 of culture $1x10^5$ Mks were harvested and allowed to adhere at 37°C and 5% CO_2. After different time points (30 minutes, 3-8-16 hours), adhering cells were washed with PBS samples, fixed in 4% PFA, permeabilized with 0.1% Triton X-100, and stained for immunofluorescence evaluation with anti–α-tubulin antibody (1:700), TRITC-conjugated phalloidin (1:2500) and/or CD61 (1:100), as previously described.[3,4] The cover-slips 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). At least 50 fields per sample were analyzed. Adhesion was evaluated as average of the number of adherent cells per field. Cytoskeletal reorganization was recognized in cells displaying microtubule and stress fibers assembly. Proplatelet forming megakaryocytes were identified as cells

displaying long filamentous structure ending with platelet-sized tips. Both results are expressed as percentage of adherent cells, as previously described.[3-5] In some experiments, before being seeded, cells were pre-incubated with the following substances, at the indicated final concentrations: apyrase 1 U/ml, ADP 25 μM, 2-APB 20 μM, U-73122 10 μM, BTP-2 20 μM. For these experiments, results are compared with respect to the same cells treated with vehicle alone. At least 5 independent experiments were performed.

$[Ca^{2+}]_i$ measurements

Intracellular $[Ca^{2+}]_i$ measurements were performed as previously described.[6] Briefly, 12 mm glass coverslips were coated with 100 μg/ml fibrinogen or fibronectin 25 μg/ml, overnight at 4°C. Mks at day 13 of culture were harvested and plated onto substrate-coated cover-slips in 24-wells plates ($1x10^5$ cells/well). After 60 minutes at 37°C and 5% CO_2, Mks were loaded with 4 μM fura-2 AM in PSS for additional 30 minutes. 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. 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. The experiments were performed at room temperature.

In order to analyze Mks activation on flow conditions, 24x50 mm glass coverslips were coated with 100 µg/ml fibrinogen, overnight at 4°C. Mks at day 13 of culture were harvested and plated onto substrate-coated coverslips. After 60 minutes at 37°C and 5% CO_2, Mks were loaded with 5 mM FLUO-3 AM for additional 30 minutes. Then, the coverslips were placed in a modified Hele-Shaw flow chamber[7] and positioned on the stage of a laser-scanning confocal microscope (Nikon, Eclipse TE300). A sample of physiological salt solution and ADP 25 µM, was aspirated through the chamber with a syringe pump (Harvard Apparatus, Boston, MA) at a flow rate of 50 sec^{-1}. The experiments were performed at 37°C. Images obtained through a Nikon Plan Fluor DICH 40x NA 1,30 oil immersion objective were acquired in real time with a digital camera (iXon^{EM}, Andor ^{TM} TECHNOLOGY, Belfast); the area of the field of view seen through the 40X objective was 41943 mm^2.

Study of store operated Ca^{2+} entry effectors expression in human megakaryocytes

In order to evaluate the expression of the putative mediators of store operated Ca^{2+} entry (SOCE) in human megakaryocytes, $1x10^6$ cells/condition at day 13 of culture 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, Na_3VO_4 1 mM, 1 µg/ml leupeptin, 1 µg/ml aprotinin), as previously described.[5] In some experiments, before being lysed, cells were pre-incubated with ADP 25 µM or CPA 10 µM for 30 minutes at 37°C. Lysis was performed on ice for 30 minutes and lysates clarified by centrifugation at 15700xg at 4°C for 15 minutes. Finally, protein concentration was measured by the bicinchoninic acid assay (Pierce, Milan, Italy). Immunoprecipitation was performed, as previously described.[5]

Briefly, cellular lysates were precleared by incubation with protein A-Sepharose and then incubated with 2 µg of anti-STIM1 at 4°C for 4 hours on a rotatory shaker. Thereafter, lysates were incubated with 100 µl of 50 mg/ml protein A-Sepharose on the rotatory shaker at 4°C. After 2 hours beads were washed three times with lysis buffer and samples were eluted with Laemmli buffer at 90°C for 5 minutes. 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 affinity-purified antibodies against STIM1 (1:1000), Orai1 (1:1000), TRPC1 (1:1000) and CD61 (1:500), following the conditions recommended by the manufacturers. Immunoreactive bands were detected by horseradish peroxidase-labeled secondary antibodies using enhanced chemiluminescence reagent (Merck Millipore, Milan, Italy). Prestained protein ladders were used to estimate the molecular weights (Bio-Rad, Milan, Italy). At least 3 independent experiments were performed.

Study of ADP-induced SOCE activation on signal transduction pathways and cytoskeleton reorganization in human megakaryocytes

$1x10^6$ megakaryocytes/condition at day 13 of culture were harvested and suspended in physiological salt solution (PSS) or in Ca^{2+}-free solution ($0Ca^{2+}$), and pre-treated or not with BAPTA-AM (20 µM), for 20 minutes at 37°C and 5% CO_2. Subsequently, megakaryocytes were stimulated or not with ADP 25 µM, at 37°C and 5% CO_2. After an additional 30 minutes, samples were lysed, as described above. Samples containing equal amounts of proteins were subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF Membrane (BioRad, Milan, Italy). Membranes were incubated using affinity-purified antibodies against phospho-FAK (1:500), phospho-Src Family (1:1000), phospho-Syk (1:1000), phospho-Akt (1:1000), phospho-ERK (1:1000), phospho-MLC (1:1000), CD61 (1:500) or β-actin (1:5000), following the conditions

recommended by the manufacturers. In some experiments, after treatment megakaryocytes were let to adhere on fibrinogen and fibronectin coated cover-slips and analyzed by immunofluorescence microscopy or lysed, as described above. At least 3 independent experiments were performed for each assay.

RNA Isolation and Retro-Transcription

Cord blood derived-$CD61^+$ Mks at day 13 of maturation were separated using the immunomagnetic beads technique (Miltenyi Biotec, Bologna, Italy) and total cellular RNA was extracted using the Mammalian GeneElute Total RNA Kit (Sigma-Aldrich), as previously described.[4] Retrotranscription (RT) was performed in a final volume of 20 µl reaction mixture in the presence of: 1 µg RNA, 1x PCR buffer, 5 mM MgCl2, 4 mM of each dNTP, 0.625 µM oligo d(T)16, 1.875 µM Random Hexamers, 20 U RNase Inhibitor, 50 U MuLV reverse transcriptase (all from Applera, Monza, Italy). The conditions for the reverse transcription were as follows: 25°C for 10 minutes, 42°C for 45 minutes, 99°C for 5 minutes. The RT samples were diluted up to 50 µl with ddH_2O. Previously published PCR primers were used.[6] The amplification reaction was performed in 25 µL using the MJ Min Personal Thermal Cycler (Biorad, Milan Italy). 20 µL PCR products were electrophoresed in 1% agarose gel or 20% polyacrylamide gel stained with ethidium bromide. At least 3 independent experiments were performed.

Cell Migration Assay

Cell migration assay was performed, with the trans-well migration chamber system (Merck Millipore, Milan, Italy), as previously published.[5,8,9] Briefly, 96-well plates with polycarbonate inserts having 0.3 cm^2/well membrane area with 8 µm pore size were coated with 100 µg/ml of fibrinogen, as described above. Megakaryocytes at the end of the culture were harvested and $20x10^3$ cells/well were suspended in 100 µl of Stem Span

medium and seeded in the upper chambers. In some experiments, before being seeded, cells were preincubated with the following substances, at the indicated final concentrations: apyrase 1 U/ml, ADP 25 μM, 2-APB 20 μM, U-73122 10 μM, BTP-2 20 μM. The lower chambers were filled with 150 μl of Stem Span medium supplemented with 100 ng/ml of SDF1-α (Peprotech, London, UK). The cells were left to migrate for 16 hours at 37°C and 5% CO_2. At the end of the incubation cells that had passed through the filters in the outer wells, were recovered, counted by inverted microscope and characterized by flow cytometry, as described above. Data are expressed as numbers of total migrated cells per insert. Thereafter, cells remaining on the upper face of the filters were removed by a cotton wool swab. Inserts were then washed three times with PBS and cells on the underside of the membrane were fixed with 4% PFA and stained, as described above. Finally, the membranes were washed again, cut out with a scalpel, and mounted onto glass slides. The assays were performed in triplicate wells for each condition described and each experiment was performed at least three times. Images were acquired using a Olympus BX51 microscope (Olympus, Deutschland GmbH, Hamburg, Germany). The number of cells that had migrated was counted by analyzing the entire area of the membranes. Data are expressed as percentages of cells migrated related to that of the control samples.

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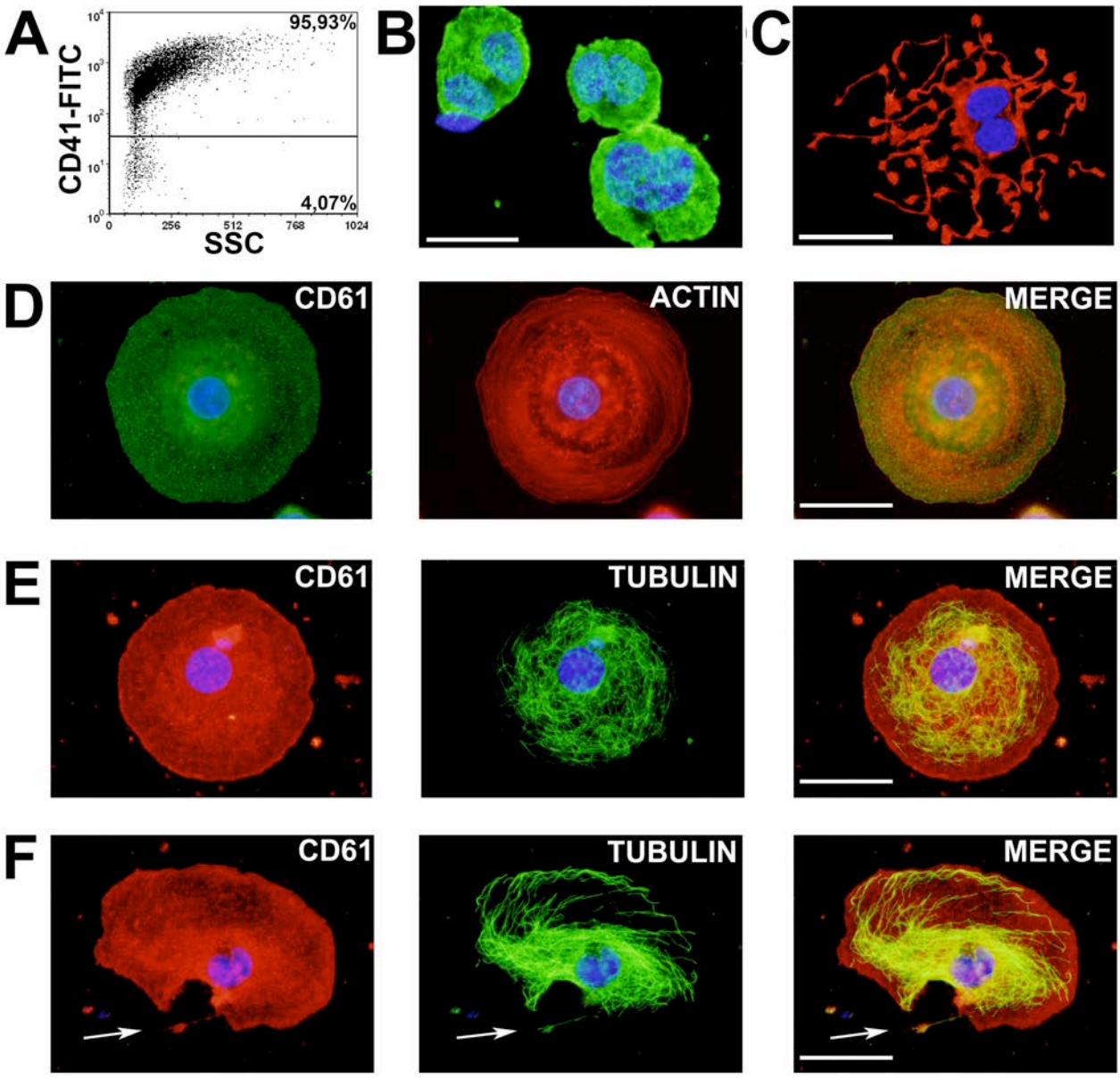
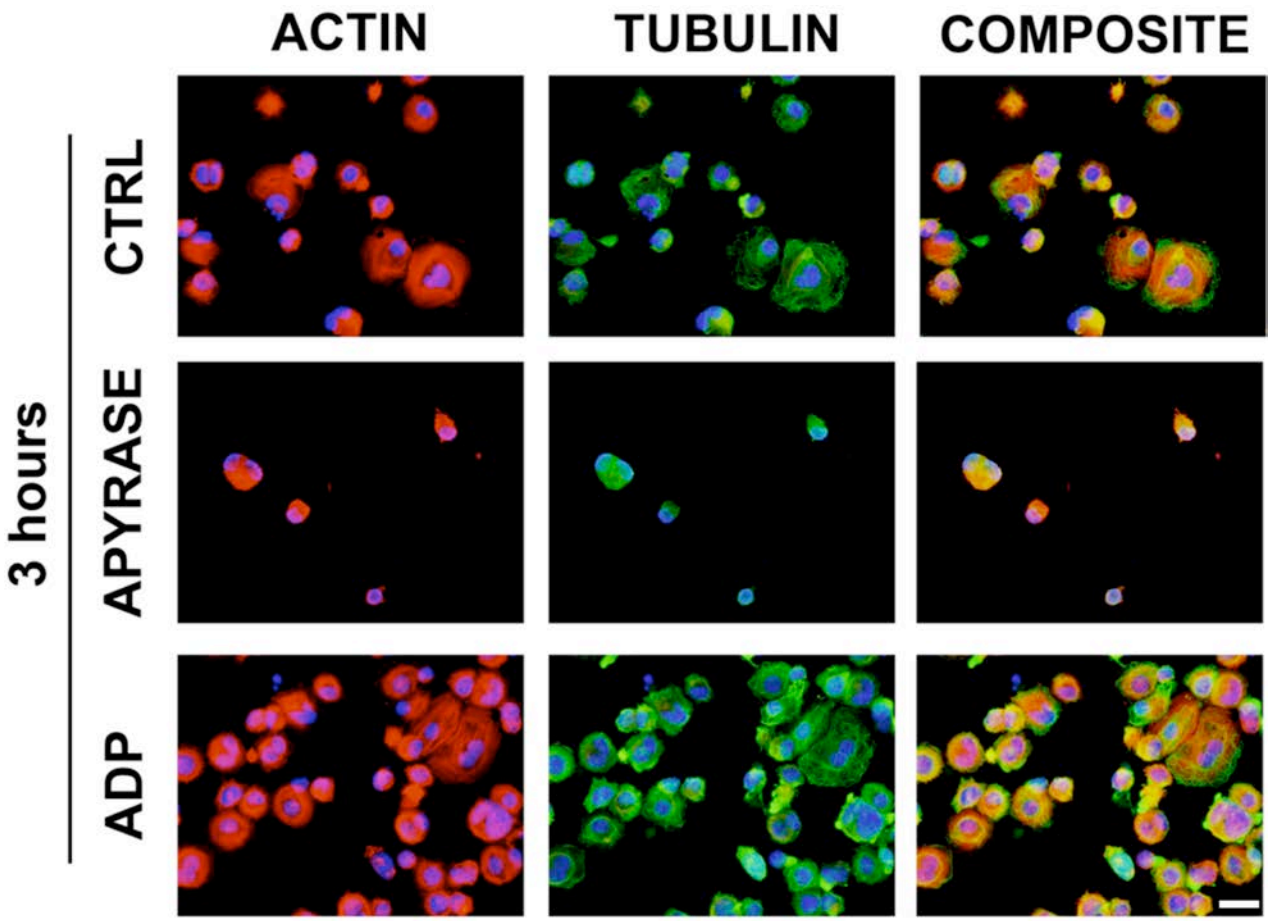
SUPPLEMENTAL FIGURE



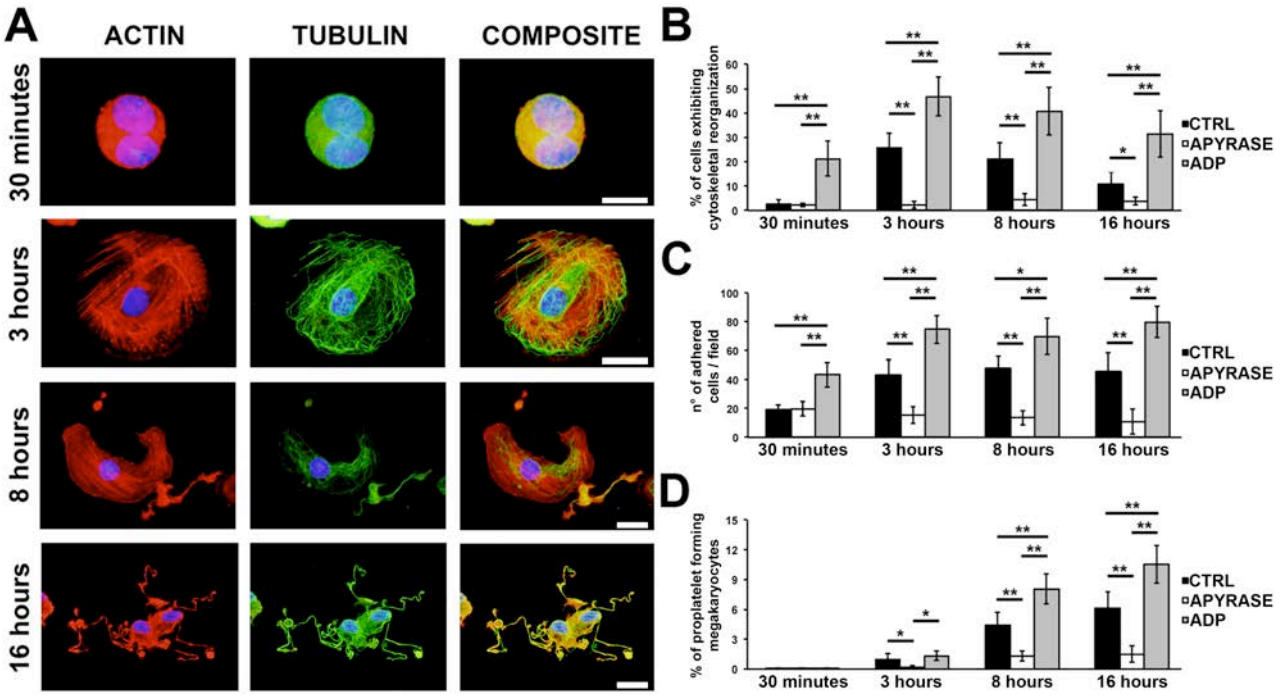
Supplemental Figure 1. Characterization of differentiation marker expression in cultured megakaryocytes. (A) Representative flow cytometry analysis of CD41 expression in human megakaryocytes after 13 days of differentiation. (B) Representative immunofluorescence staining of CD61 (green) in mature Mks at the end of culture. (C) Representative immunofluorescence image of a $CD61^+$ proplatelet forming Mk (red) on glass coverslips coated with fibrinogen. (D) Representative immunofluorescence image of a $CD61^+$ Mk (green) which shows stress fibers formation and focal contacts (red, TRITC-

phalloidin) after 3 hours adhesion on fibrinogen. (E-F) Representative immunofluorescence staining of $CD61^+$ Mks (red) which show microtubule assembly throughout the cytoplasm (green) after 3 and 8 hours adhesion on fibrinogen, respectively. Arrows indicate the appearance of the first proplatelet like pseudopod ending with platelet-sized tip. In all immunoflourescence staining nuclei were counterstained with Hoechst 33258 in blue. Images were acquired by an Olympus BX51, magnification 100X, scale bar=20 μm, n=5 independent experiments).

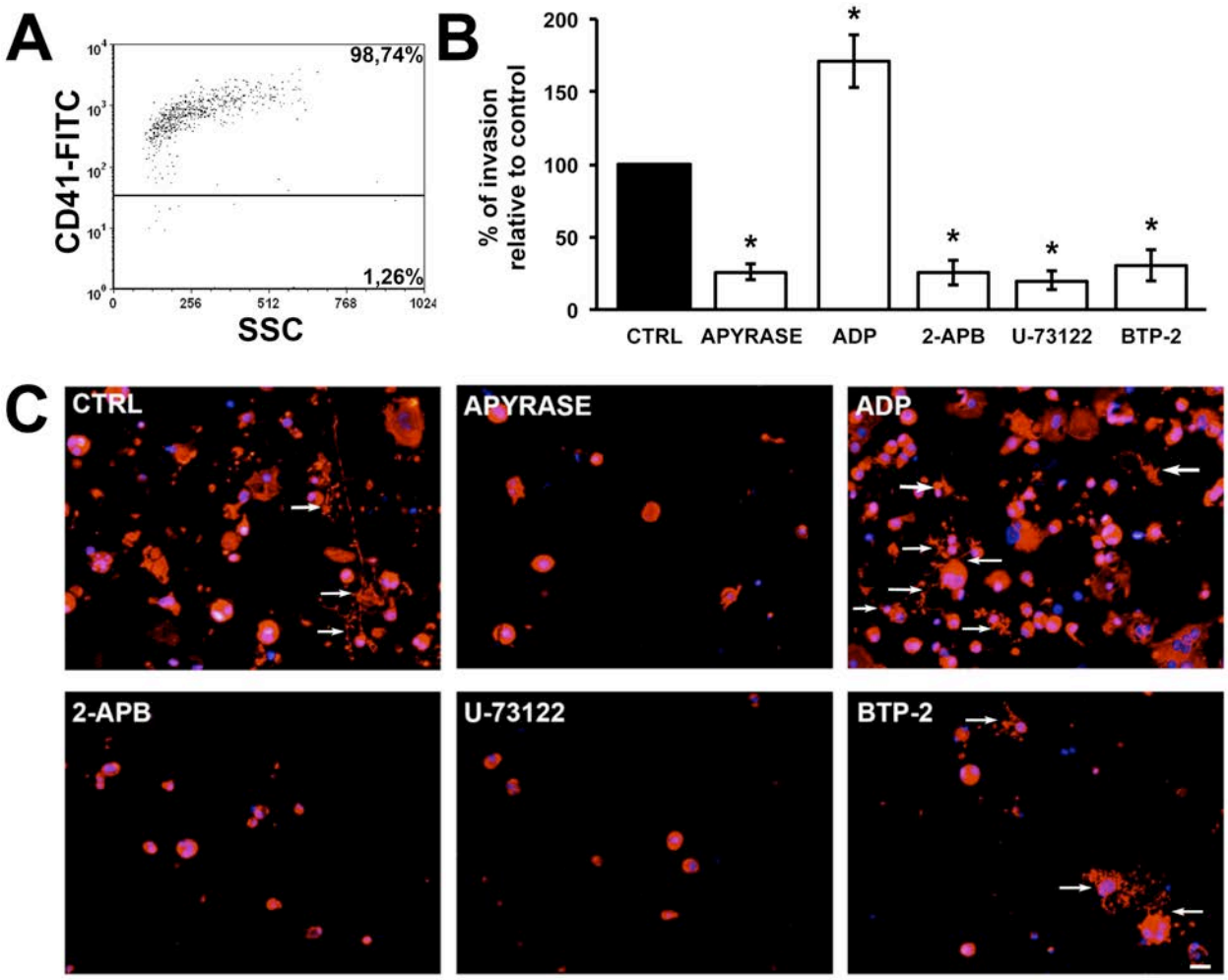


Supplemental Figure 2. Role of ADP in regulating megakaryocytes-extracellular matrix components interaction. Megakaryocytes at day 13 of culture were plated on fibrinogen coated cover-slips. In some experiments, before being seeded, cells were treated with apyrase (1U/ml) or ADP (25 μM). After 3 hours incubation adherent cells were fixed and stained for immunofluorescence analysis with TRITC-phalloidin (red) and

antibody against α-tubulin (green). Nuclei were counterstained with Hoechst 33258 (blue). Images were acquired by an Olympus BX51, magnification 40X, scale bar=30 μm, n=5 independent experiments.



Supplemental Figure 3. Analysis of Mk interaction with fibronectin during proplatelet formation. Fully differentiated Mks were plated on fibronectin-coated cover-slips, at 37 °C in a 5% CO_2. (A) As described for fibrinogen cells were fixed and stained for immunofluorescence analysis after 30 minutes and 3-8-16 hours (TRITC-phalloidin in red; α-tubulin in green). Nuclei were counterstained with Hoechst 33258 in blue. Images were acquired by an Olympus BX51, magnification 60X and 100X, scale bar=20 μm. In some experiments Mks were seeded in the presence or absence of the ADP scavengers apyrase (1 U/ml) or ADP (25 μM) and analyzed in the same conditions. (B) Cytoskeletal reorganization, (C) adhesion and (D) proplatelet formation (B) were analyzed with respect to non-treated controls (CTRL) (mean±SD, n=5 independent experiments, *p<0.05, **p<0.01).



Supplemental Figure 4. Store Operated Ca^{2+} Entry regulates ADP-mediated megakaryocytes migration on extracellular matrix components. (A) Flow cytometry analysis of $CD41^{+}$ population after migration in the trans-well chamber system. Representative of 3 independent experiments. (B) Mks adhering on the lower side of the trans-well filter were fixed and stained with TRITC-phalloidin (in red) and then counted by fluorescence microscopy. Results are reported as means±SD with respect to not treated controls (mean±SD, n=3 independent experiments, *p<0.01). (C) Representative images of mature megakaryocytes after 16 hours migration in the Transwell chamber system. Nuclei were counterstained with Hoechst 33258 in blue. In some experiments before being seeded cells were pretreated with apyrase, ADP, 2-APB, U-73122, or BTP-2. The arrows indicate proplatelet bearing Mks. Images were acquired by an Olympus BX51, magnification 20X, scale bar=20 μm, n=3 independent experiments.