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The importance of calcium in the regulation of megakaryocyte function

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Running heads: SOCE regulates megakaryocyte function.

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ABSTRACT

Platelet release by megakaryocytes is regulated by a concert of environmental and autocrine factors. We previously showed that constitutively released adenosine diphosphate by human megakaryocytes leads to platelet production. Here we show that adenosine diphosphate elicits, in human megakaryocytes, an increase in cytosolic calcium concentration, followed by a plateau, which is lowered in absence of extracellular calcium, suggesting the involvement of Store-Operated Calcium Entry. Indeed, we demonstrate that megakaryocytes express the major candidates to mediate Store-Operated Calcium Entry, stromal interaction molecule 1, Orai1 and canonical transient receptor potential 1, which are activated upon either pharmacological or physiological depletion of the intracellular calcium pool. This mechanism is inhibited by phospholipase C or inositol-3-phosphate receptor inhibitors and by a specific calcium entry blocker. Studies on megakaryocyte behavior, on extracellular matrix proteins that support proplatelet extension, show that calcium mobilization from intracellular stores activates signaling cascades that trigger megakaryocyte adhesion and proplatelet formation and promotes extracellular calcium entry which is primarily involved in the regulation of contractile force responsible for megakaryocyte motility. These findings provide the first evidence that both calcium mobilization from intracellular stores and extracellular calcium entry specifically regulate human megakaryocyte functions.
INTRODUCTION

Megakaryopoiesis is the process by which bone marrow megakaryocytes (Mks) are derived from pluripotent hematopoietic stem cells to produce platelets. During differentiation Mks migrate from the osteoblastic to the vascular niche in response to Stromal-derived factor-1α (SDF-1α). Once in proximity of the vasculature, Mks convert their cytoplasm into branching filaments called proplatelets, which protrude into the sinusoid lumen, where platelets are released. A growing body of evidence indicates that the characteristics of the extracellular matrix (ECM) structure and composition surrounding Mks play an important role in the regulation of platelet production. Moreover, early during megakaryopoiesis, Mks develop platelet-specific granules, which release their contents supporting Mk development. Among these, we recently demonstrated that Mks constitutively release adenosine diphosphate (ADP) which promotes proplatelet formation by interacting with its receptor P2Y13. Most importantly, we described that patients with Delta-Storage Pool Deficiency (δ-SPD), a congenital bleeding diathesis characterized by deficiency of dense granules and their constituents (including ADP) in Mks and platelets, display in vivo a significantly higher prevalence of thrombocytopenia than those observed in other disorder of primary hemostasis. However, the exact mechanisms by which matrix components and soluble factors coordinate to regulate platelet release are still unknown. The increase in cytosolic calcium concentration ([Ca^{2+}]) has been described to be required for interaction with the extracellular environment in different human cell types. Interestingly, extracellular nucleotides and purinergic receptors are critically involved in mediating this interaction with changes in [Ca^{2+}]. Mks express all P2Y G-protein-coupled receptors (GPCRs) for purine and pyrimidine nucleotides and all G-proteins coupled to Ca^{2+} signaling activation. Among the known P2Y receptors that bind ADP only P2Y_{1} and P2Y_{13} have been recognized to mediate an increase in [Ca^{2+}], via the phospholipase C (PLC) pathway. Specifically, PLC activation leads to the generation of
the second messengers inositol 1,4,5-trisphosphate (IP3) which diffuses within the cytosol to bind to and activate the IP3 receptors (IP3Rs) in the endoplasmic reticulum (ER), the most abundant intracellular Ca\(^{2+}\) store.\(^{18}\) This results in cytoplasmic Ca\(^{2+}\) elevation that can be separated into two distinct phases. In the first phase, Ca\(^{2+}\) is released from intracellular stores via the IP3Rs. In the second phase, the decrease in ER Ca\(^{2+}\) content causes the activation of plasma membrane Ca\(^{2+}\) channels resulting in the influx of extracellular Ca\(^{2+}\) inside the cells, a mechanism that has been termed Store-Operated Ca\(^{2+}\) Entry (SOCE).\(^{19}\) The prime candidates to mediate SOCE in hematopoietic cells are STIM and Orai proteins.\(^{19}\) More specifically, STIM1 (stromal interaction molecule 1) is the transmembrane ER Ca\(^{2+}\) sensor that translocates in close proximity to the plasma membrane upon store depletion and activates Orai1 (also known as calcium-release-activated calcium-modulator, CRACM), the pore-forming subunit of store-operated Ca\(^{2+}\) channels.\(^{19}\) The role of the additional STIM1 and Orai1 paralogues, i.e. Stim2 and Orai2-3, is far from being fully understood in naïve cell systems, albeit they may recapitulate SOCE when ectopically expressed.\(^{19}\) Moreover, several evidences demonstrated that members of the canonical transient receptor potential (TRPC) family of cation channels may represent additional candidates for SOCE.\(^{20}\) Agonist-induced elevation of intracellular Ca\(^{2+}\) levels is essential for platelet activation. To this regard, STIM1 and Orai1 proteins have been showed to be key players in human platelet SOCE during aggregation,\(^{21,22}\) whereas TRPC involvement has been questioned.\(^{23,24}\) Importantly, it has been demonstrated that SOCE plays a major role in mediating adhesion and motility onto ECM components of different cell types, including hematopoietic stem cells.\(^{9,25}\) Thus, we hypothesized that purinergic signaling and SOCE may be responsible for Mk interaction with the ECM environment and consequent regulation of platelet production. Our results demonstrate that ADP induces both intracellular Ca\(^{2+}\) mobilization and extracellular Ca\(^{2+}\) inflow in human Mks, which in turn support the cytoskeletal reorganization responsible for cellular
adhesion and migration and final proplatelet formation on ECM components that promote such a dynamic process, such as fibrinogen and fibronectin. These findings provide the first evidence that Ca\(^{2+}\) signaling is a fundamental regulator of human Mk functions.

**METHODS**

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.\(^4\)

At the end of cell culture, Mks were harvested and plated onto glass cover-slips previously coated with different ECM components in order to evaluate cell adhesion, migration and proplatelet formation. All images were acquired by Olympus BX51 microscope (Olympus, Deutschland GmbH, Hamburg, Germany). 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.

We employed Ca\(^{2+}\) imaging to investigate the expression and functionality of SOCE on the same extracellular matrix components. Specifically, Mks were loaded with 4 µM fura-2 acetoxyethyl ester (AM) or 5 mM FLUO-3 AM and observed using an upright epifluorescence AxioLab microscope (Carl Zeiss) equipped with a Zeiss X63 Achromplan objective or a laser-scanning confocal microscope (Nikon, Eclipse TE300).

For all the experiments values are expressed as mean ± SD. Student’s t-test was performed for paired observations. ANOVA, followed by the post-hoc Bonferroni t-test, was performed for grouped observations. Values of p<0.01 or p<0.05 were considered
statistically significant. All experiments were independently replicated at least 3 times, unless specified otherwise.

A completely detailed methods section is provided in Supplemental Methods.

RESULTS

ADP regulates proplatelet formation in adhesion on extracellular matrix components

During differentiation human Mks release ADP that support proplatelet formation. In order to demonstrate whether ADP plays a role also in regulating Mk interaction with extracellular matrix components, Mks were differentiated in vitro starting from human cord blood derived CD34+ cells. At day 13 of culture, the percentage of mature CD41+ Mks was 93±5% and cells were able to extend long and branched proplatelet structure ending with platelet-sized tips (Supplemental figure 1A-C). Fully differentiated Mks were plated on two different bone marrow ECM components, fibrinogen and fibronectin, known to support Mk maturation and proplatelet formation. A time course analysis revealed that on fibrinogen Mks exhibit actin stress fibers, focal contacts and convoluted microtubules throughout the cytoplasm after 3 hours incubation, while, after 8 hours, they started to extend proplatelet-like pseudopods that became more branched prolonging the incubation to 16 hours (Figure 1A, Supplemental figure 1D-F). The addition of the ADP scavenger apyrase 1 U/ml, markedly inhibited cytoskeletal reorganization and Mk adhesion (Figure 1B-D and Supplemental Figure 2). Conversely, treatment with ADP 25 µM augmented the number of adherent Mks exhibiting stress fibers and microtubule assembly (Figure 1B and 1C), with consequent significant increase of adherent proplatelet forming Mks after 16 hour incubation (Figure 1D). Similar results were obtained on fibronectin (Supplemental Figure 3). Conversely, no significant differences were observed in the same conditions in adhesion on type I collagen, which has been described to inhibit platelet release, or
polylysine, a neutral control substrate (data not shown). Thus, these results suggested that ADP promotes proplatelet formation in adhesion on extracellular matrix components that support this process.

**ADP induces the activation of Ca$^{2+}$ signaling in human megakaryocytes**

Human Mks were exposed to ADP (25 µM) in physiological salt solution (PSS) containing 1.5 mM Ca$^{2+}$ and fluorescence measurements of [Ca$^{2+}$]$_i$ were carried out in Mks loaded with the Ca$^{2+}$ sensitive fluorochrome Fura-2. We showed that ADP elicited a large increase in [Ca$^{2+}$]$_i$ with a clear plateau following the initial Ca$^{2+}$ peak (Figure 2A). Pre-incubation with MRS 2179 (10 µM), a highly specific inhibitor of P2Y$_1$, or MRS 2211 (10 µM), the specific P2Y$_{13}$ inhibitor, significantly affected both the initial peak, and the extent of the plateau (Figure 2A and 2E). However, the strongest inhibition was obtained by the latter, confirming a primary role for P2Y$_{13}$ in mediating Mk response to ADP.7

Subsequently, we focused on the study of Ca$^{2+}$ signaling in order to understand its role in regulating cell response to agonist stimulation. Thus, Mks were pre-incubated with BAPTA-AM (20 µM), a membrane-permeable intracellular Ca$^{2+}$ chelator. Here ADP stimulation did not evoke any detectable Ca$^{2+}$ signal (Figure 2B and 2E). Interestingly, when ADP was applied in the absence of extracellular Ca$^{2+}$ (0Ca$^{2+}$) the initial increase in [Ca$^{2+}$]$_i$ remained unchanged, while the plateau phase totally disappeared (Figure 2C and 2E). Thus, intracellular Ca$^{2+}$ release plays a major role in eliciting the initial Ca$^{2+}$ peak induced by ADP, while extracellular Ca$^{2+}$ influx determines a sustained Ca$^{2+}$ signaling, the hallmark of SOCE activation.26 In accordance with this evidence, removing extracellular Ca$^{2+}$ during the plateau phase, in the presence of ADP, caused a rapid drop of [Ca$^{2+}$]$_i$ to the base line (Figure 2D).
mRNA and protein expression of the putative mediators of SOCE in human megakaryocytes

We investigated, in human Mks, the expression of the molecules that have been proposed to mediate SOCE. Specifically, we analyzed the expression of mRNA encoding for the recently cloned Orai (Orai1, Orai2 and Orai3) and STIM (STIM1 and STIM2) genes and for all the known TRPC expressed in humans (TRPC1, TRPC3, TRPC4, TRPC5, TRPC6, TRPC7). Orai1, Orai2, Orai3, STIM1, STIM2, TRPC1 and TRPC6 transcripts were detectable in human Mks (Figure 3A-B), whereas we didn’t detected significant amount of mRNAs for TRPC3/4/5/7. Expression of STIM1, Orai1, and TRPC1, the best characterized effectors of SOCE, was also confirmed in human CD61+ Mks by western blot (Figure 3C).

SOCE is functional in human megakaryocytes

In order to demonstrate the presence of a functional SOCE in human Mks, we exposed cells to cyclopiazonic acid (CPA, 10 µM), a widely employed activator of this mechanism. Specifically, CPA blocks Sarco-Endoplasmic Reticulum Ca2+ ATPase (SERCA) activity, thereby preventing Ca2+ sequestration into the stores and leading to their depletion and SOCE activation. Figure 3D shows that STIM1 co-immunoprecipitates with both Orai1 and TRPC1, with a significant increase in cells subjected to store depletion as compared to not treated controls, thus suggesting the formation of a molecular complex among the three molecules under these conditions. In order to further confirm this evidence, we next carried out fluorescence measurements of [Ca2+]. Depleting the stores with CPA (10 µM), without extracellular Ca2+ (0Ca2+), evoked a transient rise in [Ca2+], because of passive emptying of Ca2+ stores through leakage channels in ER membrane. Thereafter, Ca2+ levels dropped to the baseline as the plasma membrane transporters (i.e., plasma membrane Ca2+-ATPase and Na+-Ca2+ exchanger) extruded Ca2+ from the cytosol. Then, when external Ca2+ was restored to 1.5 mM, a second increase in intracellular Ca2+ levels was
observed due to activated influx through store-operated channels (Figure 3E). Acute application of BTP-2 (50 µM), a specific inhibitor of SOCE, strongly inhibited CPA-dependent Ca\(^{2+}\) inflow (Figure 3E). Accordingly, 30 minutes pre-incubation with BTP-2 (20 µM) prevented SOCE (Figure 3F), whereas it did not affect the ER Ca\(^{2+}\) content (Figure 3F and 3G).

SOCE is engaged by human megakaryocytes in response to ADP and regulates cell adhesion and migration

SOCE recruitment by GPCRs is mediated by the PLC\(\beta\)/IP3 pathway. Thus, human Mks were treated with the selective pan PLC\(\beta\) inhibitor, U-73122 (10 µM), and that of IP3Rs, 2-APB (20 µM), at a concentration that did not affect SOCE. U-73122 has been shown to prevent Ca\(^{2+}\) release by interfering with SERCA-dependent Ca\(^{2+}\) sequestration into ER lumen. However, the acute addition of this drug never caused the slow increase in [Ca\(^{2+}\)] which is typical of SERCA-inhibiting drugs (see, for instance, the tracing displayed in Figure 4B). Moreover, we have previously utilized 10 µM U-73122 to selectively impair PLC activity in a variety of cell types. Both molecules prevented ADP-induced [Ca\(^{2+}\)] elevation, compared to control (Figure 4A-C), demonstrating that the IP3-dependent Ca\(^{2+}\) pool shapes initial Mk response to ADP. Importantly, Mk pre-treatment with BTP-2 (20 µM) for 30 minutes prevented the plateau phase of the Ca\(^{2+}\) signal after ADP stimulation without affecting the transient Ca\(^{2+}\) peak (Figure 4D). SOCE activation following treatment with ADP was also confirmed by the evidence that STIM1 association with Orai1 and TRPC1 was notably enhanced upon stimulation with the agonist (Figure 4E).

The aforementioned inhibitors were also tested for their effects on Mk functions. After 16 hours, the percentages of Mks displaying cytoskeletal reorganization on fibrinogen was significantly inhibited by 2-APB (20 µM), U-73122 (10 µM) and BTP-2 (20 µM) (Figure 4F) with a decreased number of both adherent and proplatelet forming Mks on ECM
components (Figure 4G and 4H). Interestingly, after treatment with 2-APB and U-73122 adherent proplatelets exhibited altered structure and decreased branching, while, in the presence of BTP-2, the few adherent Mks were still able to extend normal structured and branched proplatelets (data not shown). Therefore, we hypothesized that the observed role of Ca$^{2+}$ inflow was above all to promote Mk interaction with ECM components, suggesting a possible role for SOCE in triggering in vivo Mk migration at the site of platelet release. Actually, ADP enhanced migration of CD41$^+$ Mks in response to SDF-1$\alpha$ compared to control, while addition of apyrase, 2-APB, U-73122 or BTP-2 significantly reduced it (Figure 4I and Supplemental Figure 4A and 4B). Interestingly, some of the Mks that had passed through the filter in presence of BTP-2 elongated normal branched proplatelet, further supporting the hypothesis that extracellular Ca$^{2+}$ entry regulates mainly Mk active interaction with the ECM components, while intracellular Ca$^{2+}$ release is sufficient to activate proplatelet formation (Supplemental Figure 4C). The same results were obtained on fibronectin (data not shown).

**ADP-induced Ca$^{2+}$ mobilization activates megakaryocyte downstream signaling**

To assess the role of ADP-induced Ca$^{2+}$ signals we investigated the activation of molecules relevant for proplatelet formation and/or Mk adhesion and motility (i.e. the serine/threonine-specific kinase Akt, the mitogen-activated protein kinases ERK, the tyrosine kinases FAK, Src Syk and the myosin light chain, MLC).$^{7,32-35}$ We showed that, upon stimulation with ADP (25 µM), the extent of phosphorylation of all tested molecules increased in adherent Mks on both fibrinogen and fibronectin substrates (Figure 5A). We next focused the attention exclusively on the role of Ca$^{2+}$ on ADP signaling. We confirmed that ADP drives the activation of these pathways (Figure 5B, lane 2) over the basal level (Figure 5B, lane 1). At the same time, some Mks were incubated in presence of the intracellular Ca$^{2+}$ chelator BAPTA-AM (20 µM) to prevent the overall Ca$^{2+}$ activation, or in
absence of extracellular Ca\(^{2+}\) (0Ca\(^{2+}\)) to selectively abrogate Ca\(^{2+}\) influx and thus SOCE activation. Interestingly, intracellular Ca\(^{2+}\) buffering prevented ADP-mediated activation of all molecules, with the exception of Syk (Figure 5B, lane 3). Conversely, 0Ca\(^{2+}\) prevented only phosphorylation of MLC (Figure 5B, lane 4).

We examined the cytoskeleton structure under the same conditions upon adhesion on fibrinogen. Treatment with ADP induced actin stress fiber formation and microtubule assembly, whereas pre-treatment with BAPTA abolished these responses (Figure 5C). Removal of extracellular Ca\(^{2+}\) prevented actin stress fibers development, but did not affect microtubule assembly (Figure 5C). Results were perfectly comparable on fibronectin (data not shown). Importantly, Mk behavior was monitored, during stimulation with ADP (25 µM), using a flow chamber system. During perfusion experiments, in the presence of ADP, Mks showed a sustained Ca\(^{2+}\) oscillation with different spatial and temporal organization of Ca\(^{2+}\) in an observation time frame of about 5 minutes (Figure 6A). The same Mks showed remodeling of their cytoplasm as shown in Figure 6B.

**Inhibition of extracellular Ca\(^{2+}\) inflow affects megakaryocyte interaction with type I collagen**

Stimulation or inhibition of ADP signaling in human Mks didn’t affect interaction with type I collagen, as described above. However, it has been demonstrated that human Mks display a significant increase in [Ca\(^{2+}\)]\(_i\) during interaction with this ECM component.\(^{36}\) Importantly, we demonstrated that only type I collagen is able to inhibit proplatelet formation by sustained phosphorylation of MLC in human Mks.\(^{34}\) Thus, we decided to test the role of SOCE on Mk behavior on this ECM component. Interestingly, treatment with BTP-2 (20 µM) significantly inhibited Mk adhesion with a decreased number of cells displaying cytoskeleton reorganization with respect to not treated controls (Figure 7A-C).
DISCUSSION

Mk maturation and proplatelet formation in the bone marrow are consequent to an integrated concert of signals belonging to both extracellular environment and Mks themselves. Importantly, it is known that different autocrine loops are fundamental modulator of Mk development. To this regard, we demonstrated that ADP is constitutively released by human Mks in vitro and it is a crucial molecule in the regulation of platelet production. Importantly, we also described that patients with Delta-Storage Pool Deficiency, display in vivo a significantly higher prevalence of thrombocytopenia than those observed in other disorders of primary hemostasis. Despite this knowledge, it has not been described yet whether or not soluble factors, that promote platelet release, can regulate Mk interaction with ECM components that support such a dynamic process.

In this study we pursued to unravel the mechanism underlying platelet production within a bone marrow ECM environment. We demonstrated that ADP promotes Ca\textsuperscript{2+} release from intracellular stores which is responsible for the regulation of proplatelet formation and for the activation of SOCE. This latter promotes Mk adhesion and migration.

First, we showed that Mk interaction over fibrinogen and fibronectin requires the co-ordination of different cellular processes which operate in a cycle that can be divided into four critical steps: (1) early passive adhesion to ECM components; (2) active reorganization of the cytoskeleton and assembly of focal adhesions; (3) dynamic contraction of the cytoplasm with appearance of proplatelet-like pseudopod; (4) final proplatelet branching in adhesion on ECM components. Interestingly, all these events were regulated by constitutively released ADP, whereas its scavenger apyrase significantly inhibited them. Noteworthy, exogenous administrated ADP markedly enhanced adhesion of proplatelet forming Mks. Thus, ADP exerts a role in favoring the establishment of the tight interaction with extracellular substrates that support proplatelet formation and together with them help this process.
ADP-dependent platelet activation relies on the increase in cytosolic Ca\(^{2+}\) concentration, accomplished by SOCE activation.\(^{21}\) The same mechanism is responsible for promoting interaction with ECM environment in different human cell types.\(^{9,25}\) In order to investigate the role of Ca\(^{2+}\) signaling in regulating ADP-dependent human Mk functions, we performed real time measurements of intracellular Ca\(^{2+}\) movements. We demonstrated an increase in [Ca\(^{2+}\)]\(_i\) upon ADP stimulation in static conditions and a direct correlation between Ca\(^{2+}\) signaling and Mk cytoskeleton remodeling under flow conditions.

The application of MRS 2211, a selective P2Y\(_{13}\) antagonist, inhibited the ADP-induced Ca\(^{2+}\) mobilization at a higher extent with respect to MRS 2179, a selective P2Y\(_{1}\) antagonist. These results confirmed our previous data that P2Y\(_{13}\), which is expressed by human Mks but not by human platelets, represents a crucial and specific modulator of Mk function.\(^7\) Interestingly, ADP elicited a large increase in [Ca\(^{2+}\)]\(_i\) with a clear plateau following the initial peak. Treatment with BAPTA-AM totally abolished this response, suggesting that first mobilization is sustained by intracellular Ca\(^{2+}\) release. Conversely, exposure to ADP in absence of extracellular Ca\(^{2+}\) prevents the plateau phase. Thus, in human Mks ADP-induced depletion of the intracellular stores activate Ca\(^{2+}\) inflow, a feature that hinted at SOCE involvement.

We demonstrated that human Mks express both mRNA and protein for the most characterized SOCE mediators, STIM1, Orai1 and TRPC1. Moreover, we showed that Mks express also mRNA for Stim2 and Orai2 and 3, which are involved in SOCE and for two members of the TRPC family, TRPC1 and 6.\(^{19,20}\) However, whether or not TRPC1 is an additional candidate for SOCE is still matter of debate.\(^{21,23}\) Conversely, TRPC6 activation is completely independent of store depletion.\(^{24}\) Importantly, we demonstrated that SOCE was functionally activated in response to the emptying of the IP3-sensitive Ca\(^{2+}\) reservoir. Specifically, passive depletion of the ER Ca\(^{2+}\) content with CPA, led to the formation of a ternary molecular complex between STIM1, Orai1 and TRPC1 which is
likely to be involved in SOCE. For instance, in platelets, Orai1 mediates the store-dependent interaction between STIM1 and TRPC1 which regulates SOCE in response to both pharmacological (i.e. SERCA inhibition) and IP3-dependent depletion of the ER Ca²⁺ content. BTP-2, a selective inhibitor of store-dependent Ca²⁺ inflow, abrogated CPA- and ADP-induced Ca²⁺ inflow. Moreover, blocking PLC or the IP3Rs, prevented depletion of the ER Ca²⁺ pool and abrogated SOCE. Interestingly, PLC and IP3Rs inhibitors prevented both adhesion and proplatelet formation, while BTP-2 significantly decreased Mk-ECM interaction, but exerted a minor impact on Mks that, if adherent, were still able to extend normal branched proplatelets. Consistently, ADP-dependent IP3-dependent Ca²⁺ mobilization was sufficient to activate all signaling molecules known to be relevant for both Mk adhesion and proplatelet formation, including FAK, Src, Akt and ERK, indicating that intracellular Ca²⁺ release can promote these events. Conversely, MLC activation was selectively driven by store-dependent Ca²⁺ entry, as more extensively discussed below.

Recently, Chen et al. described the unique role of STIM1-dependent Ca²⁺ signaling in controlling cell migration by the regulation of actomyosin contractility. A number of studies have demonstrated SOCE involvement in biological and pathological migration of multiple cell types, including CD133⁺ hematopoietic stem cells and blood cells. Moreover, it is well known the critical role for extracellular nucleotides and purinergic receptors in mediating cell motility. Dynamic regulation of Mk migration, within the bone marrow environment, is critical for platelet production. Actually, patients with MYH9-related disease, caused by mutations of the gene for the heavy chain of the nonmuscle myosin, present thrombocytopenia, probably caused by impaired migration and premature proplatelet formation within the osteoblastic niche due to the lack of proper interaction with ECM components. Here, we showed that the ADP-mediated Ca²⁺ inflow is required for MLC phosphorylation, actin polymerization and formation of focal complexes in human Mks, but not for microtubule assembly. It has been well described that actomyosin
contraction plays a major role in regulating the focal complexes structure which serve as
cytoskeletal organizing centers as well as surface-sensing entities that coordinate cellular
migration. Thus, we hypothesized that the functional counterpart of the observed role
of ADP-induced SOCE could be to support actomyosin cytoskeleton reorganization
responsible for Mk migration. Consistently, we showed that absence of ADP or SOCE
inhibition strongly impaired Mks motility toward SDF-1α. Thus, these results dissect the
role of Ca^{2+} compartmentalization in regulating different Mk function: Ca^{2+} mobilization
from intracellular stores drives both Mk adhesion and proplatelet formation (by activating
FAK, Src, ERK and Akt), while Ca^{2+} inflow following store depletion is primarily responsible
for promoting cytoskeletal reorganization and subsequent migration on ECM components
(through MLC-mediated remodeling of actomyosin cytoskeleton). Thus, intracellular and
extracellular Ca^{2+} are both needed to ensure Mk functions.
Importantly, rare patients with mutated STIM1 and Orai1 accompanied by
thrombocytopenia have been described, though there are no reports regarding bone
marrow phenotype and/or Mk development and function. Stim1^{-/-} or Orai1^{-/-} mice have also
been described. Both these models present normal platelet counts. However mice, as
well as humans, express different STIM, Orai and TRPC isoforms that mediate SOCE
activation. Thus, we expect neither for mice nor for humans that single knock-down could
totaly affect SOCE. Actually, both Stim1^{-/-} and Orai1^{-/-} platelets aggregate normally to
ADP even at very low concentrations of agonists and ADP is still able to induce unaltered
 cellular activation despite the defect in [Ca^{2+}]i signaling. However, the importance of [Ca^{2+}]i
in the regulation of platelet release in vivo has been recently suggested by two different
groups that described new somatic mutations of calreticulin, a protein that binds Ca^{2+} in
the ER contributing to Ca^{2+} homeostasis, in patients affected by myeloproliferative
neoplasms. Interestingly, these mutations impair Ca^{2+} binding affinity and are expressed in
all haemopoietic progenitors, including megakaryocyte–erythroid progenitors. Given
the evidence that all these patients are characterized by an excessive platelet production it is possible that an impaired Ca^{2+} homeostasis in bone marrow Mks may be responsible for the altered platelet production.

In summary, we report the first evidence that SOCE activators and Ca^{2+} mobilization are involved in the regulation of human Mk functions. These data, along with the most recent clinical discoveries on calreticulin mutations, open new perspectives in the study of the signals that \textit{in vivo} concur in promoting platelet production.

**AUTHORSHIP AND DISCLOSURES**

CADB, MB performed the experiments; LDM, MM, RM, FT analyzed the data and edited the manuscript; CADB, FM, AB designed the experiments, analyzed the data and wrote the manuscript. AB coordinated the research. The authors report no potential conflicts of interest.

**REFERENCES**


FIGURE LEGENDS

Figure 1. Analysis of Mk-matrix interaction during proplatelet formation. Cord blood derived-Mks at day 13 of culture were plated on fibrinogen-coated cover-slips, at 37 °C in a 5% CO₂. (A) After different time-points (30 minutes, 3-8-16 hours) 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 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 scavenger apyrase (1 U/ml) or ADP (25 µM) and analyzed as described for control. (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).
Figure 2. ADP elicited Ca\textsuperscript{2+} mobilization in human Mks. (A) ADP (25 µM) evoked an initial rise in [Ca\textsuperscript{2+}]\textsubscript{i} (peak) followed by a signal of lesser magnitude (plateau) in human Mks (CTRL). Pre-incubation with the P2Y1 inhibitor, MRS 2179 (10 µM) and the P2Y13 inhibitor, MRS 2211 (10 µM) inhibited ADP-induced Ca\textsuperscript{2+} signaling. (B) Pre-treatment with BAPTA-AM caused the complete lack of Ca\textsuperscript{2+} response to ADP. (C) When ADP was applied in the absence of extracellular Ca\textsuperscript{2+}, the intracellular Ca\textsuperscript{2+} displayed a fast kinetic with no plateau phase. (D) Removal of extracellular Ca\textsuperscript{2+} (0Ca\textsuperscript{2+}) caused the rapid recovery of [Ca\textsuperscript{2+}]\textsubscript{i} to resting levels. (E) Statistical evaluation of the effect of the different treatment on the amplitudes of the two phases of Ca\textsuperscript{2+} signaling (n=5 independent experiments, *p<0.01).

Figure 3. Expression and function of molecular mediators of SOCE in human Mks. (A-B) Gel electrophoresis of the PCR products for Orai, STIM and TRPC expression. PCR products were of expected size: Orai1, 257bp; Orai2, 334bp; Orai3, 159bp; Stim1, 347bp; Stim2, 186bp, TRPC1, 307bp, TRPC6, 341bp. No signal was observed for TRPC3/5/6/7. Representative of 3 independent experiments. (C) Protein expression of STIM1, Orai1 and TRPC1 in human CD61\textsuperscript{+} Mks at day 13 of culture. (D) Mature Mks were treated (+) or not (−) with CPA (10 µM) and lysed. Lysates were immunoprecipitated (IP) with an anti-STIM1 antibody and subjected to Western blotting. Membranes were stained with antibodies against Orai1 and TRPC1 and re-blotted with antibody against STIM1 to ensure equal immunoprecipitation of the protein. Representative of 3 independent experiments. (E) Intracellular Ca\textsuperscript{2+} pools were depleted by exposing the cells to CPA (10 µM) in 0Ca\textsuperscript{2+} solution. Re-addition of extracellular Ca\textsuperscript{2+} led to an increase in [Ca\textsuperscript{2+}]\textsubscript{i} which was indicative of SOCE. Subsequently, acute application of BTP-2 (50 µM) inhibited CPA-elicited Ca\textsuperscript{2+} inflow, thus confirming the store-dependent nature of Ca\textsuperscript{2+} entry. (F) Pre-incubation with BTP-2 (20 µM) prevented CPA-induced Ca\textsuperscript{2+} signal on Ca\textsuperscript{2+} readdition to extracellular...
solution. (G) Statistical analysis of the effect exerted by BTP-2 on the peak amplitudes of both Ca\(^{2+}\) release and Ca\(^{2+}\) entry stimulated by CPA (mean±SD, n=5 independent experiments, *p<0.01).

**Figure 4. ADP induces SOCE in human Mks.** (A) ADP (25 µM) evoked the described [Ca\(^{2+}\)]\(_i\) signaling in control Mks. Sample pre-treatment with U-73122 (B) or 2-APB (C) caused the complete lack of response to ADP. (D) Twenty-minute pre-incubation with BTP-2 inhibited ADP-elicited Ca\(^{2+}\) inflow, but not intracellular Ca\(^{2+}\) mobilization, confirming the store-dependent nature of the response to ADP. (E) Mature Mks were treated (+) or not (−) with ADP (25 µM) and lysed. Lysates were immunoprecipitated (IP) with an anti-STIM1 antibody and subjected to Western blotting. Membranes were stained with antibodies against Orai1 and TRPC1 and re-blotted with an antibody against STIM1 to ensure equal immunoprecipitation of the protein. Representative of 3 independent experiments. Mks at day 13 of culture were pre-treated or not with 2-APB, U-73122 and BTP-2, before being seeded on fibrinogen. The percentage of Mks displaying cytoskeletal reorganization (F), the number of adherent Mks (G) and the percentage of proplatelet formation in adhesion on fibrinogen (H) were analyzed compared to untreated controls (CTRL) (mean±SD, n=5 independent experiments, *p<0.01). (I) Mks at day 13 of differentiation were treated with the same compounds described above and left to migrate in a Transwell plate, previously coated with fibrinogen, for 16 hours. Mks that had passed in the lower chamber were collected and counted by phase contrast microscopy (n=3 independent experiments, *p<0.01).

**Figure 5. The role of intracellular and extracellular Ca\(^{2+}\) in regulating Mk response to ADP.** (A) Human Mks at day 13 of culture were stimulated (+) or not (−) with ADP and let to adhere on fibrinogen or fibronectin before being lysed. (B) Parallel samples were
suspended in the PSS or Ca\textsuperscript{2+} free solution (0Ca\textsuperscript{2+}) and pre-treated (+) or not (−) with BAPTA-AM, before being stimulated (+) or not (−) with ADP and finally lysed. All samples were subjected to Western blot analysis for evaluation of FAK, Src Syc, Akt, Erk and MLC phosphorylation. Samples were also probed with anti-CD61 and anti–β-actin antibodies to ensure equal loading. (C) Some cells were let to adhere in the same conditions on fibrinogen. Mks were fixed and stained for immunofluorescence analysis with TRITC-phalloidin (red) and antibody against α-tubulin (green). Nuclei were counterstained with Hoechst 33258 in blue. Images were acquired by an Olympus BX51, magnification 100X, scale bar=20 µm. Representative of 3 independent experiments.

**Figure 6. ADP elicited Ca\textsuperscript{2+} mobilization in human Mks in flow conditions.** Mks at day 13 of culture were plated on fibrinogen-coated cover-slips, at 37 °C in a 5% CO\textsubscript{2} atmosphere and then loaded with 5 µM FLUO 3-AM. Perfusion experiments were started at a shear rate of 50 sec\textsuperscript{-1} and Mks behavior was followed for 22 minutes. (A) Images of Mks in pseudo-colors captured in an observation time of 5 minutes and 23 seconds. Associated single-cell calcium flux recording demonstrate the duration and amplitude of the calcium response. Shear rate and ADP (25 µM) evoked an initial intracellular Ca\textsuperscript{2+} mobilization and a subsequent spatiotemporal redistribution of Ca\textsuperscript{2+}. (B) The same frames were recorded in green fluorescence to better demonstrate the remodeling of human Mks cytoplasm in those cells undergoing to calcium fluxes.

**Figure 7. A role for Ca\textsuperscript{2+} inflow in regulating adhesion on type I collagen.** Cord blood derived-Mks at day 13 of culture were plated on type I collagen-coated cover-slips, at 37°C in a 5% CO\textsubscript{2} in presence or absence of BTP-2. (A) After 16 hours 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 in blue.
Arrows indicate Ms exhibiting cytoskeletal reorganization. Images were acquired by an Olympus BX51, magnification 20X, scale bar=20 µm). The percentages of Mk displaying reorganized cytoskeleton (B) and the numbers of adherent Ms (C) were analyzed compared to untreated controls (CTRL) (mean±SD, n=5 independent experiments, *p<0.01).
Figure 4

Panel A: U-73122, ADP
Panel B: ADP
Panel C: 2-APB, ADP
Panel D: BTP-2, ADP
Panel E: IP:STIM1, Orai1, TRPC1, reblot:STIM1
Panel F: % of cells exhibiting cytoskeletal reorganization
Panel G: n° of adhered cells/field
Panel H: % of platelet forming megakaryocytes
Panel I: n° of migrated cells X100
FIGURE 7

A

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B

% of cells exhibiting cytoskeletal reorganization

CTRL          | BTP-2

C

n° of adhered cells / field

CTRL          | BTP-2

* indicates statistical significance.
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-pyrrrole-2,5-dione (U-73122), 1,2-Bis(2-aminothoxy)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\(_2\)HPO\(_4\), 20 mM NaH\(_2\)PO\(_4\), 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\(^6\) 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, $2 \times 10^5$ 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 $1 \times 10^5$ 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.\textsuperscript{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.

\textbf{[Ca\textsuperscript{2+}]}\textsubscript{i} measurements

Intracellular [Ca\textsuperscript{2+}]\textsubscript{i} measurements were performed as previously described.\textsuperscript{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\textsuperscript{5} cells/well). After 60 minutes at 37°C and 5% CO\textsubscript{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 Achromat 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\textsuperscript{2+}]\textsubscript{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 $[\text{Ca}^{2+}]_i$ 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 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$^\text{EM}$, Andor$^\text{TM}$ 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$_3$VO$_4$ 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% CO2. Subsequently, megakaryocytes were stimulated or not with ADP 25 µM, at 37°C and 5% CO2. 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 ddH₂O. 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.⁵,⁶,⁹ Briefly, 96-well plates with
polycarbonate inserts having 0.3 cm²/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 20x10⁵ 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% CO₂. 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.

REFERENCES


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 coverslips, at 37 °C in a 5% CO₂. (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\textsuperscript{2+} Entry regulates ADP-mediated megakaryocytes migration on extracellular matrix components. (A) Flow cytometry analysis of CD41\textsuperscript{+} 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.