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Human thrombopoiesis depends on Protein kinase Cδ/protein kinase Cε functional couple

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ABSTRACT

A deeper understanding of the molecular events driving megakaryocytopoiesis and thrombopoiesis is essential to regulate *in vitro* and *in vivo* platelet production for clinical applications. We previously documented the crucial role of PKCε in the regulation of human and mouse megakaryocyte maturation and platelet release. However, since several data show that different PKC isoforms fulfill complementary functions, we targeted PKCε and PKCδ, which show functional and phenotypical reciprocity, at the same time as boosting platelet production *in vitro*. Results show that PKCδ, contrary to PKCε, is persistently expressed during megakaryocytic differentiation, and a forced PKCδ down-modulation impairs megakaryocyte maturation and platelet production. PKCδ and PKCε work as a functional couple with opposite roles on thrombopoiesis, and the modulation of their balance strongly impacts platelet production. Indeed, we show an imbalance of PKCδ/PKCε ratio both in primary myelofibrosis and essential thrombocythemia, featured by impaired megakaryocyte differentiation and increased platelet production, respectively. Finally, we demonstrate that concurrent molecular targeting of both PKCδ and PKCε represents a strategy for *in vitro* platelet factories.

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Introduction

Platelets are circulating anucleate elements derived from megakaryocytes (MK), with major roles in hemostasis, thrombosis and inflammation.[1,2] Appropriate numbers and function of circulating platelets are essential in hemostasis. Indeed, uncontrolled platelet release and activation is associated with thrombotic risk.[3,4] On the contrary, low levels of platelets, as well as their functional defects, might compromise the healing of wounds, resulting in bleeding.[5] The therapeutic strategy to prevent severe bleeding is platelet transfusion; however, the use of platelet units derived from human donors has several limitations.[6,7] Consequently, both scientific and technological efforts are currently active for generating large platelet supplies, including *in vitro* platelet producing systems and pharmacological treatments able to modulate *in vivo* thrombopoiesis and platelet production.[8,9] A deeper understanding of the molecular regulation of thrombopoiesis clearly plays a key role in this context.

Thrombopoiesis is a complex process resulting in the generation of thousands of platelets from a single megakaryocyte which, following polyploidization, forms elongated cellular processes called proplatelets (proPLT).[10] Several molecules, including transcription factors and their intermediates, have been found to be involved in the regulation of this process and the perturbation of the expression and activity of these proteins leads to alterations in platelet number, morphology or function.[11-15]

Protein kinase C (PKC) is a family of serine-threonine kinase involved in many cellular functions, including cell death, proliferation, migration and differentiation.[16,17] Protein Kinase C epsilon (PKCε) and Protein Kinase C delta (PKCδ) are both members of the novel sub-family of PKCs, which can be considered as "yin and

yang" because of their antithetical roles in several cellular functions.[18] PKCε is largely considered as an oncogene because of its anti-apoptotic and pro-proliferative functions,[19] whereas PKCδ generally slows down proliferation and induces cell cycle arrest and apoptosis.[20,21] In the heart, they are among the most widely expressed PKC isoforms, playing an opposite role in ischemic-reperfusion preconditioning.[22,23] In the hematopoietic system it has been demonstrated that protein expression levels of ε and δ isoforms are opposite during erythroid differentiation.[24-26] Moreover, while PKCε down-regulation sensitizes primary acute myeloid leukemia (AML) blasts to the apoptogenic and pro-differentiative effects of TRAIL,[27] PKCδ activation mediates pro-differentiative and antileukemic effects of statin and INF-α in AML blasts, including acute promyelocytic leukemia cells.[28-30]

We previously demonstrated that PKCε has a key role in human megakaryocytopoiesis *in vitro*[27,31] and platelet function *in vivo*,[3] as well as in proPLT production in the murine model.[32] Specifically, PKCε levels increase in the early phase of *in vitro* human megakaryocytic (MK) differentiation and decrease in the late phase before platelets release,[31] and a forced PKCε overexpression prevents MK full maturation,[31] while its down-regulation increases MK differentiation.[33] Additionally, more recently we have demonstrated that primary MK from myelofibrotic (PMF) patients express higher levels of PKCε than those from healthy donors (HD), and that PKCε inhibition in PMF restores a *bona fide* normal MK differentiation.[33] Although in murine models it has been demonstrated that PKCδ deficiency enhances megakaryopoiesis,[34] its role in human megakaryocytopoiesis still remains unexplored. The few data available from the literature shows increased levels of the delta isoform in K562 and HEL cell lines when committed to megakaryocytic differentiation.[35-37]

On these bases we hypothesized that PKCε and PKCδ may have an antithetical role in human MK differentiation and platelet formation. Therefore we investigated herein the role of PKCδ during *in vitro* human normal and malignant megakaryocytopoiesis and the effects of PKCε and PKCδ modulation on platelet release, in the translational perspective of clinical applications.

Methods

CD34+ cell isolation and cell culture

Primary CD34+ cells were isolated from peripheral blood of healthy donors, primary myelofibrosis (PMF) patients, and essential thrombocythemia (ET) patients. Samples were collected following written informed consent and approval by the Ethical Committee of Parma University Hospital, Italy. Clinical and laboratory characteristics of PMF and ET patients are reported in Table 1. Cells were cultured for up to 14 days in serum free X-Vivo medium supplemented with recombinant human thrombopoietin, recombinant human stem cell factor and recombinant human interleukin-3. For details see the *Online Supplementary Material.*

shRNA cell infection

For shRNA-based gene silencing we used a pLKO.1 lentiviral vector encoding short hairpin RNAs (shRNA) against human PKCδ and, as control, a MISSION pLKO.1-puro Non-Target

Table 1. Clinical and laboratory characteristics of PMF and ET patients.

PMF patients										
Code	Sex, age	JAK2 V617F	WBC mm³	Hb g/dL	PLT mm³	Blast %	Spleen Ø cm	IPSS*	DIPSS#	Constitutional symptoms
PMF1	F, 65	pos	10,130	11.6	360,000	0	18,5	-	Int-1	yes
PMF2	M, 35	pos	30,510	11.2	257,000	3	28,5	-	High	yes
PMF3	M, 64	pos	66,200	8.2	525,000	1	22	-	High	yes
PMF4	M, 33	pos	27,260	10.9	210,000	2	23.5	High	-	yes
PMF5	M,55	neg	5,650	13.6	784,000	0	9	Low	-	no
PMF6	M,66	pos	5,000	7.6	107,000	8	12		High	yes
PMF7	F, 68	nd	8,740	10.5	1,080,000	0	8	Int-1	-	no
PMF8	M, 65	pos	25,100	10.8	843,000	1	14	-	Int-1	no
PMF9	M, 59	pos	6,120	15.6	950,000	0	12.6	Low	-	no
ET patients										
Code	Sex, age	JAK2	WBC	Hb	Ht	PLT	Spleen Ø	IPSET		thrombosis
		V617F	mm3	g/dl	(%)	mm3	cm			
ET1	F, 12	nd	11,390	14.4	43.2	661,000	11.5	int		no
ET2	F, 77	pos	7,920	14	43.4	633,000	10.5	high		yes
ET3	F, 55	pos	8,200	13.8	41	689,000	9.6	low		no
ET4	F, 66	pos	13,380	15.4	45.7	951,000	8	high		no
ET5	F, 73	neg	6,860	13.9	41.3	642,000	8.5	int		no
ET6	F, 47	pos	11,210	13.8	41.4	565,000	9	int		no
ET7	F, 85	pos	10,120	15.3	46.6	736,000	12	int		no

**IPSS (International Prognostic Scoring System) has been calculated for patients with newly diagnosed disease at time of sample collection. #DIPSS (Dynamic International Prognostic Scoring System) has been calculated for patients with ongoing disease at time of sample collection. IPSET (International Prognostic Score for ET), has been calculated for patients with newly diagnosed disease at time of sample collection. Int-1: intermediate-1; Int: intermediate; neg: negative; nd: not determined; pos: positive.*

shRNA Control Plasmid, containing an shRNA insert that does not target any known genes from any species. Cells were infected at Day 8 of TPO-culture, selected according to puromycin-resistance cells and cultured for up to 14 days. For details see the *Online Supplementary Material*.

Pharmacological inhibition and activation of PKCδ and PKCε activity

PKCδ and PKCε activities were inhibited by δV1-1 (SFNSYELGSL) and by εV1-2 (CEAVSLKPT) peptides, respectively, whereas PKCδ or PKCε activities were enhanced by using ψδRACK (MRAAEDPM) or ψεRACK (CHDAPIGYD) peptides.[23,38] For details see the *Online Supplementary Material*.

Morphological evaluation of MK differentiation

At 14 days of culture, cells were analyzed using a phase contrast microscope (40X/0.5NA). The percentage of megakaryocytes extending proPLT and cell diameter were determined using ImageJ software analyzing a minimum of 100 cells for each treatment from at least 4 independent experiments. For details see the *Online Supplementary Material*.

Flow cytometric analysis

Flow cytometry analyses were performed at day 14 of culture.

Cell culture viability was assessed by FITC conjugate Annexin V (ACTIPLATE; Valter Occhiena, Torino, Italy) in Ca^{2+} and PI staining buffer, following manufacturer's protocol.

For ploidy analysis, cells were permealized with 70% ethanol overnight and incubated in PBS containing PI $80*10^{-6}$ mmol/L and RNAse-A $7*10^{-3}$ mmol/L for 15 minutes before flow cytometry analysis.

Platelets produced in culture were quantified by staining with anti-CD41-RPE and Calcein AM and adding a fixed volume of calibration beads at known concentration, as previously described.[27,39,40]

Analysis of the samples was performed by a FC500 flow cytometer and the Expo ADC software (Beckman Coulter). For details see the *Online Supplementary Material*.

Western blot

Cultured cells were collected on days 0, 3, 6, 9 and 14 for healthy donors and on day 14 for PMF and ET patients. Cells were lysed and 25 μg of proteins from each sample were run on SDS-acrylamide gels, blotted onto nitrocellulose membranes and incubated with specific primary antibodies. Specifically, we used mouse monoclonal anti-PKCδ antibody, rabbit polyclonal anti-PKCε antibody, rabbit polyclonal anti-Bax antibody, rabbit polyclonal anti-Bcl-xL antibody and monoclonal anti-GAPDH antibody, and secondary antibody peroxidase-conjugated anti-rabbit or peroxidase-conjugated anti-mouse IgG. Proteins were resolved by a chemiluminescence detection method and densitometric analyses were performed by using the ImageJ software system.

Statistical analysis was performed using a t-test or analysis of variance (ANOVA) and Tukey's test, when applicable. For details see the *Online Supplementary Material*.

Results

PKCδ/PKCε and Bax/Bcl-xL expression levels are differently modulated during MK differentiation

In agreement with our previous studies in human megakaryocyte cultures,[31] PKCε protein expression increases during the early phases of MK differentiation, declining in the final steps of this process. On the contrary, herein we find that human PKCδ levels rise at the beginning of megakaryocytopoiesis, remaining high throughout the entire maturation process (Figure 1A,B).

On the basis of the previous results obtained by our[31,33] and other groups,[41] we proceeded to assess the level of expression of Bcl-xL and Bax, involved in both normal and neoplastic megakaryocytic differentiation and known as downstream mediators of PKCε anti-apoptotic and PKCδ pro-apoptotic effects.[42]

We found that both Bcl-xL and Bax expressions are significantly modulated in differentiating MKs, with a kinetic similar to PKCε and PKCδ, respectively (Figure 1A,C).

PKCδ down-regulation reverses the normal expression of Bcl-xL and Bax

We previously demonstrated that during the late phases of MK differentiation the forced expression of PKCε induces Bcl-xL up-regulation.[31] Taking advantage of PKCδ-specific shRNA, we sought to determine whether PKCδ expression was necessary to keep Bcl-xL and Bax expression at the levels required for a successful megakaryocytopoiesis. Therefore we used recombinant lentiviral vectors to introduce and stably express shRNA that specifically target PKCδ into MK differentiating cells at day 8 of culture. Analysis of puromicyn-selected megakaryocyte cultures at day 14 (day 5 post-infection) revealed that abrogation of PKCδ was specific, not modifying the expression of PKCε (Figure 2A,B). However, the selective down-regulation of PKCδ dramatically reduces Bax while, to the contrary, boosting Bcl-xL expression (Figure 2A,B). The densitometric analysis (Figure 2B) of Western blot assays clearly shows the significant modulation of the tested proteins only in the presence of PKCδ-specific shRNA (shPKCδ), as compared to the samples infected with control shRNA (shCT), which are similar to uninfected controls.

PKCδ down-regulation impairs MK differentiation and platelet formation

We previously demonstrated that in mouse MK differentiation the PKCε down-regulation impairs proplatelet production.[32] Furthermore, Kostyak and colleagues have shown that, in a mouse model, PKCδ down-regulation reinforces MK differentiation and platelet production.[34] Since it is well documented that PKCε and PKCδ have opposite expression and function in mouse *versus* human platelets,[43] we hypothesized that high levels of PKCδ are necessary for adequate human MK differentiation and platelet release.

Indeed, analysis of puromycin-selected human MK cultures at day 5 post-infection revealed that abrogation of PKCδ impaired MK differentiation (Figure 3). We showed that PKCδ-specific shRNA (shPKCδ) infected cells resulted more viable (Figure 3A), smaller (Figure 3B) and less polyploid (Figure 3C,D), as compared to controls (Uninfected and shCT).

Moreover, although few residual branched protrusions could still be observed, proPLTs generated by shPKCδ-infected cells were characterized by few abortive branches (Figure 4A). On the contrary, shCT proPLTs, as well as uninfected samples, were characterized by the presence of proPLTs formation (Figure 4A).

Platelet release in the culture medium is the terminal step of MK differentiation and, in our system, we

observed a reduction of greater than 50% in platelet numbers in PKCδ knockout cultures (Figure 4B).

The PKCδ and PKCε balance is altered in human pathological megakaryocytopoiesis.

In our model, PKCε and PKCδ have opposite expression levels at the end (day 14) of MK differentiation (Figure 1). We hypothesized that the proper expression of both PKC isoforms could be critical for terminal megakaryocytopoiesis and platelet production. In order to test our speculation, CD34⁺ cells were isolated from the peripheral blood of both patients affected by PMF and ET, which are hematologic neoplasms characterized by abnormal MK differentiation and platelet production. Specifically, MKs generated *in vitro* from PMF CD34⁺ cells show an impaired differentiation and proplatelet formation; conversely, an increase in proplatelet formation is normally observed in ET CD34⁺ cell cultures.[33,44] PMF, ET and HD isolated CD34⁺ cells were therefore cultured up to day 14 in the presence of TPO, in order to induce MK differentiation, and then collected for Western blot analysis (Figure 5). As compared to HD, PKCε and Bcl-xL expression was significantly higher in PMF (in agreement with published data[33,45]), and significantly lower in ET (Figure 5A). On the contrary, PKCδ and Bax showed an opposite modulation, being significantly increased in ET and almost halved in PMF, (Figure 5A), hinting again at an antithetical role of PKCε and δ on thrombopoiesis.

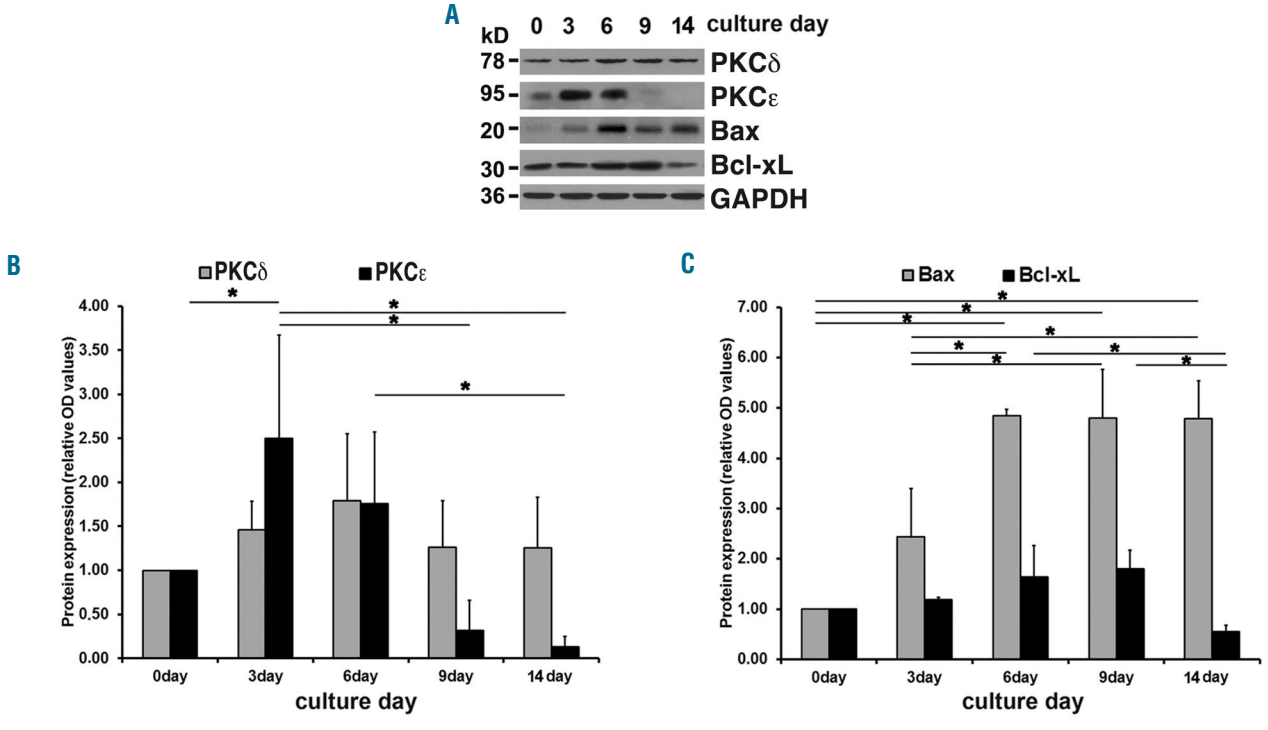

Figure 1. PKCδ/PKCε and Bax/Bcl-xL expression levels are differently modulated during MK differentiation. (A) Western blot detection of PKCδ, PKCε, Bax, Bcl-xL protein expression in CD34+-derived MK cultures. GAPDH was monitored for protein loading. (B) Relative PKCδ and PKCε protein expression during megakaryocytic differentiation of human CD34⁺ cells normalized for GAPDH expression levels. Densitometric measurements of Western blots from 3 replicates were performed by ImageJ software (means ± SD; **P*<0.05 ANOVA and Tukey's Tests), Error Bar = SD. (C) Relative Bax and Bcl-xL protein expression during megakaryocytic differentiation of human CD34⁺ cells normalized for GAPDH expression levels. Densitometric measurements of Western blots from 3 replicates were performed by ImageJ software (means ± SD; * *P*<0.05 ANOVA and Tukey's Tests), Error Bar = SD.

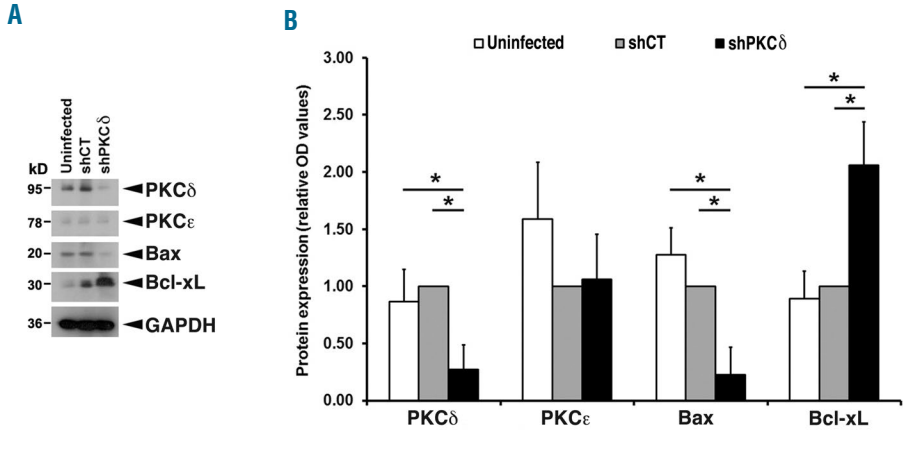

Figure 2. PKCδ down-regulation reverses the normal expression of Bcl-xL and Bax. (A) Western blot detection of PKCδ, PKCε, Bax and Bcl-xL in uninfected CD34⁺-derived MK cultures (Uninfected) and in puromycin-selected CD34+-derived MK cultures infected with PKCδ-specific shRNA (shPKCδ), and with Non-Target shRNA control (shCT) at day 5 post-infection. GAPDH was monitored for protein loading. (B) Densitometric analyses of proteins expression, normalized for GAPDH expression levels and expressed as fold increase of shCT, were performed using ImageJ software. Densitometric measurements of Western blots from 4 replicates (means ± one SD; **P*<0.05 ANOVA and Tukey's Tests), Error Bar = SD.

In summary, at day 14 of culture decreased PKCδ/PKCε and Bax/Bcl-xL ratios typify diseases –like PMF- characterized by impaired MK differentiation and proplatelet formation. On the contrary, these ratio values increase in MK culture characterized by enhanced megakaryocytopoiesis and increased proplatelet and platelet formation, like in ET (Figure 5B).

The amount of platelet production can be modified by modulating PKCε/PKCδ function

Given these results, we asked whether the pharmacological modulation of PKCε and PKCδ activity might impact platelet formation in normal and pathologic conditions. MK differentiating cells were treated with specific activatory and/or inhibitory peptides at day 8 and then cultured for a further 5 days. In MK precursors, both the concomitant inhibition of PKCε and activation of PKCδ, or activation of PKCε and inhibition of PKCδ activity affect thrombopoiesis (Figure 6). Indeed, in normal MK precursors, the simultaneous inhibition of PKCδ and activation of PKCε (δV1-1/ψεRACK) halves the percentage of MKs producing proplatelets (Figure 6A) and the number of platelets released in culture (Figure 6B). Conversely, the concurrent PKCδ activation and PKCε inhibition (ψδRACK /εV1-2) significantly increase both the percentage of MKs producing proplatelets and platelets release (Figure 6 C,D).

We then tested whether PKCε and PKCδ pharmacological modulation could affect the expression levels of the downstream effectors Bcl-xL and Bax.

As expected, the combination of peptides that reduces platelet output, (δV1-1/ψεRACK), is also capable of reducing Bax and increasing Bcl-xL expression levels, while the combination of peptides that increase platelet production, (ψδRACK /εV1-2), has the opposite effect on Bax and Bcl-xL (Figure 6E). This data further reinforces the PKCδ/PKCε and Bax/Bcl-xL axis in the context of thrombopoiesis.

Finally, we investigated whether pharmacological modulation of the two studied novel PKC isoforms could impact on *in vitro* platelet production in PMF and ET malignant megakaryocytopoiesis. We found that the combination of PKCε inhibition and PKCδ activation was capable of increasing platelet release from PMF-derived MK and, conversely, PKCε activation combined with PKCδ inhibition was able to reduce platelet output from ET-derived MK (Figure 6F).

Collectively, this data shows that platelet production can be modulated *in vitro* by tuning PKCε/PKCδ activity, likely *via* Bax and Bcl-xL.

Discussion

Megakaryocytopoiesis is the process by which hematopoietic stem cells differentiate into megakaryocytes, eventually capable of releasing mature platelets into the bloodstream through a process called throm-

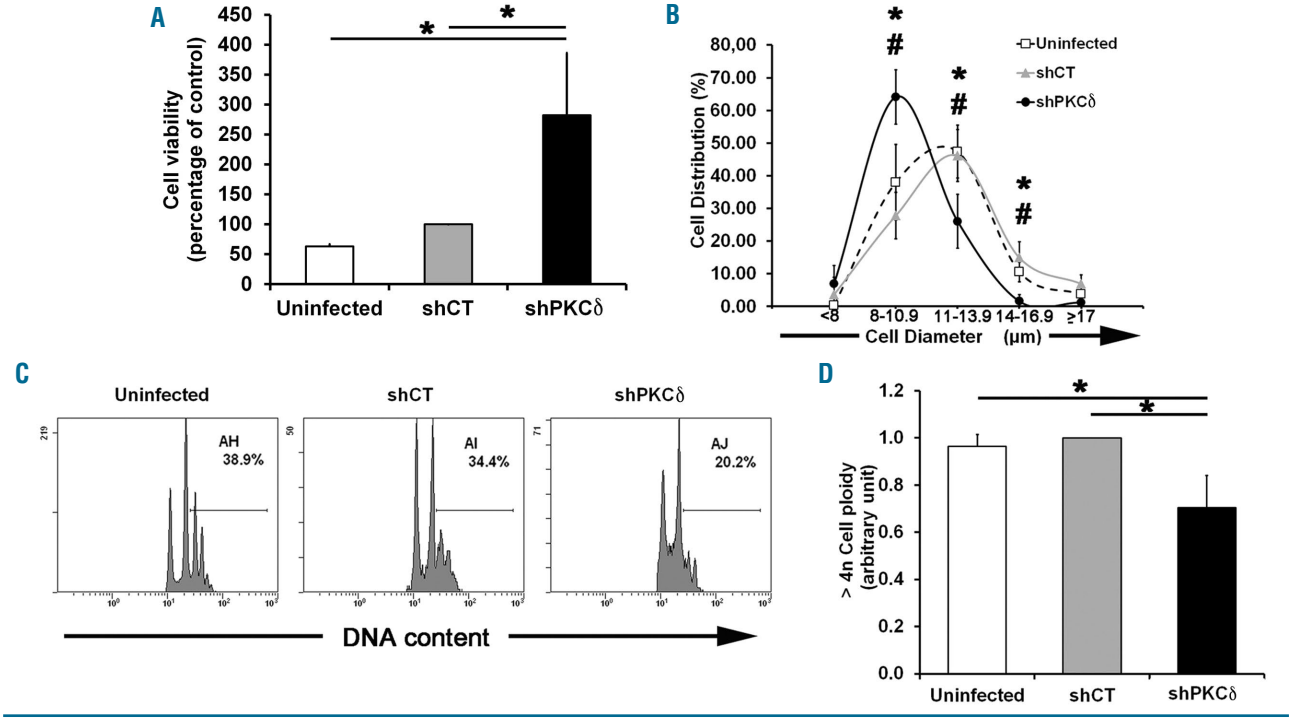

Figure 3. PKCδ down-regulation impairs MK differentiation. (A) Cell viability analysis of uninfected CD34⁺-derived MK cultures (Uninfected) and puromycin-selected CD34⁺-derived MK cultures infected with PKCδ-specific shRNA (shPKCδ), and with Non-Target shRNA control (shCT) at day 5 post-infection. Percentage of Annexin V-/Propidium Iodide- cells from 3 replicates (cells data expressed as percentage of shCT; means ± one SD; **P*<0.05 ANOVA and Tukey's test), Error Bar = SD. (B) Analysis of size distribution within uninfected CD34⁺-derived MK cultures (Uninfected) and puromycin-selected CD34⁺-derived MK cultures infected with PKCδ-specific shRNA (shPKCδ), and with Non-Target shRNA control (shCT) at day 5 post-infection. Percentage of cells with a given diameter range of 3 replicates. (mean ± one SD; **P*<0.05 shPKCδ *vs.* Uninfected, #*P*<0.05 shPKCδ *vs.* shCT, ANOVA and Tukey's test), Error Bar = SD. (C) Representative histogram of cell ploidy analysis of uninfected CD34⁺-derived MK cultures (Uninfected) and puromycin-selected CD34⁺-derived MK cultures infected with PKCδ-specific shRNA (shPKCδ), and with Non-Target shRNA control (shCT) at day 5 post-infection. (D) Percentage of cells with a DNA content >4N of uninfected CD34⁺-derived MK cultures (Uninfected) and puromycin-selected CD34⁺-derived MK cultures infected with PKCδ-specific shRNA (shPKCδ), and with Non-Target shRNA control (shCT) at day 5 post-infection. Cells data were obtained from 3 replicates and expressed as an arbitrary unit of shCT (means ± one SD; **P*<0.05 ANOVA and Tukey's test), Error Bar = SD.

bopoiesis. The entire process is characterized by a progressive increase of cellular dimensions, DNA content and, finally, proplatelet formation and fragmentation.[46]

A deeper understanding of the molecular events driving megakaryocytopoiesis and thrombopoiesis is essential: i) to develop new drugs able to overcome the cellular metabolic key nodes of MK maturation and platelet production that characterize primary thrombocytopenias, thrombocytoses, or accompany different hematopoietic disorders; ii) to achieve massive platelet production *in vitro*. Indeed, *ex vivo* MK cultures and *in vivo* MK infusion are being developed as strategies to obtain an unlimited, donor-independent supply of platelets for clinical applications.[8,9]

Solid data emerged from our and other groups in recent years showing that PKCε has a specific role in the regulation of human megakaryocytopoiesis.[47] Nevertheless, sparse data, particularly in non-human hematopoietic and other systems, also show that PKC functions may not be necessarily confined to one specific isoform, whereas they can also be surrogated by members of the same protein family. For instance, human mature platelets do not express PKCε but do express PKCδ, whereas mouse platelets do exactly the opposite.[48] Such characteristics complicate the translation of basic science discoveries in

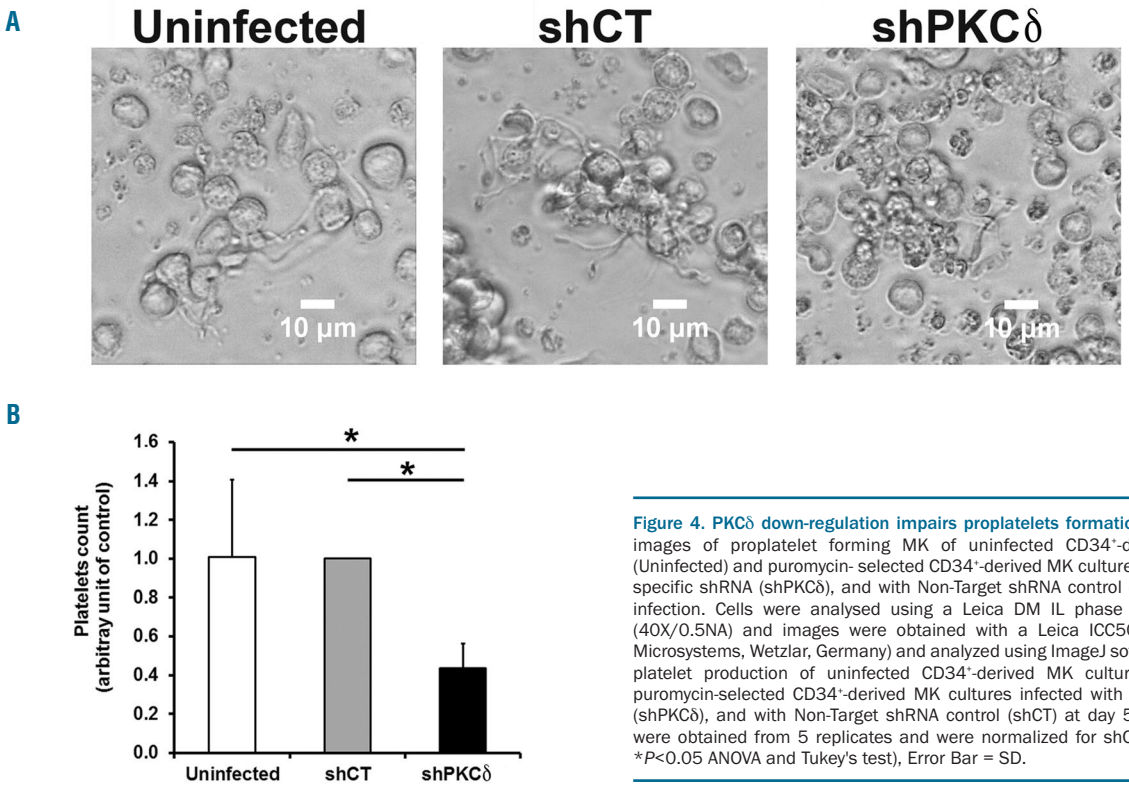

Figure 4. PKCδ down-regulation impairs proplatelets formation. (A) representative images of proplatelet forming MK of uninfected CD34⁺-derived MK cultures (Uninfected) and puromycin- selected CD34⁺-derived MK cultures infected with PKCδ-specific shRNA (shPKCδ), and with Non-Target shRNA control (shCT) at day 5 post-infection. Cells were analysed using a Leica DM IL phase contrast microscope (40X/0.5NA) and images were obtained with a Leica ICC50 HD camera (Leica Microsystems, Wetzlar, Germany) and analyzed using ImageJ software. (B) Analysis of platelet production of uninfected CD34⁺-derived MK cultures (Uninfected) and puromycin-selected CD34⁺-derived MK cultures infected with PKCδ-specific shRNA (shPKCδ), and with Non-Target shRNA control (shCT) at day 5 post-infection. Data were obtained from 5 replicates and were normalized for shCT (means ± one SD; *P<0.05 ANOVA and Tukey's test), Error Bar = SD.

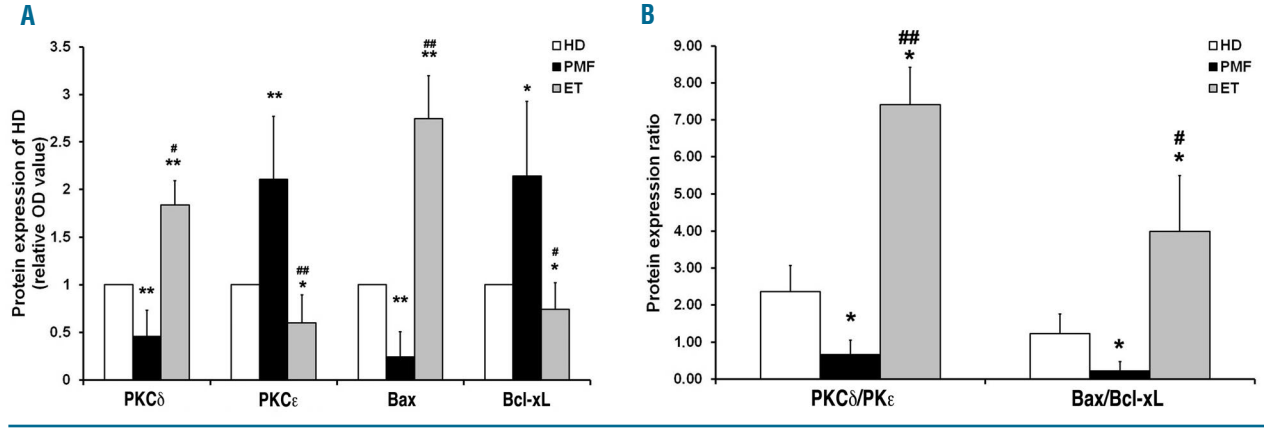

Figure 5. The PKCδ and PKCε balance is impaired in human pathological megakaryocytopoiesis. (A) Densitometric analysis of PKCδ, PKCε, Bax and Bcl-xL in human CD34⁺-derived MK at day 14 of differentiation of healthy donor (HD), PMF and ET patients. Protein expression is normalized for GAPDH expression levels, and expressed as fold increase of HD. Densitometric measurements of Western blots from 4 replicates is performed using ImageJ software (means ± one SD; *P<0.05 *vs.* HD t-test; **P<0.01 *vs.* HD t-test; #P<0.05 *vs.* PMF t-test; ##P<0.01 *vs.* PMF t-test), Error Bar = SD. (B) Densitometric analysis of PKCδ, PKCε, Bax and Bcl-xL in human CD34+-derived MK at day 14 of differentiation of of HD, PMF and ET patients. Protein expression is normalized for GAPDH expression levels, and expressed as the ratio between PKCδ and PKCε, and between Bax and Bcl-xL. Densitometric measurements of Western blots from at least 4 replicates is performed using ImageJ software (means ± one SD; *P<0.05 *vs.* HD t-test; #P<0.05 *vs.* PMF t-test; ##P<0.01 *vs.* PMF t-test), Error Bar = SD.

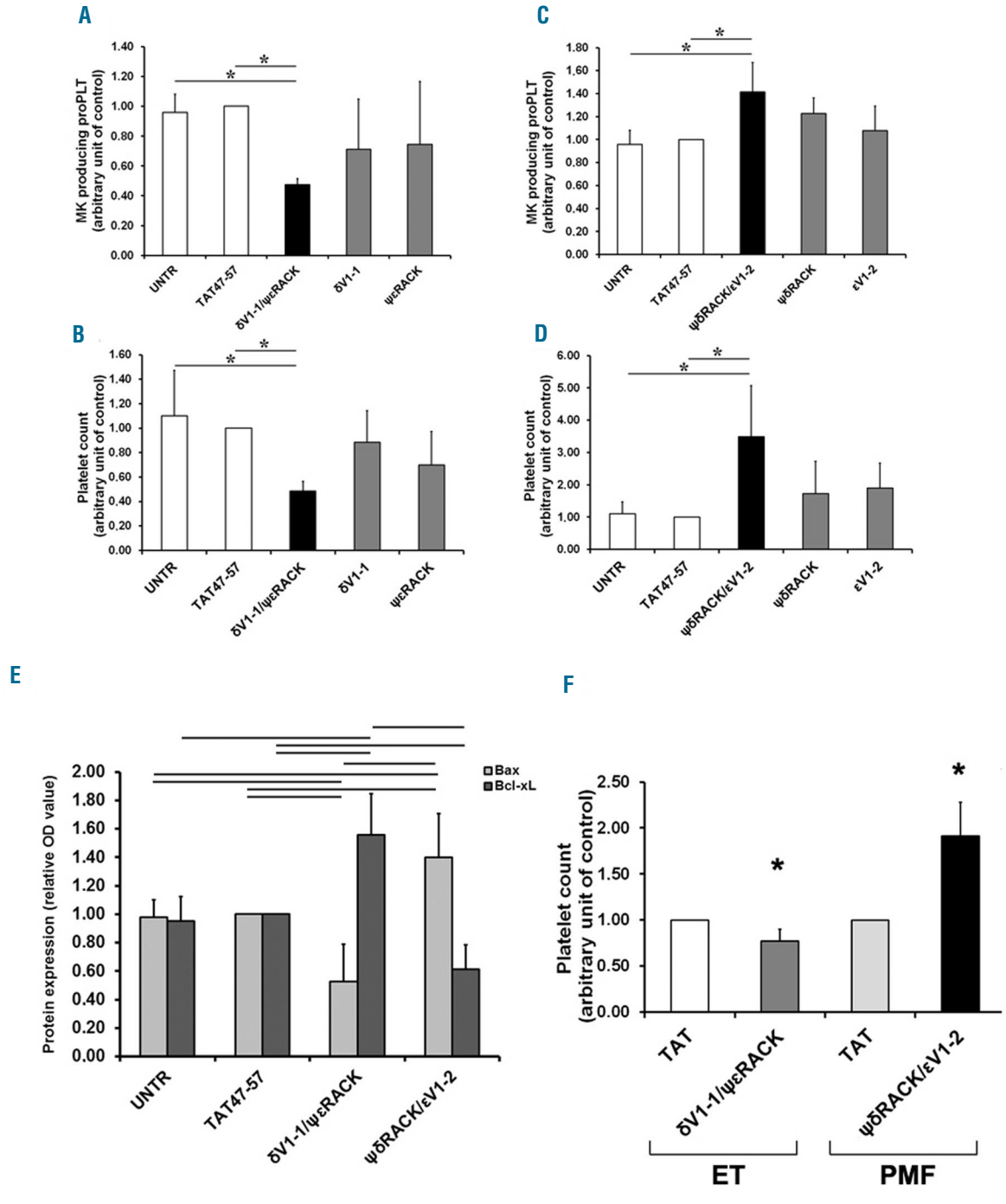

Figure 6. The amount of platelet production can be revised by modulating PKCε/PKCδ function. (A) Analysis of proplatelet producing megakaryocytes of untreated (UNTR), treated with TAT_{47-57} peptide (TAT_{47-57}), PKCδ inhibitor peptide, PKCε activator peptide alone (δV1-1 and ψεRACK, respectively) or in combination (δV1-1/ ψεRACK) cultures, at day 5 post-treatment. Data are expressed as percentage of megakaryocyte extending proplatelet analysing 100 cells for each treatment from at least 3 independent experiments. (means ± one SD; **P*<0.05, ANOVA and Tukey's Test), Error Bar = SD. (B) Analysis of platelet production of untreated (UNTR), treated with TAT_{47-57} peptide (TAT_{47-57}), PKCδ inhibitor peptide, PKCε activator peptide alone (δV1-1 and ψεRACK, respectively) or in combination (δV1-1/ ψεRACK) cultures, at day 5 post-treatment. Data are expressed as an arbitrary unit of control (TAT_{47-57}) from at least 3 independent experiments. (means ± one SD; **P*<0.05, ANOVA and Tukey's Test), Error Bar = SD. (C) Analysis of proplatelet producing megakaryocytes of untreated (UNTR), treated with TAT_{47-57} peptide (TAT_{47-57}), PKCδ activator peptide, PKCε inhibitor peptide alone (ψδRACK and εV1-2, respectively) or in combination (ψδRACK / εV1-2) cultures, at day 5 post-treatment. Data are expressed as percentage of megakaryocyte extending proplatelet analysing 100 cells for each treatment from at least 3 independent experiments. (means ± one SD; **P*<0.05, ANOVA and Tukey's Test), Error Bar = SD. (D) Analysis of platelet production of untreated (UNTR), treated with TAT_{47-57} peptide (TAT_{47-57}), PKCδ activator peptide, PKCε inhibitor peptide alone (ψδRACK and εV1-2, respectively) or in combination (ψδRACK / εV1-2) cultures, at day 5 post-treatment. Data are expressed as an arbitrary unit of control (TAT_{47-57}) from at least 3 independent experiments. (means ± one SD; **P*<0.05, ANOVA and Tukey's Test), Error Bar = SD. (E) Densitometric analysis of Bax and Bcl-xL in human CD34$^+$-derived MK at day 14 of differentiation of healthy donor untreated (UNTR), treated with TAT_{47-57} peptide (TAT_{47-57}), with PKCδ inhibitor- and PKCε activator peptide (δV1-1/ ψεRACK), with PKCδ activator- and PKCε inhibitor peptide (ψδRACK / εV1-2). Protein expression is normalized for GAPDH expression levels, and expressed as fold increase of TAT_{47-57}. Densitometric measurements of Western blots from 4 replicates were performed by ImageJ software (means ± SD; ANOVA and Tukey's Tests, statistical significant variations are indicated by the horizontal lines), Error Bar = SD. (F) Analysis of platelet production of human CD34$^+$-derived MK at day 14 of differentiation in ET and PMF patients. ET patients were treated with control peptide (TAT_{47-57}) or with PKCδ inhibitor- and PKCε activator peptide (δV1-1/ ψεRACK), PMF patients were treated with control peptide (TAT_{47-57}) or with PKCδ activator- and PKCε inhibitor peptide (ψδRACK / εV1-2). Data are expressed as an arbitrary unit of control (TAT_{47-57}) from 3 independent experiments. (means ± one SD; **P*<0.05 *vs.* TAT_{47-57}, t-test), Error Bar = SD.

this field to the therapy, and might be the theoretical reason for the limited success of the related clinical trials.[23] Starting from the above mentioned observation of functional and phenotypical "reciprocity" of PKCε and PKCδ in the megakaryocytopoietic systems of mouse and man, we therefore changed our methodological approach and started thinking in terms of "PKC couples" playing a role in a specific cellular pathway of maturation, which, in the case of megakaryocytopoiesis, could most likely be represented by PKCε and PKCδ.

We already know that: i) PKCε increases in the early phases of MK differentiation and then decreases to undetectable levels in the late phases; ii) forced expression of PKCε reduces MK maturation and platelet release.[31] Since PKCε and PKCδ have antithetical roles in many cellular systems,[18] we hypothesized that they may also mediate opposing effects on MK differentiation, concurring, however, to the final success of the process.

Our results show that PKCδ has opposite kinetics and functional roles in megakaryocytopoiesis when compared to PKCε. In fact it is: i) constantly expressed during MK differentiation; ii) high levels of PKCδ are required in the final steps of megakaryocytopoiesis to allow full MK maturation and PLT production. Indeed, PKCδ down-modulation during the later phase of differentiation impairs MK maturation reducing cell dimensions, polyploidization and platelet production: exactly the same alterations induced by PKCε overexpression, and previously described.[31]

To summarize, successful human megakaryocytopoiesis requires both late PKCε down-regulation in the presence of persistently high levels of PKCδ. Of course, our subsequent question was about their downstream effectors. Consistent with our previous data[24,31] and with our theoretical expectations, the experiments with megakaryocytes *in vitro* showed that the downstream effectors of PKCε and PKCδ are represented by two Bcl2-family members, Bcl-xL and Bax. Given the well documented role of apoptosis in MK differentiation,[41,49,50] this result was nicely predictable: proapototic Bax lays downstream PKCδ and is up-regulated in the late phases of megakaryocytopoiesis, whereas antiapoptotic Bcl-xL, that lays downstream PKCε, is down-regulated. Interestingly however, forced PKCδ down-modulation in MKs not only down-regulates Bax but also up-regulates Bcl-xL, further confirming that the two upstream PKCs work as a functional couple.

To both reinforce our hypothesis and give a translational perspective to our findings, we then took advantage of two human haematological disorders characterized by thrombocytopenia or thrombocytosis, where we would expect to find an imbalance between PKCδ and PKCε expression during MK differentiation. We very recently demonstrated that primary myelofibrosis (PMF)-derived megakaryocytes express higher levels of PKCε as compared to healthy subjects, and that its forced down-modulation or inhibition restores a normal MK maturation and platelet formation.[33] On this basis, we herein studied the expression levels of PKCδ, PKCε, Bax and Bcl-xL in human primary myelofibrosis (PMF) (characterized by a platelet count reduction), and in essential thrombocythemia (ET) (characterized by an enhanced MK maturation and platelet production).

As expected, at day 14 of culture we found that both the PMF and the ET CD34$^+$- derived megakaryocytes expressed altered levels of all these target proteins. Indeed, PKCδ/PKCε and Bax/Bcl-xL ratio values were significantly decreased in PMF while, to the contrary, significantly increased in ET, as compared to healthy subjects.

Eventually, we tested the possibility to modulate platelet production both in normal and pathologic MK differentiation, by using PKCδ and PKCε specific, commercially available, activatory and inhibitory peptides already in use for clinical trials.[23] In MKs derived from healthy subjects, the combined inhibition of PKCδ and activation of PKCε significantly reduced platelet production *in vitro*, reducing Bax levels and increasing Bcl-xL levels; conversely, the concurrent activation of PKCδ and inhibition of PKCε boosted platelet production, *via* up-regulation of Bax and down-regulation of Bcl-xL levels.

Additionally, in disease models of abnormal MK differentiations (i.e., PMF and ET), the simultaneous modulation of these two PKC isoforms was capable of reverting, *in vitro*, the altered thrombopoiesis. In fact, the combined inhibition of PKCδ and activation of PKCε significantly reduced platelet production in ET patients; conversely, the concurrent activation of PKCδ and inhibition of PKCε boosted platelet output in PMF patients, proving that a fine pharmacological tuning of both kinases can revert the thrombocytotic phenotype in ET and the thrombocytopenic phenotype in PMF.

Collectively this data show that: i) during human megakaryocytopoiesis PKCδ has an opposite kinetic expression compared to PKCε and their balance is critical for adequate MK maturation and PLT production; ii) PKCδ and PKCε work as a functional couple with opposite roles on thrombopoiesis, and the modulation of their balance strongly impacts platelet production, likely *via* the pathway of Bax and Bcl-xL; iii) as far as we can now say, *ex vivo* both thrombocytopenia and thrombocytosis can be corrected acting on the PKCε/PKCδ system both in normal and pathologic conditions. On this basis, we also suggest that the modulation of both PKCδ and PKCε expression and function might represent a strategy for platelet factories under the proper conditions.

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