SUPPLEMENTARY APPENDIX

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

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Supplementary Material

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 in sodium citrate tubes after written informed consent and approval by the Ethical Committee of Parma University Hospital. Primary $CD34^+$ cells were purified by immunomagnetic positive selection using the $CD34^+$-cell isolation Kit (Milteny Biotech, Gladbach, Germany) in the magnetic field of autoMACS Pro Separator (Milteny Biotech), according to the manufacturer's protocol. Purity of $CD34^+$ cells was immediately checked by a R-phycoerythrin (RPE)-conjugated anti-CD34 mAb (Immunotech, Beckman Coulter, Miami, FL) and flow cytometry; only samples exceeding 95% purity were used for subsequent experiments.

Purified human $CD34^+$ cells were cultured up to 13 days, at an optimal cell density of $1x10^6$ cells/ml, in serum free *X-vivo* medium (Lonza Group, Switzerland) supplemented with recombinant human thrombopoietin (TPO) $11*10^{-6}$mmol/L, recombinant human stem cell factor (SCF) $3*10^{-6}$mmol/L and recombinant human interleukin-3 (IL-3) $0.2*10^{-6}$mmol/L (Peprotech, London, UK). TPO and SCF were renewed every 3 days.

shRNA cell infection.

For shRNA-based gene silencing, pLKO.1 lentiviral vector encoding short hairpin RNAs (shRNA) against human PKCdelta (transcription variant 1, NM_212539 and transcription variant 2, NM_006254; shPKCdelta) were obtained from Open-Biosystem (Thermo Fisher Scientific Inc, Waltham, MA USA). As control (shCT), we used the MISSION pLKO.1-puro Non-Target shRNA Control Plasmid, containing an shRNA insert that does not target any known genes from any species (Sigma-Aldrich, St. Louis, MO, USA). shRNA expressing viruses were produced in 293TL

cells according to standard protocols. Cells were infected at Day 8 of TPO-culture and then cultured in the presence of puromycin, to select infected, puromycin-resistant cells.

At Day 14 of culture, infected MKs were analysed for morphological evaluation by light microscopy and an aliquots from each cultures were collected for flow cytometry and western blot analysis.

Pharmacological inhibition and activation of PKCdelta and PKCepsilon activity.

Anchoring of PKCs to their anchoring proteins, Receptors for Activated C Kinase (RACK) is a required step for PKC functions. Therefore we utilized specific inhibitory peptides acting via disruption of PKC-RACK interaction and specific activatory peptides which mimic RACK binding[23]. More in details, PKCdelta and PKCepsilon activities were inhibited by the δV1-1 (SFNSYELGSL) and by εV1-2 (CEAVSLKPT) peptides, respectively, whereas PKCdelta or PKCepsilon activities were enhanced by using ψδRACK (MRAAEDPM) or ψεRACK (CHDAPIGYD) peptides. Both the PKCdelta-specific peptides (synthesized by Selleckchem, Huston, TX, U.S.A) and the PKCepsilon-specific peptides (synthesized by AnaSpec Inc, Fremont, CA, U.S.A) were coniugated to TAT_{47-57} (CYGRKKRRQRRR) by a cysteine disulfide bound, as carrier peptide. Unconjugated TAT_{47-57} peptide (synthesized by AnaSpec Inc, Fremont, CA, U.S.A) and untreated cells (Untreated) were used as control. TAT_{47-57} (CYGRKKRRQRRR) is a cargo peptide used as control because both the PKCdelta-specific peptides and the PKCepsilon-specific peptides were conjugated to TAT_{47-57} by a cysteine disulfide bound, for delivery into cells.

At Day 8, primary $CD34^{+}$-derived MK cultures, from HD, PMF, ET, were treated with the specific activatory and/or inhibitory peptides, both in combination or alone. All the peptides were used at a final concentration of 10^{-6} mol/L. Cells treated with $2*10^{-6}$ mol/L TAT_{47-57} (TAT_{47-57}) and untreated cells (Untreated) were used as controls. Cells were cultured up to 14 days and peptides were renewed every 48 hours. At Day 14 of culture an aliquots from each cultures were collected for flow cytometry and western blot analysis.

Morphological evaluation of MK differentiation.

At 14 days of culture, cells were analysed using Leica DM IL phase contrast microscope (40X/0.5NA) and images were obtained by Leica ICC50 HD camera (Leica Microsystems, Wetzlar, Germany).

Percentage of megakaryocytes extending proPLT and cell diameter were determined using ImageJ software analysing a minimum of 100 cells for each treatment from at least 4 independent experiments.

Flow cytometric analysis

Flow cytometry was used to analyse the viability and the ploidy of the differentiating cell populations 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, aliquots of cells were permealized with 70% ethanol overnight at 4°C, washed with PBS and incubated in PBS containing PI $80*10^{-6}$mmol/L and RNAse-A $7*10^{-3}$mmol/L (Sigma-Aldrich) for 15 minutes in dark at room temperature before flow cytometry analysis.

Finally, to quantify the platelet production in culture, fixed volumes of the culture supernatants were collected, incubated with anti-CD41-RPE and Calcein AM (to exclude fragments; Sigma-Aldrich) and added to a fixed volume of calibration beads (DAKO, Agilent Techonolgies, Santa Clara CA, U.S.A) at known concentration. Both the platelets and beads populations were simultaneously identified in flow cytometry on the Forward Scatter (FS) *vs* logarithmic Side Scatter (SS) dot plot. The absolute platelets count was performed on the gated $CD41^{+}$/Calcein AM^{+} platelet population in order to identify functional platelets.

Analysis of the samples was performed by an FC500 flow cytometer (Beckman Coulter) and the Expo ADC software (Beckman Coulter).

Western blot

Cultured cells were counted and 1.5 x 10^6 cells were collected on Days 0, 3, 6, 9, 14 for healthy donors, on day 14 for PMF and ET patients, and on day 14, after 5 day of puromycin selection or peptides treatment. Cells were washed in PBS and centrifuged at 200g for 10 min. Pellets were resuspended in a cell lysis buffer ($50*10^{-3}$mol/L Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 0.15 mol/L NaCl; 10^{-3}mol/L EDTA; 10^{-3}mol/L PMSF; 10^{-3}mol/L Na_3VO_4; 10^{-3}mol/L NaF) supplemented with fresh protease inhibitors and protein concentration was determined using BCA™ protein assay kit (Pierce, Thermo Scientific). Twenty five μg of proteins from each sample were ran on 4-20% SDS-acrylamide gels (Biorad), blotted onto nitrocellulose membranes, blocked and incubated with specific primary antibodies diluted as described in maunfacturers' protocols. Specifically, mouse monoclonal anti-PKCdelta antibody (BD Pharmingen, Cat. #610397) was diluted 1:500, rabbit polyclonal anti-PKCepsilon antibody (Merk Millipore, Cat.# 06-991) was diluted 1:1000, rabbit polyclonal anti-Bax antibody and rabbit polyclonal anti-Bcl-xL antibody (Cell Signaling Technology, Inc. Lake Placid, NY, Cat. # 2772 and Cat. # 2762) were diluted 1:500 and 1:1000 respectively and monoclonal anti-GAPDH antibody (Merk Millipore, Cat. # MAB374), 1:5000 dilution was used as protein loading control.

Membranes were washed and incubated for 1.5 hours at room temperature with 1:5000 peroxidase-conjugated anti-rabbit (Pierce, Thermo Fisher Scientific Inc) or with 1:2000 peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich) at room temperature and resolved by ECL Supersignal West Pico Chemiluminescent Substrate detection system (Pierce, Thermo Fisher Scientific Inc). Protein densitometric analyses from western blot assays were performed by using the Image J software system, normalized for GAPDH expression levels.

Statistical analysis was performed by t-Test or using analysis of variance (ANOVA) and Tukey's test, when applicable.