Regulated Expression of the α Isoform of the Human Thromboxane A₂ Receptor During Megakaryocyte Differentiation: A Coordinated Role for WT1, Egr1 & Sp1

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ABSTRACT
Thromboxane plays an essential role in haemostasis, regulating platelet aggregation and vessel tone. In humans, it signals through the TPα and TPβ isoforms that are transcriptionally regulated by distinct promoters, Prm1 and Prm3, respectively. Herein, the consequence of megakaryocytic differentiation on Prm1-directed TPα expression was investigated. Phorbol ester (PMA) treatment substantially increased TPα mRNA and Prm1-directed gene expression in human erythroleukemia (HEL) and K562 cells. Deletional analyses localized the major responsive element(s) to the upstream -8500 and -7504 region while mutation of four WT1/Egr1/Sp1 cis-elements therein established that each contribute to the induction. Moreover, PMA increased Egr1, but not WT1 or Sp1, expression while the NAB1 co-repressor impaired PMA-induction of Egr1 and Prm1-directed gene expression. Chromatin immunoprecipitations established that WT1 is predominantly bound in vivo to the 5’ Prm1 region in non-differentiated HEL cells. In response to PMA, there was initial induction in Egr1 and associated reduction in WT1 binding to Prm1 in vivo which was displaced by Sp1 following sustained treatment. Collectively, data establish that regulated WT1 followed by sequential Egr1 and Sp1 binding to elements within Prm1 mediate repression and subsequent induction of TPα during differentiation into the megakaryocytic phenotype, shedding significant insights into factors regulating TPα expression therein.

Key Words: thromboxane receptor, megakaryocytic differentiation, WT1, Egr1, Sp1, gene, transcription.
INTRODUCTION

Thromboxane (TX) A₂ is a potent mediator of platelet activation and aggregation, as well as acting as a constrictor of vascular and bronchial smooth muscle. It is abundantly produced in megakaryocytes/platelets, activated macrophages and monocytes and plays a key role in haemostasis and in the regulation of vascular tone. Imbalances in the level of TXA₂, or its receptor, are associated with a range of dysfunctions including thrombosis, various hypertensions, atherosclerosis and ischaemic heart disease.

In humans, TXA₂ signals through the α and β isoforms of its cognate G-protein coupled receptor, termed TP. TPα and TPβ transcripts arise by alternative splicing from a single gene located on chromosome 19p13.3. While the significance of having two distinct isoforms of TP in primates is unclear, there is accumulating evidence that TPα and TPβ have distinct physiologic roles. TPα and TPβ differ in their modes of signaling, as well as being subject to differential regulation by desensitization. Moreover, the relative expression of TPα and TPβ mRNA varies greatly in a range of cell and tissue types of vascular origin. The finding that platelets may exclusively express TPα, and that anti-aggregatory autacoids including prostacyclin and nitric oxide act as mediators of TPα desensitization, suggest that TPα may be the isoform that plays a more central role in haemostasis.

As well as displaying differences in their signaling pathways, modes of post-translational regulation and patterns of expression, TPα and TPβ are under the transcriptional control of distinct promoters within the single TP gene. TPα expression is regulated by promoter (Pṛm) 1, while Pṛm3 regulates the expression of TPβ. Hence, considering the central role of TXA₂ in normal and pathophysiologic processes, identification of the factors regulating Pṛm1 and Pṛm3 may lead to a greater understanding of the contributory roles of TPα and TPβ in health and disease. While AP1 and Oct-2 are the key factors regulating basal expression of Pṛm3/TPβ in the HEL cell line, they may also be regulated by both natural and synthetic thiazolidinediones (TZD) peroxisome proliferator-activated receptor (PPAR)γ ligands suggesting that they may have combined therapeutic benefits in the treatment of type II diabetes and associated cardiovascular disease, partly due to their suppression of TPβ expression.

Despite the recognized importance of TPα in haemostasis, contributing to platelet activation status and vascular tone, until recently the factors regulating its expression through Pṛm1 remained largely uncharacterized. Through recent studies, it has been established that the core Pṛm1 is under the control of the general transcription factors Sp1 and Egr1, as well as the more haematopoietic-specific factor NF-E2 in HEL cells. Identification and characterization of an upstream activator region (UAR) revealed central roles for GATA-1 and Ets-1 as positive regulators of Pṛm1 activity. Moreover, WT1 was identified as a transcriptional repressor, binding to three negative regulatory regions within Pṛm1. Collectively, these studies provide an extensive characterization of the factors regulating Pṛm1 and Pṛm3, as well as providing a molecular and genetic basis for understanding the role of TXA₂ and the TP isoforms in haemostasis and vascular disease.

The pluripotent human erythroleukemia (HEL) cell line is frequently employed as an early megakaryoblastic model and can be induced to undergo differentiation toward the platelet progenitor.
megakaryocytic phenotype by cytokines, growth factors and phorbol esters, such as phorbol 12-myristate 13-acetate (PMA). Phorbol ester-induced differentiation of HEL cells is associated with increased expression of megakaryocytic markers including glycoprotein (GP) Ib and GPIIb/IIIa. Moreover, in the context of the potent platelet agonist TXA₂, transcription of the TP gene is increased following PMA-treatment of K562 cells. Considering the recognized importance of TPα in platelets and in haemostasis, the initial aim of the current study was to investigate the consequence of PMA-induced megakaryocytic differentiation of HEL cells on TPα expression. It was also sought to identify the specific factors that lead to changes in TPα expression through their regulation of Prm1 during differentiation of HEL cells toward the megakaryocytic phenotype. The data herein describe a novel mechanism of regulation involving distinct, time-dependent binding patterns for the transcription factors WT1, Egr1 and Sp1 to Prm1 that change during the course of PMA-treatment of HEL cells, providing a molecular basis for understanding TPα expression during megakaryocytic differentiation. Furthermore, key experimental findings in HEL cells have been independently corroborated in the human erythromyeloblastoid leukaemia K562 cell line.
MATERIALS AND METHODS

Materials

Dual Luciferase® Reporter Assay System, pGL3Basic and pRL-Thymidine Kinase (pRL-TK) were from Promega. DMRIE-C®, RPMI 1640 culture media and fetal bovine serum were from Invitrogen. Anti-WT1 (sc-192 X), anti-Sp1 (sc-59 X), anti-Egr1 (sc-110 X), normal rabbit IgG (sc-2027) and goat anti-rabbit horseradish peroxidase (sc-2204) were from Santa Cruz. Anti-HDJ-2 antibody was from Neomarkers. AlexaFluor488-conjugated anti-rabbit antibody was from Molecular Probes® (Invitrogen).

Construction of luciferase-based genetic reporter plasmids

The plasmid pGL3b:Prm1, encoding Prm1 (-8500 to -5895, relative to the translational start codon at +1) from the human TP gene in the pGL3Basic reporter vector, in addition to pGL3b:Prm1B, pGL3b:Prm1C, pGL3b:Prm1D, pGL3b:Prm1E and pGL3b:Prm1K were previously described. The plasmid pGL3b:Prm1F was generated by PCR subcloning using pGL3b:Prm1 as template and sense (5’GAGAGGTACCCTCCAGGCGGTTGGTGCTG3’; nucleotides (nu) -6552 to -6535, where the Kpn1 cloning site is underlined) and antisense (5’AGAGACGCGTCCTTACGAGACCTCATCTCGCGGG3’; complementary to nu -5917 to -5895 of Prm1, where the Mlu1 site is underlined) primers, essentially as previously described. The plasmids pGL3Basic:Prm1GC*(8345), pGL3Basic:Prm1GC*(8281), pGL3Basic:Prm1GC*(8146), pGL3Basic:Prm1GC*(7831), pGL3Basic:Prm1GC*(8345,7831), pGL3Basic:Prm1GC*(8345,8281,7831) and pGL3Basic:Prm1GC*(8345,8281,8146,7831) were previously described.

Assay of luciferase activity

Human erythroleukemic (HEL) 92.1.7 cells and human erythromyeloblastoid leukemia K562 cells were obtained from the American Type Culture Collection and were cultured in RPMI 1640, 10% fetal bovine serum at 37 °C in a humid environment with 5% CO2. Both cell lines were co-transfected with the various pGL3Basic-recombinant plasmids, encoding firefly luciferase, along with pRL-TK, encoding renilla luciferase, using DMRIE-C® transfection reagent as previously described. The medium was supplemented with PMA (100 nM), with PMA (100 nM) plus PD98059 (10 µM) or, as a control, with vehicle [0.1% (v/v) DMSO] ~32h post-transfection. After 16h, cells were assayed for firefly and renilla luciferase using the Dual-Luciferase Reporter Assay System™. The plasmid pCMV5-NAB1, generously donated by Dr. Gerald Thiel, University of Saarland Medical Center, Homburg, Germany, was previously described. To investigate the effects of over-expression of NAB1 on the PMA-mediated induction of Prm1- and Prm3-directed gene expression, HEL or K562 cells were transiently co-transfected with pRL-TK (200 ng) plus either pGL3b:Prm1 (2 µg) or pGL3b:Prm3 (2 µg), along with either the control vector pCMV5 (1 µg) or pCMV:NAB1 (1 µg). Cells were harvested 48h post-transfection and assayed for luciferase activity.
Reverse transcriptase-polymerase chain reaction (RT-PCR) and Southern blot analysis

Total RNA was isolated using TRIzol reagent, where HEL cells or K562 cells (5 x 10⁶ approximately) were preincubated for the indicated times with PMA (100 nM) or, as a control, with vehicle [0.1% (v/v) DMSO]. RT-PCR was carried out with DNase1-treated total RNA using primers to amplify TPα (forward, 5’-GAGATGATGGCTAGCTCCT-3’; reverse, 5’-CCAGCCCCCTGAATCTCA-3’) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA sequences (forward, 5’-TGAAGGTCCGAGTCAACG-3’; reverse, 5’-CATGTGGGCCATGAGGTC-3’), as previously described. Following transfer, RT-PCR products were screened and quantified by Southern blot analysis using 5’ biotinylated probes for TPα (5’[Btn]CTGTCCCACACCAGGAGAAG3’) and GAPDH (5’[Btn]CACCCAGAAGACTGTGGATGGC3’) with detection using the Detector™ HRP Chemiluminescent Blotting Kit (KPL).

Western blot analysis

HEL cells or K562 cells were pre-incubated for the indicated times with 100 nM PMA, 10 µM PD98059, 100 nM PMA plus 10 µM PD98059 or, as a control, with vehicle [0.1% (v/v) DMSO]. Whole cell protein (60 µg per lane) was resolved by SDS-PAGE (10% acrylamide gels) and transferred to polyvinylidene difluoride (PVDF) membrane according to standard methodology. Membranes were incubated with anti-WT1, anti-Sp1, anti-Egr-1 or anti-HDJ-2 sera in 5% non-fat dried milk in 1X TBS (0.01 M Tris/HCl, 0.1 M NaCl) for 2h at room temperature, washed and screened using goat anti-rabbit horseradish peroxidase (sc-2204), followed by chemiluminescence detection as described by the supplier (Roche).

Chromatin immunoprecipitation assays

ChIP assays were performed in HEL cells, as previously described. Briefly, cells pre-incubated with vehicle [0.1% (v/v) DMSO; 16h] or 100 nM PMA for 5, 8 or 16h were harvested by centrifugation (1000 g, 10 min, 4°C), washed in ice-cold PBS and resuspended in serum-free RPMI 1640. Formaldehyde-cross linked chromatin was sonicated to generate fragments of 500 - 1000 bp. Chromatin samples were immunoprecipitated with 10 µg of anti-WT1, anti-Sp1 or anti-Egr1. All antibodies employed were ChIP-validated and have been widely used in the literature for such analyses.

The primers used for the ChIP PCR reactions, their sequences and corresponding nucleotides within Prm1 were: Kin462 (5’CGAGACCCCTGCAGGACTGAGG3’; -8460 to -8437); Kin463 (5’GAGAAGGGAGAAACTGAGCAGCAGAAG3’; -8030 to -8006); Kin456 (5’CTTCCCCAGAGGCAGGTAGGGTGTC3’; -6368 to -6344); Kin109 (5’AGAGACCGGCGTGTTCAGACAATCTGAGC3’; -5917 to -5895); Kin364 (5’TTCGAGGGCTACAGAGTGGACG3’; -1081 to -1061); Kin365 (5’GCAGACCCGCGGCGACG3’; -711 to -695).

Confocal microscopy
HEL cells or K562 cells were seeded at 2 x 10^5 cells in 2 ml RPMI 1640, 10% FBS in 6-well plates pre-coated with poly-L-lysine (0.001% for 1h) and grown at 37°C for 24h prior to incubation with 100 nM PMA for 0, 1, 5, 8, 16 and 24h. Thereafter, cells were fixed in 3.4% paraformaldehyde and permeabilized with 0.2% Triton X-100 for 10 min on ice, after which they were immunolabelled with anti-WT1 (1:1000 dilution of 2 μg/µl stock) and AlexaFluor488 conjugated anti-rabbit, followed by counterstaining with DAPI (1 μg/ml in H2O). Images were captured using Carl Zeiss Lazer Scanning System LSM510 and Zeiss LSM Imaging software for acquiring multi-channel images with filters appropriate for enhanced DAPI & AlexaFluor488.

Subcellular Fractionation

Subcellular fractionation was carried out essentially as described by Niksic et al. in HEL or K562 cells pre-incubated for the indicated times with 100 nM PMA. Briefly, cells were washed and harvested in ice-cold PBS prior to centrifuging at 4,000 rpm for 3 min at 4°C. The cell pellet was resuspended in hypotonic buffer (10 mM Tris HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2 and protease inhibitor cocktail) and incubated on ice for 10 min incubation in ice prior to adding NP40 (1% final concentration in hypotonic buffer). The cells were centrifuged at 14,000 rpm for 1 min and the resulting supernatant retained as the cytoplasmic fraction. The nuclear fraction was prepared by resuspending the pellet in lysis buffer (20 mM HEPES, pH 7.9, 600 mM KCl, 0.2 mM EDTA, 1 mM DTT, and protease inhibitor cocktail) and incubating on ice for 30 min. Thereafter, cells were centrifuged at 14,000 rpm for 10 min and the resulting supernatant retained as the nuclear fraction. Cytoplasmic and nuclear fractions were precipitated using standard TCA precipitation method. Total, nuclear and cytoplasmic fraction protein samples (60 µg per lane) were resolved by SDS-PAGE (10% acrylamide gels) and transferred to polyvinylidene difluoride (PVDF) membrane according to standard methodology. Thereafter, membranes were incubated with anti-WT1, as described above.

Statistical analysis

Statistical analysis of differences were analysed using the two-tailed Student’s unpaired t-test and two-way ANOVA. All values are expressed as mean ± standard error of the mean (SEM). P-values < 0.05 were considered to indicate statistically significant differences, where *, **, *** and **** indicate p < 0.05, p < 0.01, p < 0.001 and p < 0.0001, respectively.
RESULTS

Effect of PMA on TPα mRNA expression and Prm1-directed gene expression in HEL cells

TPα and TPβ are under the transcriptional regulation of Prm1 and Prm3, respectively, within the human TP gene. The overall aim of the current study was to investigate the consequence of PMA-induced megakaryocytic differentiation of HEL cells on TPα expression and to identify the key *trans-acting* factors and *cis*-acting elements within Prm1 that are responsible for such changes. Prm1 is defined as nucleotides -8500 to -5895 located 5’ of the translation initiation codon\(^\text{17}\) and its proximal “core” Prm1 (-6320 to -5895) is under the control of Sp1, Egr1 and NF-E2 *trans*-acting factors.\(^\text{21}\) Additionally, Prm1 contains two upstream activator regions (UAR) 1 (from -7962 to -7717) and UAR2 (from -7717 to -7504). While GATA-1 and Ets-1 were identified as key factors regulating UAR1, the factors regulating UAR2 remain to be identified.\(^\text{21}\) Moreover, WT1 acts as a key repressor of TPα expression by binding to three repressor regions (RRs), namely URR1 (from -8500 to -7962), URR2 (from -6848 to -6648) and RR3 (from -6258 to -6123) within Prm1\(^\text{22}\).

Herein, RT-PCR and Southern blot analysis established that PMA (100 nM; 0–48 h) led to a time-dependent and sustained induction of TPα mRNA expression in HEL cells (*Figure 1A - 1C*). Moreover, pre-incubation with PMA (100 nM; 16h) resulted in a 4-fold increase in Prm1-directed luciferase reporter gene expression (*p* < 0.0001; *Figure 2A & 2B*). In order to localize the key PMA-responsive regulatory domain(s), genetic reporter assays were carried out using a series of plasmids encoding 5’ deletions of Prm1 (*Figure 2A & 2B*). Initially, and consistent with the previous finding that WT1 represses Prm1 activity by binding to elements within URR1\(^\text{22}\), 5’ deletion of Prm1 (-8500) to Prm1B (-7962) resulted in a 2.1-fold increase in basal luciferase expression in vehicle-treated cells (*p* < 0.0001; *Figure 2A*). However, deletion of those nucleotides also reduced the PMA-mediated induction of Prm1-directed gene expression from 4-fold to 2.9-fold (*Figure 2A & 2B*).

Thereafter, consistent with the previous finding that GATA-1 and Ets-1 activate Prm1 by binding to specific elements within UAR1\(^\text{21}\), 5’ deletion of nucleotides from Prm1B (-7962) to generate Prm1C (-7504) resulted in a 2.4-fold decrease in basal luciferase expression of Prm1 (*p* < 0.0001; *Figure 2A*). However, deletion of those nucleotides from Prm1B (-7962) to generate Prm1C (-7504) also decreased the PMA-mediated induction of Prm1-directed gene expression from 2.9-fold to 1.4-fold (*Figure 2A & 2B*). Further 5’ deletion to generate sub-fragments Prm1D (-6848), Prm1E (-6648), Prm1F (-6552) and Prm1K (-6067) abolished PMA-responsiveness of Prm1 (*Figure 2A & 2B*). Hence, these data indicate that the increased transcriptional activity of Prm1 in response to PMA is mediated mainly by *cis*-acting elements located between -8500 and -7504, while elements between -7504 and -6848 may also play a role.

Localization of the site(s) of action of PMA within Prm1 by mutational analysis

As stated, WT1 binds to GC elements at -8345, -8281 and -8146 within URR1 and to another adjacent GC element at -7831 within UAR1 in quiescent HEL cells to repress transcriptional activity of Prm1\(^\text{22}\). Since the GC elements at -8345, -8281, -8146 and -7831 consist of putative binding sites for WT1/Egr1/Sp1 (*Table I*), it was sought to determine if factor(s) binding to these elements contribute to the PMA-mediated increase in Prm1 activity. Site-directed mutagenesis of any of the individual elements at -8345, -8281, -8146 or -7831 within

Prm1 did not substantially abrogate the PMA-induction of Prm1-directed gene expression (Figure 3A). More specifically, the sub-fragments Prm1\(^{GC*(-8345)}\), Prm1\(^{GC*(-8281)}\), Prm1\(^{GC*(-8146)}\) and Prm1\(^{GC*(-7831)}\) displayed 3.8- to 3.9-fold increases in luciferase activity in response to PMA, compared with a 4.3-fold PMA-induction of Prm1 itself (Figure 3A & 3B). To investigate whether the four GC elements act in a cooperative or an independent manner to contribute to the PMA-increase in Prm1 activity, the effect of collectively mutating the sites within Prm1 was examined (Figure 3C & 3D). The introduction of sequential mutations to generate Prm1\(^{GC*(-8345,-8281,-8146,-7831)}\) progressively reduced the PMA-mediated induction of Prm1 activity. Specifically, PMA only yielded a 2.2-fold increase in luciferase activity directed by Prm1\(^{GC*(-8345,-8281,-8146,-7831)}\) compared to the 4.3-fold increase directed by Prm1 itself (Figure 3C & 3D). Additionally, disruption of GC\(^{-7831}\) in Prm1B, which does not contain any of the other three GC elements, resulted in an attenuation of PMA-induction from 2.5-fold to 1.9-fold (Supplementary Figure 1A & 1B). Therefore, it appears that GC elements at -8345, -8281, -8146 and -7831 are responsible, at least in part, for the PMA-mediated increase in Prm1 activity in HEL cells. The finding that mutation of any of the individual GC elements resulted in only a marginal attenuation of the PMA-induction of Prm1, compared to the substantial decrease observed upon combined mutation of all four elements together, to generate Prm1\(^{GC*(-8345,-8281,-8146,-7831)}\), suggests that each elements at -8345, -8281, -8146 and -7831 acts independently to contribute to increased Prm1-directed gene expression in response to PMA.

**Effect of PMA on expression of WT1, Egr1 and Sp1 proteins in HEL cells**

In view of the finding that PMA significantly increased Prm1-directed transcriptional activity and TPα mRNA expression in HEL cells, it was sought to identify the specific transcription factors involved. As stated, mutational analysis of GC elements representing putative overlapping sites for WT1/Egr1/Sp1 at -8345, -8281, -8146 and -7831 indicated that these elements are at least partially responsible for PMA-mediated increases in Prm1-directed luciferase expression. Therefore, it was sought to determine if the levels of expression of WT1, Egr1 or Sp1 altered upon incubation of HEL cells with PMA. While pre-incubation with PMA over a 48h period did not substantially alter the expression of the WT1 doublet at 52/54 kDa (Figure 4 & Supplementary Figure 2), there was a slight decrease in expression of both forms at 24h and 48h post-induction. Concomitant with this, there was a significant increase in Egr1 expression from 1-16h, with the highest induction observed at 5h and 6h (Figure 4 & Supplementary Figure 2). At 24h post-PMA treatment, the level of Egr1 expression returned to basal levels (Figure 4 & Supplementary Figure 2). Pre-incubation with PMA over a 48h period did not result in appreciable changes in Sp1 (Figure 4 & Supplementary Figure 2), a protein expressed as a doublet corresponding to its phosphorylated and non-phosphorylated forms \(^{31}\). There were no appreciable changes over the 48h period in the expression of HDJ-2 (DNA J homologue, Supplementary Figure 2 & data not shown), a molecular chaperone protein which served as an endogenous loading control. Collectively, these data indicate that PMA-induced differentiation of HEL cells is associated with substantial, though transient, increases in Egr1 expression, but does not significantly alter overall expression levels of WT1 or Sp1 over a 48h period.
Investigation of the role of Egr1 expression in mediating increased Prm1 activity in response to PMA

To further explore the possible involvement of Egr1 in mediating increased Prm1-directed luciferase expression in response to PMA, the effect of ectopic expression of the constitutive Egr1 co-repressor NGFI-A-binding protein 1 (NAB1) was investigated (Figure 5A & 5B). While NAB1 over-expression did not significantly affect Prm1-directed luciferase activity in vehicle-treated HEL cells (p = 0.1953; Figure 5A), it significantly reduced the PMA-induction of Prm1 activity (p < 0.0001; Figure 5A & 5B). As a control, the effect of NAB-1 over-expression on the PMA-induction of an alternative promoter, namely Prm3 of the same TP gene, was investigated (Figure 5C & 5D). NAB1 did not affect the PMA-induction of Prm3 in HEL cells (p = 0.823), consistent with the previous finding that the PMA-regulation of Prm3 was actually dependent on an AP-1-, as opposed to an Egr1-dependent transcriptional mechanism 18.

To investigate the role of the extracellular signal-regulated kinase (ERK) 1/2 pathway in mediating the PMA-induction of Prm1, the effect of the MEK1 inhibitor PD98059 on Prm1-directed luciferase expression was investigated (Figure 5E). PD98059 reduced the PMA-induction of Prm1-directed luciferase expression from 4.3-fold to 2.3-fold (p = 0.0011). However, neither PMA alone (p = 0.4721) nor PMA plus PD98059 (p = 0.2693) significantly affected luciferase expression directed by Prm1K, a control sub-fragment that does not respond to PMA-induction. Moreover, PD98059 completely abolished the PMA-induction of Egr1 protein expression in HEL cells, while PMA alone, PD98059 alone, nor PMA plus PD98059 did not have any appreciable effect on WT1 expression (Figure 5F). Collectively, these data indicate that increased expression of Egr1, mediated by PMA-induced activation of ERK signaling, is at least partly responsible for the PMA-induction of Prm1-directed luciferase activity in HEL cells.

To determine the actual time required for PMA to mediate increased Prm1-directed luciferase expression, Prm1-transfected HEL cells were incubated with PMA for 0–48 h (Figure 6). Prm1-directed luciferase activity was significantly increased within 4h of incubation with PMA (p = 0.029; Figure 6) and was continuously increased for the duration of the 48h incubation (p < 0.0001). From the time course assay, PMA-induction of Prm1-transcriptional activity was somewhat multi-phasic, with initial activity reaching a plateau at 5–8h and a subsequent phase at ~12-16h post-induction. This was followed by a more sustained activity at 24-48h post-treatment.

In vivo binding of WT1, Egr1 and Sp1 to GC elements between -8460 and -8006 within Prm1

To investigate the molecular identity of the transcription factor(s) regulating Prm1 activity in response to PMA treatment in vivo, ChIP analyses were carried out using antibodies directed to endogenous WT1, Egr1 and Sp1 and chromatin was extracted from HEL cells pre-incubated with PMA for 5, 8 or 16h. Non-treated or vehicle-treated cells served as controls (Figure 7A & 7B). Initially, ChIPs analyses based on amplicons to the distal 5’ region of Prm1 (between -8460 and -8006, containing GC elements at -8345, -8281 and -8146) were carried out (Figure 7A). In non-treated (0h) or in vehicle-treated (data not shown) cells, amplicons from both input chromatin and the anti-WT1, but not from anti-Sp1 or anti-Egr1, immunoprecipitates were generated.

Figure 7A. ChIP analysis from cells pre-incubated with PMA for 5h yielded amplicons from both input chromatin and the anti-WT1 immunoprecipitate, and to a lesser extent from an anti-Egr1 immunoprecipitate. However, no amplicon was generated from an anti-Sp1 immunoprecipitate (Figure 7A; 5h). Conversely, following pre-incubation with PMA for 8h, PCR resulted in amplification of DNA recovered from both the input chromatin and from an anti-Egr1 immunoprecipitate, but not from anti-WT1 or anti-Sp1 immunoprecipitates (Figure 7A; 8h). ChIP analysis from cells pre-incubated with PMA for 16h (Figure 7A; 16 h) yielded amplicons from the input chromatin and an anti-Sp1 immunoprecipitate, but not from anti-WT1 or anti-Egr1 immunoprecipitates. Hence, it appears that following PMA-induced differentiation, there are distinct and multi-phasic patterns of binding of WT1, Egr1 and Sp1 to elements located within the -8460 to -8006 region of Prm1 chromatin in HEL cells. These patterns may account for the observed time-dependent induction in TPα mRNA expression (Figure 1) and Prm1-directed luciferase expression (Figure 6).

It has previously been established that Sp1, Egr1 and WT1 bind to GC elements in the proximal “core” Prm1 located between -6320 and -5895.21;22 Hence, ChIP analysis of this region was carried out to investigate if changes in the pattern of Sp1, Egr1 or WT1 binding occurred upon PMA-induced differentiation of HEL cells. Consistent with the finding that PMA did not lead to an induction of luciferase expression directed by Prm1E, Prm1F and Prm1K sub-fragments consisting of “core” Prm1 sequences (Figure 2A & 2B), ChIP analysis revealed similar binding patterns for WT1, Egr1 and Sp1 in non-treated quiescent cells (0h) and in cells treated with PMA for 5, 8 or 16h (Figure 7B and data not shown). Conversely, primers specific to the -1081 to -695 region of Prm3 of the TP gene, which acted as a negative control, resulted in generation of an amplicon from the input chromatin, but not from Sp1, Egr1 or WT1 precipitates (Figure 7C). Collectively, these data indicate that while PMA-treatment of HEL cells does not lead to significant changes in the relative levels of WT1, Egr1 and Sp1 binding to the proximal “core” Prm1, it leads to substantial changes in the pattern of binding of these factors to distal upstream GC-enriched elements. Specifically, in non-treated cells, WT1 binds to the 5’ Prm1 region from -8460 to -8006. However, following exposure of cells to PMA for 5h, and coincident with its increased expression (Figure 4), Egr1 appears to bind to this region in vivo, albeit to a much lesser extent than WT1. Following pre-incubation with PMA for 8h, a substantial increase in Egr1 binding and an associated decrease in WT1 binding was observed. Conversely, in cells pre-incubated with PMA for 16h, Sp1 is the predominant protein bound. Therefore, it is suggested that these distinct patterns of binding of WT1, Egr1 and Sp1 (Figure 7A & 7B) account for the initial and sustained increases in Prm1-directed luciferase expression in response to PMA-induced differentiation of HEL cells (Figure 6).

While the ChIP data herein have established that there are substantial changes in WT1 binding to the -8460 to -8006 region of chromatin in vivo, immunoblot analysis did not reveal significant changes in its expression over a 48h period following PMA treatment that might account for such changes in WT1 binding. Hence, it was sought to analyze the intracellular localization of WT1 in non-treated and PMA-stimulated HEL cells. In the absence of PMA and at 1h post-treatment, WT1 was mainly localized to the nucleus but also showed significant cytoplasmic expression. At 5h and, in particular, at 8h, there was a redistribution of WT1 to
the cytosolic fraction. Moreover, at 16 and 24h (Figure 7D; 16h & 24h), a substantially higher proportion of WT1 was located in the cytosol than in the nucleus where it appeared to be associated with/recruited into punctate vesicular structures. Analysis of WT1 expression through subcellular fractionation also confirmed that at 0 and 1h, most of it is expressed in the nuclear fraction but is also found in the cytosolic fraction. However, following PMA treatment, there was significant redistribution from the nuclear to the cytoplasmic fraction (Figure 7F). Hence, taken together these data establish that whilst there is no overall change in the level of WT1 expression as a function of PMA treatment, there is a substantial time-dependent change in its nuclear versus cytosolic intracellular localization.

To investigate whether the mechanisms of Prm1 and TPα regulation described herein are specific to HEL cells or are representative of other megakaryocytic cell lines also, key experiments were carried out in the human erythromyeloblastoid leukaemia K562 cell line. RT-PCR analysis showed that PMA led to a time-dependent and sustained induction of TPα mRNA expression in K562 cells (Figure 8A). Thereafter, genetic reporter assays using plasmids encoding 5’ deletions of Prm1 localized the PMA-responsive domain to a region between -8500 and -7504 of Prm1 (Figure 8B). Consistent with the findings reported herein for HEL cells, sequential mutations of GC elements at -8345, -8281, -8146 and -7831 to generate Prm1GC*(−8345,−8281,−8146,−7831) progressively reduced the PMA-mediated induction of Prm1 activity in K562 cells (Figure 8B). In the case of K562 cells, while expression of both Sp1 and Egr1 was increased in response to PMA, the time course of their inductions differed (Figure 8C). Similar to findings in HEL cells, Egr1 expression was rapidly increased within 1h but in a transient manner between 1 – 8h, and expression thereafter returned to basal levels. Conversely, increased expression of Sp1 began at 5h and was sustained for the duration of the PMA-treatment. Expression of WT1 was not significantly changed over the 0 – 16h period but, consistent with the HEL cell data and previous reports 32, there was an observable decline in its expression at 24h post-PMA treatment. Screening of the immunoblot with an antibody to HDJ-2, which acted as an internal protein loading control, confirmed that the observed differences in Sp1, Egr1 or WT1 was not due to differences in protein loading. Additionally, analysis of the intracellular localization of WT1 in K562 cells revealed that PMA led to substantial time-dependent changes in its distribution (Figure 8D). In the absence of PMA and at 1h post-treatment, WT1 was localized to the nucleus and in the cytoplasm while at 5h and 8h, it redistributed to the cytosolic fraction such that at 16 and 24h, a substantially higher proportion of WT1 was located in the cytosol than in the nucleus. Thereafter, the effect of NAB1 on the PMA-induction of Prm1-directed luciferase expression was investigated (Figure 8E). Consistent with findings in HEL cells, NAB1 significantly reduced the PMA-induced expression by Prm1 (Figure 8E(i) & 8E(ii)) but not by the control Prm3 (p = 0.793; Figure 8E(iii) & 8E(iv)) in K562 cells. Taken together, these data in K562 cells corroborate our findings in HEL cells and indicate that WT1, Egr1 and Sp1 play a critical role in regulating Prm1 and TPα expression in the megakaryotic and erythromyeloblastoid cell lineages.
DISCUSSION

In humans, the actions of TXA₂ are mediated by the α and β isoforms of TP. TPα and TPβ are transcriptionally regulated by distinct promoters, termed Prm1 and Prm3, respectively. While TXA₂ and/or the TP(s) have been implicated in a range of conditions including thrombosis, various hypertensions, atherosclerosis and ischaemic heart disease, the relative or collective involvement of the individual TP isoforms in such pathologies is unknown. Hence, identification of the factors that regulate Prm1 and Prm3 should not only lead to a greater understanding of the physiologic roles of the individual TPs, but also of their contribution to the aforementioned pathologies. The aim of the current study was to investigate the effect of megakaryoblastic differentiation on Prm1-directed TPα expression, using phorbol ester-induced differentiation of the haemopoietic HEL 92.1.7 and the erythromyeloblastoid leukaemia K562 cell lines as a model systems.

Herein, PMA-induced differentiation of HEL and K562 cells led to substantial time-dependent increases in TPα mRNA expression and in Prm1-directed reporter gene expression. Moreover, the major PMA-responsive cis-acting element(s) were localized to an upstream region between -8500 and -7504 within Prm1. The induction of TP gene expression was previously attributed to increased binding of Sp1 to a GC-enriched element at -8345 within Prm1. More recent studies established that this GC element at -8345, along with other adjacent GC elements at -8281, -8146 and -7831, mediate repression of Prm1 through binding of WT1 in quiescent HEL cells. While each of those GC elements represents putative overlapping binding sites for WT1/Egr1/Sp1, previous electrophoretic mobility shift and antibody supershift assays established that each has the capacity to allow Egr1 and WT1 binding. Moreover, Sp1 may also bind to GC-8345 and GC-8281. Hence, it was sought to determine if one or more of those GC elements may be responsible for the PMA-induction of Prm1 in HEL and K562 cells. While mutation of the individual GC elements each resulted in only marginal reductions in PMA-induction of Prm1, more substantial reductions were observed following sequential disruption of all four elements, generating Prm1\textsuperscript{GC*(-8345,-8281,-8146,-7831)}. These data suggested that GC elements at -8345, -8281, -8146 and -7831 act in an independent manner to mediate the PMA-induction of Prm1 transcriptional activity and TPα mRNA expression in both HEL and K562 cells.

Western blot analysis established that while expression of the 52 kDa/54 kDa forms of WT1 were not substantially altered following PMA treatment, there was rapid, yet transient, increases in Egr1 expression in both HEL and K562 cells. In addition, while the levels of expression of Sp1 were not altered in HEL cells for the duration of the PMA-treatment, there was a significant increase in its expression in K562 cells but at later times that those of Egr1. These data suggest certain differences between the HEL and K562 cells in regard to Sp1 expression. In general, PMA-mediated up-regulation of Egr1 largely occurs through activation of the extracellular signal-regulated kinase (ERK) 1/2 cascades. ERK 1/2 activation leads to phosphorylation of the ternary complex factor (TCF) including Elk-1, SAP-1 and SAP-2. Phosphorylated TCF, in complex with the serum response factor, binds to a serum response element within the Egr1 promoter to increase its expression. In addition, Egr1 may also be directly phosphorylated in response to ERK activation and phospho-Egr1 binds DNA more efficiently to regulate target gene expression. Herein, ectopic expression of the NAB1 co-
repressor of Egr1 significantly and specifically reduced PMA-mediated induction of Prm1 but had no effect on PMA-induction of gene expression by the control Prm3 in HEL or K562 cells. Moreover, the ERK 1/2 pathway inhibitor PD98059 significantly abrogated both the PMA-mediated increase in Prm1 activity and induction of Egr1 expression while having no effect on WT1 expression. Collectively, these data suggested that PMA-induced differentiation of HEL cells and K562 cells is associated with increased Egr1 expression, which leads to increased Prm1-directed gene expression. Furthermore, in the context of the TPα and TPβ isoforms, the Egr1-induction is entirely specific to Prm1 while having no effect on Prm3.

Thereafter, it was sought to investigate whether the increased Prm1-directed gene expression could be due to altered binding of Egr1 to the upstream PMA-responsive region within Prm1. While WT1, Egr1 and Sp1 proteins are abundantly expressed in HEL cells, it is WT1 that binds in a co-operative manner to GC elements at -8345, -8281, -8146 and -7831 to repress Prm1-directed gene expression in non-stimulated HEL cells. ChIP analysis confirmed that WT1 preferentially binds to the -8460 to -8006 region of Prm1 in non-stimulated HEL cells, with an absence of Egr1 or Sp1 binding (Figure 7A). Following incubation with PMA for 5h, there was no appreciable change in the level of WT1 binding but there was evidence of low-level binding of Egr1 to Prm1. However, following 8h incubation, the level of Egr1 bound to Prm1 substantially increased while WT1 binding was not detectable. Hence, collectively, the data indicate that the PMA-mediated time-dependent induction in Egr1 expression leads to its increased binding to upstream elements within Prm1, thereby up-regulating TPα mRNA expression.

The increase in Egr1 binding to Prm1, as determined by ChIP analysis, was associated with a parallel decrease in WT1 repressor binding. No substantial decreases in WT1 protein expression were observed herein in HEL or K562 cells at 0 – 16h post-PMA treatment but, at 24h, there was an observable decline in its expression consistent with previous reports that WT1 mRNA is down-regulated during erythroid and megakaryocytic differentiation of K562 cells. WT1 contains a nuclear export sequence and undergoes nucleocytoplasmic shuttling. Moreover, WT1 is subject to both protein kinase (PK)C- and PKA-induced phosphorylation, and activation of PKA is associated with its cytoplasmic retention. More recent reports also suggest that PMA may alter the intracellular localization of WT1. Herein, while immunolocalization and subcellular fractionation data suggested that, under basal conditions, WT1 showed dual expression in the nucleus and cytoplasm but substantially redistributed to the cytosolic fraction post-PMA treatment in HEL cells. Moreover, similar patterns of WT1 redistribution also occurred in K562 cells. Hence, the observed nuclear to cytoplasmic translocation of WT1 and the concomitant increased Egr1 expression may displace WT1 repressor in favour of Egr1 activator binding to the upstream GC elements. This, in turn, may account for the initial PMA-induction of Prm1-directed reporter gene expression and TPα mRNA expression.

Further ChIP analysis of HEL cells pre-incubated with PMA for a longer period (16h) revealed a decline in Egr1 binding to basal levels, whilst WT1 binding was still undetected. Conversely, substantial binding of Sp1 was now detected. Western analysis revealed that the PMA-induction of Egr1 expression decreased to near basal levels by approximately 8h in both HEL and K562 cells. However, due to high levels of Egr1 expression
in the absence and at the later times following PMA induction, such as at 16h post-PMA treatment, it is unlikely that decreased Egr1 binding to Prm1 observed in the ChIP analyses is solely due to its reduced expression levels. It is possible that post-translational modification(s), such as phosphorylation and/or ubiquitination, of Egr1 in response to sustained stimulation of HEL cells with PMA may also contribute to its decreased binding to Prm1. Moreover, since the transcriptional effects of Egr1 are largely determined by its interaction with specific co-factors, it is possible that sustained PMA-stimulation of HEL cells may eventually interfere with these interactions, thereby decreasing Egr1 binding and activation of Prm1. While further studies would be necessary to comprehensively investigate the loss of Egr1 binding upon sustained stimulation of HEL cells with PMA, the decrease is likely to be due to a combination of several of these factors.

As stated, the loss of Egr1 binding to Prm1 upon sustained incubation of HEL cells with PMA observed through ChIP analysis was associated with a substantial increase in Sp1 binding. While western blot analysis revealed that PMA-treatment did not substantially affect Sp1 expression in HEL cells, its expression was actually increased in K562 cells but at times later than that of Egr1 induction and corresponding to times where it was observed to bind to Prm1 through ChIP analysis in HEL cells. Hence, whilst the increased expression of Sp1 in K562 cells is likely, at least in part, to account for the increased binding of Sp1 to Prm1 in vivo, the molecular basis of its increased binding to Prm1 in HEL cells clearly differs. Sp1 is thought to play a structural role in transcriptional activation by binding to multiple sites on given promoters to maintain chromatin in a more accessible conformation and PMA increases ERK-mediated phosphorylation of Sp1, enhancing its DNA-binding affinity which might possibly accounting, at least partly, for the increased binding of Sp1 to Prm1 leading to sustained expression of TPα in HEL cells.

Considering the data presented herein, a model can be proposed to explain the PMA-induction of Prm1 and TPα expression. In the absence of PMA-treatment, WT1 binds to multiple upstream GC-elements to mediate repression of Prm1 and TPα expression (Figure 9A). Following incubation with PMA for approximately 5h, ERK-mediated up-regulation of Egr1 may lead to increased Egr1 activator binding to Prm1, thereby leading to induction of expression (Figure 9B). Subsequently, PMA-stimulation may lead to translocation of WT1 from the nucleus to the cytoplasm, leading to Prm1 de-repression. The decrease in WT1 binding, as well as the continued increase in Egr1 expression, would facilitate a more pronounced induction of Prm1 (Figure 9C). Thereafter, it is suggested that decreased expression of Egr1, in association with increased competition for binding from phosphorylated Sp1, would facilitate Sp1-mediated increases in Prm1 transcriptional activity in response to prolonged PMA incubations. This may be responsible for maintaining TPα expression in the differentiated state (Figure 9D). While further studies are needed to clarify certain aspects of this model, the current study indicates that the PMA-mediated induction of Prm1 is more complex than originally thought, and involves specific or regulated binding patterns of the transcriptional regulators WT1, Egr1 and Sp1 to multiple elements within Prm1 to mediate repression, induction and/or maintenance of Prm1/TPα expression in HEL cells.
Consistent with its role reported herein in regulating Prm1 and TPα expression during PMA-induced differentiation of HEL and K562 cells, it is thought that Egr1 is intimately involved in the megakaryoblastic differentiation of both K562 and HEL cell lines. Specifically, the induction of Egr1 by PMA is correlated with increased expression of megakaryocyte markers, including glycoprotein (GP) IIb/IIIa. Moreover, Egr1 mediates increased expression of Gaq during PMA-induced megakaryocytic differentiation of HEL cells. Gaq plays a central role in platelet signal transduction and platelets from Gaq-deficient mice are unresponsive to a variety of physiological platelet activators. Additionally, due to its induction by stimuli such as shear stress, mechanical injury, hypoxia and reactive oxygen species, Egr1 has been associated with the pathogenesis of several vascular diseases following injury to the vascular endothelium. Moreover, Egr1 and Egr1-inducible genes are significantly up-regulated in endothelial and smooth muscle cells within human atherosclerotic lesions, while induction of atherosclerosis in low density lipoprotein (LDL) receptor-null mice leads to increased aortic expression of Egr1. Following its up-regulation in vascular disease, it is possible that increased and prolonged transcriptional activity of Egr1 may be responsible for increased expression of TPα in various pathophysiologic conditions, including atherosclerosis and coronary artery disease. Moreover, as previously stated, in the context of the TP isoforms, it is notable that the PMA-induction of Prm3-directed TPβ expression does not involve Egr1, pointing to further critical differences between the individual TP isoforms.

Herein, it has been established that differential patterns of WT1, Egr1 and Sp1 binding to elements within Prm1 are responsible for the induction and maintenance of TPα expression during the PMA-induced megakaryoblastic differentiation of HEL and K562 cells. Importantly, the finding that several aspects of Prm1 and TPα regulation described herein are universal for both HEL and K562 cells would suggest that these mechanisms may also be relevant to megakaryocyte-erythroid progenitor cells, as well as to other model cell lines. Collectively, the data herein provide a molecular basis for understanding the regulated expression of TPα during megakaryocytic differentiation toward the platelet phenotype and may also provide potential mechanism(s) to rationalize TPα expression within the vasculature. Moreover, the mechanism involving repression, induction and sustained expression through different trans-acting factors identified for Prm1/TPα may be more generally relevant to the transcriptional regulation of other gene(s).

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References:


**FIGURES**

Figure 1: Effect of PMA on TPα mRNA expression in HEL cells. **Panel A:** RT-PCR analysis of TPα and GAPDH mRNA from HEL cells incubated with PMA (100 nM; 1–48h), where cells incubated with vehicle (v; 0.1% (v/v) DMSO; 48h) served as a control. **Panel B:** Southern blot analysis of the RT-PCR products co-screened using 5’ biotin-labeled oligonucleotide probes specific for TPα and GAPDH mRNA. Images are representative of four independent experiments. **Panel C:** Mean levels of TPα mRNA expression in vehicle- or PMA-incubated HEL cells represented as a percentage of mean levels of GAPDH mRNA (Relative expression, % ± SEM, n = 4). The asterisks (*) indicate that TPα:GAPDH mRNA expression was significantly increased in PMA- relative to vehicle-treated cells.

**Figure 2:** Effect of 5’ deletions on the PMA-mediated increase of Prm1-directed gene expression. Panels A and B: Schematic of the human TP gene spanning nucleotides -8500 to +786 encoding Prm1 (-8500 to -5895), Prm3, exon (E)1, intron (I)1 and E2, where nucleotide +1 represents the translational start site (ATG) and nucleotides 5’ of that are given a – designation. Recombinant pGL3Basic encoding Prm1 (-8500 to -5895) and its 5’ deletions Prm1B (-7962), Prm1C (-7504), Prm1D (-6848), Prm1E (-6648), Prm1F (-6552) and Prm1K (-6067) were co-transfected with pRL-TK into HEL cells. Cells were incubated ~32h post-transfection with either vehicle (0.1% DMSO) or PMA (PMA; 100 nM) for 16h. Data is presented as (Panel A) mean firefly relative to renilla luciferase activity expressed in arbitrary relative luciferase units (RLU ± SEM; n = 4) or (Panel B) fold induction of mean luciferase activity in PMA-treated cells relative to vehicle-treated cells. The asterisks (*) indicate that PMA significantly increased luciferase expression in HEL cells.
Figure 3: Identification of PMA-responsive elements within the Prm1 region from -8500 to -7504. Panels A, B, C and D: Schematic of GC elements representing putative WT1/Egr1/Sp1 binding sites within Prm1, where the 5′ nucleotide is indicated and the star symbol signifies mutated elements. Recombinant pGL3Basic encoding: Prm1, Prm1\textsuperscript{GC*(-8345)}, Prm1\textsuperscript{GC*(-8345,-8281)}, Prm1\textsuperscript{GC*(-8345,-8281,-8146)}, or Prm1\textsuperscript{GC*(-8345,-8281,-8146,-7831)} (Panels A and B) or Prm1, Prm1\textsuperscript{GC*(-8345)}, Prm1\textsuperscript{GC*(-8345,-8146)}, Prm1\textsuperscript{GC*(-8345,-8146,-7831)} or Prm1\textsuperscript{GC*(-8345,-8281,-8146,-7831)} (Panels C and D) were co-transfected with pRL-TK into HEL cells. Cells were incubated with vehicle (0.1% DMSO) or PMA (PMA; 100 nM) for 16h. Data is presented as (Panels A and C) mean firefly relative to renilla luciferase activity (RLU ± SEM; n = 4) or (Panels B and D) fold-induction of mean luciferase activity in PMA-treated cells relative to vehicle-treated cells. The asterisks (*) indicate that PMA significantly induced luciferase expression in HEL cells.
Figure 4: Effect of PMA on expression of WT1, Sp1 and Egr1 proteins in HEL cells. Immunoblot analysis of WT1, Egr1 & Sp1 expression in HEL cells pre-incubated with vehicle (v; 0.1% DMSO; 48h) or PMA (100 nM; 0–48h). The molecular sizes are indicated to the left of the panels. Images are representative of four independent experiments.
Figure 5: Effect of NAB1 co-repressor and ERK 1/2 signaling on the PMA-mediated induction of Prm1-directed luciferase expression. Panels A-D: HEL cells, transiently co-transfected with pCMV5 (control) or pCMV:NAB1 along with pRL-TK plus either pGL3b:Prm1 (Panels A & B) or pGL3b:Prm3 (Panels C & D), were incubated ~32h post-transfection with vehicle (0.1% DMSO) or PMA (PMA; 100 nM) for 16h. Data is presented as (Panels A & C) mean firefly relative to renilla luciferase activity (RLU ± SEM; n = 3) or (Panels B & D) fold induction of mean luciferase activity in PMA-incubated cells compared to vehicle-incubated cells. The asterisks (*) indicate that PMA significantly induced Prm1- or Prm3-directed luciferase expression (Panels A & C), or that over-expression of NAB1 significantly reduced PMA-induction of Prm1-directed luciferase expression (Panel B). Panel E: HEL cells, transiently co-transfected with pRL-TK plus pGL3b:Prm1 or pGL3b:Prm1K, were incubated ~32h post-transfection with vehicle (0.1% DMSO), PMA (PMA; 100 nM) or 100 nM PMA plus 10 μM PD98059 (PMA + PD98059) for 16h. Luciferase activity is expressed as mean firefly relative to renilla luciferase activity (RLU ± SEM; n = 3). Panel F: Immunoblot analysis of Egr1 and WT1 expression in HEL cells (40 μg total protein) incubated for 16h with: vehicle (v; 0.1% DMSO), 10 μM PD98059, 100 nM PMA or 100nM PMA plus 10 μM PD98059, where non-stimulated HEL cells served as an additional control (0h). The images are representative of three independent experiments.
Figure 6: Time-course of PMA-mediated induction of Prm1-directed luciferase expression. HEL cells, transiently co-transfected with pGL3b:Prm1 plus pRL-TK, were incubated with vehicle (v; 0.1% DMSO; 48h) or PMA (100 nM; 0–48h). Luciferase activity is expressed as mean firefly relative to renilla luciferase activity expressed in arbitrary relative luciferase units (RLU ± SEM; n = 4). The asterisks (*) indicate that PMA significantly induced Prm1-directed luciferase expression in HEL cells.
Figure 7: Nuclear factor binding to elements within the -8460 to -8006 region of Prm1 in vivo and PMA-induced translocation of WT1 from the nucleus to the cytoplasm. Panels A - C: ChIP analysis of WT1, Sp1 and Egr1 binding to the TP gene. Schematic of Prm1 and primers (arrows) used in PCR to amplify the -8460 to -8006 (Panel A) and -6368 to -5895 (Panel B) regions of Prm1 from input chromatin or chromatin extracted from anti-Sp1, anti-Egr1 and anti-WT1 immunoprecipitates from non-treated (0h) HEL cells, or cells treated with 100 nM PMA for 5, 8 or 16h, as specified. Primers to amplify a region of Prm3 (-1081 to -695; Panel C) were used as negative controls. Images are representative of three independent experiments. Panel D: Confocal microscopy of HEL cells pre-incubated with PMA for 0, 1, 5, 8, 16, or 24h, followed by immunolabeling with anti-WT1 antibody and AlexaFluor488 conjugated anti-rabbit IgG (green), followed by counterstaining with DAPI (red). Co-localization was observed by merging the green and the red channels (yellow). Images are representative of three independent experiments. Panel E: Immunoblot analysis of WT1 expression in nuclear and cytoplasmic fractions of HEL cells pre-incubated with PMA (100 nM; 0 – 24 h). The molecular sizes are indicated to the left of the panels. Images are representative of three independent experiments.

**Figure 8:** Investigation of Prm1 and TPα regulation in K562 cells. **Panel A(i):** RT-PCR analysis of TPα and GAPDH mRNA from K562 cells incubated with PMA (100 nM; 1–48h), where cells incubated with vehicle (v; 0.1% (v/v) DMSO; 48h) served as a control. **Panel A(ii):** Mean levels of TPα mRNA expression in vehicle- or PMA-incubated HEL cells represented as a percentage of mean levels of GAPDH mRNA (Relative expression, % ± SEM, n = 4). The asterisks (*) indicate that TPα:GAPDH mRNA expression was significantly increased in PMA- relative to vehicle-treated cells. **Panel B:** Recombinant pGL3Basic encoding: Prm1 (-8500), Prm1B (-7962), Prm1GC(-8345), Prm1GC(-8345,-7831), Prm1GC(-8345,-8281,-7831), Prm1GC(-8345,-8281,-8146,-7831) and Prm1C (-7504) were co-transfected with pRL-TK into K562 cells. Cells were incubated with vehicle (0.1% DMSO) or PMA (PMA; 100 nM) for 16h. Data is presented as (i) mean firefly relative to renilla luciferase activity (RLU ± SEM; n = 4) or (ii) fold-induction of mean luciferase activity in PMA-treated cells relative to vehicle-treated cells. The asterisks (*) indicate that PMA significantly induced luciferase expression in K562 cells. **Panel C:** Immunoblot analysis of WT1, Egr1, Sp1 & HDJ-2 expression in K562 cells pre-incubated with PMA (100 nM; 0–24h). The molecular sizes are indicated to the left of the panels. Images are representative of four independent experiments. **Panel D:** Confocal microscopy of K562 cells pre-incubated with PMA for 0 - 24h, followed by immunolabeling with anti-WT1 antibody and AlexaFluor488 conjugated anti-rabbit IgG (green), followed by counterstaining with DAPI (red). Co-localization was observed by merging the green and the red channels (yellow). Images are representative of three independent experiments. **Panel E:** K562 cells, transiently co-transfected with pCMV5 (control) or pCMV:NAB1 along with pRL-TK plus either pGL3b:Prm1 ((i) & (ii)) or pGL3b:Prm3 ((iii) & (iv)), were incubated ~32h post-transfection with vehicle (0.1% DMSO) or PMA (PMA; 100 nM) for 16h. Data is presented as ((i) & (iii)) mean firefly relative to renilla luciferase activity (RLU ± SEM; n = 3) or ((ii) & (iv)) fold induction of mean luciferase activity in PMA-incubated cells compared to vehicle-incubated cells. The asterisks (*) indicate that PMA significantly induced Prm1- or Prm3-directed luciferase expression ((i) & (iii)), or that over-expression of NAB1 significantly reduced PMA-induction of Prm1-directed luciferase expression (ii).
Figure 9: Proposed model for PMA-mediated increases in Prm1 activity

Panels A-D: Proposed model for PMA-induction of Prm1 in HEL and K562 cells. In quiescent cells, WT1 binds cooperatively to multiple neighbouring GC elements at -8345, -8281, -8146 and -7831 within Prm1 to repress transcription by impairing the initiation of transcription by the basal transcription apparatus (BTA) at the TI site (represented by an arrow; Panel A). Following exposure to PMA for ~5h, ERK-mediated up-regulation of Egr1 expression leads to increased Egr1 binding to Prm1, thereby activating Prm1-directed gene expression (Panel B). Following exposure to PMA for ~8h, a more pronounced increase in Egr1 binding occurs, with an associated decrease in WT1 binding. Translocation of WT1 from the nucleus to the cytoplasm may be responsible for this decrease in WT1 binding. This would lead to promoter de-repression and may facilitate a further increase in Egr1 binding, leading to a more pronounced activation of Prm1 by the BTA (Panel C). Following exposure to PMA for ~16h, decreased Egr1 binding occurs. PMA-mediated differentiation of cells may also lead to phosphorylation of Sp1 and/or its increased expression, enhancing its DNA binding activity. Therefore, increased affinity of Sp1 for Prm1, coinciding with decreased Egr1 expression, may facilitate binding of Sp1 to Prm1, thereby mediating a sustained increase in Prm1 activity and TPα expression as differentiation of HEL and K562 cells progresses toward the platelet phenotype (Panel D).
Table I: Sequences of GC elements within Prm1.

<table>
<thead>
<tr>
<th>Element*</th>
<th>Sequence**</th>
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<tbody>
<tr>
<td>Prm1-8345 (-)</td>
<td>5’ ctggGTGGGGCGGGGgcagtt 3’</td>
</tr>
<tr>
<td>Prm1-8281 (-)</td>
<td>5’ tcggGaGGGGCCGGcag 3’</td>
</tr>
<tr>
<td>Prm1-8146 (+)</td>
<td>5’ ggcGGGGGTTGGGGGCGGGGGCGGGGc 3’</td>
</tr>
<tr>
<td>Prm1-7831 (-)</td>
<td>5’ agatGaGGGGGCAgtga 3’</td>
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<tr>
<td>Egr1 consensus***</td>
<td>50</td>
</tr>
<tr>
<td>WTE*** 50</td>
<td>5’ gtgcGTGGgaGtgaat 3’</td>
</tr>
<tr>
<td>Sp1 consensus***</td>
<td>51</td>
</tr>
</tbody>
</table>

*The + and – designation indicates that elements are found on the sense or antisense strands of Prm1, respectively. **Base pairs underlined denote the core sequences of the elements, while base pairs in capital letters are in positions that exhibit a high conservation profile, as previously described. *** Consensus Egr1, Wilms’ Tumour Element (WTE) and Sp1 elements.
Supplementary Figure 1: Identification of a PMA-responsive element within the Prm1 region from -7962 to -7717. Panels A and B: Schematic of GC elements representing putative WT1/Egr1/Sp1 binding sites within Prm1, where the 5’ nucleotide is indicated and the star symbol signifies mutated elements. pGL3Basic plasmids encoding Prm1B, Prm1B_{GC*(-7831)}, or Prm1C were co-transfected with pRL-TK into HEL cells. Cells were incubated ~32h post-transfection with vehicle (0.1% DMSO) or PMA (PMA; 100 nM) for 16h. Data is presented as (Panel A) mean firefly relative to renilla luciferase activity (RLU ± SEM; n = 4) or (Panel B) fold induction of mean luciferase activity in PMA-incubated cells relative to vehicle-incubated cells. The asterisks (*) indicate that PMA significantly induced luciferase expression in HEL cells.
Supplementary Figure 2: Densitometric analysis of WT1, Sp1 and Egr1 expression in HEL cells in response to PMA-induced differentiation. Panels A–C: Densitometric analysis of WT1, Egr1 and Sp1 protein expression in HEL cells were pre-incubated with vehicle (v; 0.1% DMSO; 48h) or PMA (100 nM; 0–48h). The bar charts represent the expression of WT1 (Panel A), Egr1 (Panel B) and Sp1 (Panel C) relative to that of HDJ2, which acted as an endogenous loading control. The asterisks indicate that the expression of Egr1 relative to that of HDJ2 was significantly altered in the presence of PMA.