

## Chapter 11

# Transcriptional Regulation of the Human Thromboxane A<sub>2</sub> Receptor Gene by Wilms' Tumour (WT)<sub>1</sub>

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### Abstract

The prostanoid thromboxane (TX)A<sub>2</sub> plays a fundamental role in vascular haemostasis and, more recently, is increasingly implicated in various neoplasms including in prostate, breast and bladder cancers, among others. In humans, TXA<sub>2</sub> signals through the TP $\alpha$  and TP $\beta$  isoforms of the T prostanoid receptor (TP), two structurally related receptors that display both common, over-lapping but also distinct, isoform-specific physiologic roles. Consistent with this, while TP $\alpha$  and TP $\beta$  are encoded by the same gene, the *TBXA2R*, they are

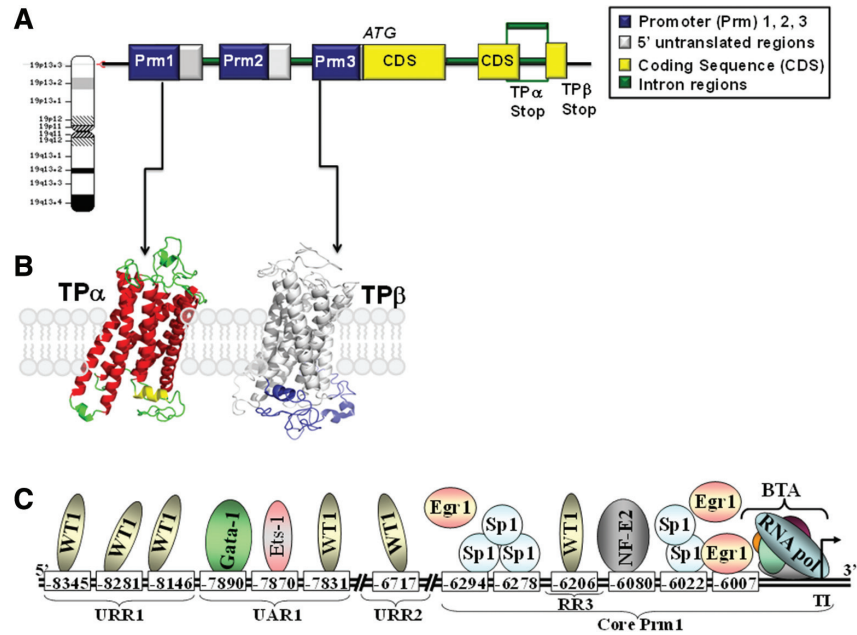
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differentially expressed due to their transcriptional regulation by distinct promoters where promoter (Prm) 1 regulates TP $\alpha$  expression and Prm3 regulates TP $\beta$ . While the clinical evidence for the role of the TXA<sub>2</sub>-TP axis in neoplastic progression is increasing, few studies to date have investigated the role of the individual TP $\alpha$ /TP $\beta$  isoforms in human cancer or indeed in most other diseases in which the TXA<sub>2</sub>-TP axis is implicated. Focusing on TP $\alpha$ , this review details the current understanding of the factors regulating its expression and transcriptional regulation through Prm1, including in prostate and breast cancers. Emphasis is placed on the *trans*-acting transcriptional regulators that bind to *cis*-elements within the core and upstream regulatory regions of Prm1 under basal conditions and in response to cellular differentiation. A particular focus is placed on the role of the tumour suppressor Wilms' tumour 1 in the regulation of TP $\alpha$  expression through Prm1 in megakaryoblastic cells of vascular origin and in prostate and breast carcinoma cells. Collectively, this review details current knowledge of the factors determining regulation of the TXA<sub>2</sub>-TP $\alpha$  axis and thereby provides a genetic basis for understanding the role of TXA<sub>2</sub> in the progression of certain human cancers.

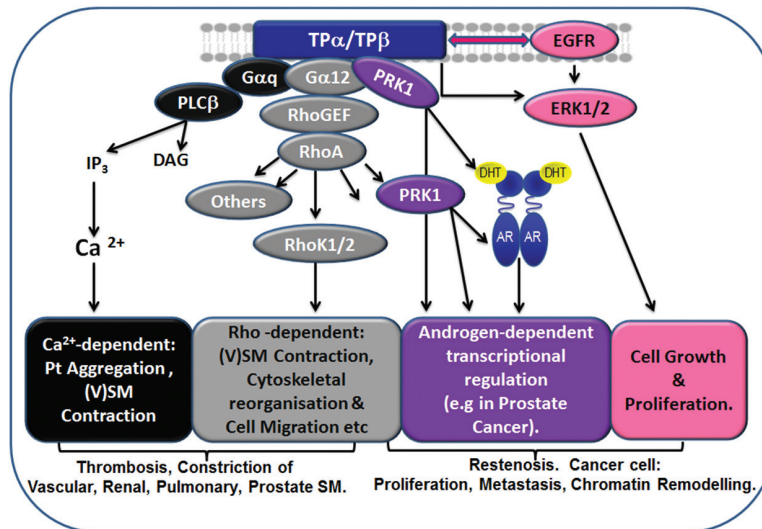
**Key words:** Cancer; Gene; Thromboxane receptor; Transcription; Wilms' tumour 1

## Introduction

Thromboxane (TX)A<sub>2</sub>, a prostanoid synthesized from arachidonic acid by the sequential enzymatic actions of cyclooxygenase (COX)-1/-2 and TXA synthase (TXS) predominantly in platelets and in monocytes/activated macrophages, plays an essential role in haemostasis regulating platelet aggregation and vascular tone (1). It also induces the constriction of various other types of smooth muscle (SM), including pulmonary, renal and prostate SM, and promotes vascular remodelling in response to endothelial injury contributing to neointimal hyperplasia and restenosis post-stenting (2-4). Accordingly, imbalances in the levels of TXA<sub>2</sub> or of its synthase (TXS) or of its receptor, the T prostanoid receptor (or, in short, the TP), are widely implicated in several cardiovascular, pulmonary, renal and prostate pathologies (1-3, 5). In humans and in other primates, the TP exists as two structurally related isoforms referred to as TP $\alpha$  and TP $\beta$ , which are identical for their N-terminal 328 amino acid residues but which differ exclusively in their intracellular C-terminal domains (6). While TP $\alpha$  and TP $\beta$  are encoded by the same TP gene, the *TBXA2R* (Figure 1), they are differentially expressed in several cell/tissue types being transcriptionally regulated by two different promoters referred to as Prm1 and Prm3, respectively, within the *TBXA2R* (7-10). Functionally, as members of the G-protein-coupled receptor (GPCR) superfamily, TP $\alpha$  and TP $\beta$  both primarily couple to G $\alpha$ q-mediated phospholipase C $\beta$  activation, raising intracellular calcium levels in response to inositol phosphate turnover, but also readily couple to G $\alpha$ 12-mediated RhoA and to extracellular signal-regulated protein kinase (ERK) activation (Figure 2) (6, 11-14). In contrast, TP $\alpha$  and TP $\beta$  undergo distinct mechanisms of agonist-induced



**Figure 1.** Structural organization of the *TBXA2R* gene. Panel A: In humans, a single *TBXA2R* gene, located on Chr19p13.3, encodes both the TP $\alpha$  and the TP $\beta$  isoforms of the T prostanoid receptor (the TP), where promoter (Prm) 1 exclusively regulates TP $\alpha$  and Prm3 regulates TP $\beta$  expression, respectively. The functional role of Prm2 is currently unclear but may contribute to the transcriptional regulation of TP $\alpha$  expression. Panel B: Structurally, TP $\alpha$  and TP $\beta$  are identical for their N-terminal 328 aa residues but differ exclusively in their intracellular C-terminal domains, where TP $\alpha$  (343 aa) and TP $\beta$  (407 aa) have 15 and 79 unique aa residues, respectively. Panel C: Schematic representation of Prm1 and the main *cis*-acting elements and *trans*-acting factors that regulate TP $\alpha$  expression through Prm1. Prm1 is located at -8500 to -5895 relative to the ATG translational initiation codon within the *TBXA2R*. Under basal or resting cell conditions, the Core Prm1 region, located between -6294 and -5895, is under the transcriptional regulation of Sp1, Egr1 and NF-E2 but also contains a repressor region (RR3) that is regulated by Wilms' tumour (WT) 1. Prm1 also contains several upstream repressor regions (URR) and upstream activator regions (UARs). The haematopoietic-specific factors Gata-1 and Ets-1 are the main *trans*-acting factors that bind and regulate UAR1 in megakaryoblastic HEL92.1.7 cells and Ets-1/2 and Oct-1 regulate UAR2 (not shown). In contrast, WT1 is the main *trans*-acting factor that binds to multiple GC-enriched *cis*-elements within URR1, URR2 and RR3 to repress Prm1-directed TP $\alpha$  expression under basal/resting cellular conditions (e.g., in non-differentiated HEL cells).



**Figure 2.** Summary of the main signalling cascades regulated by TP $\alpha$  and TP $\beta$ . 1 (Black): Both TP $\alpha$  and TP $\beta$  primarily couple to G $\alpha_q$ -mediated phospholipase (PL)C $\beta$  activation, leading to the generation of IP $_3$  and mobilization of intracellular calcium (Ca $^{2+}$ ). Elevation in intracellular Ca $^{2+}$  is the main signalling event that triggers TXA $_2$ -induced platelet activation, including thrombosis, and constriction of various types of smooth muscle (SM), including vascular (V), renal, pulmonary and prostate SM. 2 (Grey): TP $\alpha$  and TP $\beta$  also couple to G $\alpha_{12}$ -mediated activation of RhoGEF (Rho guanine nucleotide exchange factor), leading to activation of RhoA. Activated (GTP-bound) RhoA, in turn, interacts with a range of effector proteins including Rho kinase (K) 1/2, leading to Ca $^{2+}$ -independent (V)SM contraction, to general reorganizations of the cellular cytoskeleton and to a host of events that promotes tumour cell migration and metastasis. In addition, activated RhoA can also interact and activate the effector protein kinase C-related kinase (PRK) 1. PRK1 can also interact with the activated (DHT-bound) androgen receptor (AR) which, in turn, can enhance AR-dependent transcriptional activation by promoting phosphorylation of histone H3 at Thr11 and, hence, androgen-induced chromatin remodelling. 3 (Purple): It has been recently discovered that TP $\alpha$  and TP $\beta$  can directly interact with and activate PRK1 which, in turn, can also phosphorylate histone H3 at Thr11 to augment androgen-induced chromatin remodelling in response to TP $\alpha$ /TP $\beta$  signalling. 4 (Pink): TP $\alpha$  and TP $\beta$  also lead to (i) activation of the extracellular signal-regulated protein kinase (ERK) 1/2 cascades (intermediate steps not shown), as well as to (ii) transactivation of the epidermal growth factor receptor (EGFR), both of which (i and ii) account for the ability of TXA $_2$  to promote cell proliferation and mitogenesis, including tumour progression. *Note:* The inflammatory or immune-modulatory roles of the TXA $_2$ -TP $\alpha$ /TP $\beta$  axis are not shown but may also contribute to their role in certain cancers.

homologous (15, 16) and heterologous (17–19) desensitization to differentially regulate their intracellular signalling. Most notably, signalling by TP $\alpha$ , but not by TP $\beta$ , is completely desensitized/inhibited by the counter-regulatory *anti*-platelet and vasodilatory agents prostacyclin/prostaglandin I<sub>2</sub> and nitric oxide, which is mediated by direct protein kinase (PK) A and PKG phosphorylation of TP $\alpha$  at Ser<sup>329</sup> and Ser<sup>331</sup>, respectively, the very first residues within its unique carboxyl-terminal tail domain of TP $\alpha$  and divergent from those of TP $\beta$  (13, 18). The conclusion from those studies is that TP $\alpha$  is the TP isoform essential for haemostasis/thrombosis, while the role of TP $\beta$  in this pathophysiologic process remains unclear (13, 18). Hence, TP $\alpha$  and TP $\beta$  have both shared and unique patterns of expression and function to mediate the (patho)physiologic actions of the potent autocrine/paracrine mediator TXA<sub>2</sub> in human health and disease.

### The role of thromboxane in cancer

In addition to its prominent role within the vasculature, there is growing evidence highlighting a central role for TXA<sub>2</sub> in human cancers (20, 21). In recent years, evidence supporting this hypothesis has been strengthened by several longitudinal studies showing the prophylactic benefits of long-term daily use of Aspirin in reducing the risk of many prevalent cancers, predominantly gastrointestinal but also breast, lung and prostate cancers (PCa), with numerous clinical trials completed or underway testing the benefits of Aspirin and other COX-1/-2 inhibitors in chemoprevention (22–29). While those longitudinal studies do not specify which COX-1/-2-derived prostanoid metabolite(s) is actually lowered by Aspirin to account for its prophylactic benefits in cancer risk reduction, recent reports strongly suggest that some/many of its *anti*-cancer effects may be due to its ability to inhibit TXA<sub>2</sub> generation, as stated a prostanoid more typically associated with thrombosis and cardiovascular disease (20, 21). Indeed, it has long been known that platelets, the main source of TXA<sub>2</sub> and key target of Aspirin, play a key role in cancer progression promoting cancer cell metastasis, immune evasion and extravasation (30). Furthermore, increased levels of TXA<sub>2</sub> and expression of its synthase and its T prostanoid receptor, the TP, occur in a number of prevalent cancers including, for example, strongly correlating with bladder (31), prostate (32, 33), colorectal (34, 35) and non-small-cell lung cancer (36). Mechanistically, the role of TXA<sub>2</sub> in neoplastic progression is at least partly explained by the ability of the TXA<sub>2</sub>-TP axis to regulate key mitogenic/ERK- and RhoA-mediated signalling cascades that contribute to tumour development and metastasis (12, 14) and also by its ability to regulate local inflammation and immunity (37–42), including within the tumour (Figure 2; summary of TXA<sub>2</sub>-TP signalling). Hence, aside from its regulation of ERK- and RhoA-mediated processes (Figure 2) (12, 14), TXA<sub>2</sub> is a potent proinflammatory and immune-modulatory agent being abundantly produced in monocytes/activated macrophages and promotes monocyte chemoattractant protein-1 expression in tumours, recruiting tumour-associated macrophages, and negatively

regulates the interaction between T-cells and dendritic cells, a process essential for adaptive/acquired immunity (38, 39, 43, 44). Moreover, TXA<sub>2</sub> is critical for early B-cell development, also with implications for its role in tumour-infiltrating B-cells (42, 45). Hence, due to its role in tumour growth and metastasis combined with its ability to regulate local inflammation and immunity, the TXA<sub>2</sub>-TP axis can impact at multiple levels within the tumour environment.

Genome-wide association studies also reveal that certain single nucleotide polymorphisms within the TXS gene (the TBXAS1) may predispose individuals to breast cancer (46), while inhibition of TXS activity enhances apoptosis of lung carcinoma A549 cells *in vitro*, implicating a role for TXA<sub>2</sub> also in tumour cell survival (36). In the prostate, an increased expression of TXS and the TP $\alpha$ /TP $\beta$  isoforms directly correlate with the tumour Gleason score and pathologic stage (32, 33, 47), where expression of both TXS and the TP is mainly found in areas of perineural invasion, a recognized mechanism by which PCa cells invade the prostatic capsule and metastasize to other tissues (20, 32). Significantly in the context of PCa, through detailed mechanistic studies, we recently discovered that both the TP $\alpha$  and the TP $\beta$  isoforms directly interact with and regulate signalling by protein kinase C-related kinase/protein kinase novel (PRK/PKN) (48), a family of 3 AGC kinases that act immediately downstream of phosphatidylinositol 3'kinases, and are strongly, yet differentially, implicated in several cancers (49–51) and in B-cell development (52). Indeed, in addition to acting as Rho GTPase effectors, activation of the PRKs (e.g., PRK1) in response to androgen receptor (AR) signalling within the prostate catalyses phosphorylation of histone (H)3 at Thr11 (H3pThr11) which, in turn, serves as a specific epigenetic marker, and gatekeeper, of androgen-induced chromatin remodelling and transcriptional activation (48, 53–55). Hence, owing to their ability to regulate RhoA-/C-mediated responses, including metastatic processes, combined with their epigenetic priming of tumour cells, members of the PRK family are key chemotherapeutic targets particularly in castrate-resistant prostate cancer, the metastatic lethal form of PCa that occurs following androgen deprivation therapy (53, 55, 56).

Indeed, our research shows that TP $\alpha$ -/TP $\beta$ -mediated PRK1 activation not only leads to histone H3 threonine 11 phosphorylation in response to TXA<sub>2</sub> but can also cooperate with the AR to enhance the androgen-induced chromatin remodelling (H3pThr11) and transcriptional activation (48). Collectively, these studies raise the exciting possibility that TXA<sub>2</sub>, through its ability to directly regulate PRK-induced H3pThr11, may be a strong epigenetic regulator, thereby adding to the range of possible mechanisms, whereby the Aspirin-target TXA<sub>2</sub> may influence the neoplastic growth. Added to this complexity, we recently established that the TP $\alpha$  and TP $\beta$  isoforms differentially associate with and regulate signalling by the other individual members of the PRKs (PRK1/PKN $\alpha$ , PRK2/PKN $\gamma$ , PRK3/PKN $\beta$ ) (57). Furthermore, consistent with our previous studies involving PRK1 (48), *siRNA* disruption

of PRK1 and PRK2, but not PRK3, expression eliminates TP-mediated cancer cell responses (proliferation, anchorage-independent growth, migration) and H3pThr11 phosphorylation in the prostate carcinoma PC-3 cell line (57). Identification of a direct, functional interaction of both TP $\alpha$  and TP $\beta$  with the PRKs provides yet another molecular link accounting for the role of TXA<sub>2</sub> in tumour progression, particularly in prostate and other cancers in which the TXA<sub>2</sub>-TP and PRKs are increasingly implicated. Critically, as stated, it suggests that the TXA<sub>2</sub>-TP axis may serve as an epigenetic regulator, adding to the range of possible mechanisms whereby the Aspirin-target TXA<sub>2</sub> may influence neoplastic growth.

### **Factors determining transcriptional regulation of TP $\alpha$ in platelet progenitor megakaryoblastic cells**

Collectively, these and numerous other studies provide significant mechanistic insights into the role of TXA<sub>2</sub> and of the TPs (TP $\alpha$ /TP $\beta$ ) in cancer progression. However, with only limited exceptions (20, 31), few of those studies investigated the roles of the individual TP $\alpha$  or TP $\beta$  isoforms or examined their transcriptional regulation in cancer. To address this and focusing on TP $\alpha$ , the predominant isoform expressed in most cell/tissue types (10), we recently examined its expression in prostate and breast cancer and identified a key role for the tumour suppressor gene product Wilms' tumour (WT)1 in its transcriptional regulation (58). Prior to presenting and discussing these findings, it is first relevant to review knowledge on the transcriptional regulation of TP $\alpha$  in the haematopoietic system where most data and insight is available.

As stated, while TP $\alpha$  and TP $\beta$  are encoded by the same *TBXA2R* gene (Figure 1), they are differentially expressed being regulated by distinct promoters, whereby promoter (Prm)1 exclusively regulates TP $\alpha$  expression and Prm3 regulates TP $\beta$  (7-9, 59, 60). Through initial studies carried out in the platelet progenitor megakaryoblastic human erythroleukaemia (HEL) 92.1.7 and K562 cell lineages (9, 60, 61), the transcription factors Sp1 (stimulating protein 1), early growth response 1 (Egr1) and NF-E2 were identified as the key *trans*-acting factors that bind to the 'core promoter region' of Prm1 to drive basal expression of TP $\alpha$  mRNA (9). In addition, several functional upstream activator regions (UARs; UAR1 and UAR2) and upstream repressor regions (URRs; URR1, URR2 and RR3, where repressor region 3 specifically lies within the core promoter; Figure 1) were identified within Prm1 (9). While GATA-1, Ets-1, Ets-2 and Oct-1 were identified as the main *trans*-acting transcription factors that regulate the UARs in the megakaryoblastic lineages, the tumour suppressor gene product WT1 was found to bind to several GC-enriched consensus *cis*-elements within the repressor regions (URR1, URR2 and RR3) to repress Prm1, maintaining TP $\alpha$  expression at relatively low levels when cells were cultured under basal conditions (60). However, following differentiation of the pluripotent megakaryoblastic cell lineages to the platelet phenotype, it was established that TP $\alpha$  expression was strongly upregulated, and this occurred

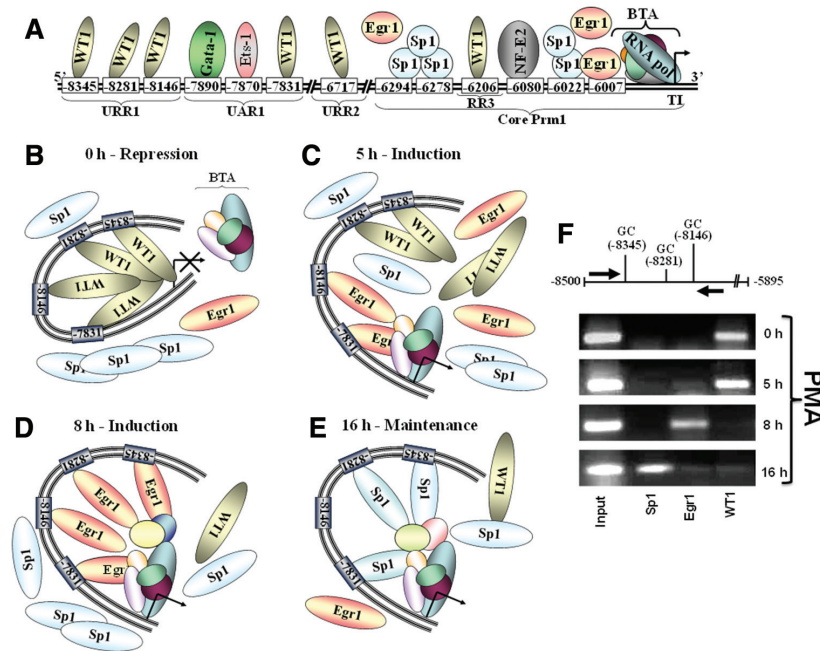
through a complex transcriptional mechanism involving coordinated: (i) *alleviation* of TP $\alpha$ /Prm1 repression by WT1 by displacement of its binding to its consensus GC-enriched *cis*-acting elements within the URRs of Prm1, (ii) *induction* of TP $\alpha$  expression by binding of the transcriptional activator Egr1 to the same *cis*-acting GC elements within Prm1, followed by (iii) *sustained upregulated expression* of TP $\alpha$  through binding of Sp1 also to the same *cis*-acting GC elements within Prm1 (Figure 3) (61). Hence, WT1 plays a central role in repressing TP $\alpha$  expression by binding to multiple *cis*-acting elements within the repressor regions (URR1, URR2 and RR3) of Prm1, maintaining TP $\alpha$  expression at low levels under basal/non-differentiated conditions. However, in response to cellular differentiation, WT1 repression is lifted in favour of sequential high-affinity binding of the transcriptional activators Egr1 followed by Sp1 to the same *cis*-acting GC elements within Prm1 to induce (by Egr1) and maintain (by Sp1) high levels of TP $\alpha$  expression following differentiation (Figure 3) (61).

In addition to its recognized role in normal and aberrant haematopoiesis (62), WT1 was initially described as a tumour suppressor in Wilms' tumour (WT), a rare form of renal cancer (63–65), but can also play an oncogenic role in certain cancers (66–69). Considering the recognized role of WT1 in WT of the kidney and in other cancers while also acting as a key transcriptional repressor/regulator of TP $\alpha$  expression in megakaryoblastic HEL and K562 cell lineages combined with the increasing awareness of the role of the TXA<sub>2</sub>-TP axis in neoplastic progression, we recently investigated the possible regulation of TP $\alpha$ /Prm1 by WT1 in prostate and breast cancer, including in the model prostate PC3 and breast MCF-7 [a model oestrogen receptor/oestrogen receptor (ER)-positive breast cancer cell line] and MDA-MB-231 (a model oestrogen receptor/ER, progesterone receptor/PR and Her2/neu triple-negative breast cancer cell line) carcinoma cell lines, respectively. In brief and consistent with the findings in the megakaryoblastic lineages, it was established that WT1 can repress Prm1-directed TP $\alpha$  expression in both the prostate and the breast cancer lineages. Overall, as elaborated upon in detail later in this chapter, the study provided a comprehensive molecular analyses of the factors regulating the TP $\alpha$  expression through Prm1 in the prostate and breast and suggested that aberrant regulation by/or dysfunction of the tumour suppressors WT1, along with hypermethylated in cancer (HIC) 1, may account at least, in part, for the increased association of TXA<sub>2</sub>/TP signalling with certain prostate and breast cancers and, potentially, in other cancers in which TXA<sub>2</sub>, WT1 and/or HIC1 are implicated (58). The reader is referred to the original study for full details on the role of HIC1 in its regulation of TP $\alpha$ /Prm1 expression (58), while this communication will mainly focus on the role of WT1.

### **Role of Wilms' tumour 1 in regulating TP $\alpha$ expression in prostate and breast cancer**

The WT1 gene encodes a zinc finger transcription factor critical for development of the genitourinary, haematopoietic and central nervous systems (62, 70). The finding that mutations

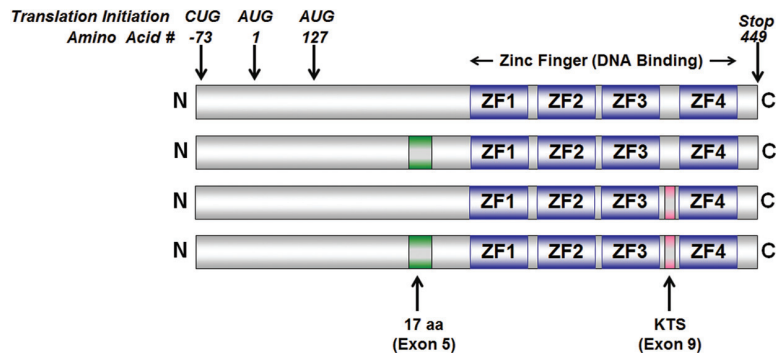




**Figure 3.** Proposed model for PMA-mediated increases in Prm1 activity. Panels A-E: Proposed model for PMA induction of Prm1/TP $\alpha$  mRNA transcriptional regulation in HEL92.1.7 and K562 megakaryoblastic cells. In resting cells, WT1 binds in a cooperative manner to multiple adjacent GC elements (at -8345, -8281, -8146 and -7831) within Prm1 to impair transcription initiation by the basal transcription apparatus (BTA) and thereby repressing TP $\alpha$  mRNA expression (Panels A and B; Repression). In response to exposure to PMA for ~5 h, the up-regulated expression of Egr1 results in increased high-affinity binding of Egr1 to Prm1, thereby activating Prm1-directed *TBXA2R* transcription to up-regulate TP $\alpha$  mRNA expression (Panel C; Induction). After exposure to PMA for ~8 h, a further, enhanced increase in Egr1 binding coincides with nuclear export and a resulting reduction in WT1 binding (Panel D; Induction). The decrease in WT1 binding results in de-repression of Prm1 and in a further increase in Egr1 binding, leading to a more pronounced transcriptional activation of Prm1 by the BTA (Panel D; Induction). Following the prolonged exposure to PMA for ~16 h, decreased Egr1 binding coincides with its rapid protein turnover (Panel E). PMA-mediated differentiation of cells can also lead to phosphorylation of Sp1 and/or its increased expression, enhancing its DNA-binding activity. Therefore, the increased affinity of Sp1 for Prm1, coinciding with the decreased Egr1 expression, facilitates binding of Sp1 to Prm1, thereby resulting in a sustained increase in Prm1 activity and TP $\alpha$  expression as the differentiation of HEL and K562 cells progresses toward the platelet phenotype (Panel E; Maintenance). Panel F: Representative chromatin immunoprecipitation (ChIP) analysis of Sp1, Egr1 and WT1 binding *in vivo* to Prm1 of the *TBXA2R* in HEL cells as a function of PMA-induced cellular differentiation. *Note:* Data presented in this figure were reproduced from our previous study (62), and the reader is referred to the original manuscript for the experimental details that led to the proposed model (panels B-E).

within the WT1 gene are a leading cause of the childhood renal cancer WT first led to the suggestion that it might serve as a tumour suppressor (64). However, the fact that non-mutated/wild-type WT1 is also abundantly expressed in a variety of cancers, including cancers of the breast (71), oesophagus (72) and pancreas (73), indicated that WT1 might also play an oncogenic role.

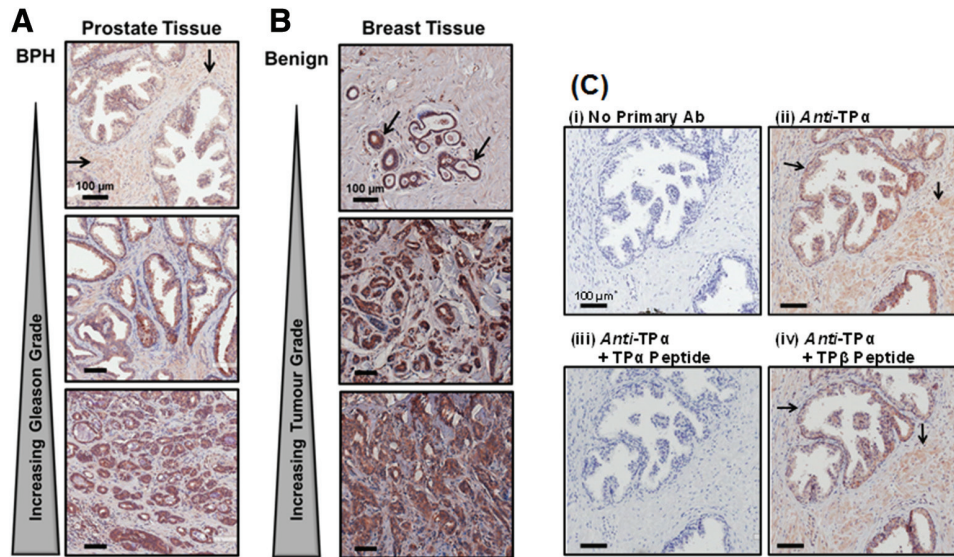
In addition to acting as a regulator of transcription, WT1 can also play a role in post-transcriptional regulation, including in RNA splicing (74, 75), and also in translation (76). Such diverse functions are likely due to fact that WT1 protein exists as multiple isoforms that arise owing to differential splicing and/or the use of multiple translational initiation sites within the WT1 gene (77). Of the most prevalent WT1 isoforms, the best characterized are the variants that differ due to the presence or absence (+/-) of exon 5 and +/-KTS (Lys-Thr-Ser) sequences (Figure 4). More specifically, differential splicing at these two sites yields four different isoforms, namely (+/+), (+/-), (-/+), and (-/-), each of which differ in respect of exon 5 and KTS sequences, respectively. While the -KTS isoforms can act as transcriptional repressors or activators, the +KTS isoforms do not readily bind DNA and, therefore, are less active in the process of transcription [reviewed in reference (62)]. WT1 has four Kruppel-like C<sub>2</sub>H<sub>2</sub> fingers within its C-terminal region that share similarity with those of the aforementioned Egr1, another prominent member of the zinc finger family of transcription factors. While the zinc finger domain of WT1 can facilitate its DNA at the consensus Egr1 DNA-binding site (consensus sequence 5'GCG(G/T)GGGCG3'), the binding affinity of WT1 for the Egr1 consensus site is significantly less than that of Egr1 itself (78). In addition to binding to WT1 and/or Egr1 *cis*-acting elements, WT1 can also bind to another motif termed the Wilms' tumour element (consensus sequence, 5'GCGTGGGAGT3') (79). Hence, depending on the cellular context and/or on the particular promoter, WT1 can therefore serve as a transcriptional repressor or activator. This is exemplified in the case of c-Myc where over-expression of WT1 in K562, a HEL cell line, and in breast cancer cells activates the c-Myc promoter (80) but WT1 represses the c-Myc promoter in HeLa cells (81). WT1 can also complex with other DNA-binding *trans*-acting co-factors, such as p53 (82), and with certain co-activators or co-repressors, such as CBP (83) or BASP1 (84), respectively, to regulate transcription. It is the identity of these co-factor-binding partners that determines whether WT1 serves as an activator or repressor during transcription. In the case of the *TBXA2R*, the overwhelming evidence is that WT1 predominantly acts as a transcriptional repressor by binding to multiple GC-enriched *cis*-acting elements within *Prm1* to suppress TP $\alpha$  expression in pluripotent megakaryoblastic cells but that in response to cellular differentiation, this repression is lifted coinciding with the increased expression and binding of Egr1 to the same GC elements to induce TP $\alpha$  expression (Figure 3) (9, 60, 61). In turn, following Egr1-mediated induction, subsequent binding of the constitutive Sp1 to the same *cis*-elements within *Prm1* maintains the expression of TP $\alpha$  at high levels in the fully



**Figure 4.** Schematic representation of Wilms' tumour (WT) 1 isoforms. WT1, encoded by the tumour suppressor gene WT1, can exist as multiple isoforms depending on the translational start site and the inclusion/exclusion of amino acids encoded by exon 5 (encodes a 17 aa sequence) or exon 9 [encodes the 3 aa sequence KTS (Lys-Thr-Ser) located between Zinc Finger (ZF) domains 3 and 4]. The best characterized isoforms are the  $\pm$  exon 5 and the  $\pm$  exon 9/KTS isoform variants, representing the four most abundantly expressed forms of WT1 with a molecular mass of 52–54 kDa. In addition, the initiation of translation can occur at an upstream CUG codon (-73), resulting in the generation of larger WT1 isoforms with a mass of 62–64 kDa or alternatively can occur at a downstream AUG codon (+127) giving rise to smaller WT1 isoforms of 32–34 kDa. WT1 has four Kruppel-like  $C_2H_2$  zinc fingers, ZF1–ZF4, within its C-terminal DNA-binding domain that share significant identity with those of the early growth response (Egr)1, another member of the zinc finger family of transcription factors. While the -KTS isoforms of WT1 can either repress or activate transcription, the +KTS isoforms have a reduced ability to bind DNA and therefore are less transcriptionally active (62). The increased affinity of Egr1 relative to that of WT1 for binding to the same *cis*-acting elements accounts for why Egr1 can displace WT1 binding when progressing from transcriptional repression by WT1 to induction by Egr1 as exemplified by the coordinated regulation of Prm1/TP $\alpha$  expression during cell differentiation (Figure 3).

differentiated state (61). The known increased affinity of Egr1 relative to that of WT1 for binding to the same *cis*-acting elements accounts for why Egr1 can displace WT1 binding when progressing from transcriptional repression by WT1 to induction by Egr1 as exemplified by the coordinated regulation of TP $\alpha$ /Prm1 during cell differentiation (Figure 3) (61, 78).

In our recent studies investigating the expression and the transcriptional regulation of TP $\alpha$  through Prm1 in prostate and breast cancer, immunohistochemical analysis confirmed that the expression of TP $\alpha$  correlated with increasing prostate and breast tissue tumour grade (Figure 5), while stimulation of the prostate (PC3) and breast (MCF-7 and MDA-MB-231) carcinoma cell lines with the TXA<sub>2</sub> mimetic U46619 increased both cell proliferation and migration (58). Collectively, these data provided further evidence of a role for the TXA<sub>2</sub>-TP signalling axis in prostate and breast cancer progression. In order to identify the factors



**Figure 5.** Analysis of TP $\alpha$  expression in the prostate and breast. Panels A and B: Immunohistochemical analysis of prostate and breast tissue (benign and increasing tumour grade) screened with an affinity-purified *anti*-TP $\alpha$  antibody (200 $\times$  magnification; counterstained with haematoxylin). The increased TP $\alpha$  expression coincides with an increased prostate and breast cancer tumour grade. Panel C: Immunohistochemical analysis of full-face benign prostate tissue screened either (i) in the absence of a primary antibody or with the (ii) affinity-purified *anti*-TP $\alpha$  antibody. The specificity of the *anti*-TP $\alpha$  antibody was confirmed, whereby the (iii) immunogenic TP $\alpha$  peptide, (iv) but not a TP $\beta$  specific-peptide, competed out the *anti*-TP $\alpha$  immune-staining. The arrows in (ii) and (iv) indicate specific detection of TP $\alpha$  expression in the prostate glandular epithelial cells and in the fibromuscular stromal smooth muscle cells of the prostate tissue. All sections were counterstained with haematoxylin, and images shown were captured at 200 $\times$  magnification. *Note:* Data presented in this figure were reproduced from our previous study (58).

regulating TP $\alpha$  in the prostate and breast through Prm1, genetic-based reporter analyses confirmed that the repressor regions, designated URR1, URR2 and RR3 and previously identified within Prm1, are functional in the prostate and breast carcinoma lineages. Furthermore, in each of the prostate and breast carcinoma lineages studied, over-expression of WT1 repressed TP $\alpha$  mRNA and Prm1-directed reporter gene expression, while chromatin immunoprecipitation analysis confirmed that WT1 binds *in vivo* to each of the consensus GC-enriched *cis*-elements within the repressor regions of Prm1. Furthermore, in the prostate and breast cellular systems, it was established that the tumour suppressor HIC1 represses TP $\alpha$  mRNA expression through its binding to a functional *cis*-element, referred to as the HIC1(b) element, within Prm1 in PC3 and MCF-7 cells, while a second HIC1 element,

referred to as HIC1(a), was identified within Prm1 through bioinformatics analyses but was not found to be functional (58).

Among the many cell-specific differences we observed in the transcriptional activity of Prm1 in the prostate and breast carcinoma-derived cell types, it was noteworthy that the UAR1/UAR2 repressor regions within Prm1, previously identified in the megakaryoblastic HEL92.1.7 and K562 cell lines (9) where they are regulated by GATA-1, Ets-1, Ets-2 and Oct-1, were not found to be functionally active in the prostate PC3 or breast MCF-7 lineages (Table 1). These observations pointed to clear cell-/tissue-specific differences in the regulation of TP $\alpha$  expression through Prm1 in the haematopoietic system *versus* prostate and/or breast tissues. Moreover, as summarized in Table 1, our analysis of Prm1 in the prostate and breast carcinoma lineages revealed additional cell-specific differences in the upstream regulatory regions. Included in this is a prostate-specific Novel URR identified in PC3 cells, a novel UAR1 in PC3 and MCF-7 cells and an additional Novel UAR2 in PC3, MCF-7 and MDA-MB-231 cells (58). Detailed bioinformatic analysis by us revealed a putative oestrogen response element (ERE) within the Novel URR (-7962 to -7859; Table 1), suggesting that this region might function as a binding site for the oestrogen and/or ARs (85). The finding that oestrogen production increases in men with age, mainly due to aromatase conversion of androgens to oestrogens, indicates that like androgens (86), oestrogens may also play a role in PC progression (87, 88). Whether the ERE within the Novel URR of Prm1 regulates the TP $\alpha$  expression in the prostate remains to be investigated.

With regard to the Novel UAR1 (-7504 to -6848; Table 1) identified in both the prostate PC3 and the ER-positive breast MCF-7 lines, but not in the triple-negative breast cancer MDA-MB-231 cell line, several putative *cis*-acting binding elements were identified through bioinformatics, including multiple EREs that lie in close proximity to each other. While remaining speculative, binding of the ER to some or all of these adjacent EREs may explain why the Novel UAR1 repressor region is functional in both the ER-positive PC3 and the MCF-7 lines but not active in the ER-negative MDA-MB-231 cell line.

In terms of the novel activator regions identified within Prm1 and confirmed to be transcriptionally active in all prostate and breast cell lineages examined, the Novel UAR2 located between -6648 and -6492 of Prm1 was of particular note (Table 1). Specifically, similar to that found in all other repressor regions (UAR1, UAR2 and RR3) within Prm1, putative binding elements for Egr1 and stimulating protein (Sp) 1 were also identified within this novel prostate-/breast-specific Novel UAR2 region of Prm1. As stated, our previous studies in differentiated megakaryoblastic HEL and K562 lineages established that both the inducible Egr1 and the constitutive Sp1 factors bind to multiple GC-consensus WT1 *cis*-elements within the UAR1, UAR2 and RR3 repressor regions of Prm1 to strongly up-regulate the TP $\alpha$  mRNA expression (9, 60, 61). Hence, it is indeed possible, if not likely, that the expression of TP $\alpha$  through Prm1 in the prostate and

**Table 1.** Activator and repressor regions within Prm1

		Cell type <sup>1</sup>			
<i>Regulatory regions initially identified in megakaryoblastic lineages<sup>2</sup></i>					
Regulatory region <sup>2</sup>	Position within Prm1 <sup>3</sup>	HEL 92.1.7	PC3	MCF-7	MDA-MB-231
URR1	-8500 to -7962	Yes	Yes	Yes	Yes
URR2	-6848 to -6648	Yes	Yes	Yes	Yes
RR3	-6258 to -6123	Yes	Yes	Yes	Yes
UAR1	-7962 to -7859	Yes	No	No	No
UAR2	-7859 to -7504	Yes	No	No	Yes
Core	-6320 to -5895	Yes	Yes	Yes	Yes
<i>Novel regulatory regions identified in prostate or breast cancer cell lineages<sup>1</sup></i>					
Regulatory region <sup>1</sup>	Position within Prm1	HEL 92.1.7	PC3	MCF-7	MDA-MB-231
Novel URR	-7962 to -7859	No	Yes	No	No
Novel UAR1	-7504 to -6848	No	Yes	Yes	No
Novel UAR2	-6648 to -6492	No	Yes	Yes	Yes

<sup>1</sup>This table has been adapted from our previous study (58).

<sup>2</sup>From references (9, 60, 61).

<sup>3</sup>The nucleotide numbers given are relative to the translational initiation codon at +1 within the *TBXA2R*.

breast may be subject to a similar type of complex transcriptional regulation involving occupancy of the common/shared and Novel prostate-/breast-specific GC-enriched *cis*-acting elements that can act as consensus binding sites for WT1, Egr1 and/or Sp1, where temporal occupancy may possibly be determined by the (patho)physiologic setting. Clarity on this matter remains to be experimentally determined and is necessary to shed further light on the transcriptional regulation of TP $\alpha$  in both the normal and the malignant prostate/breast tissue and potentially in other tissues in which the TXA<sub>2</sub>-TP axis is implicated. Critically, given that Egr1 serves as a master regulator in several key aspects of prostate and breast cancer progression (89, 90), investigation of the interplay between WT1 and Egr1 in the regulation of TP $\alpha$  expression through Prm1 within the *TBXA2R* as a function of tumour grade merits detailed investigation.

### Conclusions and future perspectives

The prostanoid TXA<sub>2</sub> plays a central role in haemostasis and is widely implicated in a range of cardiovascular, renal, pulmonary and prostate diseases (1-3, 5). In humans, TXA<sub>2</sub> signals

through TP $\alpha$  and TP $\beta$ , two structurally related TP isoforms that display both common, overlapping and isoform-specific physiologic roles (6). TP $\alpha$  and TP $\beta$  are encoded by the same gene, the *TBXA2R*, but are differentially expressed being regulated by distinct promoters where Prm1 regulates TP $\alpha$  expression, mainly involving NF-E2, Sp1, GATA-1, Ets-1, WT-1/Egr1, and Prm3 regulates TP $\beta$ , involving cFos/cJun and Oct-1/-2 (7-9, 59, 60). While the clinical evidence for the role of the TXA<sub>2</sub>-TP axis in neoplastic progression is increasing, few studies to date have investigated the role of the individual TP $\alpha$ /TP $\beta$  isoforms in human cancer or indeed in most other diseases in which TXA<sub>2</sub> is implicated (20, 31). Focussing on TP $\alpha$ , we investigated its expression and transcriptional regulation through Prm1 in prostate and breast cancer and established that the tumour suppressor protein WT1 plays a key role in regulating its expression in both tissue types. Critically, it was established that WT1 can repress TP $\alpha$  expression through binding to multiple GC-consensus *cis*-elements within previously recognized regulatory regions within Prm1, while several other prostate- and/or breast-specific novel regulatory regions were identified accounting for cell-/tissue-specific regulation of TP $\alpha$ . Taken together with previous studies in platelet progenitor megakaryoblastic lineages (9, 60, 61), the findings provide a strong genetic basis for understanding the diverse physiological roles played by TXA<sub>2</sub>-TP axis within the vasculature and other systems, including potentially in the progression of certain human cancers. These studies may also provide a mechanistic basis accounting, at least in part, for the prophylactic benefits of Aspirin in reducing certain cancer risks by lowering the overall TXA<sub>2</sub> levels.

WT1 has recently emerged as an important target in immune-therapy approaches to treat certain cancers, and ongoing clinical trials involving the WT peptide 1 vaccination are proving positive in reducing tumour growth in breast and lung cancers, in leukaemia and, more recently, in glioblastoma (91-93). As the TXA<sub>2</sub>-TP axis has been implicated in the development of prostate and breast cancer (20, 33, 47, 94, 95), the findings herein suggest that aberrant WT1 regulation of TP $\alpha$  expression may contribute to such cancers and potentially to WT itself. In addition, bearing in mind that the TP $\alpha$  and TP $\beta$  isoforms display a number of important functional similarities but also differences in terms of their signalling (6) and regulation including in certain cancers (96, 97), coupled with the fact that they are regulated by distinct promoters within the *TBXA2R* gene, it will be of considerable interest to investigate the expression and transcriptional regulation of TP $\beta$  in human cancers, in particular in prostate and breast cancer.

### Abbreviations

ADT, androgen deprivation therapy; AR, androgen receptor; ChIP, chromatin immunoprecipitation; COX, cyclooxygenase/prostaglandin G2/H2 synthase; CRPC, castrate-resistant prostate cancer; C-tail, carboxyl-terminal tail; Egr1, early growth response 1; ER, oestrogen receptor; ERE, oestrogen response element; ERK, extracellular signal-regulated protein kinase; GPCR, G-protein-coupled receptor; HEL, human erythroleukaemia; H3Thr11, histone H3 threonine 11; IHC,

immunohistochemistry; PCa, prostate cancer; PDK-1, 3-phosphoinositide-dependent protein kinase-1; PG, prostaglandin; PI3K, phosphatidylinositol 3'kinase; PK, protein kinase; PRK, protein kinase C-related kinase; RR, repressor region; SDM, site-directed mutagenesis; Sp1, stimulating protein 1; TP, thromboxane prostanoid receptor; TX, thromboxane; TXS, TXA synthase; UAR, upstream activator region; URR, upstream repressor region; WT1, Wilms' tumour 1.

### Conflict of Interest

The author declares no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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