Epigenetic Inactivation of the Placentally Imprinted Tumor Suppressor Gene *TFPI2* in Prostate Carcinoma

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Abstract. *Background: Imprinted genes are often arranged in clusters epigenetically controlled by differentially methylated regions (DMR) containing bivalent histone modifications. Both DNA hypermethylation and hypomethylation in cancer can therefore disturb imprinted gene expression. We have studied expression, DNA methylation and histone modifications of TFPI2, a presumed tumor suppressor, and that of other genes in the 7q21 imprinted gene cluster in prostate cancer. Materials and Methods: TFPI, TFPI2, SGCE and PON2 expression were assessed by qRT-PCR in prostate cancer tissues and cell lines. DNA methylation and histone modifications were investigated by bisulphite sequencing and chromatin immunoprecipatation. Results: TFPI2 was highly variably expressed in cancer tissues, in contrast to TFPI, and did not correlate to unchanged SGCE and significantly elevated PON2 expression. TFPI2 expression variations were unrelated to global DNA hypomethylation, but were associated with promoter methylation. PC3 cells with high expression retained normal methylation and bivalent histone modifications at DMR and promoter, whereas low-expressing LNCaP cells presented aberrant DNA methylation and more repressive histone modifications. Conclusion: Epigenetic disturbances in the 7q21 cluster affect imprinted genes in a non-coordinate manner suggesting an unstable epigenetic state prone to selection for specific expression changes.*

Epigenetic aberrations accompany the development of many cancer types, including prostate carcinoma (1, 2). The best characterized change is hypermethylation of certain tumour suppressor genes, but global hypomethylation is also prevalent in many cancer types (3).

While global methylation loss mainly results in the hypomethylation of multicopy repetitive genomic elements, such as *LINE-1* and *Alu*, some CpG islands associated with genes, inactivated in a tissue-specific manner, can also become targets of DNA hypomethylation. This latter process can lead to inappropriate expression of genes, which then can drive misdifferentiation, disturb cell identity and promote metastasis (4-6).

Imprinted genes may be affected by both hypermethylation and hypomethylation in cancer, since they often contain differentially methylated regions (DMR) in their imprinting control regions that control the expression of individual genes or entire gene clusters. These present hot spots for hypomethylation in cancer, in inborn imprinting disorders and in assisted reproduction technology (ART)-derived human and animal offspring (7-10). Since DMRs are often crucial for the complex epigenetic regulation of parent-specific monoallelic expression of several genes in a cluster, their hypomethylation may affect the expression of multiple neighbouring imprinted genes (11).

The chromatin of differentially methylated regions is reciprocally enriched with active and inactive histone modifications. The methylated allele is usually enriched with H3 lysine 9 and lysine 27 methylation, whereas the active allele lacks DNA methylation and is enriched with H3 lysine 9 acetylation and lysine 4 methylation (12-15). This chromatin constellation resembles the bivalent histone modification profile, namely the juxtaposition of active and inactive histone marks that is found in pluripotent stem cells on many genes functioning in early embryonic development (16). Bivalent chromatin has been suggested to predispose genes for later tumour-specific *de novo* DNA methylation (17, 18).

Many imprinted genes have growth related function, which makes them potential targets for changes during tumourigenesis (7, 10, 19). In the placenta, the maternally expressed genes typically limit placental growth and invasion of the trophoblast. On the other hand, paternally expressed

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genes increase the efficiency of the placenta and promote fetal growth (20-22). The presence of both paternally and maternally expressed genes in imprinted clusters implies a functional opposition and coordinated control. Because of their potential tumour-suppressive function, maternally expressed genes are often down-regulated in different cancer types. Therefore epigenetic aberrations associated with tumour progression may contribute to inactivation of such tumour suppressors (23, 24).

The maternally expressed *TFPI2* gene is specifically imprinted in the placenta, where it is thought to limit trophoblast invasion into the decidua (25, 26). It is part of the imprinted gene cluster on human chromosome 7q21 (mouse chromosome 6), which is situated around two paternally expressed genes – paternally-expressed gene 10 (*PEG10*) and sarcoglycan epsilon (*SGCE*) (27).

Together with its homologue TFPI, TFPI2 can inhibit the coagulation-initiating protease tissue factor (TF) which exerts additional haemostasis-independent functions during embryonal and placental morphogenesis, but also in oncogenic growth, tumour cell migration, angiogenesis and metastasis (28). TF is a component of prostate secretions that can accumulate in the disorganized carcinoma interstitium and contribute to stroma remodelling and angiogenesis (29, 30). TF levels positively correlate with Gleason score (31). Therefore, tight control of TF by TFPI and TFPI2 may be an important tumour-suppressing mechanism in prostate carcinoma. TFPI2 has been shown to decrease the invasive potential of the LNCaP prostate cancer cell line, but it is not known to what extent the TFPIs are misregulated in prostate cancer tissues and which mechanism might underlie any change (32).

TFPI2 has been reported to be down-regulated in several types of cancers, where its restoration inhibits cancer migration and invasion (32-36). Loss of TFPI2 function may, therefore, promote metastasis. Among the mechanisms implicated in *TFPI2* down-regulation are promoter DNA hypermethylation, histone deacetylation, chromosome loss and aberrant splicing (37-41).

Global hypomethylation of DNA correlates with prostate cancer progression and is most pronounced in advanced, metastatic cases (1, 5, 42, 43). One might, therefore, ask whether global hypomethylation in prostate cancer affects the methylation pattern of the DMR and the expression regulation of the 7q21 imprinted locus and especially of the *TFPI2* gene. The *TFPI2* promoter and the nearby DMR have been shown to be associated with allele specific active H3K4me3 and inactive H3K9me3 histone marks in the mouse placenta (13, 27). As argued above, this bivalent-like chromatin conformation could moreover target the *TFPI2* promoter for hypermethylation in cancer.

On the basis of these considerations, we have studied the expression of *TFPI2* in prostate carcinoma tissues and cell lines, its correlation with changes in other genes in the cluster as well as DNA methylation and histone modifications in the *TFPI2* promoter and 7q21 imprinting cluster DMR.

Materials and Methods

Patient samples and cell lines. The array of prostate carcinoma samples and benign areas from cancer-carrying prostates was obtained and has been characterized for multiple clinical and molecular features as described elsewhere (42-44). Patient consent was obtained and the study approved by the Ethics Committee of the Medical Faculty of the Heinrich Heine University. The prostate carcinoma cell lines LNCaP, 22Rv1, PC3, and DU145 were cultured and treated with 5-aza-2'-deoxycytidine (5-aza-dC; Sigma, Taufkirchen, Germany) as described (45). 5-aza-dC was supplied at 2 μM every 24 h for 3 days.

DNA and RNA extraction. DNA and RNA were extracted from identical powdered tissues as described previously (43).

Quantitative RT-PCR. Messenger RNA expression of *TFPI, TFPI2, PON2,* and *SGCE* was quantified using QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) and QuantiTect (Qiagen) primer assays (Hs_TFPI_1_SG #QT00086149, Hs_TFPI2_1_SG #QT00062804, Hs_PON2_1_SG #QT00095690, and Hs_SGCE_1_SG #QT00052507) on an ABI 7900 instrument. TBP served as internal control (Hs_TBP_1_SG #QT00000721). Each run was standardized using a dilution series of cDNA from a strongly expressing cell line or normal tissue. Experimental variation for each sample was below 10%.

Bisulphite sequencing. Isolated DNA was subjected to bisulphite modification using the EZ DNA methylation-Gold Kit (Zymo Research, Hiss Diagnostics, Freiburg, Germany) according to the manufacturer's instructions. The *TFPI2* promoter and DMR regions were then amplified using HotStarTaq DNA polymerase (Qiagen) with the primers FWD 5'-GGTTAGATATTTGTTGGTTTTTGAG-3', REV 5'-CTCTCCCTCTTACACAATTTAC-3'; DMR FWD 5'-GTGTTATGTTTTATAAATAGATAAG-3', REV 5'-AACTCATATA CCTCTACAATTC-3') published by Monk *et al*. 2008 (27) using the following conditions: hot start at 95˚C for 15 min followed by 35 cycles of PCR (94˚C for 30 s, annealing temperature for 30 s, and 72˚C for 45 s) with a final extension for 5 min at 72˚C. Annealing temperature was 54˚C for *TFPI2* and 48˚C for DMR. Lack of amplification of control genomic DNA proved specificity for bisulphite-treated DNA. Prior to sequencing, the PCR products were cloned into pCR4-TOPO vector (TOPO TA Cloning Kit for Sequencing; Invitrogen, Carlsbad, USA). The vectors were then used to transform competent *E. coli* cells (One Shot TOP10 Competent Cells, Invitrogen). After antibiotic selection, plasmids were isolated using the 5 Prime Kit (5 Prime, Hamburg, Germany) and the correct size of the inserted fragment was controlled by *EcoRI* digestion. Four to eight plasmid clones per gene and sample were then sequenced with M13 uni primer (5'-TGT AAA ACG ACG GCC AGT-3') by the central sequencing facility (BMFZ) at our institution.

Chromatin immunoprecipitation. ChIP was performed with the ChIP-IT Express Kit (#53008; Active Motif, Brusseles, Belgium) according to the instructions of the manufacturer. In brief, intact

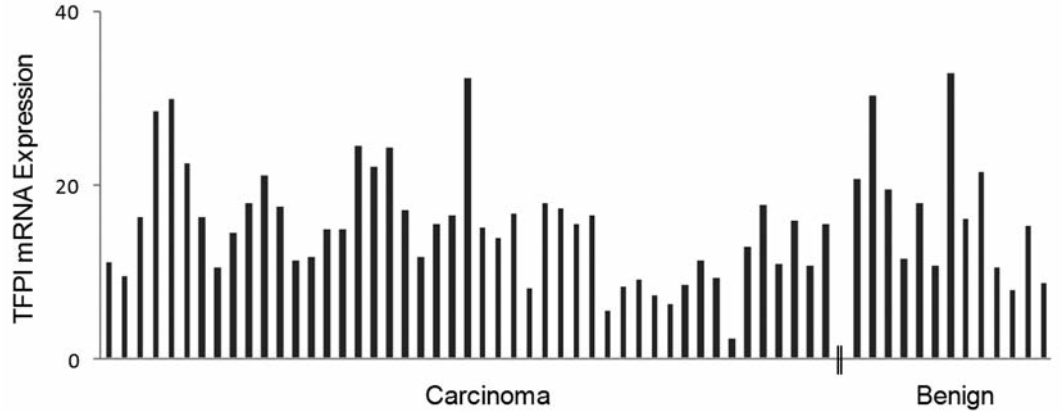

Figure 1. *TFPI mRNA expression relative to TBP in 45 prostate carcinoma and 13 benign prostate tissue samples measured by quantitative RT-PCR.*

cells were fixed with 1% formaldehyde to preserve protein-protein/DNA interactions. The cross-linked chromatin was sheared by sonication to obtain fragments in the range 200-1500 bp. The DNA was purified with QIAquick PCR Purification Kit (Qiagen) and the concentration determined on a Nanodrop instrument. Fragment size was controlled on a 1% agarose gel. Estimated 7 μg of sheared chromatin per reaction was immunoprecipitated overnight with protein-G-coated magnetic beads, anti-sera against H3K4me3 (#ab8580), H3K9ac (#ab4441), H3K9me3 (#ab8898) (Abcam, Cambridge, UK), H3K27me3 (#39535, Active Motif) or positive (RNA Polymerase II antibody) or negative (IgG antibody) control antibodies (CHIP-IT Control Kit- Human #53010, Active Motif), in the presence of proteinase inhibitor cocktail. After washing out unbound proteins from the beads, the bound chromatin was eluted, cross-links were reversed, and DNA was recovered after treatment with proteinase K. Before DNA was used for PCR analysis, it was treated with a proteinase K inhibitor (ChIP-IT Express Kit, Active Motif). DNA from a sample from the non-precipitated sheared chromatin was purified in parallel to the ChIP reactions, and was used to create a standard curve in the QPCR analysis.

Analysis of immunoprecipitated DNA by real-time QPCR. The bound ChIP fractions were quantified by real-time PCR amplification using SYBR Green PCR mix (Qiagen) and the following primer pairs: *TFPI2* FWD 5'-CTCCGCCGGTTG GGGAGAGA-3', REV 5'-GGGCCGCCTGGAGCAGAAAG-3'; DMR FWD 5'-AATGTGCCAGTGGTCGCGGG-3', REV 5'-GCCCGCCGCTAGAGGGAGTA-3'; *CTCFL* FWD 5'-GAACAG CCCATGCTCTTGGAG-3', REV 5'-CAGAGCCCACAAGCC AAAGAC-3'; and *GAPDH* primers from ChIP-IT Control Kit (Active motif) with the following conditions: a hot start at 95˚C for 15 minutes, followed by 45 cycles of PCR (94˚C for 15 s, 60˚C for 30 s, and 72˚C for 30 s), followed by a dissociation step (95˚C for 15 s, 60˚C for 15 s, 95˚C for 15 s). The 'Input DNA' purified from the sheared unprecipitated chromatin was used to make a standard curve. The relative quantity of the measured active histone modifications (H3K4me3 and H3K9ac) on the *TFPI2* and DMR genomic regions were normalized versus the enrichment of those modifications on the housekeeping gene *GAPDH*. Analogously, inactive histone modification (H3K9me3 and H3K27me3) enrichment on the regions of interest was normalized to the respective enrichment on the testis-specific gene *CTCFL* (*BORIS*). This procedure not only minimizes the handling errors during purification of DNA from the bound fractions, but also allows for a quantitative comparison of chromatin conformation of genes in different cell lines.

Statistical analysis. The significance of statistical comparisons was evaluated by means of Mann-Whitney *U* and Kruskal-Wallis tests. Gene expression was correlated to the presence of LINE-1 hypomethylation, tumour stage (pT2 *vs*. pT3 + pT4), Gleason score (<7 *vs*. 7 *vs*. >7), the presence of lymph node metastasis, and biochemical recurrence in the tumour samples. A value of $p<0.05$ was considered significant.

Results

Analysis of *TFPI, TFPI2, SGCE* and *PON2* expression in prostate cancer tissues and cell lines. The mRNA levels of *TFPI* and *TFPI2* were assessed by means of real time RT-PCR in 47 prostate carcinoma tissue samples and 13 benign prostate tissues. *TFPI* mRNA levels were found to be relatively stable in both carcinoma and normal samples (Figure 1). In contrast, *TFPI2* expression was highly divergent in cancerous as well as benign tissues (Figure 2). Overall, neither *TFPI* nor *TFPI2* mRNA levels significantly differed from those in normal tissues. They neither correlated with tumour stage, Gleason score, lymph node metastasis status nor recurrence.

TFPI2 is an imprinted gene within a gene cluster on chromosome 7q21, and could therefore be affected by global DNA hypomethylation. However, the presence of global hypomethylation in the carcinoma samples, as measured by *LINE-1* hypomethylation, did not significantly correlate to *TFPI2* levels (Figure 3).

Although it is not known to what extent the genes of the imprinted cluster on 7q21 are co-regulated, their clustering hints at a common regulation. The arrangement of several genes of the cluster is shown in Figure 4. We measured the

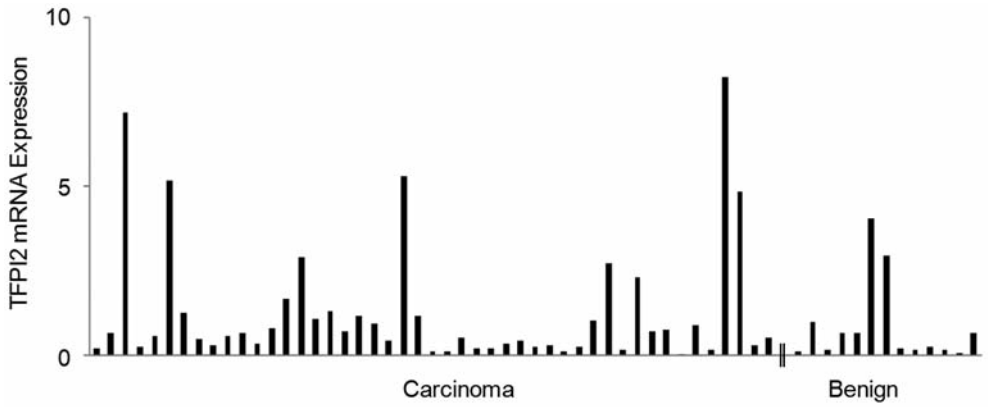

Figure 2. *TFPI2 mRNA expression relative to TBP in 45 prostate carcinoma and 13 benign prostate tissue samples measured by quantitative RT-PCR.*

expression of the paternally expressed *SGCE* and the flanking gene *PON2* (Figure 5) and assessed their correlation with the expression of *TFPI2*. Neither of the two genes was significantly co-expressed with *TFPI2* (for SGCE p=0.087; for PON2 p=0.076). Therefore, we conclude that the assessed genes are independently expressed in prostate carcinoma and normal prostatic tissue.

Interestingly, *PON2* was highly significantly overexpressed in the carcinomas in comparison to the normal samples (p=0.003), whereas *SGCE* expression did not differ significantly (Figure 5). Neither *PON2* nor *SGCE* expression was statistically different between high stage (pT3 + pT4) and lower stage (pT2) carcinomas or related to other clinical parameters.

As in the tissue samples, *TFPI2* expression was also highly divergent among prostate cancer cell lines. Whereas PC3 and DU145 cells expressed significant levels, LNCaP, 22Rv1 and MDA-PCA 2B cells showed only very low expression (Figure 6). In order to study the mechanisms leading to *TFPI2* down- and up-regulation, we chose LNCaP and PC3 as representatives of cell lines with low and high expression, respectively.

Analysis of CpG methylation of TFPI2 promoter and 7q21 differentially methylated region in selected prostate cancer tissues and cell lines. The *TFPI2* promoter has been reported to be frequently hypermethylated in various tumour types. Bisulphite sequencing was applied to assess *TFPI2* promoter methylation in normal prostate epithelial cells, normal urothelial cells, each two high- and low-*TFPI2*-expressing prostate carcinoma tissues, as well as PC3 and LNCaP cells. All normal cells as well as the highly expressing carcinomas and the PC3 line lacked *TFPI2* promoter methylation. In contrast, the promoter was significantly, albeit partially, hypermethylated in LNCaP cells and the two low-*TFPI2*-expressing carcinomas (Figure 7 left panel).

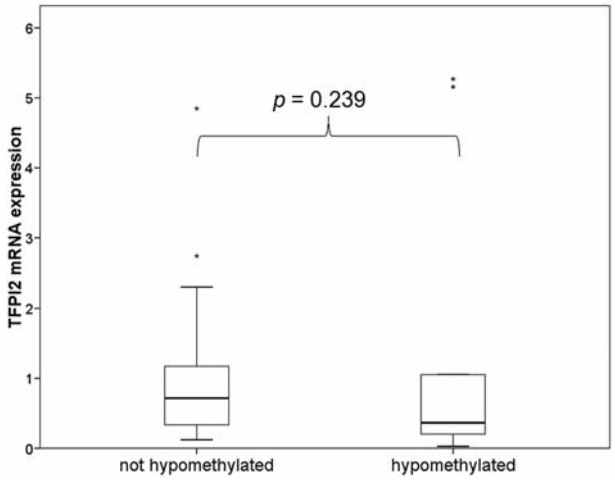

Figure 3. *TFPI2 expression in 45 carcinoma samples, of which 14 displayed significant LINE-1 hypomethylation, indicating global hypomethylation (right) compared to 31 samples with relatively normal global methylation (left). Mann-Whitney U test was used to assess the statistical significance of the difference. Asterisks represent outliers.*

The methylation of the DMR was assessed by bisulphite sequencing in the same samples for which *TFPI2* promoter methylation was measured. In a normal state, the typical pattern of the DMR consists of fully methylated and fully unmethylated alleles. This pattern was correctly preserved in all high- and low-expressing carcinomas and normal tissues, as well as in the PC3 cell line and normal cells. In contrast, the methylation pattern of the DMR in LNCaP cells was severely disturbed, with predominant partially methylated alleles in which the methylation clustered in the 5' end of the assessed region. Notably, no fully methylated alleles were detected (Figure 7 right panel).

Treatment of the prostate cancer cell lines LNCaP, 22Rv1, PC3 and DU145 with the DNMT inhibitor 5-aza-2'-

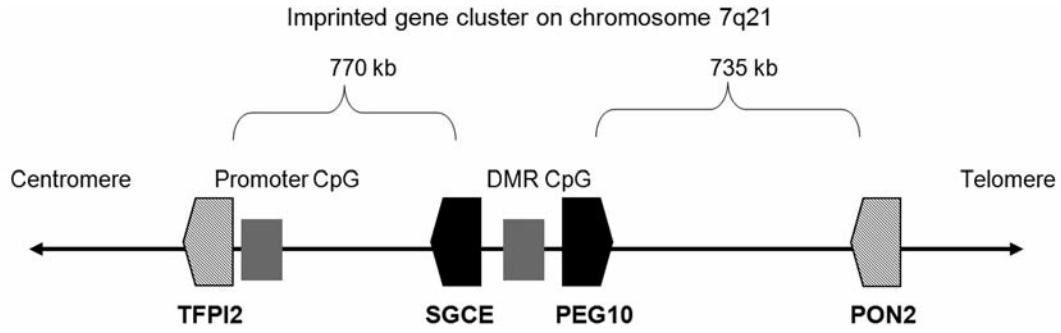

Figure 4. *Schematic representation of a selection of genes in the imprinted gene cluster on human chromosome 7q21. Paternally expressed genes are represented by filled black boxes, placental imprinted maternally expressed genes by hatched boxes. The relative positions of the TFPI2 promoter and the DMR analysed by bisulphite sequencing and chromatin immunoprecipitation are designated by grey boxes. Distances in the figure are not to scale.*

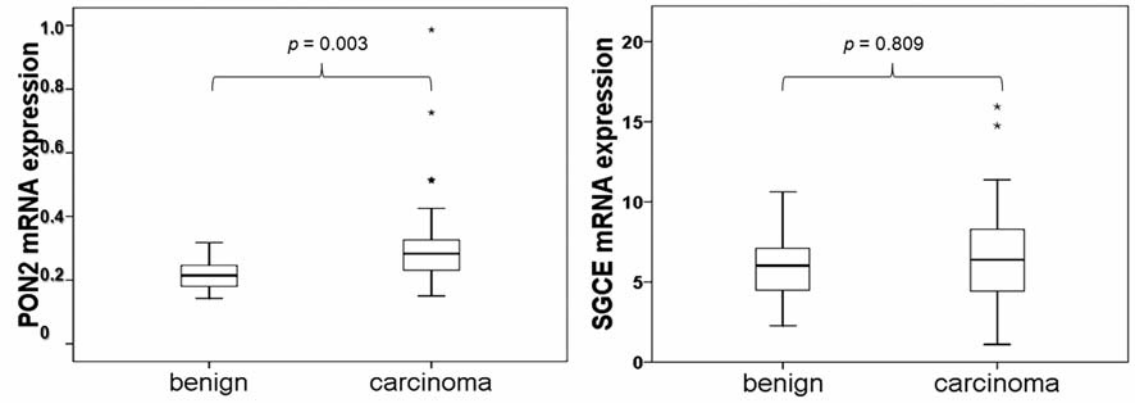

Figure 5. *PON2 (left) and SGCE (right) mRNA expression relative to TBP in 45 prostate carcinoma compared to 13 benign prostate tissue samples measured by quantitative RT-PCR. Mann-Whitney U test was used to evaluate the statistical significance of the differences. Asterisks represent outliers.*

deoxycytidine induced *TFPI2* mRNA expression by approximately two-fold in LNCaP, three-fold in PC3, and 5-fold in 22Rv1 and DU145. Nevertheless, the expression of *TFPI2* in the initially low-expressing LNCaP and 22Rv1 lines remained much lower than the basal *TFPI2* levels of the high-expressing PC3 and DU145 cells (data not shown).

Enrichment of histone modifications at the TFPI2 promoter and 7q21 differentially methylated region in LNCaP and PC3 cells. We assessed the chromatin conformation of the DMR and *TFPI2* promoter by means of chromatin immunoprecipitation in PC3 and LNCaP. As expected, the promoter was more enriched with active H3K4me3 and H3K9ac marks in the strongly *TFPI2*-expressing PC3 cells, while the repressive H3K9me3 and HeK27me3 modifications were more strongly represented in the low-*TFPI2*-expressing LNCaP cells (Figure 8A).

The DMR of both LNCaP and PC3 cell lines was enriched with active histone modifications, while the levels of the repressive H3K9me3 mark were slightly higher in PC3 (Figure 8B). In general, one may conclude that the chromatin conformation of the DMR is relatively open in both cell lines. This region is the promoter of the paternally expressed *SGCE*, which is expressed in PC3 but not in LNCaP cells.

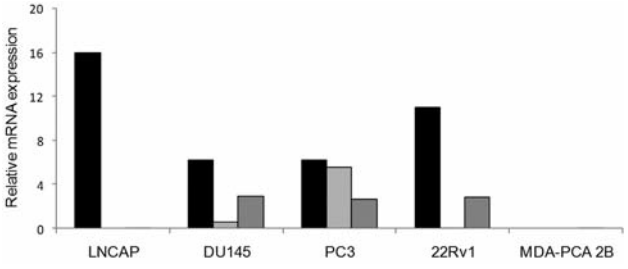

Figure 6. *TFPI (filled), TFPI2 (hatched) and SGCE (checkered) mRNA expression relative to TBP in the prostate cancer cell lines LNCaP, DU145, PC3, 22Rv1, and MDA-PCA 2B, measured by quantitative RT-PCR. Missing bars represent undetectable expression.*

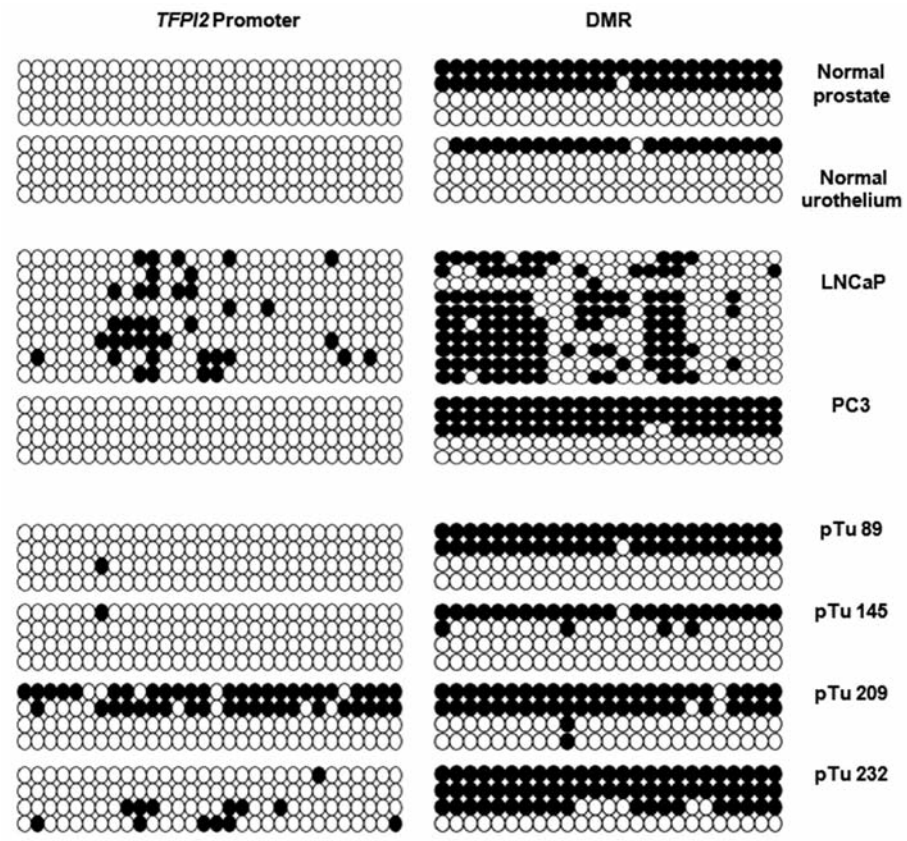

Figure 7. *Methylation status of TFPI2 promoter and 7q21.3 DMR. CpG methylation was assessed by sequencing of bisulphite-converted DNA from normal prostate epithelial cells, normal urothelial cells, the prostate cancer cell lines LNCaP and PC3, two high TFPI2-expressing (pTu89 and pTu145) and two low TFPI2-expressing (pTu209 and pTu232) primary prostate carcinomas. Each line corresponds to one cloned PCR product; open circles represent unmethylated and closed circles methylated CpG sites.*

Discussion

According to online expression databases, such as Gene Expression Omnibus, Oncomine, and ArrayExpress, the *TFPI* gene is ubiquitously expressed in normal human tissues, whereas high *TFPI2* levels are confined to the placenta. Although the two molecules are structurally homologous, they behave differently in cancer (46). TFPI levels have been shown to be elevated in the plasma of patients with a number of solid tumours, where it is hypothesized to inhibit intra-tumour coagulation (47, 48). In contrast, the expression of *TFPI2* often appears to diminish with an increasing degree of malignancy and loss of differentiated features (49). Since transduction studies with non-expressing cell lines suggested that TFPI2 negatively regulates metastasis and angiogenesis, while inducing apoptosis, it is often considered a potential tumour suppressor (34, 50, 51).

We found *TFPI* mRNA expression to be relatively stable in prostate carcinoma tissues, without significant differences from benign tissues (Figure 1). In comparison, *TFPI2* expression was highly variable in carcinoma but also in benign prostate tissues (Figure 2). This raises the possibility that both high and low states of *TFPI2* expression could in different ways be involved in prostate tumourigenesis. The variable *TFPI2* expression observed in benign samples from cancer-carrying prostates may hint at its potential involvement in early events during prostate carcinogenesis. It may also be the result of heterogeneity of TFPI2 expressing cells, since the presence of populations of either TFPI2-positive or -negative cells has been detected in breast, gastric, endometrial and colon carcinomas, and may be indicative of a progenitor cell population (49). In the placenta, likewise, *TFPI2* gene expression has been shown to be specific for a subpopulation of the trophoblast cells (26, 52). The possibility of cell subtype-specific *TFPI2* expression in prostate carcinoma tissues might explain the lack of correlation with tumour stage, grade, metastasis potential and recurrence. Immunohistochemical comparisons relating *TFPI2* distribution with that of multiple stem-cell specific markers should answer this issue in future studies.

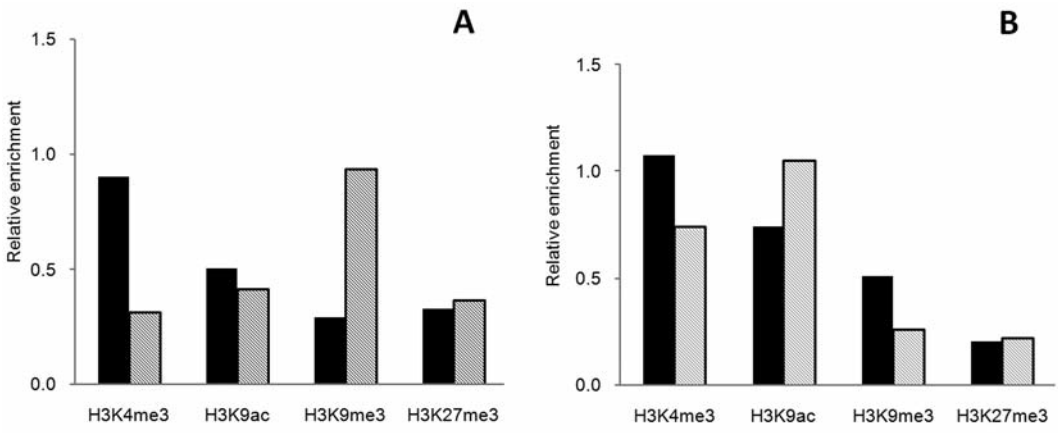

Figure 8. *Chromatin immunoprecipitation analysis of histone modifications H3K4me3, H3K9ac, H3K9me3 and H3K27 on the TFPI2 promoter (A) and the 7q21.3 DMR (B) in PC3 (filled bars) and LNCaP (hatched bars) cells. Data represent qPCR results normalized as described in Materials and Methods.*

Previous studies, in which the promoter of a high *TFPI2*-expressing cell line was transfected into a low *TFPI2*-expressing cell line, argued that the persistent low *TFPI2* expression might be the result of a defect in a transcription activator protein (41). Although it is not clear which proteins might be involved, vascular endothelial growth factor (VEGF) and serum growth factors such as fibroblast growth factor (FGF) have been shown to induce the expression levels of *TFPI* and *TFPI2* (53). Furthermore, increases of TF might also induce its inhibitors, in a control feedback mechanism. Therefore, uncoupling of the regulation by potentially oncogenic factors such as TF and VEGF might be the cause of subsequent *TFPI2* deregulation.

Repression of *TFPI2* expression has been reported to be accompanied by CpG hypermethylation of its promoter in a number of cancer lines and primary carcinoma tissues (38, 39, 41, 54-56). Accordingly, we found partial promoter methylation in the context of low *TFPI2* mRNA expression in LNCaP cells and primary prostate carcinomas, whereas normal prostate epithelial and urothelial cells, prostate cell lines and cancer tissues with high expression showed no CpG methylation (Figure 7 left panel).

Inhibition of the DNA methylation machinery induced *TFPI2* levels in both highly expressing PC3 and DU145, and low-expressing LNCaP and 22Rv1 cells. However, the induced levels in the latter cell lines were much lower than the uninduced *TFPI2* mRNA levels of PC3 and DU145 cells. Taken together with previous studies (*e.g.* (41)), this observation would suggest that mechanisms in addition to CpG methylation are involved in the repression of *TFPI2* expression in prostate cancer cell lines. Simultaneous or sequential treatment of cancer cell lines with inhibitors of histone deacetylation and DNA methylation have been shown to synergistically de-repress *TFPI2* expression, but the effect might also involve the de-repression of proteins involved in its activation (55-58). The binding of the transcription factor KLF6 to the *TFPI2* promoter has been reported to be induced by 5-aza-2'-deoxycytidine treatment in a breast cancer cell line with a methylated *TFPI2* promoter (39). This potential *TFPI2* activator has been proposed to participate in the regulation and maintenance of the basal expression of pregnancy-specific glycoprotein genes (59). *KLF6* is a candidate tumour suppressor gene mutated in prostate cancer (60). Therefore, its loss of function in combination with the presence of *TFPI2* promoter methylation may contribute to low *TFPI2* expression in some prostate carcinomas.

The expression of genes in imprinted clusters is usually co-ordinately regulated by complex mechanisms resulting in differential accessibility of maternal and paternal alleles for transcription. Many of the genes of the *TFPI2* cluster have functions in placental and foetal development and their regulation is best studied in those tissues (20, 22, 61, 62). In the placenta, the flanking maternally expressed genes of the *TFPI2* cluster are co-ordinately regulated by the centrally situated ICR, containing the promoters of the two paternally expressed genes *SGCE* and *PEG10* (27, 63, 64). Little is known about the regulation of these genes as a cluster in adult tissues. In prostate carcinomas and normal tissues, the expression of *TFPI2* did not change in coordination with the flanking *PON2* or with the central *SGCE* gene. The expression of other genes in the cluster was low according to microarray data and was therefore not studied in this context. Therefore, we may conclude that the mechanisms leading to the variability of *TFPI2* expression do not involve other genes of the cluster in primary prostate cancer. In LNCaP cells, *SGCE* was rather like *TFPI2* very weakly expressed (Figure 6). Therefore, in this cell line a more drastic aberration of the chromatin structure might indeed affect the whole cluster.

As suggested by the highly aberrant DNA methylation pattern and chromatin modifications, we hypothesized that global hypomethylation could potentially affect the methylation pattern of the DMR and thereby disturb the correct regulation of the genes in the cluster. However, the extent of global hypomethylation in prostate carcinoma tissues, as measured by the methylation state of the most prominent retrotransposon in the genome, *LINE-1*, did not correlate with *TFPI2* expression (Figure 3). A more extensive hypomethylation present in individual prostate cancer tissues or cell lines such as LNCaP may still have a graver effect on the integrity of the cluster. Such an effect is indicated by the observation that the typical fully methylated alleles of the DMR were missing in LNCaP (Figure 7 right side), which has the greatest level of global hypomethylation among prostate cancer cell lines.

The DMR had a relatively open conformation in both the high *TFPI2*-expressing PC3 and the low-expressing LNCaP (Figure 8B). In contrast, the *TFPI2* promoter of LNCaP cells was more enriched with the inactive H3K9me3, while the active H3K4me3 and H3K9ac histone modification marks prevailed in PC3 cells (Figure 8A). These differential chromatin modifications may underlie a corresponding accessibility of the transcription machinery to the *TFPI2* promoter. The lack of *SGCE* expression in LNCaP, regardless of the open conformation of its promoter, may, therefore, be the direct consequence of the disturbed methylation pattern inhibiting the binding of a methylation-sensitive transcription factor. Which mechanisms lead to DMR CpG methylation pattern disruption, and how this affects the expression of flanking cluster genes, still remains to be resolved.

Lack of transcriptional activation is proposed to mechanistically precede CpG hypermethylation of certain genes, but their specificity is governed by additional factors (65-67). The presence of simultaneous active and inactive histone marks, the so-called bivalent chromatin, is suggested to be such a factor. It is characteristic for genes with functions in early development which can be repressed upon differentiation at later stages. Genes targeted for DNA hypermethylation in cancer are often associated with polycomb protein complexes which contain or recruit some of the histone modifying enzymes responsible for bivalent chromatin marks (16-18). The heritability of modified histones would impose an inherent predisposition of imprinted genes to cancer-specific CpG hypermethylation. However, the decisive step leading to repression of the target genes may depend on the integrity of regulatory feedback loops and transcriptional activators.

Because of the predominance of epigenetic mechanisms in their normal physiological regulation, imprinted genes may be particularly susceptible to epigenetic disturbances in cancer, acting at the gene itself or at distant regulatory elements such as the DMR. In support of this idea, it has been shown that cellular stress can create permanent epigenetic alterations of imprinted genes (68). It has been proposed that cancer development may involve an increased plasticity of epigenetic states that are subject to selection for changes favouring tumour progression (4, 69, 70). The variously high or low expression of *TFPI2* in prostate cancer tissues and cell lines associated with according epigenetic states could represent a good example of such a phenomenon at work. Our data are well explained by the assumption of an epigenetic labile state during prostate carcinogenesis from which cells with high or low *TFPI2* gene expression are selected by factors depending on the cell and tissue context in an individual cancer.

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