

Methylation of TFPI-2 gene is not the sole cause of its silencing

CHILUKURI N. RAO¹, TAKEHIKO SEGAWA², JASON R. NAVARI³, LINDA XU²,
SHIV SRIVASTAVA², JUDD W. MOUL² and BENETTE PHILLIPS³

¹Department of Molecular Pharmacology, Division of Biochemistry,
Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, MD 20910;

²Department of Surgery, Uniformed Services University of Health Sciences, Center for Prostate Disease Research,
1530 East Jefferson Street, Rockville, MD 20852; ³Department of Obstetrics and Gynecology,
Northwestern University Medical School, 303-E. Chicago Avenue, Chicago, IL 60611, USA

Received October 21, 2002; Accepted December 10, 2002

Abstract. TFPI-2 (tissue factor pathway inhibitor-2) is a serine protease inhibitor that may suppress tumor cell invasion and metastasis. TFPI-2 expression is often lost in cells derived from tumors of diverse organs. We have examined whether aberrant hypermethylation of the 5' end of the TFPI-2 gene is associated with its loss of expression. After 5-azacytidine treatment of three cell lines lacking TFPI-2 expression (HT1080 fibrosarcoma cells, MCF-7 breast carcinoma cells, and LNCaP prostate carcinoma cells), TFPI-2 transcripts could be detected by RT-PCR. In these three cell lines, methylation of the 5' end of the TFPI-2 gene was detected, while two prostate carcinoma cell lines in which the TFPI-2 gene was expressed, PC-3 and DU-145, showed no methylation. However, all the three cell lines which lacked TFPI-2 expression also contained unmethylated TFPI-2 alleles. Furthermore, a transiently transfected TFPI-2 promoter was non-functional in the three cell lines, but function was attained following treatment with 5-azacytidine. Our results indicate that while methylation of the TFPI-2 gene is associated with its silencing, it is not the sole cause, and we suggest that one or more components of pathways regulating TFPI-2 expression have also undergone methylation-associated silencing in these cell lines.

Introduction

Tissue factor pathway inhibitor-2 (TFPI-2), a secreted protein bound to the extracellular matrix (ECM), is a Kunitz-type serine protease inhibitor with broad specificity (1-3). TFPI-2 either directly or indirectly regulates trypsin, plasmin, chymotrypsin, cathepsin G, plasma kallikrein, and factor VIIa-tissue factor complex and matrix metalloproteinases -1, -3, -7, and -9 (4-7). It appears to play a major role in physiological processes associated with matrix remodeling such as wound healing, fibrinolysis, and angiogenesis and in pathological processes such as tumor invasion and metastasis. The TFPI-2 gene is expressed in most human tissues, but tumors arising from these tissues and cell lines derived from such tumors often show reduced or undetectable expression (2). In several tumor-derived cell lines showing reduced TFPI-2 expression, e.g., C32 melanoma cells, LNCaP prostate carcinoma cells, JAR choriocarcinoma cells, and SNB19 glioblastoma cells, ectopic expression of TFPI-2 decreased their invasive behavior as measured by *in vitro* assays (8-11). Moreover, an inverse correlation between TFPI-2 levels and the aggressiveness of human gliomas has been documented (12). Taken together, these results suggest that TFPI-2 expression restrains the invasive behavior of tumors.

In this study, we have tested the hypothesis that aberrant hypermethylation underlies the silencing of the TFPI-2 gene in tumor cell lines. Our results suggest that while hypermethylation is associated with silencing of the TFPI-2 gene in the three cell lines that we examined, it is not the sole cause of TFPI-2 gene silencing.

Materials and methods

Cell culture, 5-azacytidine treatment. LNCaP, PC-3, and DU-145 prostate carcinoma cells, MCF-7 breast carcinoma cells, and HT1080 fibrosarcoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained according to ATCC recommendations. To evaluate TFPI-2 re-expression post 5-azacytidine (5-azaC) treatment, cells were treated with the drug at a final

Correspondence to: Dr Chilukuri N. Rao, Division of Biochemistry, Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, MD 20910, USA
E-mail: nageswararao.chilukuri@na.amedd.army.mil

Abbreviations: TFPI-2, tissue factor pathway inhibitor-2; ECM, extracellular matrix; 5-azaC, 5-azacytidine; RT-PCR, reverse transcription-polymerase chain reaction

Key words: TFPI-2, methylation, silencing, 5-azacytidine, sodium bisulfite, promoter construct

concentration of 0.1, 0.5, or 1 μ M for 5 to 7 days, harvested, and RNA isolated for analysis by reverse transcription-polymerase chain reaction (RT-PCR).

RT-PCR. Total RNA was extracted using RNazol (Invitrogen, Carlsbad, CA), and 1 μ g of total RNA was reverse transcribed using a GeneAmp RNA PCR kit (Perkin-Elmer, Foster City, CA). A 430 bp region of TFPI-2 gene was amplified using the primers 5'-CGATGCTGCTCAGGAG CCAAC-3' and 5'-CATTGGCAGAGCACAGTCC-3'. As a control for RNA integrity, a 190 bp region of GAPDH was co-amplified with TFPI-2, using the primers 5'-GGGGAGCC AAAAGGGTCATCATCT-3' and 5'-GAGGGGCCATCC ACAGTCTTCT-3'. Amplification conditions were: 1 cycle of 95°C, 10 min; 45 cycles of 94°C, 0.75 min, 60°C, 0.75 min, 72°C, 1.5 min; 1 cycle of 72°C for 10 min.

Sodium bisulfite treatment, COBRA, bisulfite sequencing. Genomic DNA from each of the cell lines (2 μ g) was treated with sodium bisulfite for 16 h at 55°C (13), and the recovered DNA was dissolved in 100 μ l water. Nested primers were used to amplify the 5' end of the gene. All primers were designed from regions devoid of CpGs and reflect the conversion of unmethylated Cs to Us during bisulfite treatment. Primers used for the first amplification were: 5'-TAATCTAAA AACAAAAACAAATCTC-3' and 5'-GTTAAAATTTA TTAAATTGTTTATTT-3'. Primers used for the second amplification were 5'-AAAAACCTCTACAAAAAATA CAACTTA-3' and 5'-TATTTTTAATTTAATTTGAAGG TTTATTGT-3'. All amplifications employed Ampliwax PCR gems for 'hot start' (Perkin-Elmer, Foster City, CA) and contained 1X PCR buffer (50 mM KCl, 10 mM Tris, pH 8.8) 2.5 mM MgCl₂, 0.2 μ M each dNTP, 9.3 μ M each primer, 2.5 U Taq polymerase, and 3 μ l of bisulfite treated DNA in a total volume of 75 μ l. After completion of the first round of amplification, 3 μ l was withdrawn and used as template for the second round of amplification. Conditions for the first round of amplification were 1 cycle of 94°C, 2 min; 5 cycles of 94°C, 1 min, 52°C, 2 min, 72°C, 3 min; 25 cycles of 94°C, 0.5 min, 52°C, 2 min, 72°C, 1.5 min; and 1 cycle of 72°C for 6 min. The amplified products were extracted with phenol-chloroform, concentrated by ethanol precipitation, and electrophoresed on 0.9% Seaplaque agarose gel. DNA was recovered from gel slices using a QIAquick gel extraction kit (Qiagen, Chatworth, CA). Products were recovered and analyzed by COBRA (14) and were also sequenced directly. For COBRA, amplicons were digested with AciI restriction endonuclease, whose recognition site is 5'-GCGG-3'. Digestion of the amplicons with the AciI restriction endonuclease was carried to completion by performing all reactions in the presence of 50 U AciI per μ g of DNA for 2 h or longer at 37°C. Digests were electrophoresed on agarose gels and the products were visualized using ethidium bromide. Sequencing was performed with a Thermosequenase kit (Amersham, Piscataway, NJ).

Reporter construct. A 1.6 kb (-1611 bp to +1) genomic fragment encompassing the TFPI-2 promoter was amplified from PC-3 genomic DNA with the primers 5'-CTTCAGAA CTCCCAAGGCTA-3' and 5'-GGTGCAGGGGGTCCG GCG-3', which were designed based on the sequence of

human BAC clone GS1-345D13 (accession no: 002076). The product was inserted into *KpnI* and *XhoI* sites of the promoterless and enhancerless luciferase vector pGL3-Basic (Promega, Madison, WI), thus positioning it upstream of the luciferase gene, to generate pTFPI-2-luc. The sequence of the TFPI-2 promoter in this construct was confirmed by automated sequencing (Applied Biosystems Inc., Foster City, CA) using a Taq Dye Deoxy terminator cycle sequencing kit.

Luciferase assays. Transient transfection of reporter plasmids was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the protocol supplied by the manufacturer. Briefly, 1x10⁵ cells seeded in 24-well tissue culture plates were exposed to transfection mixtures containing 0.5 μ g of pGL3-Basic or TFPI-2 reporter plasmids and 50 ng of pRL-TK, a vector in which *Renilla* luciferase cDNA is driven by an HSV thymidine kinase promoter (Promega, Madison, WI), for 4 h at 37°C. Growth medium (2 ml) was then added to the cells, followed by incubation for an additional 24 or 48 h. Cells were harvested, and *firefly* and *Renilla* luciferase activities were measured with a Dual-Luciferase reporter assay system (Promega, Madison, WI). For each extract, *firefly* luciferase activity was normalized to the levels of *Renilla* luciferase activity.

Western and Northern blot analyses. ECM was isolated and subjected to Western blotting for assaying TFPI-2 levels as previously described (15). For Northern blotting, 10 μ g of total RNA were electrophoresed on a 1% agarose-formaldehyde gel, transferred to a nylon membrane, and UV cross-linked (16). A 0.68 kb TFPI-2 cDNA probe, labeled with [³²P]-dCTP using random primers (Amersham, Piscataway, NJ), was hybridized to the filters at 65°C. The filters were then washed in 0.5% x standard saline citrate (SSC; 3 M sodium chloride, 0.3 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) for 2x20 min at room temperature, then for 15 min at 65°C and exposed to X-ray film at -70°C. After stripping, the membranes were rehybridized with GAPDH cDNA. Quantification was performed using densitometry.

Results

Expression of TFPI-2 is lost in a subset of tumor-derived cell lines. We reported previously that HT1080 fibrosarcoma cells lacked TFPI-2 expression (17). To determine whether loss of expression is a common occurrence in tumor-derived cell lines, we examined expression of the TFPI-2 gene at the RNA and protein levels in DU-145, PC-3, and LNCaP prostate cancer cells and MCF-7 breast carcinoma cells. ECM extracts were used to measure TFPI-2 protein levels because most (>90%) of the 32 kDa mature TFPI-2 and its 27 and 31 kDa glycosylation variants are secreted into the ECM. By Western blotting, moderate to high-levels of TFPI-2 proteins with molecular sizes of 33, 31, and 27 kDa were detected in the ECM of DU-145 and PC-3 cells. In contrast, TFPI-2 proteins were not detected in the ECM of LNCaP prostate carcinoma cells and MCF-7 breast carcinoma cells (Fig. 1A). A similar disparity between the four cell lines was seen when TFPI-2 transcripts were measured by Northern blotting. As shown in Fig. 1B, DU-145 and PC-3 cell lines expressed moderate to

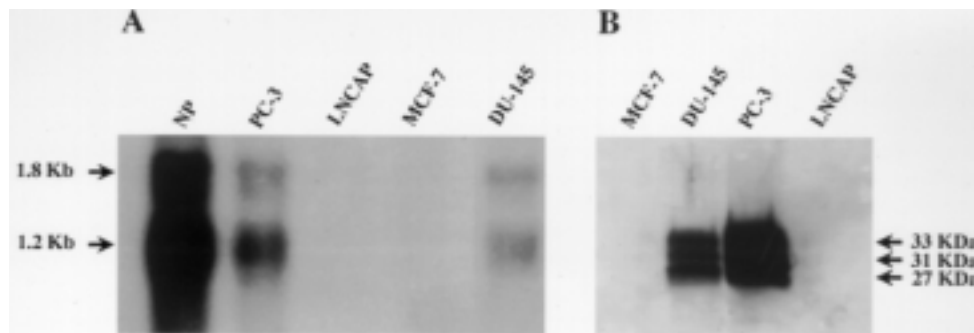


Figure 1. TFPI-2 expression in tumor cell lines. (A), Western blot analysis of TFPI-2 protein expression in the indicated cell lines. Extracellular matrix (ECM) extracts were prepared and equal quantities of proteins were run on 12% SDS-polyacrylamide gels. Separated proteins were transferred onto nitrocellulose membranes and probed with an anti-TFPI-2 antibody. The antibody reactive proteins of molecular sizes 33, 31 and 27 kDa were shown. (B), Northern blot analysis of TFPI-2 mRNA levels in tumor cell lines. Total cellular RNA was extracted and 10 μ g portions were electrophoresed in agarose-formaldehyde gels, transferred to a nylon membrane and hybridized with a 32 P-labeled TFPI-2 cDNA probe. TFPI-2 transcripts with molecular sizes 1.8 and 1.2 kb are shown.

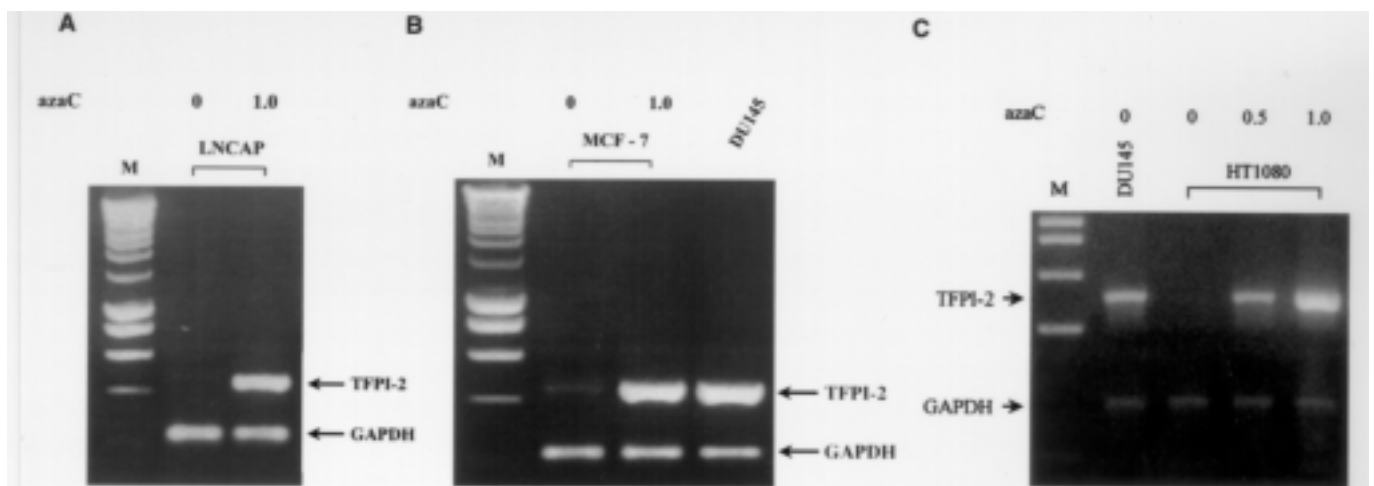


Figure 2. Restoration of TFPI-2 expression in cell lines lacking TFPI-2 expression by treatment with 5-azacytidine. Total RNA was isolated from untreated cells and from cells treated with the indicated concentrations of 5-azacytidine. TFPI-2 and GAPDH transcripts were detected by RT-PCR. RNA from DU-145 and PC-3 cells, which express TFPI-2, was included as positive controls. 430 bp TFPI-2 transcripts and 190 bp GAPDH transcripts are shown.

high levels of TFPI-2 mRNAs of 1.8 and 1.2 kb sizes, as did normal prostate epithelium (Fig. 1B, lane 1); however, these transcripts were barely detectable in LNCaP prostate carcinoma cells and MCF-7 breast carcinoma cells. GAPDH transcripts were very similar in these four cell lines (data not shown).

TFPI-2 expression is restored by 5-azaC treatment. Aberrant loss of gene expression during tumorigenesis or establishment of cells in culture is frequently accompanied by aberrant hypermethylation. We therefore tested whether expression of the TFPI-2 gene could be restored by treatment of HT1080, MCF-7, and LNCaP cells with the methyltransferase inhibitor 5-azaC. Using RT-PCR to detect transcripts, TFPI-2 expression was readily apparent in all three cell lines treated with 5-azaC (Fig. 2).

Hypermethylation of the 5' end of the TFPI-2 gene correlates with diminished expression. To directly examine whether loss of expression and hypermethylation were correlated in the five cell lines where TFPI-2 expression had been evaluated, we examined the methylation status of a 723 bp region at the 5' end of the TFPI-2 gene. This CpG-rich region comprises

440 bp of sequence upstream of the transcription start site, all of exon 1 (143 bp), and 140 bp of intron 1; a CpG density map of the region is shown in Fig. 3A. To obtain a preliminary assessment of the methylation status, we utilized combined bisulfite treatment and restriction enzyme analysis (COBRA). Genomic DNAs from the five cell lines were treated with sodium bisulfite, which converts all unmethylated Cs to Us while sparing methylated Cs from conversion. The 723 bp region of interest was then amplified, and the amplicons were digested with AciI. Nine of the 54 total CpGs in the region are contained within AciI sites (Fig. 3A). Bisulfite treatment of cellular DNA destroys these sites unless the CpGs within them were methylated in the genomic DNA and resistant to conversion. The susceptibility of the amplicon to AciI digestion thus provides an initial assessment of whether methylation is present or absent. AciI digested the amplicons derived from MCF-7, LNCaP, and HT1080 cells, which lacked TFPI-2 expression, although a fraction of the amplicon population derived from each of the lines was not cleaved (Fig. 3B). By contrast, no cleavage of the amplicons derived from the two cell lines which expressed TFPI-2, DU-145 and PC-3, was detectable (Fig. 3B).

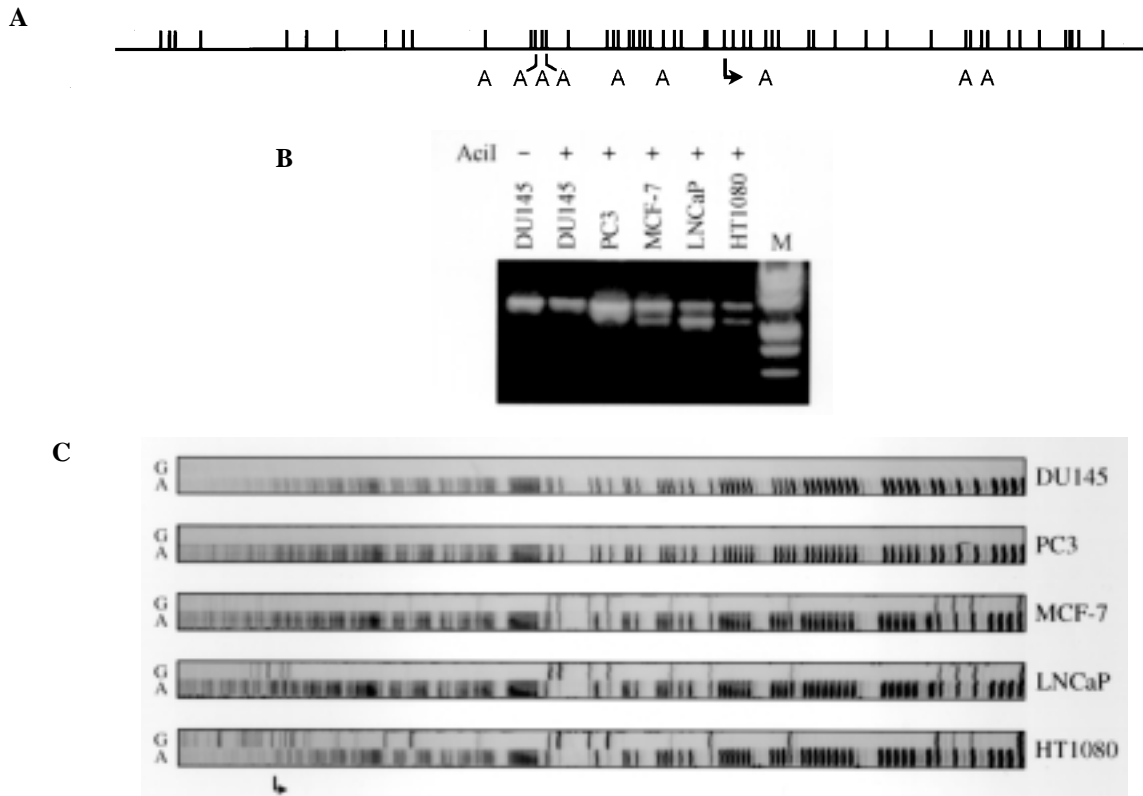


Figure 3. Methylation analysis of the 5' end of the TFPI-2 gene. (A), CpG density map of the region of the TFPI-2 gene examined, which extends from -410 to +280 relative to the transcription start site (bent arrow). As denote AcI sites, used in COBRA. (B), Determination of the methylation status by COBRA. Genomic DNAs from the indicated cell lines were bisulfite treated and amplicons corresponding to the 5' end of the TFPI-2 gene were digested with AcI restriction endonuclease and electrophoresed on an agarose gel. (C), Determination of the methylation status of the 5' end of TFPI-2 using bisulfite sequencing. Genomic DNAs from the indicated cell lines were bisulfite treated and amplicons corresponding to the 5' end of the TFPI-2 gene were sequenced using the downstream amplification primer. For brevity, only the G and A bands of the sequencing gels are shown. The relative intensities of co-migrating G and A bands indicate the relative degree of methylation at that position. A bent arrow denotes the transcription start site; sequences on the right of the gel are the furthest downstream of the transcription start site.

To confirm these results and to obtain a comprehensive view of the methylation patterns, we completely sequenced the top strand of the amplicons from each of the five cell lines. Bands present in the G lane of the sequencing ladders correspond to Cs which were methylated in the genomic DNAs and which are still present in the top strands of the amplicons (Fig. 3C, for brevity, only the G and A lanes of the sequencing gels are shown). The relative intensities of each pair of comigrating G and A bands indicate the relative degree of methylation at that position in the entire allelic population. We saw no bands in the G lane when the DU-145 and PC-3 amplicons were sequenced; however, bands in the G lanes, corresponding to positions of C^{me}G in the genomic DNA, were seen after sequencing the MCF-7, LNCaP, and HT1080 amplicons (Fig. 3C).

While sequencing confirmed that TFPI-2 silencing and methylation are correlated, it is apparent that the extent of the methylation differs among the three lines. In the case of MCF-7 cells, methylation was confined to CpGs downstream of the transcription start site and was most prevalent in the sequences furthest downstream from the transcription start (ts) site, i.e., in intron 1. LNCaP cells exhibited additional methylation in CpGs flanking the transcription start site, while HT1080 cells exhibited at least some degree of methylation at every one of 54 CpGs in the region examined (Fig. 3C). Thus, while MCF-7, LNCaP, and HT1080 cells all exhibited

methylation in the 5' end of the gene and all showed severe deficits in TFPI-2 expression, the extent to which putative regulatory sequences in the gene had undergone hypermethylation in the three lines differed considerably. Notably, MCF-7 cells showed very little methylation in sequences upstream of the ts site, where methylation is typically seen in genes that are silenced by methylation.

A second observation is inconsistent with the notion that loss of TFPI-2 expression can be solely explained by its aberrant hypermethylation. If lack of methylation at the 9 AcI sites is indicative of a complete absence of methylation in the 5' end, then unmethylated alleles, represented by the uncut amplicons in Fig. 3B, comprise a significant fraction of the allelic population in all of the cell lines lacking expression. However, the unmethylated alleles appear to be silenced as well.

Transfected TFPI-2-luciferase constructs are not expressed in cell lines which exhibit silencing of the endogenous gene, but 5-azaC treatment allows their expression: in an attempt to resolve the conundrum raised by our results, we decided to examine whether expression of a reporter construct in which the 5' end of the TFPI-2 gene was placed upstream of luciferase cDNA was linked to expression of the endogenous gene. Constructs newly introduced into cells and present as episomes are presumably unmethylated. Therefore, if no factor other than methylation of the endogenous TFPI-2 gene itself

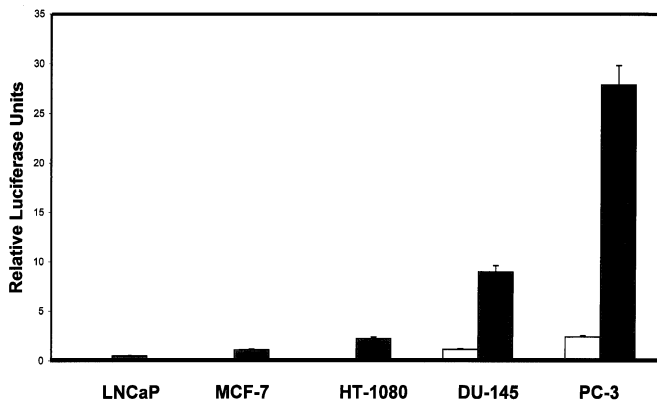


Figure 4. Expression of a transfected TFPI-2 promoter construct in tumor cells. *Firefly* and *Renilla* luciferase activities were measured in extracts of cells prepared 24 h (open bars) and 48 h (closed bars) after cotransfection with a TFPI-2 promoter construct and pRL-TK control plasmid for normalization purposes. Bars represent *firefly* luciferase activity normalized to *Renilla* luciferase activity. No *firefly* luciferase activity was detected in cells transfected with the parental vector, pGL3-basic. The average of three different experiments are shown.

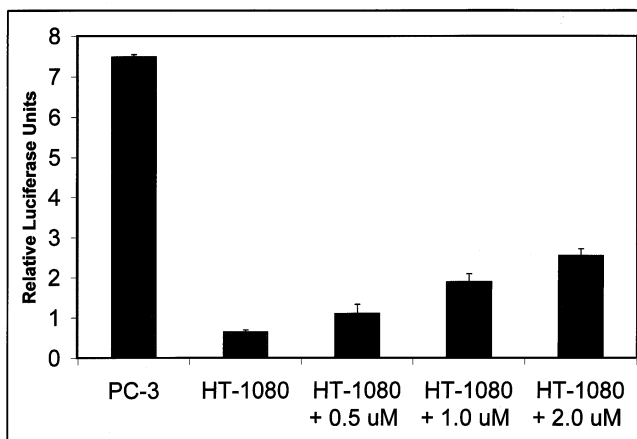


Figure 5. Detection of luciferase activity in transfected HT1080 fibrosarcoma cells after treatment with 5-azacytidine. HT1080 cells were treated with the indicated concentration of 5-azacytidine (azaC) or with DMSO only for 5 days prior to transfection with a TFPI-2 promoter construct and pRL-TK control plasmid. *Firefly* luciferase activity in cell lysates prepared 24-h post-transfection was measured and normalized to *Renilla* luciferase activity. The average of three different experiments are shown.

plays a role in its silencing, this reporter construct should be expressed in all three of the lines which lacked TFPI-2 expression. However, as shown in Fig. 4, reporter construct activity was negligible in HT1080, MCF-7, and LNCaP cells, but readily detectable in PC-3 and DU-145 cells. Thus, reporter gene activity levels correlated with the endogenous TFPI-2 levels in these cell lines. Since 5-azaC was able to restore expression of the endogenous gene in the three cell lines we examined, we next asked whether treatment of HT1080 cells with 5-azaC prior to transfection would allow expression of the reporter construct. As shown in Fig. 5, 5-azaC treated HT1080 cells supported expression of the reporter construct, and this effect was more pronounced in cells treated with a

higher dose of the demethylating agent. One interpretation consistent with these observations is that genes whose products regulate TFPI-2 expression are also silenced in HT1080 cells, but can be reactivated by demethylation.

Discussion

Within the last several years, a panoply of clinically important tumor suppressor genes with roles in growth control, DNA repair, adhesion, apoptosis, and inhibition of angiogenesis have been shown to have both lost expression and acquired aberrant hypermethylation in tumors and in tumor-derived cell lines (18,19). Typically, hypermethylation in cell lines is demonstrated both by the ability of a methylation inhibitor such as 5-azaC to restore gene expression and by detection of methylation in the 5' end of the gene. These criteria are met in the case of three cell lines with silenced TFPI-2 genes. Yet the presence of unmethylated alleles in all three cell populations, the inability of a transiently transfected TFPI-2 promoter to function in these silenced lines, and the silencing of the TFPI-2 gene in another of the lines despite the absence of methylation in both exon 1 and sequences upstream of the ts site, suggested that the absence of TFPI-2 expression in the three cell lines we examined was not a simple case of methylation-associated transcriptional silencing. Our further determination that exposure of HT1080 cells to 5-azaC not only restored expression of the endogenous TFPI-2 gene but also allowed a transfected TFPI-2 promoter to drive transcription of a downstream reporter gene suggested an explanation which would reconcile all of our observations, viz., that in all three of the cell lines containing silenced and hypermethylated TFPI-2 genes, one or more components of pathways regulating TFPI-2 expression has also undergone methylation-associated silencing. The silencing of this putative regulatory pathway component(s) appears to result in repression of the TFPI-2 promoter irrespective of its methylation state; therefore, the methylation of TFPI-2 which we observed in all three cell lines may be a secondary event which takes place subsequent to and as a consequence of its transcriptional inactivity. Examination of TFPI-2 silencing in additional cell lines using the same battery of approaches will be necessary to determine how widely applicable our findings are.

An important question raised by our findings is the identity of the putative regulatory gene(s) whose methylation-associated silencing so profoundly impacts TFPI-2 gene expression. Unfortunately, little is presently known regarding transcriptional regulation of the TFPI-2 gene. Transient transfection of a set of reporter plasmids containing up to 3.5 kb of TFPI-2 upstream sequences into both transformed bone marrow endothelial cells and glioblastoma cells revealed that sequences between -224 and -139, termed the minimal promoter, were critical for transcription (20). The reporter used in our study comprised 1.6 kb of upstream sequence, and it will be important to determine whether similar results are obtained using a reporter containing this minimal promoter. If similar results are obtained, then the elucidation of regulatory pathways which target these critical sequences may be facilitated by the application of methylomics technologies (19) to cell lines in which TFPI-2 expression can be reactivated by 5-azaC.

Our results underscore the value of a comprehensive approach to analyzing the possible involvement of methylation in gene silencing. When applied to cell lines, this approach should include the determination of the expression status of a transfected reporter construct. Our results demonstrate that the absence of methylated CpGs in a silenced gene does not preclude the involvement of methylation in its silencing and that, conversely, the detection of methylation, even in regulatory sequences, may not suffice to fully account for the silencing.

Acknowledgements

We are grateful to Drs Walter Kisiel and Donald C. Foster for the human anti-TFPI-2 antibody.

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