Methylation Silencing of Transforming Growth Factor-β Receptor Type II in Rat Prostate Cancers

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Abstract

To identify methylation-silenced genes in prostate cancers, a microarray analysis for genes up-regulated by treatment with a demethylating agent, 5-aza-2′-deoxycytidine, was performed using three rat prostate cancer cell lines. Eight genes (*Aebp1, Dysf, Gas6, LOC361288, Nnat, Ocm, RGD1308119*, and *Tgfbr2*) were re-expressed at 16-fold or more, and their promoter CpG islands were shown to be densely methylated in the cancer cell lines. From the eight genes, *Tgfbr2*, a key mediator of transforming growth factor-β (TGF-β) signaling that has been strongly implicated in human and rat prostate carcinogenesis, was selected, and its silencing in primary samples was analyzed further. *Tgfbr2* was methylated and markedly down-regulated in three of seven 3,2′-dimethyl-4-aminobiphenyl–induced invasive adenocarcinomas in the dorsolateral lobe of the rat prostate. In humans, marked down-regulation of TGFBR2 protein was observed in 12 of 20 high-grade prostatic intraepithelial neoplasia and 36 of 60 prostate cancers. DNA methylation of the human *TGFBR2* promoter CpG islands repressed transcription, if present, but neither methylation nor mutation were detected in 27 human prostate cancers analyzed. Methylation silencing of rat *Tgfbr2* was associated with histone H3 lysine 9 trimethylation, whereas decreased expression of human *TGFBR2* was mainly due to decreased transcription activity, sometimes in concert with histone deacetylation and H3 lysine 27 trimethylation. The identification of methylation silencing of *Tgfbr2* in rat prostate cancers, in accordance with *TGFBR2* down-regulation in human prostate cancers, will enable us to analyze how aberrant methylation is induced *in vivo* and identify factors that promote and suppress the induction of aberrant methylation. [Cancer Res 2008;68(7):2112–21]

Introduction

Gene silencing due to DNA methylation of promoter CpG islands (CGIs) is one of the major mechanisms of tumor-suppressor gene inactivation, along with mutations and loss of heterozygosity (1). Many methylation-silenced tumor-suppressor genes have been identified, and more will be revealed by genome-wide procedures (2). In contrast, limited information is available on the mechanism of how methylation silencing is induced *in vivo* and on the factors that promote or suppress aberrant methylation. For example, although chronic inflammation is known to be an inducer of aberrant methylation in humans (3), the exact effector cells and molecular changes in target cells are unknown. To address these questions, animal models are indispensable. However, because we select models by the presence of dense methylation of a promoter CGI in cancer and by the meaningful expression of its downstream gene in the corresponding normal tissue, only a limited number of methylation-silenced genes have thus far been identified in animal models (4–7).

Prostate cancer is one of the leading causes of cancer death in men in most developed countries (8). To analyze molecular, cellular, and physiologic events in prostate carcinogenesis, rodent models have been used. Particularly in rats, prostate cancers can be induced in an age-dependent manner in ACI/Seg and Lobound-Wistar strains, or by chemical carcinogens, and the effects of androgens have been clearly shown (9). If methylation-silenced genes involved in prostate carcinogenesis are found in rat prostate cancers, they will enable us to analyze the molecular processes of how aberrant methylation is induced *in vivo* as well as the factors, including hormones, that influence the process.

To identify methylation-silenced genes in rat prostate cancers, a chemical genomic screening method (2) was adopted for its efficiency. This method screens genes re-expressed after treatment with the demethylating agent 5-aza-2′-deoxycytidine (5-aza-dC), using a microarray. It is technically simple, and effective in identifying methylation-silenced genes using cell lines. Three rat prostate cancer cell lines, PLS10, PLS20, and PLS30 have been established from three prostate cancers in the dorsolateral lobes independently induced by 3,2′-dimethyl-4-aminobiphenyl (DMAB) plus testosterone in male F344 rats (10, 11).

Here, we report the results of a chemical genomic screening using PLS10, PLS20, and PLS30 cell lines. Among the genes whose methylation silencing was confirmed, the transforming growth factor-β (TGF-β) receptor type II gene (*Tgfbr2*), a key mediator of TGF-β signaling that has been strongly implicated in human and rat prostate carcinogenesis (12–19), was identified. We further analyzed *Tgfbr2* methylation and expression both in rat and human prostate cancers.

Materials and Methods

Cell lines and their 5-aza-dC or trichostatin A treatment. PLS10 (well-differentiated adenocarcinoma), PLS20 (poorly differentiated adenocarcinoma), and PLS30 (well-differentiated adenocarcinoma) were established from three independent transplantable tumor lines induced by DMAB plus testosterone propionate in the dorsolateral lobes of F344 rats, and maintained as reported (11). Human prostate cancer cell lines (PC3, LNCaP, DU145, MDA-PCa-2b, and 22Rv1) and prostatic epithelial cells immortalized by papillomavirus 18 (RWPE-1) were purchased from the American Type Culture Collection.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org).

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Table 1. Eight genes silenced in rat prostate cancer cell lines

Gene symbol	CpG island (bp)	Methylation and expression induction			Gene title
		PLS-10	PLS-20	PLS-30	
Aebp1	500	M*	M	M	AE binding protein 1 (predicted)
Dysf	300	M	M*	M	Dysferlin (predicted)
Gas6	500	M*	M	M †	Growth arrest–specific 6
LOC361288	500	M*	U	U	Similar to FUN14 domain containing 2 (predicted)
Nnat	500	M*	M †	M †	Neuronatin
Ocm	300	M*	M*	M/U*	Oncomodulin
RGD1308119	500	M*	M	M	Similar to F-box protein FBL2
Tgfbr2	500	M/U	M*	M †	TGF-β receptor II

Abbreviations: M, methylated; U, unmethylated.
*≥16-fold increase.
† ≥4-fold increase.

For treatment with 5-aza-dC, 2×10^5 cells (1×10^5 cells for PLS-10)/10 cm dish were seeded on day 0, and exposed to freshly prepared 10 μmol/L 5-aza-dC (Sigma) for 24 h on days 1 and 3. This dose suppressed cellular growth rates to approximately half of nontreated cells. After each treatment, the cells were placed in fresh medium and harvested on day 4. For treatment with trichostatin A (TSA), cells were seeded at a half-confluent density, and exposed to 100, 300, and 1,000 nmol/L of TSA (Sigma) for 24 h until harvest. Genomic DNA was extracted by standard phenol/chloroform procedures. Total RNA was extracted using ISOGEN (Nippon Gene) and purified using an RNeasy Mini kit (Qiagen).

Primary prostate cancers and immunohistochemistry. To induce prostate cancers, 6-week-old male F344 rats underwent subcutaneous injection of 100 mg/kg of testosterone propionate and 50 mg/kg of DMAB, which was repeated 10 times in a 2-week cycle, followed by the subcutaneous implantation of a Silastic tube containing 40 mg of testosterone propionate (10). The prostate was resected *en bloc*, examined for gross abnormalities, and fixed in 10% buffered formalin. One sagittal slice was prepared for each lobe, and embedded in paraffin. A 4-μm-thick section was stained with H&E. Organ-confined prostate cancers were obtained from 60 patients (ages 49–77, stage II–IV, Gleason pattern 2–5) who underwent prostatectomy. None of these cancer patients had previously undergone chemotherapy, radiotherapy, or hormonal therapy. All histologic diagnoses were made by experienced pathologists (S. Takahashi and T. Shirai). DNA from formalin-fixed, paraffin-embedded tissue sections was extracted by heating the sections at 100°C for 20 min under pH 12 (20). The animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation at the National Cancer Center.

TGFBR2 immunohistochemistry in rat and human prostate cancers was performed using polyclonal anti-TGFBR2 antibody (L-21, Santa Cruz Biotechnology). The areas with TGFBR2 protein expression were quantitatively measured by an Image Processor for Analytical Pathology (IPAP-WIN, Sumika Technoservice), and regions that had an absorbance of one-third or less of the normal prostate were considered to have TGFBR2 down-regulation.

Oligonucleotide microarray analysis and database search. Oligonucleotide microarray analysis was performed using a GeneChip Rat Genome 230 2.0 Array (Affymetrix) and GeneChip Operating Software as in our previous studies (21, 22). Database searches were carried out at a GenBank web site, and CGI were searched for based on (*a*) CpG score ≥ 0.65, (*b*) G + C content ≥ 55%, and (*c*) length (≥200, ≥300, or ≥500 bp).

Methylation-specific PCR and bisulfite sequencing. DNA from cell lines was digested by *Bam*HI and 1 μg of digested DNA was denatured in 0.3 N NaOH at 37°C for 15 min. DNA from formalin-fixed, paraffin-embedded tissue sections was used without digestion (0.2–0.5 μg each). The samples in 3.6 N sodium bisulfite (pH 5.0) and 0.6 mmol/L of hydroquinone underwent 15 cycles of 30-s denaturation at 95°C and 15-min incubation at 50°C, desalted and desulfonated with Zymo-Spin IC Columns (Zymo Research), and were dissolved in 16 to 40 μL of TE buffer.

Methylation-specific PCR (MSP) was performed with a primer set specific to the methylated or unmethylated sequence (M or U set), using 0.5 μL (2.0 μL for DNA from formalin-fixed tissue) of the sodium bisulfite–treated DNA. DNA methylated with *Sss*I methylase (New England Biolabs) and DNA amplified by a GenomiPhi DNA amplification kit (GE Healthcare Bio-Sciences) was used as fully methylated and unmethylated control DNA, respectively (23). Bisulfite sequencing was performed with primers common to methylated and unmethylated DNA sequences, using 0.5 μL (1.0 μL for DNA from formalin-fixed tissue) of the sodium bisulfite–treated DNA (22). Primer sequences are shown in Supplementary Table S1.

Quantitative reverse transcription-PCR and 5′-rapid amplification of cDNA ends. cDNA was synthesized from 1 μg of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen) with a random primer. Real-time PCR was performed using the 7300 Real-Time PCR System (Applied Biosystems) with SYBR Green Real-Time PCR Master Mix (Toyobo; ref. 22). The copy number of a target gene was normalized to that of *GAPDH* in human and cyclophilin A (*Ppia*) in rat (24). Primer sequences are shown in Supplementary Table S2.

Rapid amplification of 5′ complementary DNA ends (5′ RACE) was performed using a GeneRacer kit (Invitrogen) on cDNA from AT6.3 and MAT-LyLu rat prostate cancer cell lines that abundantly expressed *Tgfbr2*. After the first and second PCR using LA Taq (Takara Bio), the PCR product was cloned into a pGEM-T Easy Vector (Promega), and a total of 54 clones were sequenced using a DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare Bio-Sciences) and an ABI310 DNA sequencer (Applied Biosystems).

Chromatin immunoprecipitation analysis. Cells (1.5×10^6) were treated with 1% formaldehyde for 10 min at room temperature for cross-linking, and the reaction was quenched by adding glycine. Cells were lysed in the SDS lysis buffer containing protease inhibitors (Upstate), and DNA was sonicated to a size of 100 to 3,000 bp by Bioruptor UCD-250 (Cosmo Bio). To the sonicated solution, anti-K4 dimethylated histone H3 (H3K4me2, Upstate), anti-K9 trimethylated histone H3 (H3K9me3, Upstate), or anti-K27 trimethylated histone H3 (H3K27me3, Upstate) was added, and the mixture was incubated at 4°C overnight with rotation. The resultant immune complexes were collected using Dynabeads protein G (Invitrogen Dynal AS), and washed with Immune Complex Wash Buffer (Upstate). The cross-link was reversed by incubation for 5 h at 65°C in the presence of 0.3 mol/L of NaCl. DNA was recovered by treatment with RNase and proteinase K,

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phenol/chloroform extraction, and isopropanol precipitation. The number of DNA molecules precipitated from a specific starting volume of the sonicated solution was compared with the number of DNA molecules in the same volume of the sonicated solution (whole cell extract). The number of DNA molecules was quantified by real-time PCR (primer sequences in Supplementary Table S1).

Luciferase reporter assay using a promoter with DNA methylation of a specific region. The 5′ region of human *TGFBR2* was amplified using an upper primer (5′-CCAGGAATGTCTTGGGCAAA-3′) and a lower primer (5′-CCAGCGCAGCGGACG-3′) and cloned into a *Sma*I site of the pGL3-Basic vector (Promega). To methylate a specific region within the reporter plasmid, the region was excised and methylated twice by *Sss*I methylase. The methylated DNA fragment and the mock-treated DNA fragment (treatment without *S*-adenosylmethionine) were ligated back into the remaining arm using Ligation high (Toyobo). Nonessential regions within the reporter plasmid were digested with *Sac*I, *Bam*HI, and *Fsp*I. Then,

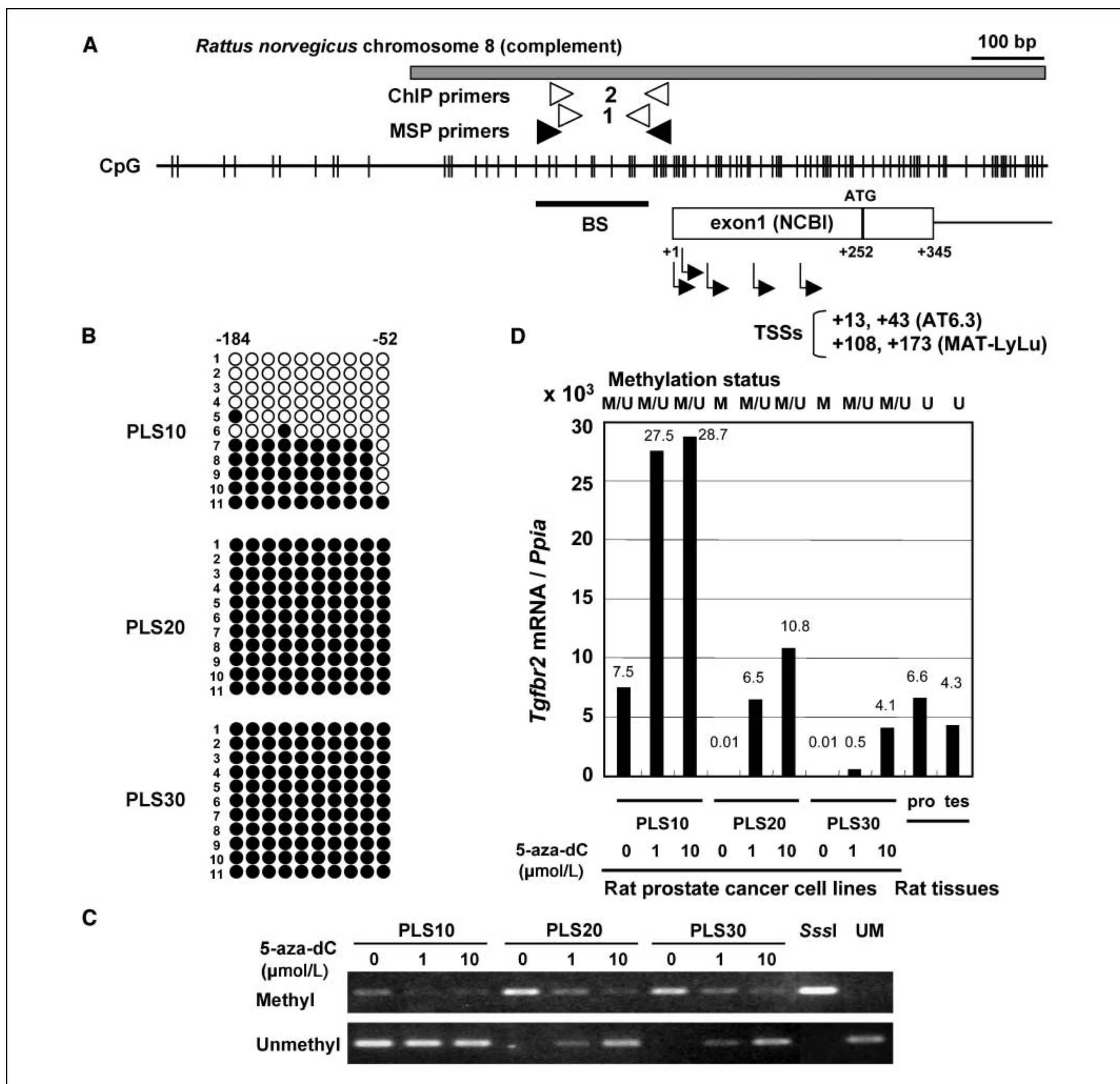

Figure 1. Methylation-silencing of *Tgfbr2* in rat prostate cancer cell lines induced by DMAB and testosterone. *A,* map of a promoter CGI, TSSs, and exon 1 of rat *Tgfbr2*. The TSSs were identified by 5′ RACE of AT6.3 and MAT-LyLu cell lines. *+1, Tgfbr2* TSS in the National Center for Biotechnology Information database (NC_005107.2, 120680453). *Vertical lines,* individual CpG sites; *gray box,* CGI region; *open boxes,* noncoding and coding exons; *arrows,* TSSs; *thick line,* the area analyzed by bisulfite sequencing; *arrowheads,* positions of MSP and ChIP primers. *B,* results of bisulfite sequencing in rat prostate cancer cell lines. The presence of dense methylation of the promoter CGI was confirmed for PLS20 and PLS30. *C, Tgfbr2* methylation status in rat prostate cancer cell lines analyzed by MSP. Demethylation was induced by 5-aza-dC in PLS20 and PLS30. *SssI,* genomic DNA methylated with *Sss*I methylase; *UM,* unmethylated control. *D,* quantitative mRNA expression analysis of rat *Tgfbr2. Tgfbr2* was expressed in the normal prostate (*pro*), testes (*tes*), and PLS10 that had unmethylated DNA molecules.

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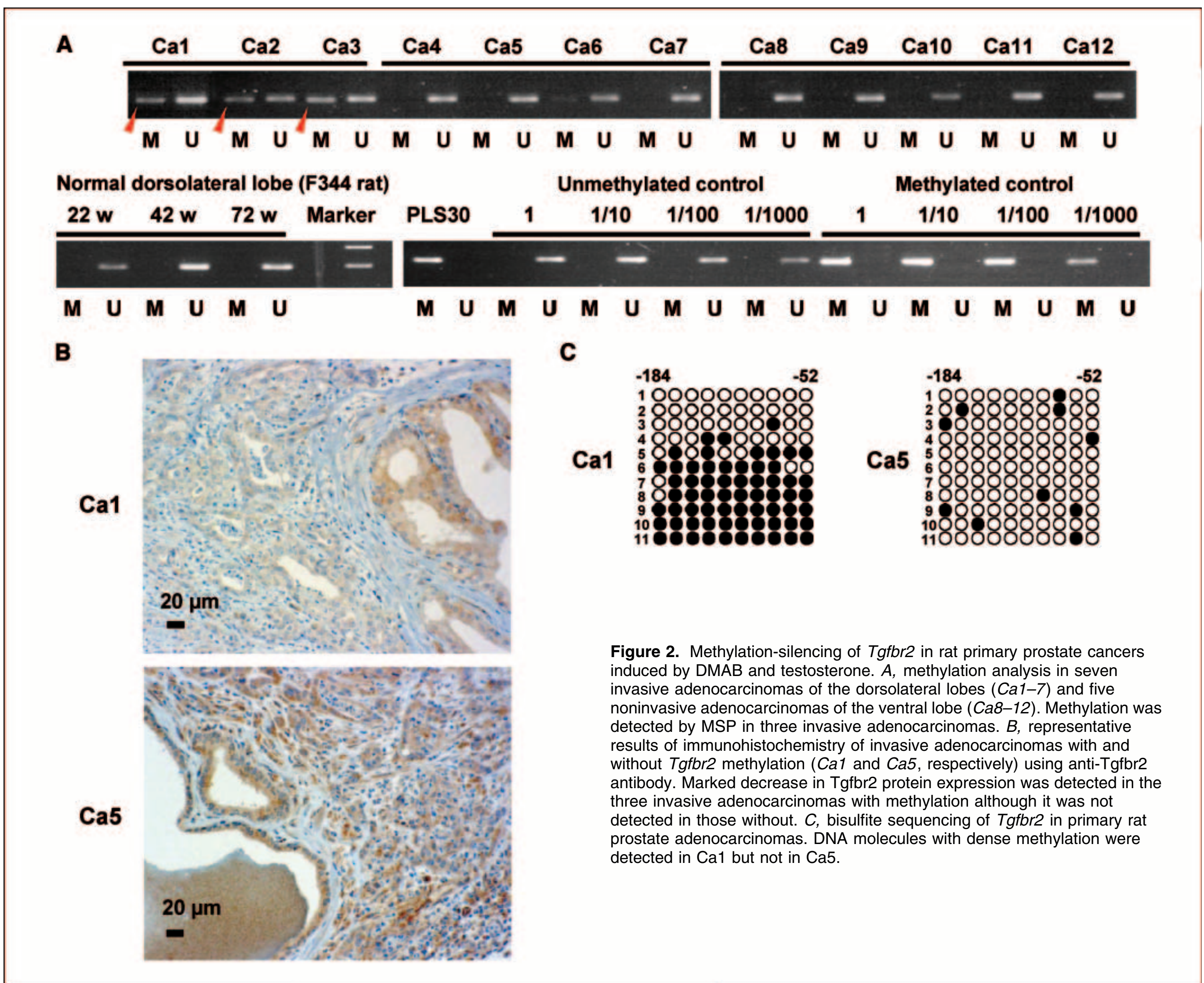

Figure 2. Methylation-silencing of *Tgfbr2* in rat primary prostate cancers induced by DMAB and testosterone. *A,* methylation analysis in seven invasive adenocarcinomas of the dorsolateral lobes (*Ca1–7*) and five noninvasive adenocarcinomas of the ventral lobe (*Ca8–12*). Methylation was detected by MSP in three invasive adenocarcinomas. *B,* representative results of immunohistochemistry of invasive adenocarcinomas with and without *Tgfbr2* methylation (*Ca1* and *Ca5*, respectively) using anti-Tgfbr2 antibody. Marked decrease in Tgfbr2 protein expression was detected in the three invasive adenocarcinomas with methylation although it was not detected in those without. *C,* bisulfite sequencing of *Tgfbr2* in primary rat prostate adenocarcinomas. DNA molecules with dense methylation were detected in Ca1 but not in Ca5.

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a *Sac*I-*Bam*HI fragment that contained *TGFBR2* promoter and luciferase cDNA was recovered after electrophoresis in an agarose gel.

Along with a control plasmid for transfection efficiency (3 ng phRL-TK; Promega), 30 ng of the *Sac*I-*Bam*HI fragment was transiently transfected into PC3 cells using Lipofectamine 2000 transfection reagent (Invitrogen) with Opti-MEM I Reduced-Serum Medium (Invitrogen) in a 96-well format. At 24 h after transfection, cells were harvested, and luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega) in a Lumat LB 9507 (Berthold Technologies). Each transfection and measurement was performed in triplicate.

Sequencing analysis for mutation analysis of human *TGFBR2*. The polyadenylic acid tract in exon 3 of *TGFBR2* (nucleotides 831–840 of NM_001024847), the target region of microsatellite instability (25), was amplified using Phusion high-fidelity DNA polymerase with HF Buffer (New England Biolabs). The product was sequenced with inner primers (Supplementary Table S2).

Results

Genes up-regulated by 5-aza-dC treatment and their methylation analysis. Three rat prostate cancer cell lines (PLS10, PLS20, and PLS30) were treated with 10 μmol/L of 5-aza-dC, and up-regulated genes were searched for using an oligonucleotide microarray. Among >28,000 genes and expressed sequence tags analyzed by the microarray, 47, 13, and 10 annotated genes (59 nonredundant annotated genes), respectively, were up-regulated at 16-fold or more (signal log ratio $\geq$4) in the three cell lines (Supplementary Tables S3 and S4). The presence of a putative promoter CGI was examined by a database search, and 10, 3, and 1 genes (12 nonredundant genes) were found to have CGIs that spanned 300 bp or more (Supplementary Table S3). Genes with these CGIs were considered as candidates for novel methylation-silenced genes in rat prostate cancers.

To examine whether the induction of these genes by 5-aza-dC treatment was due to demethylation of promoter CGIs, the methylation statuses of the putative promoter CGIs were analyzed by MSP. The CGIs of eight genes (*Aebp1, Dysf, Gas6, LOC361288, Nnat, Ocm, RGD1308119*, and *Tgfbr2*) were completely methylated before the treatment and demethylated after the treatment in at least one of the three rat prostate cancer cell lines (Table 1). The up-regulation of mRNA expression of these eight genes detected by

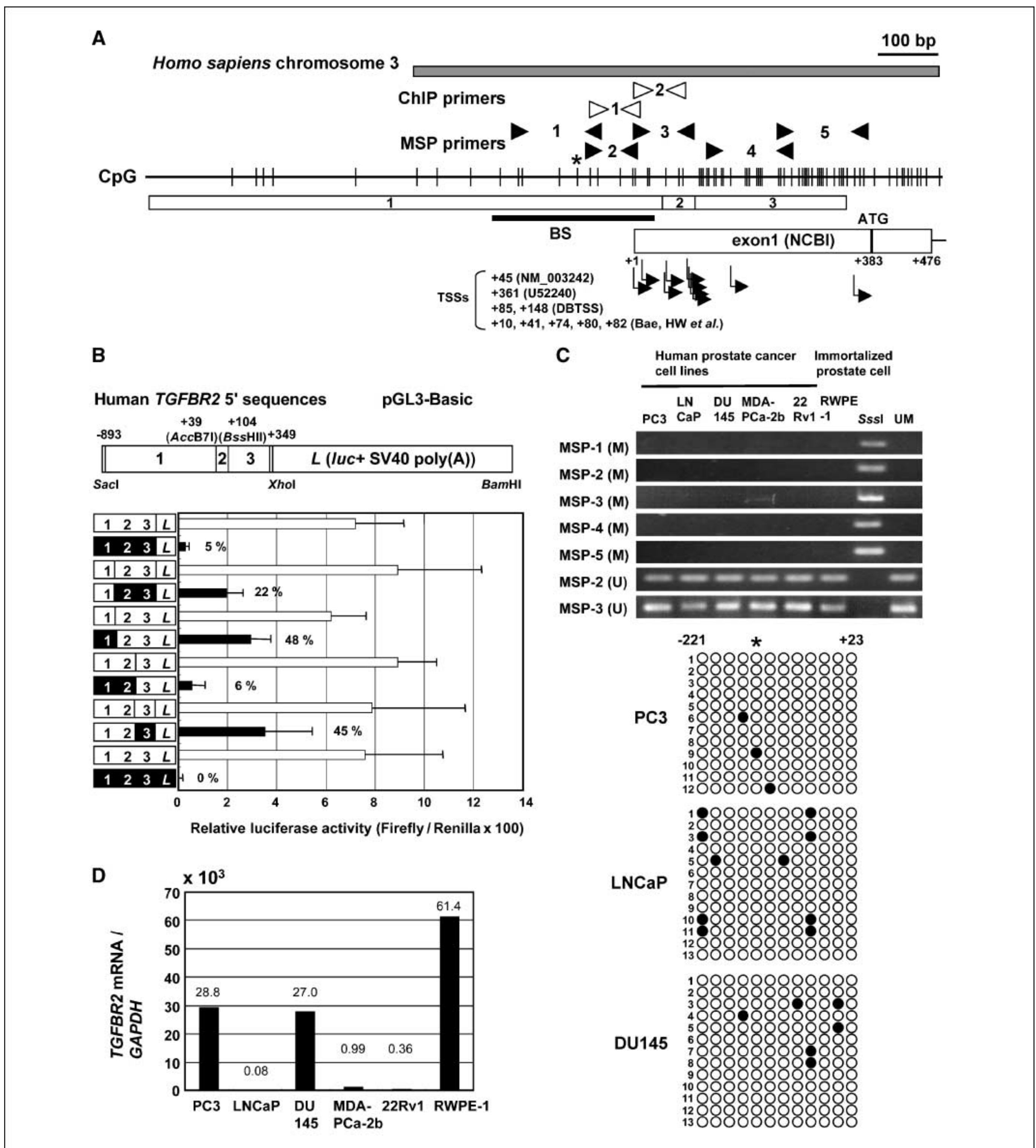

Figure 3. Human *TGFBR2* silencing due to dense methylation of its CGI, and its rare occurrence among human cancer cell lines. *A,* map of the promoter region and a CGI overlapping human *TGFBR2. +1,* TSS from the National Center for Biotechnology Information database (NC_000003.10, 30622998). Multiple TSSs reported (30) are also shown. **,* a specific CpG site at nucleotide −140 (nucleotide −96 in this article) reported in ref. 36. *B,* structure of the *Sac*I-*Bam*HI DNA fragment used for the luciferase reporter assay (*top*). A DNA fragment spanning from −893 to +349 of human *TGFBR2* was ligated to the luciferase reporter gene. *Bottom*, luciferase activity of the reporter constructs with and without methylation of specific regions of the *TGFBR2* promoter. *Open* and *closed boxes,* unmethylated and methylated regions, respectively. The promoter activities were normalized to the activity of the cotransfected phRL-TK vector. Compared with the control without methylation, a reporter construct with methylation of regions 1 and 2 showed a marked decrease in luciferase activity. A fragment that had methylation of the entire reporter plasmid showed no transcription activity. *Columns,* mean; *bars,* SD. *C,* MSP of *TGFBR2* in five human prostate cancer cell lines and immortalized prostate epithelial cells (*RWPE-1*). Screening of 33 human cancer cell lines in the same manner showed that *TGFBR2* methylation was rare. *D,* real-time reverse transcription-PCR analysis of *TGFBR2* mRNA expression in human prostate cancer cell lines and RWPE-1. The expression was down-regulated to $<10^{-4}$ of that of *GAPDH* in LNCaP and to $<10^{-3}$ in MDA-PCa-2b and 22Rv1.

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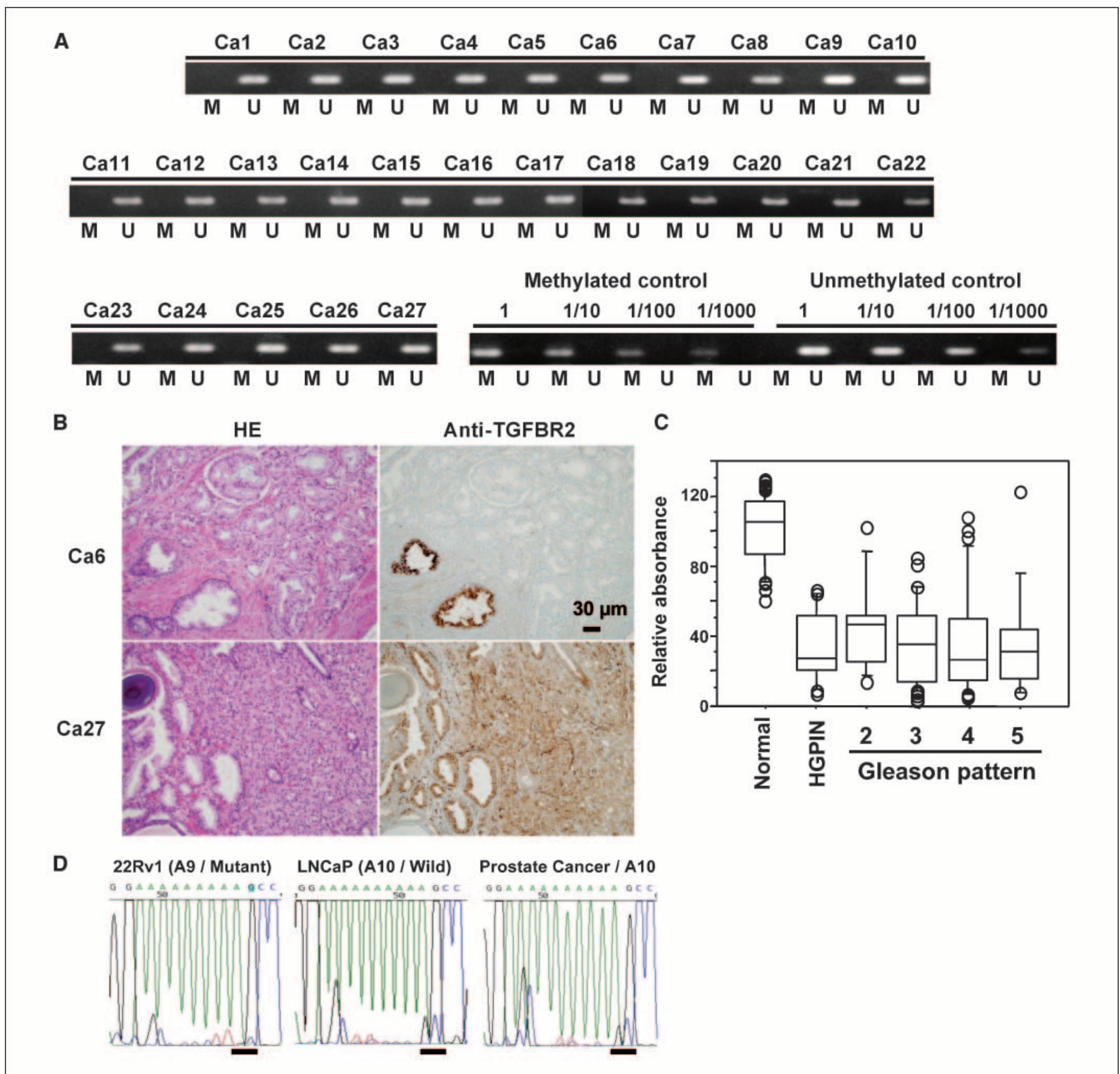

Figure 4. Absence of *TGFBR2* methylation and decreased TGFBR2 protein expression in primary human prostate cancers. *A,* methylation analysis of 27 primary human prostate cancers by MSP (MSP-3). PCR conditions were adjusted to allow detection of methylation of DNA molecules to as little as 1% of the positive control, but methylation was not detected. *B,* representative results of immunohistochemistry using anti-TGFBR2 antibody. *Ca6,* a cancer with decreased expression (*top*); *Ca27,* a cancer with positive expression (*bottom*). Decreased immunoreactivity was observed in 36 of 60 cases. *C,* TGFBR2 expression levels in HGPIN and prostate cancers with different Gleason patterns. Decreased TGFBR2 expression was already detected in HGPIN. *D,* representative results of mutation analysis. A10 (normal sequence) was deleted to A9 only in 22Rv1 and MDA-PCa-2b cell lines, and none of the 27 primary prostate cancers had a mutation.

the microarray was confirmed by quantitative reverse transcription-PCR (Supplementary Table S5). These eight genes were considered to be methylation-silenced in the rat prostate cancer cell lines.

Methylation silencing of *Tgfbr2* in rat prostate cancer cell lines. Interestingly, among the eight genes, *Tgfbr2*, a key mediator of TGF-β signaling that has been implicated in human and rat prostate carcinogenesis (12–19), was present. The methylation silencing of *Tgfbr2* was analyzed further. Generally, for methylation silencing, a dense DNA methylation of a region within a promoter CGI, specifically a region devoid of nucleosome just upstream of a transcription start site (TSS; nucleosome-devoid region), is critical (26, 27). To search for any *Tgfbr2* TSSs additional to the one reported in osteoblasts (28), we performed the 5′ RACE method using rat prostate cancer cell lines. Several TSSs were found to be located from +13 to +173 of the reported *Tgfbr2* TSS (NM_031132.3, National Center for Biotechnology Information; Fig. 1*A*), and the region analyzed by MSP was located at −178 to −22 of the multiple TSSs. Because this region

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corresponded to the critical region involved in transcription repression, the presence of dense methylation was confirmed by bisulfite sequencing. PLS20 and PLS30 had only methylated DNA molecules, and PLS10 had both methylated and unmethylated DNA molecules before the 5-aza-dC treatment (Fig. 1*B*), consistent with the results by MSP (Fig. 1*C*).

The absence of *Tgfbr2* expression before the 5-aza-dC treatment and its re-expression after the treatment were confirmed by

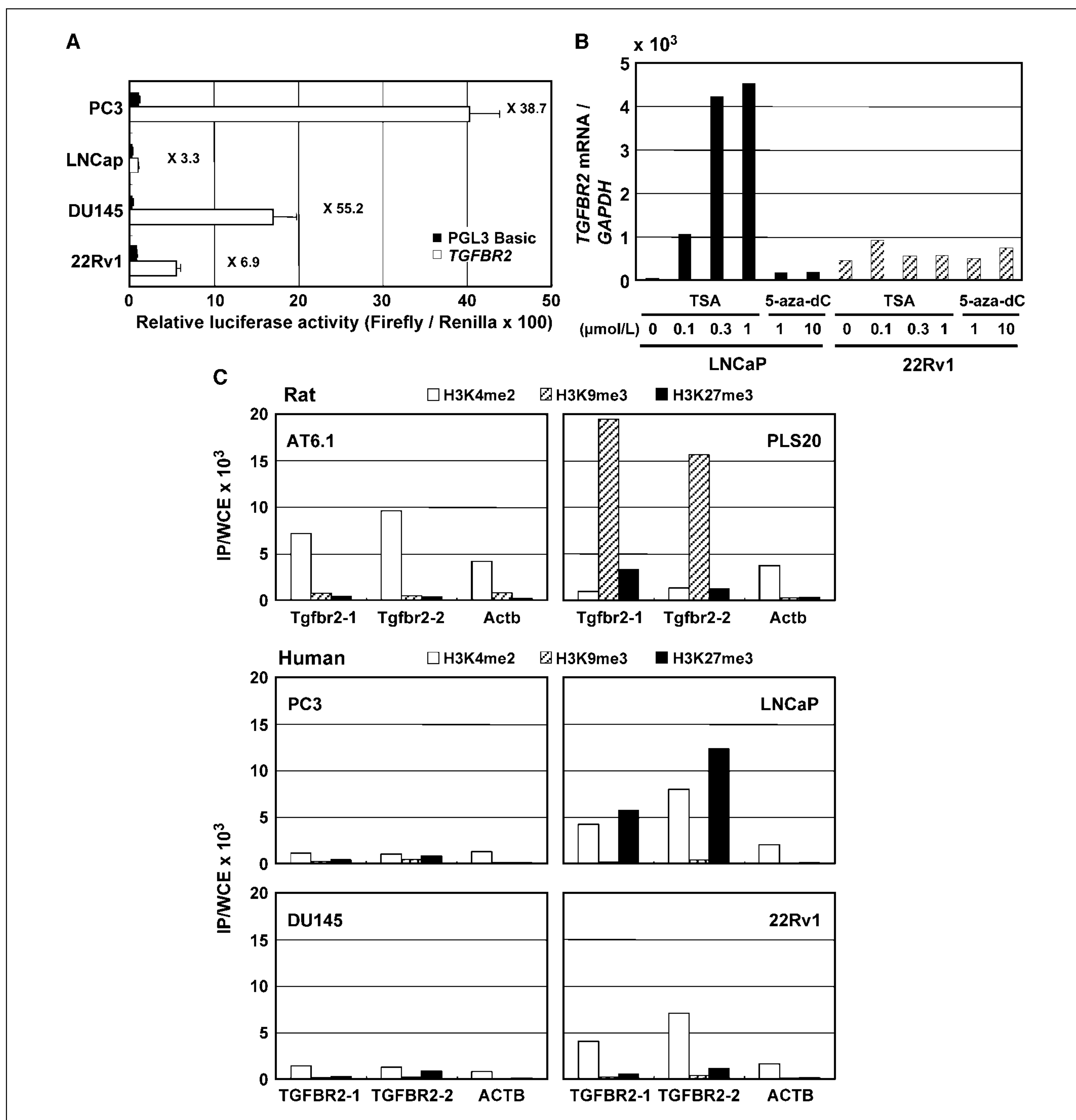

Figure 5. Transcriptional capacities and histone modifications of the *Tgfbr2* (*TGFBR2*) promoter in rat and human prostate cancer cell lines. *A,* luciferase reporter assay using a 1,242 bp DNA fragment covering the human *TGFBR2* promoter and TSSs. The DNA fragment had a 38.7-fold, 3.3-fold, 55.2-fold, and 6.9-fold higher luciferase activity in PC3, LNCaP, DU145 and 22Rv1, respectively, compared with control (pGL3-Basic vector without an inserted promoter DNA fragment). *B,* real-time reverse transcription-PCR analysis of *TGFBR2* mRNA expression in LNCaP and 22Rv1 cells with TSA or 5-aza-dC treatment. *C,* ChIP analysis of histone modifications at the *TGFBR2* and *ACTB* promoters in rat and human prostate cancer cell lines. At the *ACTB* promoter, increased H3K4me2 was observed in all rat and human prostate cancer cell lines. At the *Tgfbr2* promoter, a rat cell line with *Tgfbr2* expression (*AT6.1*) had increased H3K4me2 whereas another rat cell line without (*PLS20*) had increased H3K9me3. In contrast, human prostate cancer cell lines had both H3K4me2 and H3K27me3. Especially, a cell line with decreased *TGFBR2* expression (*LNCaP*) had H3K27me3. *IP,* immunoprecipitated; *WCE*, whole cell extract (input).

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quantitative reverse transcription-PCR in PLS20 and PLS30 cells (Fig. 1*D*). The re-expression was associated with the appearance of unmethylated DNA molecules by MSP (Fig. 1*C*). Expression of *Tgfbr2* in the normal prostate, which is important for functional gene silencing in cancer, was confirmed. We concluded that *Tgfbr2* was methylation-silenced in PLS20 and PLS30 rat prostate cancer cell lines.

***Tgfbr2* silencing in rat primary prostate cancers.** DNA methylation of the *Tgfbr2* promoter CGI and its decreased protein expression were analyzed in rat primary prostate cancers induced by DMAB and testosterone. Using MSP, methylation was detected in three of seven invasive adenocarcinomas in the dorsolateral lobe, but in none of five noninvasive adenocarcinomas in the ventral lobe (Fig. 2*A*). Using immunohistochemistry, protein expression was found to be markedly decreased in the three invasive adenocarcinomas with methylation, but was not decreased in the remaining four invasive adenocarcinomas or in any of the five noninvasive adenocarcinomas (Fig. 2*B*). The results obtained by MSP were confirmed by bisulfite sequencing of two representative samples with and without methylation (Fig. 2*C*). These data showed that the *Tgfbr2* gene was methylation-silenced in rat primary prostate cancers induced by DMAB and testosterone.

Methylation silencing of human *TGFBR2*, and its rare occurrence. In humans, silencing of *TGFBR2* due to dense DNA methylation of its promoter CGI has been reported in a limited number of cell lines (29), but has not been found in any primary cancers. Unlike that of rat *Tgfbr2*, the "promoter CGI" of human *TGFBR2* is located mainly in its first exon, based on its well-documented multiple TSSs (Fig. 3*A*; ref. 30), and CpG density becomes lower in the promoter region. It is now known that DNA methylation of a nucleosome-devoid region is critical for gene silencing (26, 27), and that methylation of a promoter region with intermediate or low CpG density does not necessarily repress the transcription of its downstream gene (31). Therefore, we decided to examine whether or not dense methylation of various regions of human *TGFBR2* could cause its silencing.

First, a 1,242 bp DNA fragment covering the human *TGFBR2* TSSs and its promoter region was cloned. Then, the entire fragment or three regions (regions 1, 2, and 3; shown in Fig. 3*A* and *B*) and their combinations were specifically methylated, and the effect was analyzed by a reporter assay. Methylation of region 1 only (−893 to +39) and methylation of region 3 only (+104 to +349) reduced the promoter activity to half of their unmethylated controls (Fig. 3*B*). On the other hand, methylation of regions 1 and 2 (−893 to +104) reduced the activity to 6% of its unmethylated control. These data showed that dense methylation of the human *TGFBR2* CGI, if present, can repress transcription, and indicated that methylation of region 2 is indispensable. Due to the short size of region 2, data on methylation of region 2 only could not be prepared.

MSP primers were designed in region 2 (MSP-3), the possible nucleosome-devoid region, encompassing most TSSs, and methylation status was screened in 33 human cancer cell lines (five prostate, six ovarian, six lung, seven pancreatic, and nine stomach cancer cell lines; data for prostate cancers in Fig. 3*C*). Only TYK-nu (ovarian) and MIAPaCa-2 (pancreas) had *TGFBR2* methylation (data not shown). The lack of methylation in three prostate cancer cell lines (PC3, LNCaP, and DU145) was confirmed by bisulfite sequencing (Fig. 3*C*). When *TGFBR2* mRNA expression was examined, it was down-regulated to $<10^{-4}$ of that of *GAPDH* in LNCaP and to $<10^{-3}$ in MDA-PCa-2b and 22Rv1 (Fig. 3*D*). These data showed that silencing of *TGFBR2* due to dense methylation of its promoter region was rare among human cancer cell lines and absent in the five human prostate cancer cell lines analyzed.

Lack of methylation of the *TGFBR2* nucleosome-devoid region, but its frequent down-regulation in primary human prostate cancers. Methylation of the possible nucleosome-devoid region (MSP-3) was analyzed in 27 primary human prostate cancers by MSP. However, none of them showed methylation (Fig. 4*A*). It has been reported that TGFBR2 expression is markedly down-regulated in human prostate cancers (14–16), and we confirmed this. Following immunohistochemical analysis of the 20 high-grade prostatic intraepithelial neoplasia (HGPIN), the 27 cancers, and an additional 33 cancers, down-regulation of TGFBR2 protein was observed in 12 of 20 HGPIN and 36 of 60 prostate cancers (Fig. 4*B* and *C*). There was no correlation between expression levels of TGFBR2 protein and histologic grade in human prostate cancers. Finally, as a possible mechanism for decreased TGFBR2 expression, its mutations were searched for. Mutations were only detected in two prostate cancer cell lines (22Rv1 and MDA-PCa-2b), but not in the 27 primary prostate cancers analyzed (Fig. 4*D*).

Contrastive histone modifications in the rat and human prostate cancer cell line with down-regulated *Tgfbr2* expression. To analyze the molecular mechanisms causing down-regulation of *TGFBR2* in human prostate cancer cell lines (LNCaP and 22Rv1), we first analyzed their transcriptional capacity by a luciferase reporter assay using a 1,242 bp DNA fragment covering the human *TGFBR2* promoter and TSSs. The transcriptional capacity of LNCaP and 22Rv1 was significantly lower than that of PC3 and DU145 (Fig. 5*A*), although precise comparison of transcription activities among different cell lines was difficult because transfection and/or luminescence efficiencies were highly variable (Supplementary Table S6). We also analyzed the histone acetylation status in rat and prostate cancer cell lines by observing the effect of 5-aza-dC or TSA, a histone deacetylase inhibitor. LNCaP showed marked re-expression of *TGFBR2* mRNA after TSA treatment whereas 22Rv1 did not (Fig. 5*B*). This showed that, in addition to decreased transcription capacity, histone deacetylation was involved in the decreased *TGFBR2* expression in LNCaP, but not in 22Rv1.

Histone methylation status was further analyzed by chromatin immunoprecipitation (ChIP) assays in the rat and human prostate cancer cell lines. A rat cell line with *Tgfbr2* methylation silencing (PLS20) had increased H3K9me3, a typical mark for inactive chromatin (32), whereas another rat cell line with *Tgfbr2* expression (AT6.1) had increased H3K4me2, a typical mark for active chromatin (ref. 32; Fig. 5*C*). In contrast, human prostate cancer cell lines had both H3K4me2 and H3K27me3, and LNCaP, which had histone deacetylation, had a marked increase of H3K27me3 (Fig. 5*C*). These suggested that the loss of *Tgfbr2* expression in a rat prostate cancer cell line (PLS20) was due to DNA methylation, accompanied by the H3K9me3 modification, and that the decreased *TGFBR2* expression in a human prostate cancer cell line was due to decreased transcriptional capacity in concert with (LNCaP) or without (22Rv1) histone deacetylation and H3K27 trimethylation.

Discussion

Silencing of *Tgfbr2* was identified in invasive adenocarcinomas of the dorsolateral lobe of the rat prostate. This is the first report

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of *Tgfbr2* silencing in animal cancers of any tissue, and of gene silencing in rat prostate cancers. In animal models, only a limited number of genes are known to be silenced by dense methylation of a region just upstream of a TSS, within a CGI, a nucleosome-devoid region (26, 27), in skin, lung, hematologic, and renal cancers (4–7). Our finding of *Tgfbr2* silencing in prostate cancers will enable us to analyze the processes of how aberrant methylation is induced *in vivo* and the factors that promote and suppress the induction of aberrant methylation, including testosterone. Mouse prostate cancers induced by the SV40 polyoma virus early region are known to be prevented by a demethylating agent, 5-aza-dC (33), but the genes responsible are still indefinite.

Functional involvement of *Tgfbr2* (TGFBR2) down-regulation in rodent and human prostate carcinogenesis is strongly supported in the literature. In rats, loss of TGF-β responsiveness in prostate epithelial cells causes malignant transformation (18), and prostate cancer sublines with high metastatic potential, MAT-LyLu and AT-3, show loss of Tgfbr2 protein (19). In mice, dominant negative Tgfbr2 mutant expression increased metastasis in the prostate of the TRAMP model (34), and conditional inactivation of *Tgfbr2* in fibroblasts resulted in intraepithelial neoplasia in the mouse prostate (35). In human prostate cancers, impaired TGF-β signaling, for which TGFBR2 is a key mediator, is likely to be deeply involved (12). Factors supporting this include, first, that TGF-β functions as an inducer of apoptosis in the normal prostate (12, 13); second, *TGFBR2* expression is reduced or lost in prostate cancers (14–16), as confirmed in this study; and third, overexpression of *TGFBR2* restores sensitivity of prostate cancer cells to apoptosis (12, 17). All these strongly indicate that *Tgfbr2* silencing is causally involved in rat prostate carcinogenesis, and suggest that *TGFBR2* down-regulation could be causally involved in human prostate carcinogenesis.

Human *TGFBR2* silencing due to dense methylation of its promoter region was first reported in lung cancer cell lines (29). Here, we showed that a critical region for its silencing was located just upstream of the human *TGFBR2* multiple TSSs (region 2, MSP-3), and that dense methylation of the region can repress its transcription. However, in human primary prostate cancers, *TGFBR2* silencing by dense methylation was not detected. The initial report on human *TGFBR2* silencing did not analyze primary cancers (29). These findings suggest that *TGFBR2* methylation silencing is very rare in human primary cancers. Methylation of a specific CpG site at −96 (nucleotide −140 in the original report) was reported to correlate with reduced *TGFBR2* expression in prostate cancer cell lines (36). However, we were not able to observe the correlation between methylation of the specific CpG site and transcription, or to detect dense methylation in any regions around the TSSs (MSP primers 1–5; Fig. 3*A* and *C*).

The rare occurrence of *TGFBR2* methylation silencing in human primary cancers was in sharp contrast with the frequent occurrence of *Tgfbr2* methylation silencing in rat invasive prostate cancers. Methylation silencing of genes other than *TGFBR2* are frequently observed in human prostate cancers (37). As a mechanism for the decreased *TGFBR2* expression, we first looked for *TGFBR2* mutations, but could not observe any. Then we analyzed transcriptional capacity and histone modifications, and revealed the presence of contrastive mechanisms between rats and humans. In the rat prostate cancer cell lines with *Tgfbr2* methylation silencing (PLS20 and PLS30), *Tgfbr2* expression levels were almost zero (Fig. 1*D*), their promoter regions were densely methylated, and had histone modification (H3K9me3) typical for inactive chromatin. In contrast, the human prostate cancer cell lines with decreased *TGFBR2* expression (LNCaP and 22Rv1) had very low levels of expression (Fig. 5*B*), decreased transcriptional capacity, and histone deacetylation and H3K27 trimethylation (LNCaP). The relative location of a CGI against the TSSs was markedly different between human and rat sequences, the human CGI mainly in exon 1 and the rat CGI mainly in the promoter region, and could be responsible for the contrastive mechanisms for the decreased *Tgfbr2* (*TGFBR2*) expression.

The induction mechanism of rat *Tgfbr2* silencing in the prostate is an interesting issue. Androgen exposure, a critical promoting factor of prostate cancers, is known to down-regulate *Tgfbr2* expression at the transcriptional level (38, 39), and transcriptional repression is known to trigger aberrant DNA methylation (3). In the rat prostate cancer model used here, a combination of an androgen (testosterone) and DMAB is important in inducing invasive prostate cancers, and thus *Tgfbr2* silencing. This suggests that not only the reduced *Tgfbr2* transcription but also some abnormality, required for induction of *Tgfbr2* silencing, is induced by testosterone and DMAB.

As for other methylation-silenced genes in the PLS rat prostate cancer cell lines, *Aebp1* is a binding partner for tumor-suppressor PTEN (40). *Gas6* and *Ocm* have oncogenic functions (41, 42). *Nnat* is known as an imprinting gene and its aberrant hypermethylation occurs frequently in pediatric acute leukemia (43). There is a possibility that silencing of these genes is related to the development and progression of rat prostate carcinoma. In human prostate cancers, two studies reported genomic screening of methylation-silenced genes (44, 45). No common genes were present between the genes identified in the two studies and the eight genes identified here. However, if we adopted a more relaxed criterion for screening of up-regulated genes in this study, *Tgfb3* (11-fold up-regulation in PLS10) was commonly identified (45). *Tgfb3* is also involved in TGF-β signaling, and is a candidate for a gene commonly methylation-silenced in both rat and human prostate cancers. Considering the number of methylation-silenced genes, it is likely that the majority of the genes silenced in PLS cells do not have causal roles in carcinogenesis.

In summary, we found *Tgfbr2* silencing due to dense DNA methylation of its promoter CGI in rat prostate cancers. This will enable us to analyze mechanisms of how methylation silencing is induced *in vivo* and identify factors that affect its induction.

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