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Epigenetic modification-dependent androgen receptor occupancy facilitates the ectopic *TSPY1* expression in prostate cancer cells

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Abstract

Testis-specific protein Y-encoded 1 (*TSPY1*), a Y chromosome-linked oncogene, is frequently activated in prostate cancers (PCa) and its expression is correlated with the poor prognosis of PCa. However, the cause of the ectopic transcription of *TSPY1* in PCa remains unclear. Here, we observed that the methylation status in the CpG islands (CGI) of the *TSPY1* promoter was negatively correlated with its expression level in different human samples. The acetyl-histone H4 and trimethylated histone H3-lysine 4, two post–translational modifications of histones occupying the *TSPY1* promoter, facilitated the *TSPY1* expression in PCa cells. In addition, we found that androgen accelerated the *TSPY1* transcription on the condition of hypomethylated of *TSPY1*-CGI and promoted PCa cell proliferation. Moreover, the binding of androgen receptor (AR) to the *TSPY1* promoter, enhancing *TSPY1* transcription, was detected in PCa cells. Taken together, our findings identified the regulation of DNA methylation, acting as a primary mechanism, on *TSPY1* expression in PCa, and revealed that *TSPY1* is an androgen-AR axis-regulated oncogene, suggesting a novel and potential target for PCa therapy.

KEYWORDS
androgen, androgen receptor, DNA methylation, prostate cancer, TSPY1

1 | INTRODUCTION

Prostate cancer (PCa) is the most prevalent male-specific malignancy and the second leading cause of cancer deaths among men worldwide.[1] Androgen ablation is an effective therapy for the treatment of advanced PCa, and many biomarkers of PCa are androgen-regulated genes, including prostate-specific antigen (PSA). However, androgen deprivation therapy invariably induces androgen independence, leading to PCa recurrence, which is referred to as castration-resistant prostate cancer (CRPCa).[2] CRPCa often exhibits enhanced androgen receptor (AR) signaling[3] due to hypersensitivity to androgen, intratumoral steroidogenesis, *AR* gene amplifications, and *AR* mutations.[4-6] For the purpose of exploring the treatment of CRPCa, it is important to identify novel AR-related pathways that drive or contribute to the growth and progression of PCa.

The human testis-specific protein Y-encoded 1 (*TSPY1*) gene is the largest tandem-repeat multicopy gene in the genome.[7-10] Normally, *TSPY1* is specifically expressed in testicular germ cells and

contributes to spermatogonial cell renewal and propagation.[11-13] Under pathological conditions, *TSPY1* is not only highly expressed in various types of germ cell tumors[8,14,15] but is also often ectopically expressed in different somatic cell carcinomas.[16-22] Many studies have focused on the role of TSPY1 during tumor progression. The results have shown that TSPY1 can interact with the cyclin B/CDK1 complex to accelerate cell G2/M phase transition[23] and interplay with the translation elongation factor eEF1A1/2 to stimulate protein synthesis during promoting cell proliferation.[24-27] Recently, our research elucidated that TSPY1 increases the formation of the TSPY1-TSPYL5-USP7 complex and decreases the p53 level so as to promote cell proliferation[28] and that TSPY1 activates the PI3K/AKT and RAS signaling through suppressing the *IGFBP3* transcription.[29] Taken together, the findings suggest an oncogene function of *TSPY1*.

In PCa cells, TSPY1 is frequently highly-abundantly expressed and positively correlated with the grade of prostate cancer.[16,17] Li et al (2017) demonstrated that TSPY1, constructing a complex with AR, exacerbates the AR-targeted gene activation and promotes PCa cell proliferation.[30] Therefore, the causes of the ectopic *TSPY1* expression in PCa deserve more attention considering the potential of TSPY1 as a diagnostic marker and therapeutic target.

To address this issue, we investigated the epigenetic regulation and androgen stimulation on *TSPY1* expression. Our observations identified that DNA demethylation of the CpG islands (CGI) and enrichment of histones with specific post–translational modifications (PTM), creating a relaxed chromatin in the *TSPY1* promoter, are two necessary conditions for the *TSPY1* activation. Interestingly, we identified an AR-binding site in the 5′ upstream region of the *TSPY1* promoter. The findings reveal that *TSPY1* is a novel AR-targeted gene, suggesting a persuasive mechanism underlying the stimulation of androgen on *TSPY1* expression.

2 | MATERIALS AND METHODS

2.1 | Human samples

Human testicular biopsies were obtained from three obstructive azoospermic patients with normal spermatogenesis. Peripheral blood samples were collected from 10 male volunteers. Six PCa tissues were selected from 56 localized PCa patients who obtained a histopathologic diagnosis from 2016-2018 at the Department of Urology, West China Hospital, Sichuan University. Written informed consent was obtained from each participant. This study was authorized by the Ethical Committee of West China Hospital, Sichuan University.

2.2 | Cell lines

Five cell lines, including a non–tumorigenic prostate epithelial cell (RWPE1), three PCa cells (LNCaP, 22RV1, and DU145), and one teratoma cell (NT2/D1), were originally purchased from the American Type Culture Collection and maintained in our laboratory. VCaP cell line was kindly gifted by Professor Qiao Zhou, Department of Pathology, West China Hospital, Sichuan University. RWPE1 cells were grown in Defined Keratinocyte-SFM medium (ThermoFisher). LNCaP and 22RV1 cells were grown in RPMI-1640 supplemented with 10% FBS. DU145, NT2/D1, and VCaP cells were maintained in DMEM supplemented with 10% FBS. All cells were incubated at 37°C with 5% CO_2.

2.3 | Antibodies

As shown in Table S1, rabbit polyclonal antibodies for acetyl-histone H3 (ab47915), mouse monoclonal antibodies for H3K9me2 (ab1220), H3K27me3 (ab6002), and H3K4me3 (ab6000) were obtained from Abcam. The rabbit polyclonal antibodies for acetyl-histone H4 (06-598) and AR (39781) were purchased from Millipore and Active Motif, respectively. Mouse and rabbit IgG were purchased from Cell Signaling Technology.

2.4 | Chemical treatments

DNA methyltransferase inhibitor 5′-aza-2′-deoxycytidine (AZA) and HDAC inhibitor trichostatin A (TSA) were purchased from Sigma-Aldrich. Cells were treated separately or simultaneously with different doses of AZA and TSA. Androgen boldenone undecylenate (B) and AR inhibitor enzalutamide (E) were purchased from MedChemExpress. Before treatment, cells were cultured in phenol red-free medium containing 5% charcoal-stripped FBS (SeraPure) for 72 hours. Then cells were cultured for an additional 6 days in the same medium supplemented with different doses of B and E. The culture media were changed every 2 days.

2.5 | RT-PCR

Total RNA was isolated from cell lines and tissues using a Super-Purity RNA Exaction Kit (BioTeke\). First-strand cDNA was reverse-transcribed using a RevertAid First-Strand cDNA Synthesis Kit (Vazyme). Quantitative RT-PCR (qRT-PCR) was performed using the AceQ qPCR SYBR Green Master Mix (Vazyme). Reactions were run using a Bio-Rad CFX96 RT-PCR Detection System. The $^{\Delta\Delta}$CT method was used for data analysis. Each assay was performed in triplicate. The *GAPDH* gene was used as an internal control. The primers that were used for RT-PCR are listed in Table S2.

2.6 | Bisulfite genomic DNA sequencing

Genomic DNA was bisulfate-treated with the DNA Methylation-Gold Kit (Zymo research) according to the manufacturer's instructions. The PCR primers used for amplifying the TSPY-CGI are shown

in Table S2. All PCR products were separated on agarose gels, recovered, and inserted into a pMD19-T vector (TaKaRa Bio). More than 20 clones for each DNA sample were sequenced.

2.7 | siRNA

Transient AR knockdown was performed using the identified siRNA oligos (#390824) obtained from Thermo Fisher. Negative control (#siN0000001-1-5) was purchased from RiboBio.

2.8 | Cell proliferation assay

The overexpressed or knockdown *TSPY1* in LNCaP cells was obtained by the infection of lentiviral vectors according to our previous report.[29] Cells were seeded into a 96-well plate (3000 cells/well) and incubated overnight in the previously described conditions. Cell numbers were measured using the Cell Counting Kit-8 (Vazyme) at different times (0, 24, 48, and 72 hours).

2.9 | ChIP and quantitative PCR

ChIP assay was performed using the ChIP-IT Express Enzymatic Kit (Active Motif) according to the manufacturer's protocol. Briefly, the cells were fixed by 1% formaldehyde. After sonication, the chromatin was immunoprecipitated with antibodies against different histone-PTM or mouse/rabbit IgG for 16 hours. The DNA purification was performed using the ChIP-IT Express Enzymatic Kit (Active Motif). A total of 5 μL of ChIP-enriched DNA was used as a template for quantitative PCR (qPCR) amplification with designated primers (Table S2). The cycle threshold (Ct) values from each run were averaged per tissue or cell, and the $^{\Delta\Delta}$CT method was then used for the data analysis, in which the value of target DNA fragments that were enriched in each tissue or cell was normalized to the value of the 5% input DNA of each sample. The qPCR data were expressed as the mean ± SD. The ChIP-qPCR assay was repeated twice to confirm the reproducibility of the results. The results were represented as fold changes compared with the control group.

2.10 | Plasmid construction

Three lentiviral vectors for overexpressing or knocking down *TSPY1* and a partial *TSPY* promoter-diverting luciferase gene were constructed by GeneCopoeia. Among them, the full-length cDNA encoding FLAG-tagged TSPY1 was synthesized and cloned into pLVX-ZsGreen1-N1 vector (Clontech). *TSPY1*-specific shRNA (shTSPY1) were synthesized and inserted into the pLKO.1 vector (Addgene). The partial *TSPY1* promoter fragment, from −1530 bp to +34 bp, was synthesized and inserted into the pEZ-LvPG04 vector (Figure S1).

In addition, the *TSPY1* promoter-deleted mutants were amplified and subcloned into the pGL3 Basic Luciferase Reporter Vector (Promega). pEZ-M13-AR encoding full-length AR were constructed by GeneCopoeia. The PCR primers for amplifying the *TSPY1* promoter are listed in Table S2. The cDNA encoding the AR-DBD domain were amplified with primers listed in Table S2 and inserted into a prokaryotic expression vector pGEX-5x-3 (GE Healthcare [Amersham Biosciences]).

2.11 | Site-directed mutagenesis

To investigate the putative androgen response element (ARE) sites in the *TSPY1* promoter, we separately mutated the ARE sequences using the specific primers (Table S2) and a site-directed mutagenesis kit (Mut Express II Fast Mutagenesis Kit V2, Vazyme).

2.12 | In vitro methylation of CpG sites in the TSPY1 promoter

The plasmids containing the partial *TSPY1* promoter were treated with a CpG methyltransferase M.Sss I and S-adenosylmethionine (New England Biolabs). The enzymes of HpaII (methylation-sensitive) and MspI (methylation-nonsensitive) were used to verify the methylation efficiency (Figure S2).

2.13 | Luciferase activity assay

After transfection with each construct of *TSPY* promoter-diverting luciferase for 48 hours, the cells were collected and lysed. The luciferase activity in the cell lysate was measured using a Dual-Luciferase Reporter Assay System (Promega). Renilla luciferase activity was used as an internal control. In addition, the Gaussia luciferase activity of the LNCaP cells infected with the lentiviral *TSPY1* promoter-luciferase vector was detected using a Secrete-Pair Dual Luminescence Assay Kit (GeneCopoeia). The activity of secreted alkaline phosphatase was used as an internal control.

2.14 | Expression and purification of GST-fused AR-DBD protein

After the construct of pGEX-5x-AR-DBD was transformed into the *Escherichia coli* host BL21 (DE3), the GST-fused AR-DBD protein was expressed under the induction with 0.1 mmol/L isopropy-β-D-thiogalactoside (Sigma-Aldrich). Then, the harvested bacterial pellet was homogenized by sonication. After centrifugation, the supernatant was purified using Glutathione Sepharose 4B (GE Healthcare). Finally, the purified proteins were detected using the Coomassie brilliant blue method after SDS-PAGE electrophoresis.

2.15 | EMSA

Biotin-labeled, biotin-unlabeled (cold competitor), and ARE-mutated (mut competitor) oligonucleotides corresponding to the *TSPY1* promoter (Table S2) were synthesized and annealed into double-stranded probes. EMSA was carried out using a Chemiluminescent EMSA Kit (Beyotime) according to the manufacturer's instructions. Briefly, AR-DBD fusion protein (2 μg) was incubated with 20 fmol of probes at room temperature for 20 minutes. The entire reaction mixtures were separated on a 6.5% nondenaturing, native polyacrylamide gel, and then transferred on a positively-charged nylon membrane (Beyotime). After the membrane was cross-linked using UV light, the biotin signals were successively developed with Streptavidin-HRP and BeyoECL Moon (Beyotime) and visualized after exposure to X-ray films.

2.16 | Statistical analyses

All data were expressed as the mean ± SD of at least three independent experiments. Statistical significance was tested using Student's *t*-test between any two groups. $P < 0.05$ was considered significant. GraphPad Prism 7 software was used for the statistical analysis.

3 | RESULT

3.1 | Identification of the negative correlation between the *TSPY1* expression level and DNA methylation in the *TSPY1* promoter

DNA methylation in the CpG-rich promoter region is the primary silencing mechanism of most genes related to prostate cancer.[20,31] The homogenous gene copies of *TSPY1* provided a chance to analyze its promoter region.[32] To investigate whether the expression of the *TPSY1* gene is regulated by DNA methylation in the promoter region, we first predicted two CpG islands in the 5′ upstream region, spanning −981 bp to −676 bp and −135 bp to +463 bp relative to the putative transcription initiation site of the *TSPY1* gene, using the bioinformatics method (http://genome.ucsc.edu/) (Figure 1A). Then, we observed that the CpG sites of the two CGI were hypermethylated, with average methylation (AM) level >0.86 in the peripheral blood cells with *TSPY1* silence (Figure 1B, Figure S3). However, the CpG sites were not frequently methylated in the *TSPY1*-expressed testicular tissues derived from three patients with obstructive azoospermia (Figure 1B, Figure S3). Further investigation of the DNA methylation status of *TSPY1* CpG sites in *TSPY1*-expressed and *TSPY1*-silent prostate cancer tissues indicated a negative correlation between the *TSPY1* expression and the CpG methylation status of the *TSPY1* promoter (Figure 1C, Figure S3), revealing that the demethylation of *TSPY1* CpG sites is necessary for its transcription activation in prostate tumors.

3.2 | In vitro *TSPY1* expression induced by DNA demethylation

We investigated the *TSPY1* expression and DNA methylation status of two CGI in five prostate cell lines (normal epithelial cells: RWPE1; cancer cells: LNCaP, 22RV1, VCaP, and DU145) and one teratoma cell line (NT2). The results showed that LNCaP was the only cell line that highly expressed *TSPY1* mRNA (Figure 2A). In LNCaP cells, the two CGI of the *TSPY1* promoter region were relatively hypomethylated. with 52.2% of the average methylation (AM) in *TSPY1* CGI1 and only 22.2% of that in CGI2 (Figure 2B). However, less *TSPY1* mRNA was detected in the cells of RWPE-1, 22RV1, DU145, VCaP,

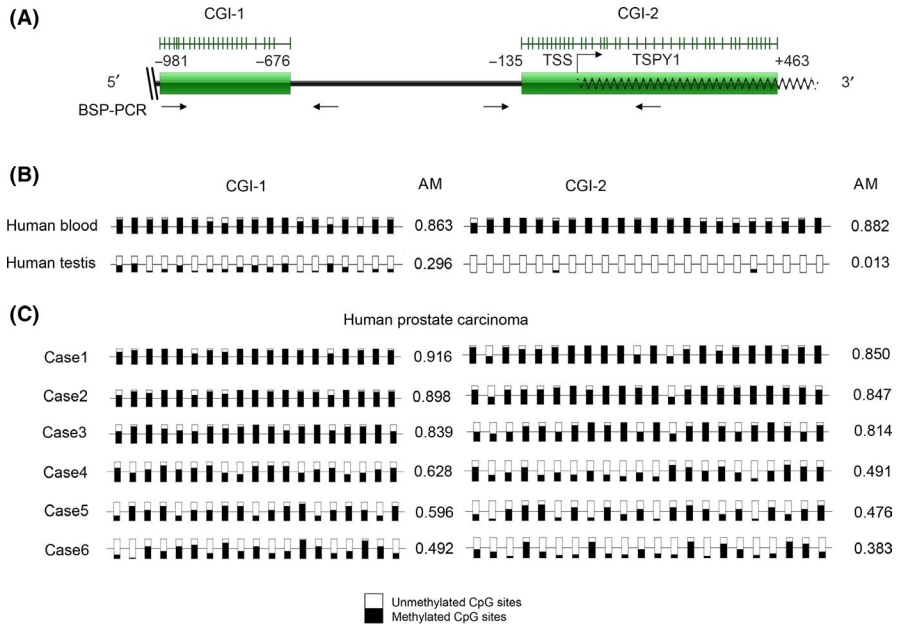

FIGURE 1 The CpG methylation status in the 5′ upstream region of the *TSPY1* gene in human samples. A, Schematic diagram of two CpG islands in the 5′ upstream region of the *TSPY1* gene. Arrows show the primers for BSP-PCR amplification. TSS, transcription start site. B, The two TSPY1 CGI are hypermethylated in the peripheral blood cells while frequently unmethylated in human testicular tissues. C, The two TSPY1 CGI are hypermethylated in TSPY1-silent while hypomethylated in TSPY1-expressed prostate cancer tissues. AM, average methylation level of CpG sites in the two TSPY1 CGI.

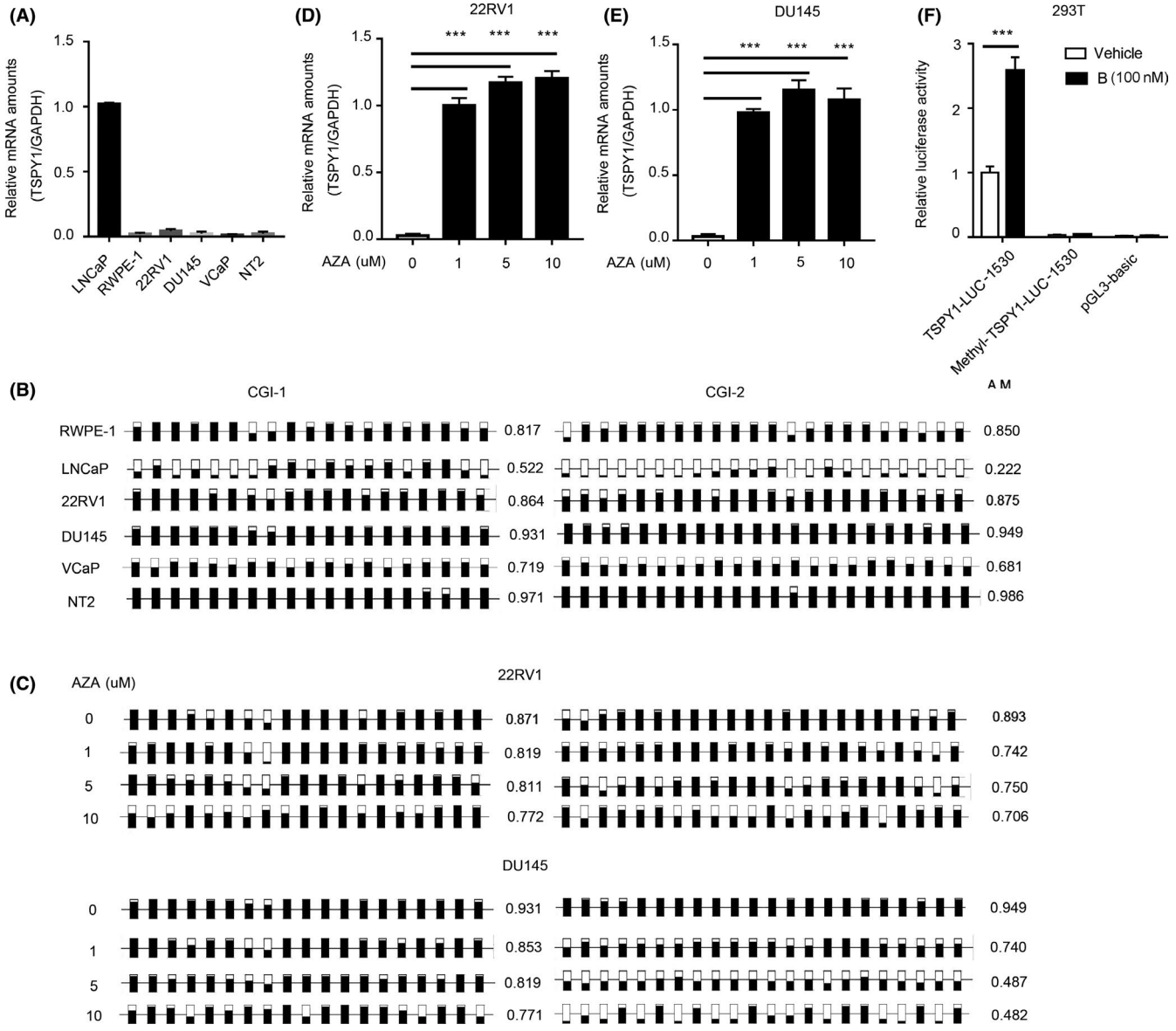

FIGURE 2 Activation of the *TSPY1* expression by DNA demethylation. A, Relative expression level of the *TSPY1* gene in different prostate cell lines. The amount of *TSPY1* mRNA in LNCaP cells was set to 1 arbitrary unit. B, The different CpG methylation profile of the *TSPY1* CGI in the prostate cells. C, Partial demethylation of the CpG sites of *TSPY1* CGI in 22RV1 and DU145 cells through the treatment with AZA. D and E, Activation of *TSPY1* gene expression in 22RV1(D) and DU145(E) cells through treatment with different concentrations (1 μmol/L, 5 μmol/L, and 10 μmol/L) of AZA. The amount of *TSPY1* mRNA in cells treated with 1 μmol/L AZA was set to 1 arbitrary unit. The expression level of the *GAPDH* gene acts as an internal control. Quantitative PCR reactions were performed in triplicate. Data were statistically analyzed using Student's *t*-test; **P*-value < 0.05, ***P*-value < 0.01, ****P*-value < 0.001. F, Luciferase activity analysis showed that the methylated *TSPY1* promoter inhibited the subsequent gene expression. B, androgen B

and NT2 (Figure 2A), and the CpG sites of the *TSPY1* CGI were heavily methylated (AM >81.7%) (Figure 2B). Then, when we treated 22RV1 and DU145 cells with 5′-aza-2′-deoxycytidine (AZA), a DNA methyltransferase inhibitor, the *TSPY1* CGI methylation decreased (Figure 2C) and the *TSPY1* expression increased (Figure 2D,E). These results suggested that DNA demethylation activated the *TSPY1* expression in vitro.

In addition, we constructed the partial *TSPY1* promoter into a luciferase reporter vector. When the *TSPY1* promoter was artificially methylated using M.SssI, a CpG methyltransferase, the promoter activity was significantly decreased in vitro (Figure 2F). The observation further supports that the methylation of *TSPY1* CGI is the primary mechanism of *TSPY1* silence.

3.3 | Regulation of histone post–translational modifications on the *TSPY1* expression

Histone-PTM affect chromatin decondensation and DNA accessibility to induce the transcriptional activation of

surrounding genes.[33-37] To investigate the influence of histone-PTM on the *TSPY1* expression, we analyzed the occupancies of acetylated histones H3 (acH3) and H4 (acH4) and methylated histones H3K4me3, H3K9me2, and H3K27me3 on the *TSPY1* promoter in different human tissues and cells. The ChIP-qPCR results showed that the amount of acH3 and acH4 binding to the *TSPY1* promoter in human testis was 2.2 and 8.9 times that in human blood (Figure 3A). The amount of trimethyl-histone H3K4me3 on the *TSPY1* promoter in testicular tissues was 4.8-fold higher than that in human blood (Figure 3A). In contrast, the occupancy of the dimethyl-histone H3K9me2 on the *TSPY1* promoter in testicular tissues were lower than that in human blood (Figure 3A). Consistently, in prostate cancer tissues ectopically expressing *TSPY1*, the amount of acH3, acH4, and H3K4me3 binding to the *TSPY1* promoter was 2.4, 4.2, and 2.9 times that in prostate cancer tissue with *TSPY1* silence, respectively (Figure 3B). Moreover, the amount of acH4 and H3K4me3 binding to the *TSPY1* promoter in the LNCaP cells was 23-fold and 8-fold higher than that in DU145 cells (Figure 3C). These findings suggested that the enrichment of acH3, acH4, and H3K4me3 created a relaxed chromatin environment and facilitated the subsequent *TSPY1* expression.

We further investigated whether TSA, a histone deacetylases inhibitor, can activate the expression of the *TSPY1* gene in

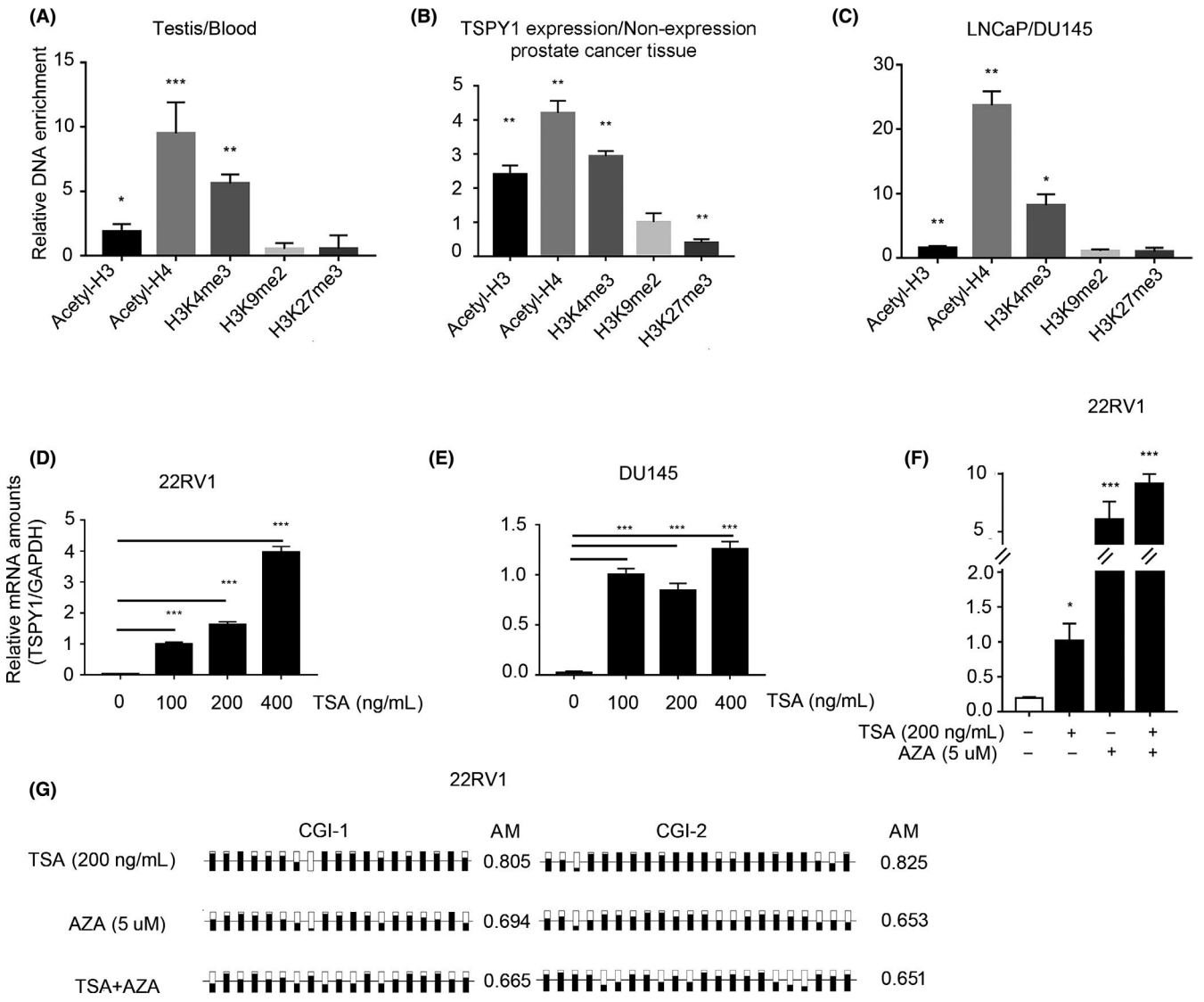

FIGURE 3 The impact of histone-PTMs on the *TSPY1* promoter activity. A-C, ChIP-qPCR analysis comparing the enrichment level of the *TSPY1* promoter region occupied by the histone-PTM in different tissues and cells, including testis and blood (A), TSPY1 expression and non-expression PCa (B), and LNCaP and DU145 (C). D and E, Expression of *TSPY1* mRNA was activated in 22RV1 (D) and DU145 (E) cells that were treated with the HDAC inhibitor trichostatin A (TSA). F, TSA treatment alone activated the *TSPY1* expression in 22RV1 cells. TSA further increased the *TSPY1* expression in 22RV1 cells to a higher level than treatment with AZA alone. G, The DNA methylation level of the *TSPY1* CGI in 22RV1 cells that were treated with TSA, AZA alone, or a combination of AZA and TSA

human cells with a hypermethylated *TSPY1* CGI. Previous reports demonstrated that AZA and TSA could act synergistically[38,39] and TSA could induce DNA demethylation.[40,41] We treated 22RV1 and DU145 cells with TSA and observed that the *TSPY1* transcription could be activated in both cell lines (Figure 3D,E). However, the expression level of *TSPY1* in 22RV1 treated by TSA was 6.0-fold lower than that treat by AZA (Figure 3F). In addition, the *TSPY1* expression level was enhanced approximately 1.5-fold when treated with a combination of AZA and TSA relative to the treatment with AZA alone (Figure 3F). Subsequent BSP sequencing showed a slight demethylation of the *TSPY1* CGI in the TSA-treated cells (Figure 3G), while the difference in the demethylation level of the *TSPY1* CGI was not observed in the cells exposed to both AZA and TSA and the cells treated with AZA alone (Figure 3G). These results further supported that the presence of acetyl-histones facilitated the *TSPY1* expression, and the upregulation was synergistically enhanced by DNA demethylation.

3.4 | Involvement of TSPY1 in androgen-induced proliferation of prostate cancer cells

Previous studies have shown that both androgen and TSPY1 can stimulate the proliferation of prostate cancer cells[30,42] and the *TSPY1* expression is upregulated by androgen.[43,44] With the constructed LNCaP cell models stably overexpressing or knocking down TSPY1, we confirmed that TSPY1 overexpression promoted and TSPY1 knockdown decreased the proliferation of LNCaP cells (Figure 4A,B). The treatment of the synthetic steroidal androgen B also increased the proliferation of LNCaP cells with TSPY1 overexpression or decreased TSPY1 expression (Figure 4A,B). However, the treatment of androgen B in the cells with decreased TSPY1 expression cannot significantly promote the cell proliferation (Figure 4B). Furthermore, we investigated whether TSPY1 overexpression can promote the proliferation of PCa cells treated with AR antagonist enzalutamide (E). We observed that the treatment of E inhibited the cell proliferation of LNCaP (Figure 4C). Then, TSPY1 overexpression

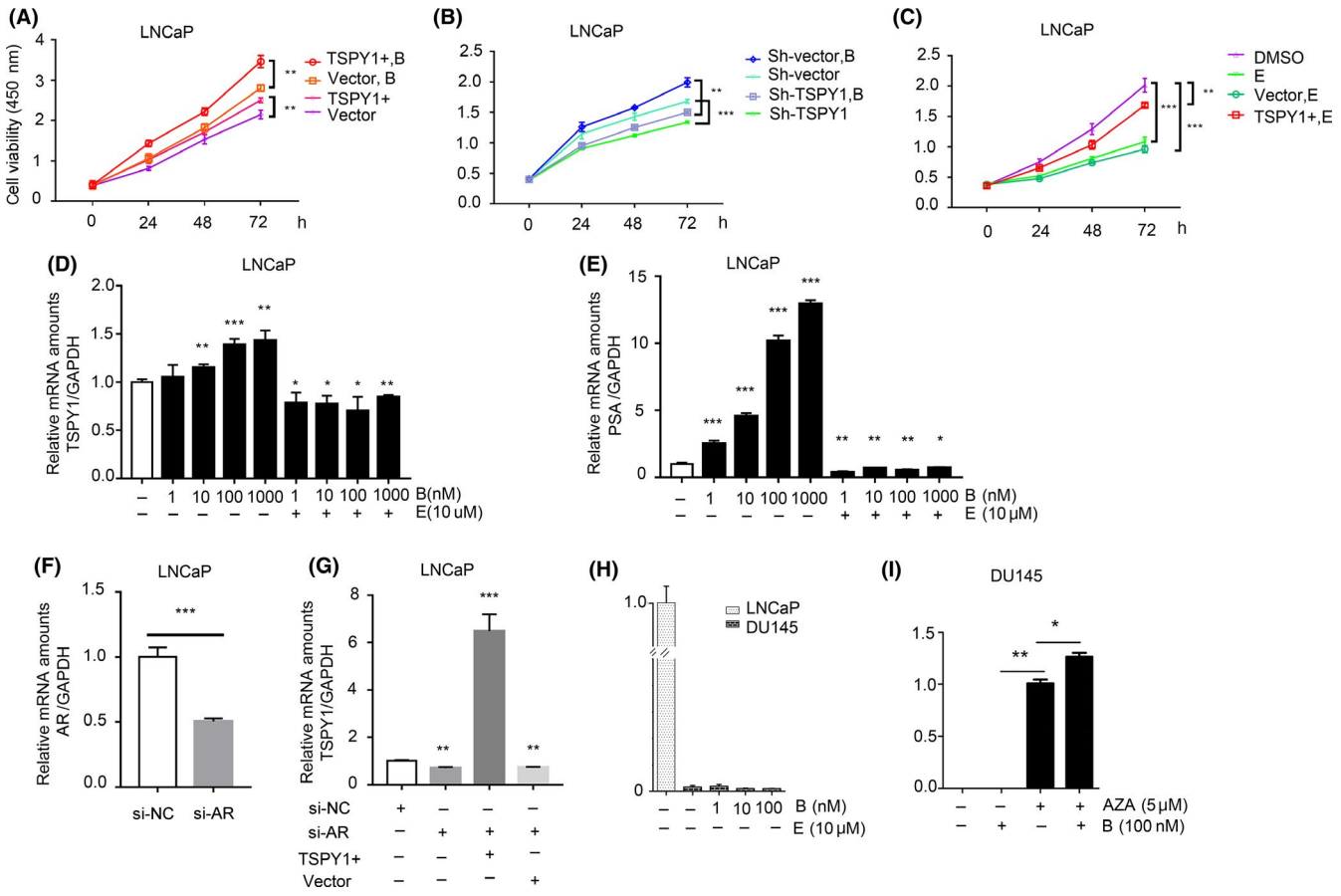

FIGURE 4 Androgen B promotes the *TSPY1* expression and the LNCaP cell proliferation. A and B, CCK-8 assays showing the increased LNCaP cell proliferation when the *TSPY1* expression was enhanced and cells were treated by androgen B. C, TSPY1 rescued the decreased cell proliferation of LNCaP treated with the androgen receptor (AR) antagonist enzalutamide (E, 1 μmol/L). D and E, The *TSPY1* and *PSA* expression level was raised or dropped in LNCaP cells following treatment with different doses of androgen B or androgen receptor inhibitor E. F, AR-targeted siRNA decreased the AR mRNA level in LNCaP cells. G, Decreased AR expression reducing TSPY1 mRNA level and the downregulation of AR not affecting the exogenous *TSPY1* expression. H, The *TSPY1* expression was not activated by androgen B in DU145 cells. I, After treatment by AZA, the *TSPY1* expression was increased by androgen B in DU145 cells

rescued the decreased proliferation trend in LNCaP cells treated with E (Figure 4C). Taken together, these observations implied that androgen-induced *TSPY1* overexpression may be involved in androgen-induced proliferation of PCa cells.

Expectedly, the expression level of the *TSPY1* gene was gradually increased when treated with androgen B in a dose-dependent manner while decreased when treated with E (Figure 4D). As a control, the expression of the AR-targeted gene *prostate-specific antigen* (*PSA*) was also significantly promoted or inhibited in LNCaP cells when treated with androgen B or AR antagonist E (Figure 4E). In addition, when we knocked down AR expression in LNCaP cells through AR-targeted siRNA (Figure 4F), we observed that the *TSPY1* transcription was reduced by approximately 30% (Figure 4G). However, the exogenous *TSPY1* expression was not inhibited (Figure 4G). Moreover, when we added different doses of androgen B to the DU145 cells, whose proliferation is known to not respond to androgen, the *TSPY1* expression was not activated (Figure 4H). Considering that DU145 contains hypermethylated *TSPY1* CGI, we then treated the cells with AZA and androgen B together, and observed that the *TSPY1* transcriptional level was increased 1.3-fold in such cells compared to cells treated with AZA alone (Figure 4I). These results suggested that androgen-mediated AR activation can induce *TSPY1* expression, and the DNA demethylation of *TSPY1* CGI is an indispensable condition for the stimulation.

3.5 | Identification of the androgen receptor binding sites in the *TSPY1* promoter region

First, we found and located six potential AR-binding elements (ARE) through an *in silico* analysis with jaspar (http://jaspar.genereg.net) and animal TFDB (V3.0) (http://bioinfo.life.hust.edu.cn/AnimalTFDB/#!/) of the *TSPY1* promoter region from −1530 bp to +34 bp (Figure 5A). After constructing a lentiviral vector containing the partial *TSPY1* promoter region (from −1530 bp to +34 bp) driving a luciferase reporter gene (Figure S1) and infecting the LNCaP cells, we observed increased expression of luciferase with androgen B treatment, while decreased expression when AR antagonist E was added to the cultured cells (Figure 5B). These observations supported our speculation that the AR may bind to the *TSPY1*

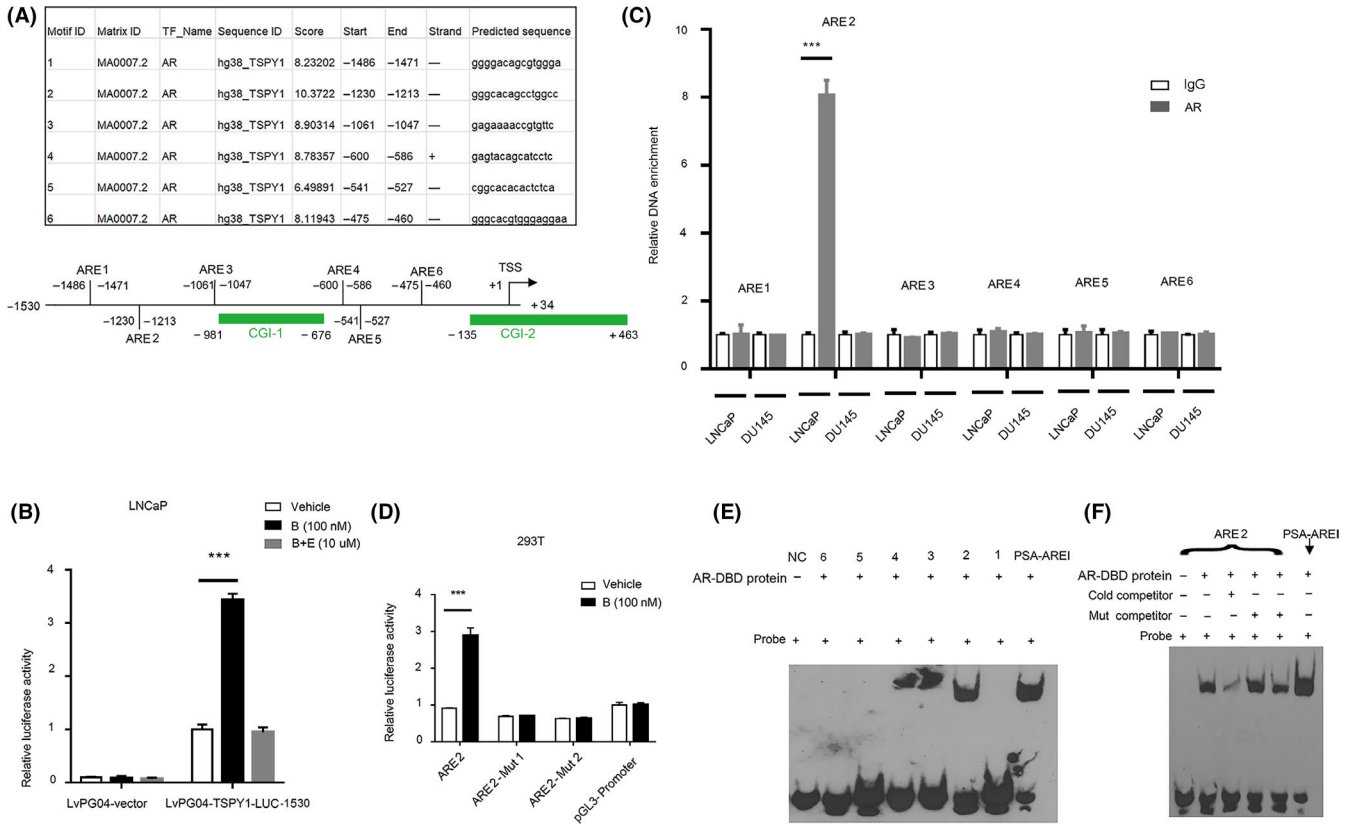

FIGURE 5 Enhancement effect of androgen receptor (AR) on *TSPY1* transcription by binding to its demethylated promoter to enhance its transcription. A, Predicted AR binding elements (ARE) in the *TSPY1* promoter; the sites of two CpG islands are shown in the green blocks. B, Relative luciferase activities of the lentiviral vector-mediated *TSPY1* promoter LUC1530 in LNCaP cells when treated by androgen B or androgen receptor inhibitor E. C, ChIP-PCR assays showing AR binding to the ARE2 of *TSPY1* promoter in LNCaP cells treated by androgen B. DU145 cells were used as a negative control. Rabbit IgG was used as a negative control. D, Relative activities of *TSPY1* promoters with different androgen response element (ARE) mutants in 293T cells when treated by androgen B. E, EMSA showing that AR-DBD protein only binds to the ARE2 of *TSPY1* promoter. F, EMSA showing that the disrupted ARE2 motif cannot bind to AR-DBD protein

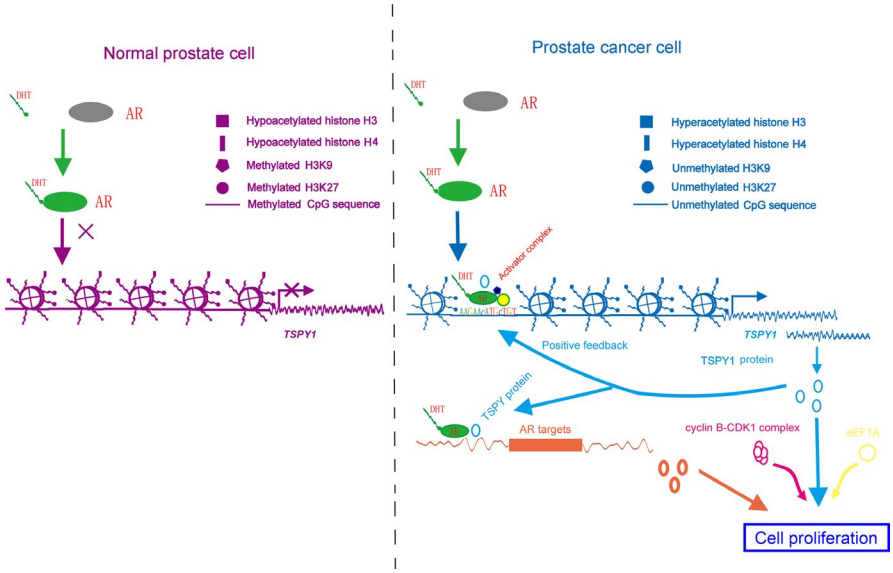

FIGURE 6 Proposed mechanism model for the ectopic expression of *TSPY1* in prostate cancer (PCa) cells

promoter. Furthermore, by conducting ChIP-qPCR and amplifying the *TSPY1* promoter sequence enriched by the AR antibodies in the cells of LNCaP and DU145, an abundant amplification of the *TSPY* promoter region from −1288 bp to −1127 bp was observed in LNCaP cells (Figure 5C). The region contains one of six potential ARE, ARE2. After constructing the mutants according to the ARE2 motif sequence and co–transfecting each mutant with AR expression vector, we observed that the activity of the *TSPY1*-promoter containing mutant ARE2 was not accelerated significantly under the stimulation of androgen B (Figure 5D), suggesting that the disruption of the consensus motif in the putative ARE2 locus results in the disappearance of the regulation of androgen B on the activity of the *TSPY1* promoter.

Moreover, we performed a gel EMSA to further assess the probability of AR binding on the *TSPY1* promoter. First, we obtained the purified GST-fused AR-partial protein, including DNA binding domain (AR-DBD) (Figure S4). Using a partial DNA sequence, each of which contained single ARE, acting as a template, we synthesized biotin-labeled and unlabeled competitor probes (Figure 5E) and then co–incubated the probes with AR-DBD proteins. The subsequent EMSA results showed that the protein-DNA complexes were formed for the ARE2 probes (Figure 5E), while not for other ARE probes, and the DNA-protein complexes disappeared completely when excess amounts of the unlabeled competitor probes were added (Figure 5F, lane 3). The levels of the DNA-protein complexes were not obviously changed when the mutated probes were added (Figure 5F, lane 4 and 5). All of this suggests that AR binds on the ARE2 site of the *TSPY1* promoter.

4 | DISCUSSION

In this study, we first identified that the methylation status of the *TSPY1* CGI was negatively correlated with its expression level, and the histone-PTM, especially acH4 and H3K4me3, bound to the *TSPY1* promoter and stimulated the *TSPY1* expression. Our findings suggest that epigenetic modifications, acting as a primary mechanism, regulate the ectopic activation of *TSPY1* in PCa. In addition, AR, acting as an important regulator in PCa progression activated by androgen, was found to accelerate the *TSPY1* transcription through occupying the *TSPY1* promoter. All of this suggested that *TSPY1* was a novel targeted gene of the transcription factor deeply involved in the occurrence and development of PCa (Figure 6).

DNA methylation, as a stable epigenetic modification mark, can silence or downregulate the following gene transcription. DNA methylation changes have been associated with a long list of pathologies of disorders, including cancers.[45] DNA hypomethylation often occurs in repeat DNA sequences and large regions of DNA in tumors.[46-49] Coincidentally, *TSPY1* is the largest tandem-repeat gene in the human genome. We proposed that the demethylation of *TSPY1*-CGI was programmed to transcribe the gene for normal spermatogenesis, while the expression of the gene contributed to the malignant behavior of PCa, although it is unknown whether the activation of the *TSPY1* gene is a result or a cause of the development of PCa.

Histone-PTM are also important epigenetic factors that determine the gene transcriptional activation or inactivation. Much evidence has revealed that the alteration of histone-PTM is involved in tumorigenesis. Recently, a histone demethylase Jumonji domain containing 1A (JMJD1A) was revealed to play a tumor-promoting role in several cancers, including PCa.[50-53] JMJD1A upregulates the activities of AR and c-Myc and promotes PCa progression.[50,51] JMJD1A also enhances alternative splicing of AR variant 7 (AR-V7),[52] a hormone-independent truncated form of AR in CRPCa. The acetylation of JMJD1A is critically important for maintaining JMJD1A stability and AR activity in CRPCa.[53] H3K9 demethylated by JMJD1A promotes

the chromatin recruitment of AR.[51] In this study, we found that H3K9me2 occupancies on the *TSPY1* promoter may be involved in keeping its silence and that the demethylated H3K9 accompanied by AR recruitment enhances the *TSPY1* transcription. Taken together, JMJD1A may be critical in the regulation of the *TSPY1* activity during PCa progression.

Androgen and its receptor are critical regulators involved in PCa development and progression. AR, activated by androgen, binds to *cis* DNA elements in the promoter or enhancer regions and promotes or suppresses the expression of target genes.[54-56] In this study, we found that AR could occupy the 5′ upstream region of the *TSPY1* gene and upregulate *TSPY1* transcription. Therefore, the importance of *TSPY1* in androgen/AR signal axis is highlighted by our finding. Previous reports on the interaction between TSPY1 and AR demonstrate that TSPY1 complexes with AR and its variant AR-V7 to exacerbate their transactivation,[30] that TSPY1 increases the AR expression in the cells derived from male hepatocellular carcinoma,[57] and that TSPY1 can amplify its self-transcription through acting on its first exon region.[58] Taken together, our finding is crucial because it completes and constructs a positive feedback loop presenting the interaction of androgen, AR, and TSPY1 (Figure 6). It is highly likely that the positive feedback mechanism drives or contributes to the tumorigenic processes in PCa with the ectopic *TSPY1* expression.

With the help of ChIP techniques combined with DNA microarray (ChIP on chip) and deep sequencing (ChIP-Seq), the AR-targeted gene network has been revealed to be deeply involved in the PCa progression.[59-61] In particular, quite a few AR-targeted genes act as critical regulators in CRPCa, including neuroendocrine PCa.[62-64] In this study, our findings suggested that *TSPY1* was a new AR-targeted gene. To the best of our knowledge, *TSPY1* is the first AR-related gene to construct a positive feedback loop with *AR*. Besides interacting with AR, TSPY1, harboring a highly conserved SET/NAP domain (SET, suppressor of variegation, enhancer of zeste, and trithorax; NAP, nucleosome assembling protein), can function as other protumorigenic regulators involved in nucleosome assembly, DNA replication, and transcriptional regulation.[13,30,58] All of this supports that TSPY1 may be a new target to use in PCa therapy.

However, due to the limitation of PCa samples, our current study is deficient for the correlation analysis of clinical outcomes of PCa patients with different *TSPY1* transcription levels. Considering the heterogeneity of PCa cells, we will further investigate whether the *TSPY1*-expressing PCa cells survive in radiotherapy and chemotherapy, or the therapy stress activates the *TSPY1* expression in PCa cells through the epigenetic alteration.

In conclusion, we found that the epigenetic modifications, including the hypomethylation status of the *TSPY1*-CGI and the histone the nearby gene expression, are necessary for the *TSPY1* transcriptional activation in PCa. More importantly, we identified that *TSPY1* was a novel target gene of AR and suggested a positive feedback loop in the androgen-AR-TSPY1 signal axis. Our work provided evidence for the potential of TSPY1 as a new therapy target for PCa.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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