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HDAC1 Inhibition by Maspin Abrogates Epigenetic Silencing of Glutathione S-Transferase Pi (GSTp) in Prostate Carcinoma Cells

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Abstract

Both maspin and glutathione s-transferase pi (GSTp) are implicated as tumor suppressors and down regulated in human prostate cancer. It is well established that GSTp down-regulation is through DNA methylation-based silencing. We report here that maspin expression in prostate cancer cell line DU145 reversed GSTp DNA methylation, as measured by methylation-specific PCR, MethyLight assay and bisulfite sequencing. The effect of maspin on GSTp expression was similar to that of the combination of a synthetic histone deacetylase (HDAC) inhibitor and DNA methylation inhibitor 5-aza-2'-deoxycytidine. Maspin expression also led to an increased level of acetylated histone 3, decreased level of methyl transferase and methyl-CpG-binding domain proteins at the site of demethylated GSTp promoter DNA. Earlier, we have shown that maspin inhibits HDAC1. In PC3 cells where both maspin and GSTp are expressed at a reduced level, maspin knockdown led to a significant reduction in GSTp expression whereas dual knockdown of maspin and HDAC1 barely increased the level of GSTp expression. Thus, HDAC1 may play an essential role in cellular response to maspin-mediated GSTp de-silencing. Maspin has been shown to increase tumor cell sensitivity to drug induced apoptosis. Interestingly, GSTp re-expression in the absence of maspin expression perturbation blocked the phosphorylation of histone 2A.X, the induction of hypoxia-induced factor 1 α (HIF-1 α), and cell death of LNCaP cells under oxidative stress. Since DNA hypermethylation-based silencing may couple with and depend on histone deacetylation, our study suggests that endogenous HDAC inhibition by maspin may prevent pathological gene silencing in prostate tumor progression.

Keywords

histone acetylation; DNA methylation; oxidative stress response; apoptosis; cellular detoxification; DNA damage repair; epithelial epigenetic homeostasis; prostate tumor progression

INTRODUCTION

DNA methylation-based pathological gene silencing is common in carcinogenesis and tumor progression. Mechanistic studies suggest that epigenetic silencing by DNA methylation may be mechanistically coupled to histone deacetylation, while maximal de-silencing effects require the combination of DNA-methylation inhibitor and histone deacetylase (HDAC)

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inhibitor [1, 2]. To date, as efforts are made to target tumor-specific DNA methylation, broad-spectrum pharmacological HDAC inhibitors have shown promising anti-tumor activity in clinical trials.

It is worth noting that the functional elucidation of HDAC has been conducted almost exclusively through the use of pharmacological HDAC inhibitors, since the mechanism of endogenous regulation of HDAC activity remains largely unknown. We have recently reported the first evidence that the tumor suppressor maspin specifically inhibits HDAC1, the most abundant nuclear (class I) HDAC in mammalian cells that is commonly upregulated in malignant diseases [3, 4]. Maspin is a member of the serine protease inhibitor (serpin) superfamily, but lacks inhibitory activity against active serine proteases [5, 6]. Nonetheless, structural considerations [7] suggest that maspin may act as a serine protein inhibitor-like molecule to interact with serine protease-like HDAC1. The activity of HDAC1 in hydrolyzing the ϵ -acetyl lysine residues in its substrate proteins [8, 9] is the function of a catalytic domain, which resembles that of metalloproteinases or serine proteases [10]. To our knowledge, maspin is the only endogenous polypeptide HDAC1 inhibitor identified thus far. Maspin is expressed in an epithelial-specific manner, thus may act as an epithelial-specific regulator of HDAC1 activity.

The biological functions of HDAC1 are thought to derive mostly from its activity in deacetylating nuclear histones [10], leading to chromatin condensation and transcription repression of target genes, such as p21, p27 and Bax [11, 12]. Consistent with the evidence that maspin inhibits HDAC1, we have shown that maspin expression in prostate epithelial cells increased the expression of p21 and Bax, restored prostate epithelial differentiation and sensitized tumor cells to drug induced apoptosis [13, 14]. The identification of HDAC1 as a maspin target [15, 16] could explain, at least in part, the evidence that nuclear maspin correlates with better epithelial differentiation and better prognosis of lung adenocarcinoma [17, 18].

In general, the biological effects of maspin are similar to those of pharmacological HDAC inhibitors [15]. Interestingly, many pharmacological HDAC inhibitors are also associated with up-regulation of detoxifying redox enzymes [19, 20], suggesting that HDAC may naturally repress the expression of these enzymes. Of particular interest is glutathione *S*-transferase pi isoform (GSTp), commonly down-regulated in prostate tumor progression, primarily by DNA methylation [2, 21]. We have previously shown that maspin expression increased total cellular GST activity in human cancer cell lines [16]. These data are consistent with our earlier observation that maspin is translocated from the nucleus to the cytosol at the step of high grade prostatic intraepithelial neoplasia (HGPIN) [22], coinciding with the GSTp silencing event in prostate tumor progression [23, 24].

The exact biological trigger of GSTp gene silencing in prostate cancer is yet to be further elucidated. Based on the emerging evidence that genetic silencing may result from coordinated histone deacetylation and DNA methylation, the current study was aimed at testing whether endogenous inhibition of HDAC1 by maspin may prevent the silencing of GSTp transcription in prostate tumor cells. We present the first evidence that maspin-mediated de-silencing of GSTp depended on HDAC1 and was associated with the reduction of GSTp DNA methylation. Our data also suggest that de-silencing of GSTp by maspin may block oxidative stress-induced DNA damage and the expression of hypoxia-induced factor 1 α (HIF-1 α). These data suggest that the loss of endogenous inhibition of HDAC1 by maspin may lead to the silencing of other tumor suppressors such as GSTp, thus representing a significant gain of function in tumor progression. Taken together, maspin-mediated GSTp de-silencing supports a mechanistic link between endogenous DNA methylation and histone deacetylation.

MATERIALS AND METHODS

Chemicals, Cell Lines and Cell Culture

Pan HDAC inhibitor M344, an analogue of classic HDAC inhibitor trichostatin A (TSA) [25, 26], was purchased from Calbiochem (La Jolla, CA). Class I HDAC-specific inhibitor LBH589 was provided by Novartis Pharmaceuticals (Cambridge, MA). DNA demethylation agent 5-Aza and hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich (Saint Louis, MO). Proteasome inhibitor MG132 was purchased from Calbiochem (San Diego, CA).

Human prostate carcinoma cell lines DU145, PC3, and LNCaP were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 media (Invitrogen, Carlsbad, CA) containing fetal bovine serum (FBS, Hyclone, Logan, UT; 5% for DU145; 10% for PC3 and LNCaP) at 37 °C with 6.5% CO_2 . DU145-derived maspin transfectant clones (M3, M7 and M10) and the mock transfectant clone (Neo) were generated and cultured as described previously [27]. Normal human prostate epithelial cell lines CF3, CF91, and MLC8891 were obtained from John Rhim (National Institutes of Health). For drug treatment, cells were cultured in 6-well plate overnight and treated with the indicated drugs or H_2O_2 for 24 h (for subsequent PCR assay) or 48 h (for subsequent WB assay).

Plasmid Construction and Stable Transfection of LNCaP Cells for GSTp Expression

GSTp cDNA (NM_000852) in MDA-MB-231 breast cancer cell line was PCR-amplified, sequence verified and used as a template for subcloning. To construct the expression vector, GSTp open reading frame was amplified using the following primers: 5'-CAGGGAATTCATATGCCGCCCTACA-3' (forward primer) and 5'-CAGTACTCGAGCTCACTGTTTCCCGT-3' (reverse primer). The amplified GSTp coding sequence was inserted in-frame into pcDNA6/HisTM A vector (Invitrogen, Carlsbad, CA) *via* EcoRI and XhoI cloning sites. The ligation product, encoding Xpress-tagged GSTp protein, was used to transform XL-10 Gold Ultracompetent cells (Stratagene, La Jolla, CA). The *E. coli* clones confirmed positive for GSTp expression plasmid were selected and used to amplify the plasmid DNA that were subsequently sequence verified. The Xpress-GSTp encoding plasmid DNA was transfected into LNCaP cells using TransFectinTM Lipid reagents (Bio-Rad Laboratories, Hercules, CA), followed by Blasticidin S HCl (BSD, Invitrogen, Carlsbad, CA) at a final concentration of 3 μ g/mL in RPMI 1640 medium. Control transfection was conducted with the vector that encodes Xpress-tagged β -galactosidase (LacZ). The resulting clonal cell lines were designated as LNCaP/GSTp and LNCaP/LacZ, respectively.

Transient Transfection

For maspin knockdown by siRNA, cells cultured in six-well plates were transfected with vehicle, a maspin specific small interference RNA SMARTpool (Mas-siRNA; Dharmacon, Lafayette, CO), or a small interference RNA with a scrambled sequence (Scr-siRNA) at 15 μ M, using the siLentFect Lipid Reagent (Bio-Rad Laboratories, Hercules, CA). Cells were continuously cultured for another 48 h, and total cell lysates were harvested for protein analyses. When cells needed to be treated with 5-Aza, cells were first cultured for 24 h after the transfection and then treated with 5-Aza at the indicated concentration for another 48 h before being harvested for further analyses.

Insufficient maspin knockdown was achieved with a single shRNA construct in our preliminary experiments. Since others have used mixtures of shRNA to achieve better gene knockdown in vitro [28], we employed this strategy. To knock down maspin expression,

four different maspin-targeting siRNA sequences (Mas-shRNAs) were separately inserted between the BamHI and HindIII restriction sites into pSilencer H1 3.0 vector (Ambion, Austin, TX). The four pairs of shRNA oligo sequences target maspin gene at 154-174, 171-191, 203-223, and 206-226, respectively. The mixture of the resulting plasmid DNAs was used to transfect PC3 cells using Fugene HD transfection reagents (Roche Applied Science, Mannheim, Germany). G418-resistant stable clones were selected.

To knock down HDAC1 expression, a GIPZ lentiviral shRNA clone RHS4430-98820597 (Thermo Scientific, Rockford, IL) was used to infect the cells at 1:1 of MOI for 48 hrs followed by three more days of culture with the maintenance medium supplemented with 0.6 mg/mL puromycin. shRNA clone RHS4430-98820597 encodes a hairpin sequence of 5'-CCC GAA TCC GCA TGA CTC ATA ATA GTG AAG CCA CAG ATG TAT TAT GAG TCA TGC GGA TTC GG-3', which targets a 158-176 sequence at 5'-terminal HDAC1 mRNA. Cells harvested were lysed, and the total cell lysates were subjected to WB.

Quantification of Cell Viability

Initially, cells were seeded in 6-well plates at 2×10^4 cells/well for vehicle treatment (control) and sub-lethal dose of M344 (0.1 μ M) treatment, and at 6×10^5 cells/well for 5-Aza (2 μ M) with or without M344 (0.1 μ M) treatment. The treatment of 5-Aza lasted for 8 days. During the 8-day treatment period, cell culture medium containing 5-Aza was replenished every two days. For combination treatment, cells were treated additionally with M344 in the last 48 h as described by Okino, et al [29]. Then, cells were washed gently to remove the floating dead cells. The remaining adherent cells were cultured in the maintenance media for another 24 h. Cells were evaluated under the microscope and photographed with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI). The cells were then harvested by trypsin digestion, resuspended in an isotonic solution, and counted using a Coulter Particle Counter (Beckman Coulter Inc., Fullerton, CA). For statistical analyses, one-tailed matched pair Student's *t* tests were done. $p < 0.001$ was considered significant.

Cell viability was also determined using the WST-1 kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instruction. Briefly, cells were seeded at the density of $4 \times 10^4/0.1$ mL/well in 96-well plate and cultured with or without a cytotoxic agent at the indicated concentrations for 48 hrs. Then, the WST assay was performed. The resulting absorbance at 450 nm (OD₄₅₀) was considered a function of cell viability. Using this method, the IC₅₀ of each cytotoxic drug was determined as the drug concentration that led to 50% of loss of the cell viability, as compared to the untreated control.

Western Blot (WB)

Protein concentration of cell lysates was determined by the BCATM protein assay kit (Thermo Scientific, Rockford, IL). Equal amounts of protein were loaded on SDS-PAGE for WB as described previously [15]. The primary antibodies used for immunoblotting were as follows: rabbit anti-GSTp (Calbiochem, La Jolla, CA), anti-Xpress McAb (Invitrogen, Carlsbad, CA), poly-ADP-Ribose-polymerase-1 (PARP; eBioscience, San Diego, CA), monoclonal antibody (mAb) against phosphorylated histone H2A.X (ser-139; p-H2A.X; Cell Signaling Technology, Danvers, MA), histone 3 (H3) acetylated at 9^{lys} residue (K9) (Upstate cell signaling solution, Lake Placid, NY), anti-maspin mAb (BD PharMingen, San Diego, CA), and mAb against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Research Diagnostic, Flanders, NJ). Corresponding secondary antibodies, were conjugated with horse-radish peroxidase, and detected by enhanced chemiluminescence (ECL) reaction.

Northern Blotting of mRNA

Total RNA (20 µg) extracted using a method previously described [15] was heat denatured at 68°C for 15-20 min followed by electrophoresis through 1.0% agarose gel containing 1.7 M formaldehyde. The fractionated RNA was transferred to a Zetaprobe membrane (Bio-Rad). The membrane was baked at 80 °C for 2 hrs. The prehybridization, hybridization and X-ray radiography were carried out as described [30, 31].

Quantitative Real-time PCR (q-RT-PCR)

Total RNA was extracted from cells using a previously described method [15]. One microgram of each RNA sample was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Triplicate q-RT-PCR reactions were routinely conducted in the laboratory as described [15]. The primers used were as follows: GAPDH, 5'-ACGGATTTGGTCGTATTGGG-3' and 5'-TGATTTTGGAGGGATCTCGC-3'; GSTp, 5'-ACCTCCGCTGCAAATACATC-3' and 5'-GGCTAGGACCTCATGGATCA-3'; GSTm3, 5'-GTGCGAGTCGTCTATGGTTC-3' and 5'-AGTTGTGTGCGGAAATCCAT-3'. The generic primers that detect a common sequence in GSTm1, m2, m4, and m5 (GSTm1245) were 5'-GGGACGCTCCTGATTATGAC-3' and 5'-GCAAACCATGGCCGCTTCCC-3'. The primers for hypoxia-induced factor 1α (HIF-1α) were 5'-TCAAGCAGTAGCGAATTGGAACATTATT-3' and 5'-TTTACACGTTTCCAAGAAAGTGATGTA-3'. The q-RT-PCR thermal profile was: 1 cycle of 95 °C/10 min, 40 cycles of 95 °C/30 sec→55 °C/1 min→72 °C/30 sec, 1 cycle of 95 °C/1 min, and finally 41 cycles of $95^{\circ}\text{C} \rightarrow \sum_{0}^{40} (50+n)^{\circ}\text{C}/30 \text{ sec}$. Critical threshold cycle numbers (C_t) were calculated using the built-in program of the Stratagene Mx4000™ Multiplex Quantitative PCR System. The fold differences in mRNA were calculated by $2^{-\Delta\Delta C_t}$ method [32].

Methylation-specific PCR (MS-PCR)

MS-PCR was performed using an established method with minor modifications [29, 33]. Briefly, to amplify the GSTp promoter sequence from 1001 to 1302 bp, genomic DNA was isolated from the cultured cells with a FlexiGene DNA kit (QIAGEN, Cambridge, MA). The genomic DNA was then treated by bisulfite using the EpiTech Bisulfite Kit (QIAGEN, Cambridge, MA). After the bisulfite treatment, DNA was purified by using the Wizard Plus Minipreps DNA purification system (Promega, Madison, WI). This DNA, along with untreated genomic DNA (as a control), was subjected to PCR amplification. PCR amplification was conducted using the Phusion PCR amplification kit (New England Biolabs, Ipswich, MA). The primers for a 272 bp methylated GSTp promoter fragment were 5'-GAAAGAGGGAAAGGTTTTTTCGG-3' (forward) and 5'-GAAAAACGAACCGCGCTACT-3' (reverse). The primers for a 276 bp un-methylated GSTp promoter fragment that could potentially be subjected to methylation were 5'-GGAAAGAGGGAAAGGTTTTTTTG-3' (forward) and 5'-CCCCAAAAACAAACCACATACT-3' (reverse). The primers for total control GSTp promoter fragment were 5'-CCTGGGAAAGAGGGAAAGGC-3' (forward) and 5'-TCACTGGTGGCGAAGACTGC-3' (reverse). The PCR cycling parameters were: 98 °C for 2 min followed by 35 cycles of 98 °C/30 sec→60 °C/30 sec→72 °C/1 min with a final extension step of 72 °C for 5 min. The PCR products were resolved by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

MethLight PCR

MethyLight PCR was carried according to the specific instruction of EpiTect MethyLight PCR kit from Qiagen (Cambridge, MA). Briefly, methylation specific primers and TaqMan

Probes for GSTp [34] and internal reference control MYOD-1 [35] were synthesized by Applied Biosystems (Foster City, CA). To quantify methylated GSTp DNA (GenBank X08058) in the promoter region (from 894 to 1116 bp), two rounds of real-time PCR were carried out with a genomic GSTp DNA template using the Mastercycler ep realplex² (Eppendorf North America Inc., Hauppauge, NY). The PCR cycling program was: 95 °C/5 min followed by 50 cycles of 95 °C/15 sec→60 °C/1 min [34, 35]. The percentage of methylation (POM) was calculated as $100/[1+2^{(Ct_{CG}-Ct_{TG})}]$ %, as described in the manufacturer's manual.

Bisulfite DNA Sequencing

Bisulfate DNA sequencing was performed using the method of Okino *et al.* [29]. Briefly, bisulfate-converted DNA was amplified by two rounds of PCR reaction using PhusionTM High-Fidelity PCR kit (New England BioLabs, Inc., Ipswich, MA) with the cycling conditions described for MS-PCR, except that the annealing step was set at 43 °C. The PCR products were resolved by 2% agarose gel electrophoresis, excised from the gel, and sequenced by the Applied Genomics Technology Center of WSU (Detroit, MI).

Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assay were performed by the EZ-ChIPTM kit (Millipore; Billerica, MA) according to the protocol recommended by the manufacturer. Briefly, cells grown to 80% confluence were fixed by 1% formaldehyde and incubated for 15 min to allow protein-DNA cross-linking. Then, the cells were harvested, washed with cold PBS and resuspended to the density of 5×10^6 cells/500 μ L in lysis buffer. Cell suspensions were sonicated (to shear chromatin DNA into 200 to 700 bp fragments), and centrifuged for 10 min at 12,000g at 4 °C. The resulting supernatants were incubated with protein G-agarose beads (Upstate Biotechnology, Charlottesville, VA) for 1 hr and centrifuged at 2,000 g for 30 sec to remove the agarose slurry. The supernatant of each sample was equally divided into six aliquots. One aliquot was used as an input control, while the other five aliquots were used for immunoprecipitation with HDAC1 McAb, Rabbit anti-Ac-H3 (K9) antibody, rabbit anti-DNMT1 antibody, rabbit anti-DMB_{2/3} antibody (Upstate Cell Signaling Solution, Lake Placid, NY), and normal rabbit immunoglobulin G mixture (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. The resulting mixtures were gently agitated overnight at 4 °C. The immuno-complexes were collected by incubation with protein G-agarose beads (Upstate Biotechnology) for 1 h at 4 °C. After the beads were washed in the sequence of low salt, high salt, LiCl and Tris-EDTA (TE) buffer, the protein-DNA cross-links were reversed by heating at 65 °C for 4 h in 3.5 M NaCl, followed by RNase A treatment for 30 min at 37 °C and proteinase K treatment overnight. The purified DNA samples were PCR amplified using the Phusion PCR amplification kit with the primers designed for a 133 bp fragment in the GSTp promoter region [36]. The primer sequences for p21 promoter in the ChIP were previously described [37]. The PCR products were visualized by 1.5% agarose gel electrophoresis.

Miscellaneous

The quality of RNA was determined based on the integrity of 18S and 28S ribosomal RNAs on agarose gel electrophoresis. For statistical analyses, one-tailed matched pair Student's *t*-tests were performed. *p* values smaller than 0.01 were considered significant.

RESULTS

Maspin De-silences GSTp in Prostate Cancer Cell Lines

Both maspin and GSTp are epigenetically down regulated in human prostate tumor progression [15, 38-40]. Interestingly, the level of maspin expression correlated with that of GSTp in a panel of prostate epithelial cell lines (Fig. 1A). Earlier, we also showed that maspin expression in both breast and prostate cancer cell lines increased the total cellular GST enzyme activity, raising the possibility that maspin may positively regulate the expression of GSTp [16]. Indeed, when maspin was endogenously expressed in a prostate cancer cell line DU145 via stable transfection, GSTp was significantly and dose-dependently up-regulated (Fig. 1B). To examine whether maspin expression altered the expression of other GST isoforms, quantitative real-time PCR (q-RT-PCR) was performed using the primers specific for GSTp, GSTm3 and a consensus sequence shared by GSTm1, m2, m4 and m5 (collectively designated as GSTm1,2,4,5). As shown in Fig. 1C, GSTp mRNA was the only significantly up-regulated in M7 cells, as compared to that in the mock transfected control Neo cells. To verify the specificity of the maspin effect on GSTp expression, prostate cancer cell line PC3 that expresses both maspin and GSTp at a moderate level, as compared normal immortalized prostate epithelial cells, was subjected to small interference RNA (siRNA)-mediated maspin knockdown. As shown in Fig. 1D and 1E, either permanent or transient transfection-mediated maspin down-regulation led to a significant down-regulation of GSTp, suggesting that the effect of maspin on GSTp expression was specific.

Transcriptional down-regulation of GSTp in human prostate tumor and in established prostate cancer cell lines have been shown to result, at least in part, from DNA methylation [39, 41]. Thus, the observed GSTp up-regulation upon maspin transgene expression, and GSTp down-regulation upon maspin knockdown, suggest that maspin may prevent and/or reverse DNA methylation-based transcriptional silencing of GSTp. Indeed, as determined by methylation-specific PCR (MS-PCR), the GSTp promoter DNA in maspin transfected DU145 cells was significantly hypomethylated as compared to that in the Neo control cells (Fig. 2A). The ratios of methylated to unmethylated GSTp promoter DNA was significantly lower in multiple maspin transfected DU145 clones than in Neo cells as confirmed by quantitative MethyLight analysis (Fig. 2B) and by bisulfate DNA sequencing (Fig. 2C). The extent to which GSTp methylation was reduced in different clonal cell lines varied slightly among the three different assays, largely due to the fact that a different set of primers targeting different GSTp genomic DNA sequences were used. Nonetheless, the effect of maspin on GSTp DNA de-methylation was dramatic. In fact, all four putative CpG methylation sites in the region of -163 bp to -120 pb of GSTp promoter were de-methylated in M7 cells. In contrast, these four sites remain methylated in Neo cells.

Accumulated evidence suggests that histone acetylation is required for DNA demethylation. Thus, maximal de-silencing of many epigenetically silenced genes may be achieved only through simultaneous inhibition of HDAC activity and DNA methylation. As expected, a significant increase of GSTp expression was detected when Neo cells were treated with the combination of 5-Aza (a DNA methyltransferase inhibitor) and M344 (an HDAC inhibitor) (Fig. 3A) or the combination of 5-Aza and LBH589 (Fig. 3B). The induction of GSTp upon the treatment of Neo cells with either M344 or LBH589 was modest, much less than that observed with maspin transfected cells. The GSTp induction following the combination treatment of Neo cells with 5-Aza and HDAC inhibitors was comparable as that in maspin transfected DU145 cells (M7 clonal line). Previously, we showed that tumor cell sensitivity to pharmacological HDAC inhibitors was enhanced by endogenous expression of maspin [15]. We speculated that endogenous inhibition of HDAC1 by maspin may reduce the level of targetable HDACs and generally lower the cytotoxic concentrations of pharmacological

inhibitors. In this study, we found that maspin further enhanced the de-silencing effects of pharmacological inhibitors of DNA methylation and/or HDAC. As shown in Fig. 3A-B, when M7 cells were treated with the combination of 5-Aza and LBH589 (or 5-Aza plus M344), GSTp expression was significantly further elevated. To verify the effect of endogenous maspin on the effects of DNA de-silencing drugs, PC3 cells that were transiently transfected with maspin siRNA oligonucleotides were treated with 5-Aza. As shown in Fig. 3C, maspin knockdown in PC3 cells reduced the de-silencing effects of 5-Aza on GSTp.

Since maspin acts as an endogenous HDAC1 inhibitor, we next tested whether HDAC1 was required for the maspin effects of GSTp de-silencing. As compared to the effect of maspin knockdown, maspin/HDAC1 double knockdown (1D5/HDAC1-shRNA cells) slightly increased the level of GSTp expression (Fig. 4A). Nonetheless, the level of GSTp expression in 1D5/HDAC1-shRNA cells was significantly lower than that in PC3 cell-derived cells that expressed either a scrambled siRNA sequence or the HDAC1 shRNA. These data suggest that maspin and HDAC1 exert opposing effects on GSTp expression, and support an antagonistic functional link between the two proteins. Earlier, we showed that maspin expression increased the expression of HDAC1 target genes p21, Bax, maspin and cytokeratin 18 [15]. Consistently, using the chromatin immunoprecipitation (ChIP) condition described by Kobayashi et al, [37], the HDAC1 antibody pulled down the promoter sequence of the p21 gene (Fig. 4B). ChIP was also performed to test whether maspin was directly involved in the binding of GSTp DNA with enzymes that control DNA methylation and/or histone acetylation. Not surprisingly, M7 cells exhibited a higher level of association between GSTp promoter and acetylated histone 3 (Ac-H3) than Neo cells (Fig. 4B-C). M7 cells also had less GSTp promoter DNA associated with DNA methylating enzymes, DNA (cytosine-5)-methyltransferase 1 (DNMT1) and methyl-CpG binding domain family of proteins MBD_{2/3}, which has been shown to be directly targeted by HDAC1-mediated gene repression [42, 43]. It was noted that the GSTp promoter DNA was not associated with HDAC1 in either Neo or M7 cells. It is possible that maspin and HDAC1 regulate DNA methylation through histone modification, rather than directly participating in the DNA-modifying enzyme complex.

Maspin Regulates GSTp in the Balance between Apoptosis Sensitivity and Oxidative Stress Resistance

Maspin has been shown to inhibit tumor invasion and metastasis and sensitizes tumor cells to drug-induced apoptosis [13, 31, 44], whereas the biological effects of GSTp are poorly understood except that its glutathione-dependent enzyme activity has been implicated in the reduction of disulfide bonds in JNK and the regulation of JNK signaling [45]. Although the inhibitory effect of maspin on HDAC1 may potentially de-silence other methylated genes, our earlier evidence that maspin increased cellular redox capacity and GST activity [16] suggests an important functional connection between maspin and GSTp transcriptional regulation. To address the potential biological consequences of GSTp de-silencing, we stably transfected prostate carcinoma cell line LNCaP with plasmid vector that encodes wither an Xpress-GSTp fusion protein (LNCaP/GSTp cells) or Xpress-LacZ fusion protein (LNCaP/LacZ cells, as control). The LNCaP cell line was of choice for this experiment because it expresses maspin at a low level and does not express GSTp (Fig. 1A). In addition, others have shown that the loss of GSTp expression in LNCaP cells was due to DNA hypermethylation [29].

Based on the enzymatic activity of GSTp, we speculated that GSTp may confer drug resistance or oxidative stress resistance. Indeed, when treated with LBH589, LNCaP/GSTp cells were more resistant than LNCaP/LacZ cells (Fig. 5A). The IC₅₀ of LNCaP/GSTp and LNCaP/LacZ cell lines were -0.1 and 0.02 μ M, respectively. Consistently, as shown in Fig.

5B, when treated with LBH589, LNCaP/GSTp cells underwent less apoptosis than LNCaP/LacZ as judged by the extents of PARP cleavage. In both cell lines, LBH589 induced the expression of maspin and increased the level of histone 3 acetylation (Ac-H3). While this result further supports an earlier notion of the positive feedback loop between maspin and HDAC inhibition [15, 46-48], we also noted that the increase of both maspin and Ac-H3 in response to LBH589 was more significant in LNCaP/GSTp than in LNCaP/LacZ cells. This maspin induction may not be due to the change in protein stability which was not affected by proteasome inhibitor MG132 (at a non-toxic concentration) or by the combination of MG132 and LBH589 in LNCaP/GSTp or M7 cells (Fig. 5C). On the other hand, since the inhibitory effects of maspin on HDAC1 may be further enhanced by a GST, as shown in our earlier report [16], these data raised the possibility that GSTp is not only up-regulated by maspin but also enhances the inhibitory effects of maspin on HDAC activity.

LNCaP/GSTp cells were also significantly more resistant than LNCaP/LacZ cells to oxidative stressor H₂O₂ (Fig. 6A), as judged by the level of histone 2A.X phosphorylation (p-H2A.X, a marker for DNA-damage). As compared to LNCaP/LacZ cells, LNCaP/GSTp cells treated with H₂O₂ underwent less detachment and rounding (Fig. 6B). In addition, H₂O₂ was less effective in inducing expression of hypoxia-induced factor 1 α (HIF-1 α) in LNCaP/GSTp cells than in LNCaP/LacZ cells (Fig. 6C). Since HIF-1 α is a major transcription activator of vascular endothelial growth factor-A (VEGF-A), which is known for promoting tumor angiogenesis [49], our data suggest that sustained GSTp expression in the presence of maspin may block angiogenesis in an oxidatively stressed tumor microenvironment. This is consistent with our earlier reports that maspin blocks H₂O₂-induced VEGF-A expression *in vitro* [16] and blocks tumor angiogenesis *in vivo* [13].

Because of the mechanistic link between histone acetylation and DNA methylation, maspin, an endogenous HDAC inhibitor, may regulate the expression of HDAC target genes such as p21 and Bax [12, 50-52], as well as those that are subjected to DNA methylation such as GSTp. While p21 and Bax serve as a rheostat of cell apoptotic sensitivity, GSTp may prevent cellular damage in toxic or stressful tissue microenvironment. To investigate how the pro-apoptotic effects of maspin and the GSTp-dependent anti-apoptotic effects of maspin may be potentially coordinated in response to cancer-targeted chemotherapy, we compared the cytotoxicity of M344, 5-Aza and the combination of the two drugs in DU145-derived transfected cells. Due to the potential mixed mechanisms of cell death, we quantified the number of viable cells by the particle counting method. As shown in Fig. 7A, the viable number of M7 cells was significantly lower than that of Neo cells by 5-Aza alone, and it was further reduced by the combination of 5-Aza and M344. In parallel, apoptotic-specific PARP cleavage and DNA-damage responsive histone 2A.X phosphorylation [53] were monitored. As compared to Neo cells, M7 cells exhibited a significantly greater sensitivity to M344-induced apoptosis (Fig. 7B), and a significantly elevated level of p-H2A.X upon 5-Aza treatment (Fig. 7C). Thus, despite the increased GSTp in the presence of maspin, maspin expressing cells were still more readily committed to cell death.

DISCUSSION

Maspin is an epithelial-specific tumor suppressive protein. We have previously shown that maspin re-expression in tumor cells restored epithelial differentiation [13, 15], while blocking tumor-induced angiogenesis and sensitizing tumor cells to a variety of apoptosis-inducing drugs [44, 50], including HDAC inhibitor M344 [15]. These biological effects may primarily stem from the inhibitory effects of maspin on HDAC1, a major class I histone deacetylase that hydrolyzes the ϵ -acetyl lysine residues in histone proteins, leading to gene transcription repression. Consistently, maspin expression in prostate tumor cells led to increased expression of several genes normally repressed by the HDAC1 activity, including

p21, Bax, cytokeratins 8 and 18, and maspin [15, 54] [38, 55]. To date, maspin is the only known endogenous polypeptide inhibitor of HDAC1. Our current study is aimed at testing the hypothesis that epigenetic gene silencing is a result of endogenous coordinated histone deacetylation and DNA hypermethylation.

Based on the emerging consensus that DNA methylation may depend on histone deacetylation, loss of histone deacetylation inhibition, when maspin is down-regulated, may increase pathological DNA methylation and epigenetic gene silencing. We focused on the effect of maspin on the expression of GSTp because GSTp is a bona fide down-regulated gene in prostate cancer by the mechanism of DNA hypermethylation [23, 24]. Furthermore, both maspin and GSTp are implicated as tumor suppressors and down-regulated in human prostate cancer specimens [13, 23, 41] as well as in established prostate cancer cell lines (Fig. 1). We have previously shown that in normal prostate epithelial (both secretory and basal) cells, maspin is highly expressed and is localized in the nucleus. During the progression of prostate cancer, maspin is differentially regulated both at the level of expression and subcellular localization [13, 56]. Maspin is expressed at moderate to a high level but largely translocated from the nucleus to the cytosol in high grade prostatic intraepithelial neoplasia (HGPIN). In low grade prostate carcinoma cells, maspin expression is significantly reduced. In high grade prostate carcinoma, little or no maspin is detected. On the side of GSTp, hypermethylation of GSTp promoter was found in 6.4% of proliferative inflammatory prostatic atrophy, 70% of HGPIN and 90% of prostate cancer [23]. Findings from the current study provide the first evidence that maspin may be sufficient to reverse the methylation-based GSTp silencing. Consistent with the notion that maspin directly inhibits HDAC1 [15], the effect of maspin on GSTp expression in prostate epithelial cells was similar to that of pharmacological inhibitors of HDAC, and was reduced when HDAC1 was knocked down. Our data suggest that the epigenetic gene silencing of GSTp is most likely a result of the coordinated histone deacetylation and DNA hypermethylation in the promoter region [57, 58]. It is important to note that HDAC1 is the most abundant in the nucleus, thus maspin translocation to the cytosol or down-regulation at the transcriptional level during prostate tumor progression may confer increased HDAC1 activity, and may underlie the progressive increase of GSTp DNA methylation.

We noticed that the effectiveness of maspin in de-silencing GSTp varied in different prostate cancer cell lines. Similarly, pharmacological HDAC inhibitors exhibited variable degrees of effectiveness in de-silencing GSTp. While more precise epigenetic mechanistic studies need to be performed in multiple cell lines to address the question whether the findings demonstrated here are universally applicable, we speculate that the effects of gross HDAC inhibition, either by maspin or pharmacological inhibitors, on gene expression may further depend on other endogenous factors that exert more specific regulations on GSTp DNA methylation and/or histone modifications. To this end, a current 'histone code' hypothesis emphasizes on the dynamic interplay between histone modifications [59, 60]. In general, methylation at 9^{Lys} (K9), 27^{Lys} (K27) and 20^{Lys} (K20) of histone 3 (H3) are considered hallmarks of a condensed chromatin state that represses gene expression (for a review, see [61]), whereas di/tri-methylation of 4^{Lys} (K4) of H3 and elevated levels of H3/H4 acetylation may concomitantly active gene transcription [62, 63]. Interestingly, a study with prostate cancer cell line LNCaP suggests that the lack of 4^{Lys} (K4) methylation, rather than a gain in methylation at 9^{Lys} (K9) and 27^{Lys} (K27), of histone 3 (H3) may drive GSTp silencing [29]. This data illustrates the difficulty in using the current understanding of "histone code" as a rule, and points to the need to find additional underlying mechanisms that regulate the outcome of histone modifications. As an example, recent studies have shown that lysine-specific demethylase 1 (LSD1) demethylates histone H3 at K9, and release the repression of androgen receptor target genes [64]. However, LSD1 complexed with histone deacetylases 1/2 was shown to remove simultaneously the acetyl groups from

lysine residues and the methyl group from Lys4 in H3, leading to transcription repression [65]. To date, it is not clear whether maspin also affects histone methylation. Nonetheless, the evidence that maspin and pharmacological HDAC inhibitors are effective, to various extents, in de-silencing genes from a methylated state [1, 66], suggests that histone acetylation may eventually help override other repressive histone modifications.

Our results show that synthetic HDAC inhibitors, used at sub-lethal concentrations for a prolonged period of time, were less effective than maspin in restoring GSTp expression. It is possible that the specific mechanism underlying the inhibitory effects of maspin on HDAC1 is not fully recapitulated by pharmacological HDAC inhibitors. Extensive studies are underway to investigate the structural-functional relationship of maspin in its interaction with HDAC1. In addition, several other possible differences between maspin and synthetic HDAC inhibitors need to be further investigated. First, maspin specifically inhibits HDAC1 [14, 15], as compared to synthetic HDAC inhibitors that generally have a broader spectrum of target specificity. Second, by mechanisms yet to be elucidated, maspin is also tightly regulated at the step of subcellular compartmentalization, as shown in the progression of prostate cancer [22, 67] and non-small cell lung cancer [17, 18, 68]. While the inhibitory effects on HDAC1 may be associated with nuclear maspin, the current design of small molecular weight HDAC inhibitors does not allow the control of their subcellular localization. Furthermore, endogenous maspin expression in tumor cell lines can be up-regulated by synthetic HDAC inhibitors. It is likely that this positive feedback between the maspin effect on HDAC1 and maspin expression is a self-propelling mechanism to sustain effective HDAC1 inhibition.

Studies are underway to examine the extent and efficiency of maspin in de-silencing other methylated genes, a role of maspin in the regulation of GSTp may be of significant biological relevance in prostate cancer. This epigenetic regulation of GSTp by maspin may play an important role in the maintenance of prostate epithelial homeostasis in response to oxidative microenvironment stress. In stressed or transformed epithelial cells, accumulated electrophilic metabolic intermediates undergo re-oxygenation leading to the generation of reactive oxygen species (ROS), such as H_2O_2 and superoxide (O_2^-). ROS can directly cause cell injury by damaging DNA, proteins and lipids. It is conceivable that normal cells should be equipped with sufficient defense mechanisms to maintain the genomic and epigenetic stability. GSTs, including GSTp, are important components of the cellular defense circuitry against ROS [69]. In addition to contributing to cellular detoxification, GSTp has been shown to directly reduce sulfide bridges of proteins such as JNK [70-72], thus it may also act as a regulator of signal transduction in cellular response to oxidative stress and other stress inducers. Of particular interest, our earlier evidence suggested that the interaction between maspin and HDAC1 may require a GST co-factor. Since GSTp expression in LNCaP significantly increased the effectiveness of pharmacological HDAC inhibitor LBH589 (Fig. 5), it is intriguing to speculate that GSTp may specifically interact with and enhance the activity of maspin in HDAC1 inhibition. A possible direct involvement of GSTp, if proven true, may suggest that the HDAC-dependent epigenetic regulation is connected to the cellular redox circuitry to balance between cell death and survival. As these exciting new possibilities are yet to be further investigated, it is important to note that GSTp is upregulated in several other types of cancer especially as the tumors become multi-drug resistant. It is possible that in drug resistant tumor cells, GSTp may be independently involved in drug-detoxifying mechanisms, such as the up-regulation of MDR-1/P-gp170 protein [73, 74].

Taken together, our new evidence suggests that maspin, as an endogenous HDAC1 inhibitor, may play a key role in preventing DNA methylation-based silencing of tumor suppressor genes such as GSTp, and concertedly inhibit HDAC1-mediated repression of other pro-

apoptotic genes. Our data support a new paradigm that DNA and histone modifications may be endogenously coupled both in the silencing and the de-silencing of gene transcription.

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ABBREVIATIONS

Ac-H3	acetylated histone 3
5-Aza	5-aza-2'-deoxycytidine
ChIP	chromatin immunoprecipitation
CMV	cytomegalovirus promoter
Ct	critical threshold
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSTp	glutathione s-transferase pi isoform
H2A.X	histone 2A.X
HDAC	histone deacetylase
HIF-1α	hypoxia-induced factor 1 α
LacZ	a bacterial gene encoding β -galactosidase
mAb	monoclonal antibody
Mas-siRNA	maspin-specific small interference RNA
DNMT1	DNA (cytosine-5)-methyltransferase 1
MBD_{2/3}	methyl-CpG binding domain family of proteins

MDR1	multi-drug resistance gene-1
PCR	polymerase chain reaction
MS-PCR	methylation-specific PCR
PARP	poly-ADP-ribose-polymerase-1
p-H2A.X	phosphorylated histone 2A.X
q-RT-PCR	quantitative real-time PCR
ROS	reactive oxygen species
Scr-siRNA	small interference with a scrambled sequence
siRNA	small interference RNA
TSA	trichostatin A
VEGF-A	vascular endothelial growth factor A
WB	western blotting
WST	4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene

Figure 1. Maspin Up-regulates the Expression of GSTp

A. Northern blotting of GSTp, maspin, and housekeeping gene 36B4 in prostate epithelial cell lines. CF3, CF91 and MLC8891 are immortalized normal human prostate epithelial cell lines, whereas PC3, DU145 and LNCaP are established human prostate carcinoma cell lines.

B. WB of GSTp, maspin, and GAPDH (loading control) in DU145-derived stably transfected clonal cell lines Neo, M3, M7 and M10.

C. q-RT-PCR detection of different isoforms of GST in DU145-derived stably transfected clonal cell lines Neo and M7. The critical threshold cycle numbers from triplicate assays for each gene was normalized by that of internal GAPDH control in the same sample. The relative GSTp mRNA levels represent an average of three repeats and the bars represent standard errors.

D. WB of GSTp, maspin and GAPDH in parental PC3 cells or transiently transfected PC3 cells by siRNA oligonucleotide against maspin (Mas-siRNA) or of a scrambled sequence (Scr-siRNA).

E. WB of GSTp, maspin and β-tubulin in parental PC3 cells, clonal PC3 cell line derived from stable transfection with a shRNA of scrambled sequence (PC3/Scr), and clonal PC3 cell line 1D5 derived from stable transfection with maspin shRNA.

Figure 2. Maspin Reverses the DNA Methylation-based GSTp Silencing

A. MS-PCR detection of unmethylated (U), methylated (M), and total input control (Ctr) promoter sequences of GSTp in DU145-derived stably transfected clonal cell lines Neo, M3, M7 and M10. The PCR products were resolved by electrophoresis in agarose gel and stained by ethidium bromide (top) which was semi-quantified and calculated as the ratio of unmethylated DNA to methylated DNA (bottom). **B.** The methylation rates of GSTp promoter DNA quantified by the MethyLight-PCR analysis and are normalized by that in Neo cells. Error bars represent the standard errors of three repeats of each reaction. **C.** Post bisulfite treatment DNA sequencing of GSTp in the region of -163 to -120 bp relative to the transcription starting point. Solid line arrows indicate methylated Cs (blue), whereas dashed line arrows indicate unmethylated Cs that had been converted to Ts (red).

Figure 3. Maspin Potentiates GSTp De-silencing in Response to 5-Aza, HDAC Inhibitors (M344 or LBH589) or the Combination of 5-Aza and HDAC Inhibitors

A. q-RT-PCR detection of GSTp mRNA in Neo and M7 cells that were treated with M344 (for 24 h), 5-Aza (for 8 days), or the combination of the M344 and 5-Aza (as described in MATERIALS AND METHODS). **B.** q-RT-PCR detection of GSTp mRNA in Neo and M7 cells. Before the mRNA was extracted, cells were treated with LBH589, 5-Aza, and the combination of LBH589 and 5-Aza for 24 h. In **A** and **B**, the critical threshold cycle number for each gene was normalized by that of internal GAPDH control in the same sample. Parallel detection of GSTp in vehicle-treated cells was used as a baseline control. Data represent an average of three repeats, and the bars stand for standard errors. **C.** WB of GSTp, maspin and GAPDH (loading control) in Mas-siRNA or Scr-siRNA transfected PC3 cells that were subsequently treated with or without 5-Aza for 48 h.

Figure 4. HDAC1 and Histone Deacetylation Correlate with GSTp DNA Methylation

A. WB of GSTp, HDAC1, maspin and β-tubulin in lentivirus transduced PC3/Scr or 1D5 cell lines. Two lentiviruses used encoded shRNA of a scrambled sequence (Scr) or an HDAC1-targeting shRNA (HDAC1-shRNA). **B.** Ethidium bromide stained PCR-amplified GSTp promoter fragment that was pulled down by ChIP assay using specific antibodies against Ac-H3, HDAC1, DNMT1 and MBD_{2/3}. The reaction with pre-immune IgG was used as a negative control. The input sheared DNA (input control) of Neo and M7 cells were also PCR-amplified for the same region of GSTp promoter fragment. PCR amplified p21 promoter was performed as a positive control of the ChIP with HDAC1 antibody. **C.** Densitometric analysis of the PCR products from Neo and M7 cells shown in **B**, presented as a percentage of the input DNA. All reactions were repeated three times. While **B** showed the representative ethidium bromide stained agarose gel, data in **C** represent the average of the three repeats and the bars represent the standard errors.

Figure 5. GSTp Increases Cellular Resistance to HDAC Inhibitor LBH589

A. Quantification of viable cells by the WST assay after the cells had been treated with LBH589 at the indicated concentrations for 48 hrs. Absorbance at 450 nm (OD_{450}) of each sample was normalized by that of the untreated cells. Data represent the average of three repeats, and the bars represent the standard errors. **B.** WB of PARP, maspin, Xpress-GSTp, Ac-H3 and GAPDH in LNCaP/LacZ and LNCaP/GSTp cells that were treated with HDAC inhibitor LBH589 at the indicated concentrations for 48 h. **C.** WB of PARP, maspin, GSTp and β -tubulin in LNCaP/GSTp and M7 cells that were treated with 0.1 μ M of LBH589, 0.1 μ M of MG132, or the combination of 0.1 μ M of LBH589 and 0.1 μ M of MG132 for 48h.

Figure 6. GSTp Enhances Cellular Resistance to Oxidative Stress by H_2O_2

A. WB of p-H2A.X, maspin, Xpress-GSTp and GAPDH (loading control) after the cells were treated with H_2O_2 for 48 h. **B.** phase contrast microscopic images of LNCaP/LacZ and LNCaP/GSTp cells that were treated with H_2O_2 for 48 h. **C.** q-RT-PCR detection of HIF-1 α mRNA in LNCaP/LacZ and LNCaP/GSTp cells that were vehicle-treated or treated for 24 h with 20 μM H_2O_2 and 100 μM H_2O_2 , respectively. The critical threshold cycle number for HIF-1 α mRNA was normalized by that of the internal GAPDH control in the same sample. Data represent the average of three repeats and the bars represent the standard errors.

Figure 7. The Regulation of Prostate Tumor Cell Sensitivity to Cytotoxic Treatments

A. Quantification of remaining live Neo and M7 cells after the treatment with 2 μM 5-Aza with or without additional 0.1 μM M344 as described in the section of Materials and Methods. Cells were counted by a Coulter Partial Counter (Beckman Coulter Inc., Fullerton, CA). Data represent the average of three repeats and the bars represent standard errors. **B.** WB of PARP, p-H2A.X, maspin, GSTp, Ac-H3 and GAPDH (loading control) in Neo and M7 cells that were treated with HDAC inhibitor M344. * Ten micrograms of total cell lysate protein were loaded in each lane to show the level of GSTp in linear WB detection range. All other WB were performed with 30 μg of protein loaded per lane. **C.** WB of PARP, p-H2A.X, maspin, GSTp, Ac-H3 and GAPDH (loading control) in Neo and M7 cells that were treated with 5-Aza at the indicated concentrations.