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Epigenetic regulation of the human mucin gene *MUC4* in epithelial cancer cell lines involves both DNA methylation and histone modifications mediated by DNA methyltransferases and histone deacetylases

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AQ: 1

ABSTRACT The human gene *MUC4* encodes a transmembrane mucin, ligand of ErbB2, that is associated with pancreatic tumor progression. In the normal pancreas, *MUC4* is not expressed, whereas activation of its expression is observed in the early steps of pancreatic carcinogenesis. The molecular mechanisms responsible for *MUC4* gene activation are however still unknown. The *MUC4* 5'-flanking region being GC-rich and including two CpG islands, we hypothesized that epigenetic regulation may be involved and undertook to decipher the molecular phenomena implied. By treating cancer cell lines with 5-aza-2'-deoxycytidine (5-aza) and trichostatin A (TSA), we were able to restore *MUC4* expression in a cell-specific manner. We showed by bisulfite-treated genomic DNA sequencing and chromatin immunoprecipitation that methylation of five CpG sites and establishment of a repressive histone code at the 5'-untranslated region were associated with *MUC4* silencing and impaired its activation by Sp1. Direct involvement of DNMT3A, DNMT3B, HDAC1, and HDAC3 was demonstrated by RNA interference and chromatin immunoprecipitation. Moreover, inhibition of histone deacetylation by TSA was associated with strong *MUC4* repression in high-expressing cells. In conclusion, this work shows for the first time the importance of epigenetics in regulating *MUC4* expression and may represent a new strategy to inhibit its expression in epithelial tumors—Vincent, A., Ducourouble, M.-P., Van Seuningen, I. Epigenetic regulation of the human mucin gene *MUC4* in epithelial cancer cell lines involves both DNA methylation and histone modifications mediated by DNA methyltransferases and histone deacetylases. *FASEB J.* 22, 000–000 (2008)

AQ: 2 Key Words: acetylation

DURING TUMOR DEVELOPMENT, aberrant DNA hypermethylation associated with histone deacetylation (1) is a common molecular mechanism that leads to the silencing of numerous genes implicated in cell differentiation, signaling, and DNA repair (2, 3). This provides a survival advantage to neoplastic cells and influences drug resistance and clinical outcome after

therapy (4). However, gene-specific hypomethylation occurs frequently during carcinogenesis and particularly in colonic and pancreatic cancers and correlates well with increased transcription levels (5). Detection of aberrant promoter methylation (or demethylation) may provide useful tools for early diagnosis and prognosis of cancers, including those of lung (6), pancreas (7), or colon (8). However, although common methylation markers have been clearly established during the past few years (9), identification of panels of complementary biomarkers that are cancer specific is still necessary to establish precise DNA methylation signatures that would be beneficial for patient screening. Hence, studying the methylation status of genes aberrantly expressed in cancers may help in developing these early detection tools (10).

Pancreatic carcinogenesis is a stepwise process characterized by preneoplastic tumors called pancreatic intraepithelial neoplasia (PanIN) with different grades (PanIN1/2A/2B/3) that degenerate into carcinoma (11). Thus, targeting PanIN or genes expressed in PanIN represents a potential therapeutic approach to control pancreatic tumor progression. The human gene *MUC4* encodes a transmembrane mucin characterized by a large growth factor-like extracellular domain, ligand of ErbB2, that participates in ErbB2 signaling, cell proliferation, and tumor progression (12, 13). In pancreatic cancer, *MUC4* has become an important molecular target (14), because it is not expressed in normal pancreas but is increasingly activated during the carcinogenetic sequence as early as PanINs (15). Moreover, its overexpression in pancreatic carcinomas is associated with a bad prognosis. For all these reasons, *MUC4* has been recently proposed as a potent diagnostic (16) and prognostic (17) factor as well as a tumor marker (18) in pancreatic cancer. However, despite extensive data at the cellular level, the molecular mechanisms that control *MUC4 de novo* expression in pancreatic cancer are still unknown.

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The characterization of the 5'-flanking region of *MUC4* in our laboratory showed that it is under the control of two transcriptional units and that both the 5'-untranslated region (5'-UTR) and the proximal promoter are extremely GC-rich (up to 72%), whereas the distal promoter is not as GC-rich (up to 65%) (19). Moreover, the analysis of the genomic DNA sequence indicated the presence of numerous potential methylated cytosine residues as well as two CpG islands. Considering the biological activities of the mucin *MUC4* in tumor progression, its *de novo* expression in pancreatic cancer or overexpression in early stages of numerous epithelial cancers sustained during the carcinogenic sequence, and the structure of its 5'-flanking region, we hypothesized that epigenetic mechanisms may be involved and thus undertook to decipher the molecular mechanisms responsible for *MUC4* epigenetic regulation in epithelial cancer cells. In this work, we report the identification of key methylated CpG sites and the establishment of a repressive histone code at the 5'-UTR that are associated with the silencing of *MUC4* in epithelial cancer cell lines and discuss the possible implications in cancer therapy.

MATERIALS AND METHODS

Cell lines and cell culture

The pancreatic (PANC-1, CAPAN-1, and CAPAN-2) and gastric (KATO-III) epithelial cancer cell lines used in this study were cultured as described previously (19–21). The inhibitor of methylation, 5-aza-2'-deoxycytidine (5-aza; 5 μ M, Sigma, Saint-Quentin Fallavier, France), and the inhibitor of histone deacetylase (HDAC), trichostatin A (TSA; 0.3 μ M, Sigma), were added to proliferating cells (PCs) or confluent cells (CCs) for 72 and 24 h, respectively, as described before (22). Cells were then lysed and processed for total RNA extraction or whole cellular extract preparation as described thereafter.

RNA extraction, reverse transcriptase-polymerase chain reaction (RT-PCR), and quantitative real-time PCR (Q-PCR)

Total RNA was prepared using the QIAamp RNA blood and cell reaction kit (Qiagen, Courtabouef, France). Total RNA (1 μ g) was used to prepare cDNA as described by Vincent *et al.* (22). RT-PCR was carried out on cDNA (5 μ l) using a specific pair of primers for *MUC4* (19). The ribosomal RNA 28S subunit was used as the internal control (20). Single-stranded oligonucleotides were synthesized by MWG-Biotech (Ebersberg, Germany). PCR products (10 μ l) were separated on a 1.5% agarose gel containing ethidium bromide run in 1 \times Tris-borate-EDTA buffer. Multiplex Q-PCR was performed in triplicate using the AB 7500 Fast Real-Time PCR System and the Absolute Q-PCR Rox Mix (Applied Biosystems, Courtabouef, France). The ribosomal RNA Control Reagent (Applied Biosystems) was used as an internal amplification control. Primer sets for *MUC4* were as follows: forward primer: 5'-TCAGCTGAGGCCTTGCCCTT-3', reverse primer: 5'-TCAGTCACCTTCCCTTTTCCA-3', and probe: ^{FAM}5'-TAAG-GCGCCATTGCTTTTGGGAGA-3'^{Tamra}. Relative transcript levels were calculated using the $2^{-\Delta\Delta CT}$ method. Untreated cells were used as reference samples.

Immunohistochemistry

PANC-1 cells were passed at 1.5×10^6 cells/75 cm² flask and grown under standard conditions until they reached confluency, at which time they were treated either for 48 h with 5-aza (5 μ M) or 24 h with TSA (0.3 μ M). Cells were then trypsinized, centrifuged, and washed once with 1 \times PBS. The cell pellet was fixed in 4% (w/v) formaldehyde and embedded in paraffin, and 3 μ m sections were prepared. Immunohistochemistry, including positive and negative (omitting primary antibody) controls, was performed using an automatic immunostainer (ES; Ventana Medical System, Illkirch, France), as described previously (23). The monoclonal anti-*MUC4* antibody, which recognizes the tandem repeat region of the apomucin (24), was used at a 1/2000 dilution.

Whole cell extract preparation and Western blotting

Total cellular extracts from CAPAN-1 and KATO-III cells were prepared using standard procedures as described previously (25). Protein content was measured using the bicinchoninic acid method as described in the manufacturer's instruction manual (Pierce, Perbio Sciences, Brebières, France). *MUC4* protein (>900 kDa) and β -actin (42 kDa) expression were respectively analyzed on 2% (w/v) SDS-agarose gels and 10% SDS-PAGE followed by transfer on nitrocellulose membrane as described in Piessen *et al.* (25). Prestained protein molecular weight standards were from Life Technologies (Cergy-Pontoise, France). The monoclonal anti-*MUC4* antibody was used at a concentration of 1 μ g/ml. The *MUC4*/ β -actin ratio was calculated after scanning protein bands with GelAnalyst-GelSmart software.

Sodium bisulfite-treated genomic DNA sequencing

Genomic DNA was extracted from the three cell lines used in this study with the Blood and Cell Culture DNA Maxi Kit (Qiagen). DNA content was quantified at 260 nm and stored at +4°C until use. Sodium bisulfite conversion was performed using 5 μ g of genomic DNA from cell lines as described by Ghoshal *et al.* (26). The promoter sequences of interest were amplified by PCR using AmpliTaq Gold polymerase (Applied Biosystems) as described previously (26). Primer sequences covering the 5'-UTR, the proximal and distal promoters, as well as the two CpG islands were designed using the MethPrimer software (<http://www.urogene.org/methprimer/>; **Table 1**). PCR products were cloned into pCR2.1 vector (Invitrogen, Cergy Pontoise, France). At least five positive clones per site were selected for plasmid preparation using QIAprep 8 Mini-prep Kit (Qiagen) and sequenced on both strands on an infrared based ABI 3730 XL sequencer (GATC, Konstanz, Germany) using T7 and M13-RP universal primers. The percentage of conversion of non-CpG cytosines was used as an index of overall bisulfite reaction efficiency. Clones with a conversion efficiency of >97% were included in the study.

Site-directed mutagenesis, *in vitro* methylation of *MUC4* proximal promoter and 5'-UTR, and cell transfection studies

QuickChange site directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands) was used to generate site-specific mutations. Oligonucleotides containing the desired mutations are shown in **Table 2**. Wild-type (WT) and mutated *MUC4*-pGL3 constructs (-461/-1 region) were digested with *SacI*-*MluI* restriction enzymes (Roche Diagnostics, Meylan, France), and the insert was gel-purified as described by

T1

T2

TABLE 1. Sequences of the pairs of primers used for bisulfite sequencing studies

Primer pair	Forward primer (5'-3')	Reverse primer (5'-3')	Primer position (PCR product size)	Annealing temperature (°C)
MUC4 (I)	GGGATAGTGTGTGGTTTAAAAAGTT	CCCTAATCAATAACCCCAACATAAAA	Distal prom. -3600/-3380 (221 bp)	56
MUC4 (II)	TTTAATTTTGGAAAATGGGTATATTG	AACCAACCCAAAATACAAAAAATC	Distal prom. -2801/-2595 (207 bp)	50
MUC4 (III)	AGGTGTATTTTTATTTTATAGGTGAA	ACAAATAACTAACCTCTTTCCCATAC	CpG island II -2400/-2211 (190 bp)	50
MUC4 (IV)	TTTATTTAGAGTTGGAGGGATTGTT	ACTCAAATTTCTACATTGCCAAAC	Prox. prom. -1595/-1370 (226 bp)	50
MUC4 (V)	TTATGGGTTTGGGGTTTGTAT	AAACAAAAACAAAATACACTATATACC	CpG island I -787/-532 (256 bp)	50
MUC4 (VI)	GTTTAGGTTGATGAGAAGTAGAGTAA	CAACAACCTACAATATAAAAAACAAAC	5'UTR -265/-47 (219 bp)	50

PCR product size and primer position refer to the first ATG (19).

Perrais *et al.* (21) before being methylated (10 µg) with 20 U of mSssI (CG) and mHpaII (CCGG) enzymes (New England Biolabs, Ozyme, St-Quentin en Yvelines, France) for 3 h at 37°C. The degree of methylation of the fragment was confirmed by testing its resistance to HpaII digestion. The methylated fragments were then ligated into pGL3 basic vector. DNA concentration was measured at 260 nm before being used in transfection experiments in CAPAN-2 cells as described by Perrais *et al.* (19). Influence of methylation of WT and mutated MUC4 promoter (1 µg) on its transactivation by Sp1 was studied by carrying out cotransfections experiments in the presence of pCMV-Sp1 (0.5 µg) expression vector as described in Van Seuning *et al.* (27). Samples were tested in triplicate in at least three independent experiments.

Chromatin immunoprecipitation assay

Cells (1.0×10⁶ per antibody) were fixed in 1% (v/v) formaldehyde, and chromatin was sonicated and immunoprecipitated as described in Vincent *et al.* (22) with either 5 µg of specific antibodies against histone H3 (antiacetylated lysine, methylated lysine 9, and trimethylated lysine 27; Upstate, Hampshire, UK) or with specific antibodies against chromatin modifier enzymes: DNMT1, DNMT3B, HDAC1, HDAC2, HDAC3 (Abcam, Paris, France), and DNMT3A (Imgenex, CliniSciences, Montrouge, France) or with normal rabbit IgGs (Upstate) at +4°C. Immunoprecipitated chromatin (50

ng) was then used as a template for PCR using the following primers: forward primer: 5'-TTTTGTCTCTTCCAG-GTTC-3' and reverse primer: 5'-TGGCTGCGGCAAAAGTCC-3', covering the -153/+1 region of MUC4 proximal promoter and 5'-UTR. PCR was performed using AmpliTaq Gold polymerase as described in Piessen *et al.* (25) with an annealing temperature of 55°C. PCR products (20 µl) were separated on a 2% agarose gel containing ethidium bromide run in 1× TBE buffer.

Small interfering RNA (siRNA) assays

KATO-III cells were seeded the day before transfection in 24-well tissue culture plates at a density of 3 × 10⁵ cells/well in antibiotic free medium. Cells were transfected with either 100 nM of DNMT1, DNMT3A, DNMT3B, HDAC1, HDAC2, or HDAC3 ON-TARGETplus SMARTpool siRNA using 1 µl of DharmaFECT4 transfection reagent (Dharmacon, Brebières, France) as described in Piessen *et al.* (25). Controls included mock-transfected cells and cells transfected with siControl nontargeting siRNA or siControl GAPD siRNA. Total RNA was isolated 48 h after transfection, and RT-PCR was performed as described above. Primers used for amplification of the internal control GAPDH, and chromatin modifier enzymes are described in Table 3. Samples were tested in triplicate in at least three independent experiments. The MUC4/GAPDH ratio was calculated as described above.

T3

TABLE 2. Sequences of the pairs of primers used for site-directed mutagenesis

Primer pair	Sequence (5'-3')	Position
Sp1 WT	CCCAGGTTCCCTGGCCCTTCGGAGAAACGC	-129/-122
Mut#1	CCCAGGTTCCCTGGC <u>AT</u> CTTCGGAGAAACGC	
Sp1 WT	CCTGGTGGGGTAGTGGGGTGGGGCTGAGGAGAGAAAAGGG	-205/-193
Mut#2	CCTGGTGGGGTAGT <u>G</u> ATGTGG <u>T</u> GCTGAGGAGAGAAAAGGG	

Mutated nucleotides are italicized and underlined. Antisense oligonucleotides were also synthesized and used for site-directed mutagenesis as described in Materials and Methods.

TABLE 3. Sequences of the pairs of primers used for RT-PCR studies

Primer pair	Forward primer (5'-3')	Reverse primer (5'-3')	PCR product size (bp)	Annealing temperature (°C)
DNMT1	AGT CGA TGA TAA CAT CCC AG	CAG AAG ATC TCT TTG ATC CG	891	60
DNMT3A	GTT CTC CCT GCC AAA AAG G	TTT TAT TTG CTC CAG GTG GG	255	60
DNMT3B	GTG TCC TTC CAC CCT CTC TT	CCT ACC TTT ATG CCC AAC TC	467	55
HDAC1	CTG CTT AGT AGC TTT GGA	TAT CTC AAA AAG GAA ACT AGA CT	236	55
HDAC2	CCC TGA ATT TGA CAG TCT CAC C	CAC AAT AAA ACT TGC CCA GAA AAA C	173	60
HDAC3	TTC ATA TCC TCC CCA CAC TTG	GAA CCC AGA GAT TTT TGA GGG	201	55
MUC4	CGC GGT GGT GGA GGC GTT CTT	GAA GAA TCC TGA CAG CCT TCA	596	60
GAPDH	TGAAGGTCGGAGTCAACGGATTTGGT	CATGTGGCCATGAGGTCCACCAC	980	60

Statistics

All values in this article are mean ± SD. When indicated, data were analyzed by Student's *t* test using GraphPad Prism 4 software (GraphPad, San Diego, CA, USA) with differences *P* < 0.05 considered significant.

RESULTS

Influence of DNA methylation and histone deacetylation on MUC4 expression in epithelial cancer cells

To study the role of methylation and histone acetylation on *MUC4* expression, one nonexpressing pancreatic cancer cell line (PANC-1) and one high-expressing pancreatic cancer cell line (CAPAN-1) were treated with either the demethylating agent 5-aza (Fig. 1A) or the HDAC inhibitor TSA (Fig. 1B). As an intermediate

model, the low-expressing gastric cancer cell line (KATO-III) was used. Studies were conducted in PCs and CCs to evaluate epigenetic regulation of *MUC4* in both cellular situations.

In nonexpressing pancreatic PANC-1 PCs and CCs, treatment with 5-aza and TSA induced *MUC4* expression at the mRNA level (Fig. 1A, B, lanes 2 and 4). Induction of *MUC4* protein expression was also observed in a few cells by immunohistochemistry after either 5-aza or TSA treatment (Fig. 1C).

In low-expressing KATO-III PCs, 5-aza and TSA treatments strongly increased *MUC4* expression both at the mRNA (4.07- and 3.03-fold increase by Q-PCR, respectively, Fig. 1A, B, lane 2) and protein (Fig. 1D) levels. In KATO-III CCs, DNA demethylation with 5-aza still induced a 3-fold activation (Q-PCR) of *MUC4* mRNA expression and inhibition of histone deacetylation with TSA induced a 2.39-fold activation (Fig. 1A, B, lane 4 and Q-PCR).

F1

FIGURE 1

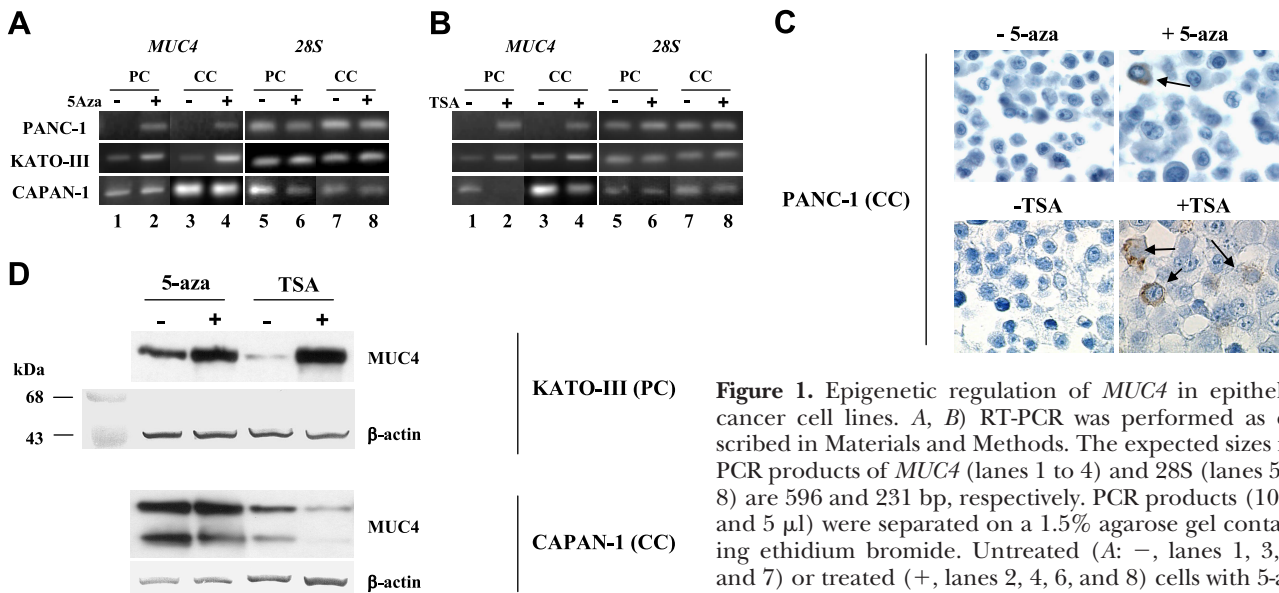


Figure 1. Epigenetic regulation of *MUC4* in epithelial cancer cell lines. A, B) RT-PCR was performed as described in Materials and Methods. The expected sizes for PCR products of *MUC4* (lanes 1 to 4) and 28S (lanes 5 to 8) are 596 and 231 bp, respectively. PCR products (10 µl and 5 µl) were separated on a 1.5% agarose gel containing ethidium bromide. Untreated (A: -, lanes 1, 3, 5, and 7) or treated (+, lanes 2, 4, 6, and 8) cells with 5-aza (5 µM, 72 h) or TSA (B: 0.3 µM, 24 h). C) *MUC4*

immunohistochemical detection in untreated (left panel) and 5-aza- or TSA-treated (right panel) PANC-1 cells was performed as described in Materials and Methods. View ×400. D) Influence of 5-aza and TSA treatment on *MUC4* apomucin expression in KATO-III PCs and CAPAN-1 CCs. Untreated (-) and 5-aza- or TSA-treated cells (+). Total cellular extracts were prepared as described in Materials and Methods; 20 µg of proteins was loaded on a 2% (w/v) SDS-agarose gel (*MUC4*) or 10% SDS-PAGE (β -actin) before being transferred onto nitrocellulose membrane and processed for *MUC4* and β -actin immunohistochemical detection. *MUC4*/ β -actin ratios are indicated, ratio corresponding to untreated cells was arbitrarily set to 1.

In high-expressing pancreatic CAPAN-1 PCs and CCs, 5-aza treatment did not affect *MUC4* mRNA level (Fig. 1A and as determined by Q-PCR), whereas TSA treatment induced a strong inhibition of *MUC4* mRNA expression (3.22- and 1.87-fold decrease, respectively, Fig. 1B, lanes 2 and 4 and Q-PCR) correlated to a strong decrease of the apomucin level in the cells (Fig. 1D).

Mapping of methylated CpG sites within *MUC4* promoters and 5'-UTR

F2

In silico analysis of *MUC4* 5'-flanking region using the MethPrimer software indicated the presence of two CpG islands at -2370/-2236 (CpG island II) and -738/-593 (CpG island I), both located in the proximal promoter (Fig. 2A). The 5'-flanking region has a very high GC content (up to 72%) almost exclusively concentrated within the proximal promoter and 5'-UTR. Bisulfite-treated DNA sequencing was used to map methylated cytosines within the distal and proximal promoters and 5'-UTR of *MUC4* in the three cell lines studied (Fig. 2A). Primer information is given in Table 1. Our results indicate that the two CpG islands are heavily methylated regardless of the level of *MUC4* expression in cells. Most of the CpG sites studied are also heavily methylated in the three cell lines except for cytosines in the -239/-73 region of the proximal promoter and 5'-UTR (Fig. 2A). Five of these CpG sites, all located in the 5'-UTR (at -81, -93, -102, -113, and -121) were significantly more methylated in the nonexpressing cell line PANC-1 (92% methylation) compared with the expressing cell line CAPAN-1 (4% methylation). As expected, in low-expressing KATO-III cells, these CpG sites showed an intermediate methylation profile (16% methylation). A more remote cytosine of the proximal promoter at -1432 was also

significantly less methylated in CAPAN-1 cells compared with PANC-1. Thus, the pattern of methylation of these six CpG sites is well correlated to the mRNA level of *MUC4* in the cells. Among them, the five CpG sites clustered in the 5'-UTR showed a methylation pattern strictly correlated to *MUC4* expression. We thus focused the rest of the work on these five CpG sites. To identify among these five CpG sites which ones would be sensitive to 5-aza and thus demethylated after cell treatment with that drug, we submitted nonexpressing PANC-1 CCs to 5-aza treatment and compared their methylation profile to genomic DNA from untreated cells. Our results indicate that DNA demethylation by 5-aza was associated with demethylation (60%) of cytosines at -81 and -93 (Fig. 2B). This suggests that activation of *MUC4* expression in 5-aza-treated PANC-1 cells is correlated to demethylation of these two CpG sites.

Study of histone H3 modifications at *MUC4* 5'-UTR

To establish histone H3 status in *MUC4* 5'-UTR, ChIP assays were carried out with chromatin from the three cell lines used in this study (Fig. 3). In nonexpressing PANC-1 cells, *MUC4* 5'-UTR DNA covering the -153/+1 region was associated with deacetylated histone H3 (lane 2) and trimethylated K27H3 (lane 4), whereas K9H3 was not methylated (lane 3). In low-expressing KATO-III cells, histone H3 was also deacetylated at *MUC4* 5'-UTR (lane 2), which was enriched in methylated K9H3 (lane 3). In high-expressing CAPAN-1 cells, *MUC4* expression was correlated to both acetylation and demethylation of histone H3 (lanes 2-4). Thus, inhibition of *MUC4* expression in these three epithelial cancer cell lines is associated with

F3

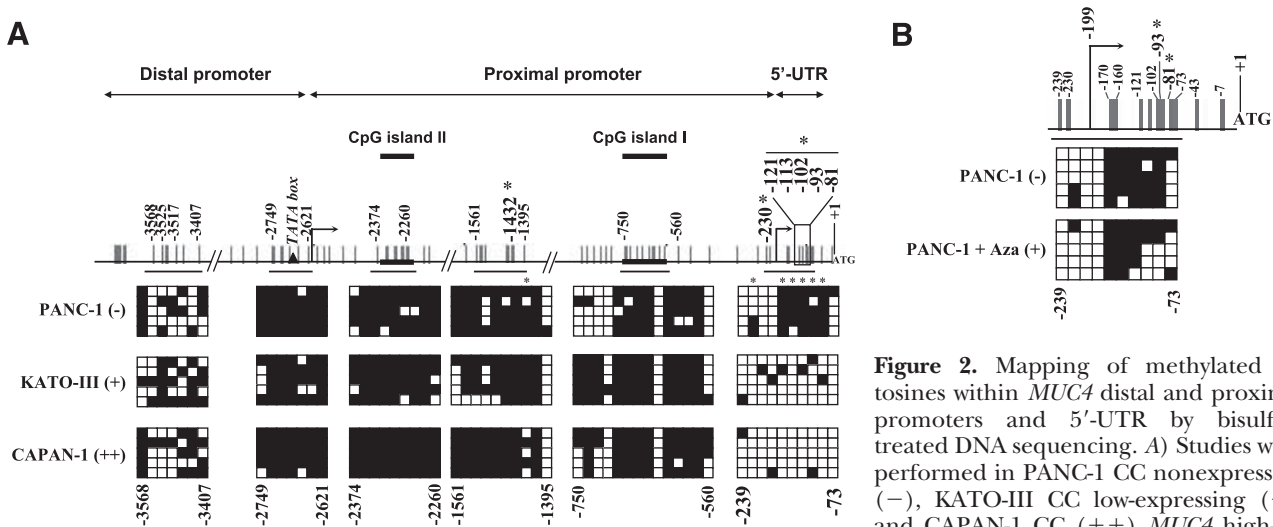


Figure 2. Mapping of methylated cytosines within *MUC4* distal and proximal promoters and 5'-UTR by bisulfite-treated DNA sequencing. A) Studies were performed in PANC-1 CC nonexpressing (-), KATO-III CC low-expressing (+), and CAPAN-1 CC (++) *MUC4* high-expressing cells. Numbering refers to the first ATG (+1) of *MUC4* gene (19); broken arrows indicate the transcription start sites situated at -199 and -2603, determining the proximal and the distal promoters of *MUC4* (19). Vertical thin bars indicate CpG sites. Horizontal thick bars indicate the positions of the two CpG islands. At least 5 individual clones were analyzed per CpG site (vertical bars) and per cell line. Black squares indicate methylated CpG sites; white squares indicate unmethylated CpG sites. Key CpG methylated sites are topped with a star. B) Mapping of methylated CpG sites of *MUC4* 5'-UTR in untreated (-, nonexpressing) and 5-aza-treated (+, *MUC4*-expressing) PANC-1 cells.

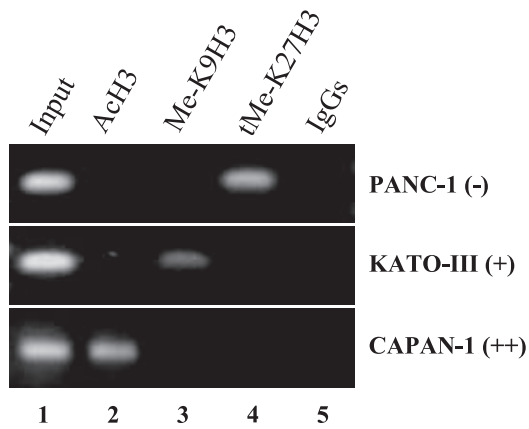


Figure 3. Histone modification status of *MUC4* promoter in PANC-1, KATO-III, and CAPAN-1 cell lines. ChIP assays were performed as described in Materials and Methods. Three histone H3 modifications were analyzed: acetylated lysine (AcH3), monomethylated lysine 9 (Me-K9H3), and trimethylated lysine 27 (tMe-K27H3). Input DNA was used as a positive amplification control; IgG indicates ChIP performed using rabbit IgGs as an isotopic antibody control.

establishment of a repressive histone code, including histone H3 deacetylation and methylation.

DNMT3A, DNMT3B, HDAC1, and HDAC3 chromatin modifier enzymes regulate *MUC4* silencing

To identify which DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B) and class I HDACs (HDAC1, HDAC2, and HDAC3) regulate *MUC4* endogenous expression, knockdown assays were performed with specific siRNAs in the KATO-III cell line (Fig. 4A). DNMT1 and HDAC1 knockdown led to a 1.8- and

2.9-fold increase of *MUC4* expression in KATO-III cells (Fig. 4B). Knocking down of DNMT3A, DNMT3B, HDAC2, and HDAC3 also led to an increase of *MUC4* mRNA expression that was significant (4.4-fold, $P < 0.05$; 4.6-fold, $P < 0.05$; 3.7-fold, $P < 0.005$; and 4.7-fold, $P < 0.05$, respectively).

To show whether DNMTs and HDACs may directly modify the methylation profile and chromatin structure at *MUC4* 5'-UTR, ChIP assays were then carried out with chromatin from the three cell lines used in this study (Fig. 4C). In nonexpressing PANC-1 cells, binding of DNMT3A (lane 4), DNMT3B (lane 5), and HDAC1 (lane 6) to DNA covering the -153/+1 region of *MUC4* 5'-UTR was observed. In low-expressing KATO-III cells, we found binding of DNMT3B (lane 5) and a strong binding of HDAC3 (lane 8). On the contrary, in high-expressing CAPAN-1 cells, neither DNMTs nor HDACs were found to bind *MUC4* 5'-UTR. Thus, DNMT3A, DNMT3B, HDAC1, and HDAC3 are directly involved in *MUC4* silencing by binding to its 5'-UTR in a cell-specific manner.

Effect of DNA methylation on *MUC4* promoter basal activity and regulation by Sp1

Having shown that methylation was involved in *MUC4* repression, we undertook to study whether promoter methylation could interfere with *MUC4* promoter basal activity and regulation by the transcription factor Sp1, a known regulator of *MUC4* transcription (19). These studies were performed in *MUC4*-expressing pancreatic CAPAN-2 cell line in which *MUC4* transactivation by Sp1 was previously shown to be the strongest (19). In that part of the 5'-flanking region (-461/-1), which includes part of the proximal promoter and the 5'-

F4

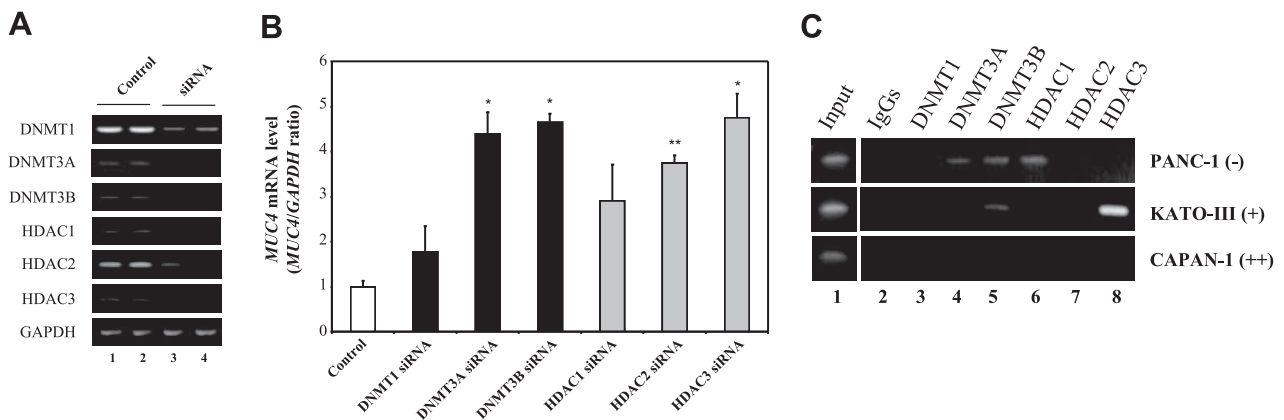


Figure 4. Role of chromatin modifier enzymes (DNMT1, DNMT3A, DNMT3B, HDAC1, HDAC2, and HDAC3) on the endogenous expression of *MUC4*. *A*) siRNA knockdown of chromatin modifier enzymes in KATO-III cell line was carried out as described in Materials and Methods. Expression level for each DNMT and HDAC was evaluated by RT-PCR using specific primers (Table 3). Levels of expression of the different genes are shown for cells transfected with nontargeting siRNA (control, lanes 1 and 2) or with specific siRNA (lanes 3 and 4). *B*) *MUC4* and *GAPDH* mRNA levels were assessed by RT-PCR. PCR products (10 μ l *MUC4*, 6 μ l *GAPDH*) were analyzed on a 1.5% agarose gel. Diagram shows data expressed as *MUC4*/*GAPDH* ratio. Control corresponds to the mean value from mock cells and cells transfected with nontargeting siRNA. Transfections were carried out in triplicate in at least three independent experiments. * $P < 0.05$; ** $P < 0.005$. *C*) Binding of chromatin modifier enzymes to *MUC4* 5'-UTR was assessed by ChIP assays using specific antibodies against DNMT1, DNMT3A, DNMT3B, HDAC1, HDAC2, and HDAC3.

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UTR, three Sp1 cis-elements and one Sp1-binding CACCC box have been previously identified (Fig. 5A, black and hatched squares; ref. 19). Among these four cis-elements, those situated at $-129/-122$ (Sp1) and $-205/-193$ (CACCC box) do not contain any CpG site (Fig. 5A, hatched squares) and thus are insensitive to DNA methylation.

In vitro methylation of the two other CpG-containing Sp1 sites ($-276/-271$ and $-166/-156$, black squares) was carried out with two methylases: mSssI, which methylates cytosine residues in CpG dinucleotides, and mHpaII, which methylates the second cytosine residue in the CCGG sequence. In the *MUC4*-461/-1 region, only one mHpaII-sensitive element was found at -346 , next to the four Sp1 binding sites (Fig. 5A). We then mutated the two CpG-less sites to inactivate them to study the direct effect of methylation of the two cis-elements potentially sensitive to methylation at $-276/-271$ (Sp1) and $-166/-156$ (Sp1) (Fig. 5A, black squares) on *MUC4* promoter activation by Sp1.

In vitro methylation of the WT *MUC4* proximal promoter by mSssI methylase resulted in a total inhibition of its basal activity (Fig. 5B, white bar) whereas methylation of cytosine at -346 by mHpaII only led to 40% decrease of *MUC4* promoter activity (Fig. 5B, gray bar). Mutation of the cis-element at $-129/-122$ (Mut#1) induced a 44% decrease of *MUC4* promoter basal activity, while mutation of the cis-element at

$-205/-193$ (Mut#2) induced a 30% decrease and mutation of both sites (Mut#3) almost totally abolished *MUC4* promoter basal activity (78% decrease) (Fig. 5B, black bars). As expected, methylation of the three mutants (Mut#1, Mut#2, and Mut#3) by mSssI resulted in a total inhibition of their basal activity (Fig. 5B, white bars). Methylation of the cytosine at -346 by mHpaII did not further alter transcriptional activity of the three mutants, as the decrease was similar to that observed with the WT construct when compared with respective unmethylated constructs (30–60% inhibition; Fig. 5B, compare gray bars to black bars).

When cotransfection was performed in the presence of WT *MUC4* promoter and an expression vector encoding Sp1, a 4-fold activation was observed (Fig. 5C, black bar). Methylation of the *MUC4* proximal promoter by mSssI significantly altered its activation by Sp1 (50% decrease, Fig. 5C, white bar), whereas methylation of the cytosine at -346 by mHpaII had no effect (Fig. 5C, gray bar). Mutation of the methylation-insensitive Sp1 binding sites, either one site at a time (Mut#1 and Mut#2) or both together (Mut#3), did not further alter *MUC4* promoter activation by Sp1 when unmethylated (Fig. 5C, black bars) or when the cytosine at -346 was methylated by mHpaII (Fig. 5C, gray bars). This indicates that 1) the two sites at $-129/-122$ and $-205/-193$ do not interfere with *MUC4* regulation by

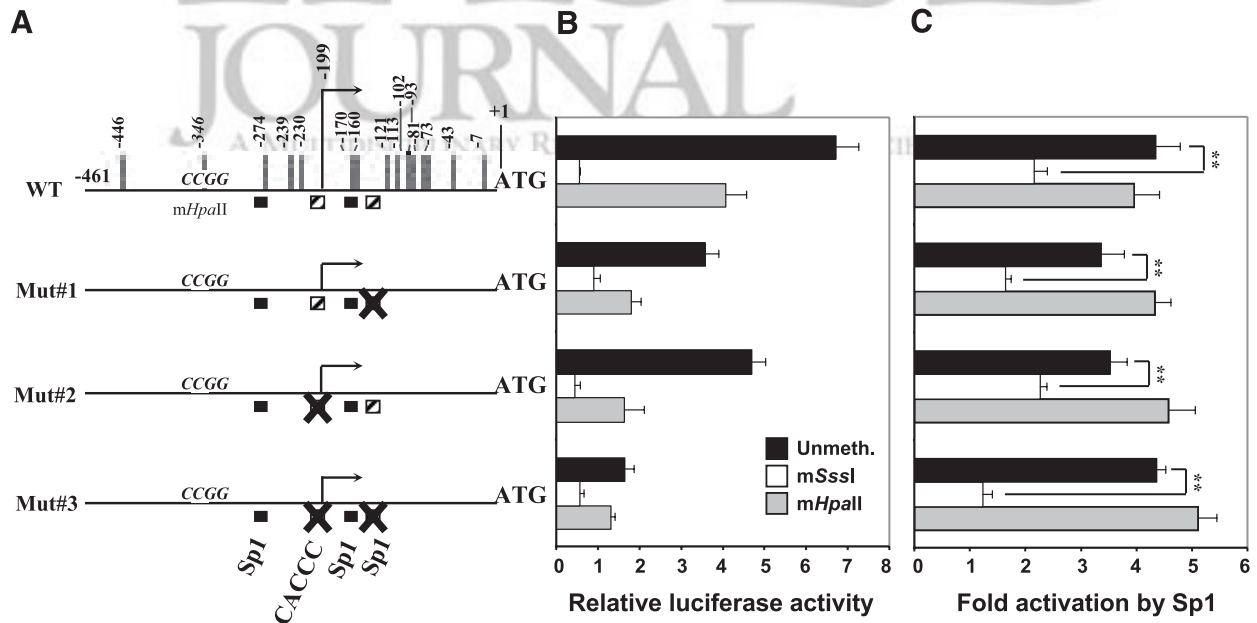


Figure 5. Influence of methylation on *MUC4* promoter basal activity and regulation by Sp1. A) Schematic representation of the deletion mutant covering the $-461/-1$ region of *MUC4* proximal promoter and 5'-UTR. Numbering refers to the first ATG (+1) of *MUC4* gene; broken arrow indicates the transcription start site situated at -199 (19). Black squares indicate CpG-containing Sp1 cis-elements; hatched squares are CpG-less Sp1 binding sites (19). Vertical bars indicate CpG sites. The mHpaII sensitive site is indicated (-346 , CCGG). B) Luciferase diagram showing the effect of *MUC4* promoter methylation by mSssI (CG) or mHpaII (CCGG) on its activity in pancreatic CAPAN-2 cell line. Unmethylated pGL3-*MUC4* ($-461/-1$) (black bars) and corresponding mSssI- (white bars) or mHpaII-methylated (gray bars) promoter constructs. C) Study of the effect of promoter methylation on *MUC4* transactivation by Sp1. Results are expressed as relative luciferase activity of the unmethylated pGL3-*MUC4* constructs (black bars) or the corresponding methylated promoter construct with either mSssI (white bars) or mHpaII (gray bars) cotransfected with pCMV4-Sp1 expression vector. Transfections were carried out in triplicate in 3 independent experiments. $**P < 0.005$.

Sp1, and 2) methylation of the adjacent cytosine at -346 does not alter activation by Sp1 either.

Promoter methylation by mSssI (Fig. 5C, white bars) did not further alter *MUC4* activation by Sp1 when the binding site at -129/-122 (Mut#1) or at -205/-193 (Mut#2) was mutated when compared with the corresponding unmethylated constructs (51 and 35% decrease, respectively). On the contrary, activation by Sp1 was significantly altered with almost total inhibition when the two CpG-less binding sites were mutated (Mut#3, 72% decrease). This indicates that direct methylation of the two CpG-containing Sp1 binding sites at -276/-271 and -166/-156 (Fig. 5A, black squares) dramatically impairs *MUC4* activation by Sp1.

DISCUSSION

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In this study, we have investigated the role of epigenetics in the regulation of *MUC4* mucin gene expression. Our results show that *MUC4* is regulated by DNA demethylation and establishment of a derepressive histone code at its 5'-UTR involving DNMT3A, DNMT3B, HDAC1, and HDAC3. Moreover, the use of three cell lines with different levels of *MUC4* expression allowed us to propose a model for *MUC4* epigenetic regulation in epithelial cells (Fig. 6). We propose that in nonexpressing cells, 5'-UTR methylation and a repressive histone code cooperate to silence *MUC4* mucin gene, whereas in low-expressing cells, progressive de-

methylation of the promoter but a repressive histone code allows low expression of *MUC4*; in high-expressing cells, selective hypomethylation and permissive chromatin allow binding of factors such as Sp1 and full transcription of *MUC4*.

The use of demethylating agent 5-aza and HDAC inhibitor TSA in three cancer cell lines showing different levels of *MUC4* expression indicated that DNA methylation and histone deacetylation play an important role in *MUC4* silencing. In nonexpressing and low-expressing cells, treatment with the two drugs induced a strong increase in *MUC4* expression. This result differs from the paradigm that, in the collaboration between DNA methylation and histone deacetylation in silencing gene expression, DNA methylation is dominant (28), whereas TSA alone is unable to reactivate genes with densely methylated CpG islands (29). Our results clearly show that both epigenetic mechanisms of DNA methylation and histone deacetylation are involved in the silencing of *MUC4*, which is consistent with other studies reporting the influence of histone acetylation on the methylation status of genes (30).

Interestingly, we observed that inhibition of histone deacetylation by TSA specifically induced the decrease of both *MUC4* gene and apomucin expression in high-expressing cells. We and others (22, 31, 32) have already shown that HDAC inhibitors are able to inhibit mucin gene expression in cancer cells. In a previous study, Ferguson *et al.* (33) showed that occupancy levels of the Sp1 transcription factor on the promoter of *Hmga2*, a gene involved in the control of chromatin structure, decreased significantly after TSA treatment. As Sp1 is an activator of *MUC4* transcription (19), the TSA-mediated inhibition of Sp1 DNA binding may explain the decrease in expression observed in this study. However, TSA and other HDAC inhibitors, such as butyrate, have been shown to 1) influence cell maturation by repressing secretory markers such as the mucin gene *MUC2* (32), 2) induce apoptosis and cell cycle arrest, and 3) enhance the response to chemotherapeutic agents in pancreatic cancer cells (34). As *MUC4* is implicated in cell proliferation, invasion, and metastasis in pancreatic cancer (13), the TSA-mediated inhibition of *MUC4* we observed next to that of other genes (34) may thus represent the molecular basis for the antitumoral activity of this drug in pancreatic cancer. Moreover, TSA has also been shown to repress expression of the oncogenic *ErbB2* (35). Because *MUC4* is the ligand of *ErbB2*, it participates in *ErbB2*-mediated intracellular signaling, its localization, and trafficking (12, 36); therefore, the concerted inhibition of both *MUC4* and *ErbB2* by TSA could also contribute to the antitumor activities of this drug in epithelial cancers implying *MUC4*-*ErbB2* complex formation, a mechanism known to promote tumor proliferation and progression (37). Finally, because *MUC4* is often overexpressed in epithelial cancers and is considered as one of the most attractive targets for novel therapeutic strategies (14, 38), another application would be to

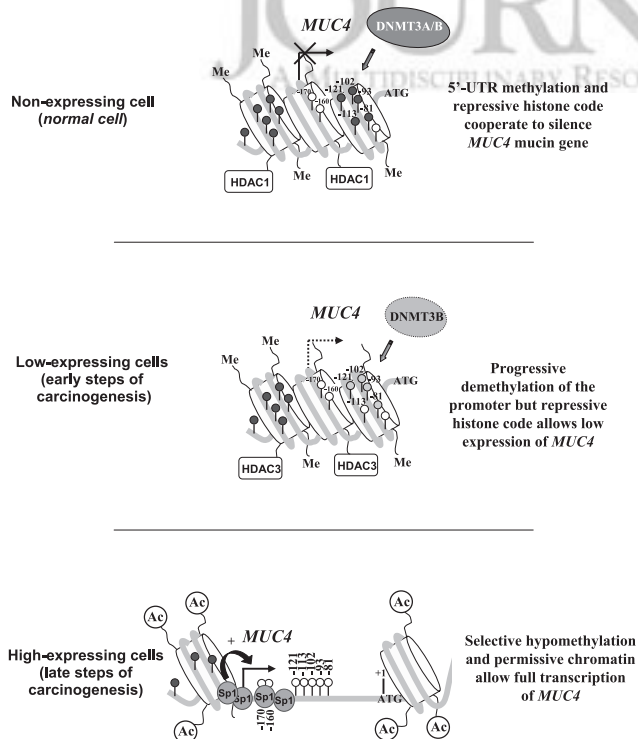


Figure 6. Model for epigenetic regulation of *MUC4* expression in epithelial cells based on mechanistic studies in epithelial cancer cell lines expressing different levels of *MUC4* gene. Me, methylated cytosine; Ac, acetylated histone.

target *MUC4* high-expressing cells by using pharmacological drugs such as TSA.

The results obtained in pancreatic cancer cell lines (this study) are in accordance with a previous study in which Singh *et al.* (39) suggested that DNMT and HDAC activity may be involved in the down-regulation of *MUC4* in prostate cancer cell lines. However, no mechanisms regarding direct silencing of *MUC4* by methylation of CpG sites and/or histone modifications have been studied. It will be interesting in the future to assess whether the mechanisms identified in pancreatic cells are also active in prostate cancer cells.

In this study, we demonstrate that most of the CpG sites in both promoters and 5'-UTR are highly methylated. Interestingly, the two CpG islands were hypermethylated in the three cancer cell lines studied regardless of the level of *MUC4* expression. Hypermethylation of CpG islands is a common characteristic of cancer cells (28), and we recently showed that promoters of other mucin genes, encoding secreted mucins *MUC5B* and *MUC6*, also have hypermethylated CpG islands whether these genes are expressed or not in the cancer cells of interest (22).

Among the six differentially methylated CpG sites that were identified in this study, five CpG sites located in the 5'-UTR at -81, -93, -102, -113, and -121 appear as key CpG sites to target in *MUC4*-expressing tumors because 1) their methylation pattern directly corresponds to the expression level of *MUC4* in the cells and 2) *in vitro* methylation of the DNA region encompassing these CpG sites totally represses *MUC4* promoter activity. Moreover, we demonstrated that 5-aza treatment, leading to activation of *MUC4* expression was associated with demethylation of two of these CpG sites. To our knowledge, this is the first time that such a mechanism involving direct regulation of *MUC4* by methylation of a short region of the 5'-UTR has been reported.

We found differential profile of DNMT binding to *MUC4* 5'-UTR in nonexpressing cells (DNMT3A and DNMT3B directly bound to *MUC4* 5'-UTR in which CpG sites were highly methylated) compared with low-expressing cells in which only DNMT3B was found to bind *MUC4* 5'-UTR (Fig. 6). This result suggests that binding of DNMT3A is essential for the establishment of the hypermethylated state of *MUC4* 5'-UTR. Surprisingly, DNMT1, which is known to be responsible for maintaining DNA methylation through cell divisions and which is involved in repression of numerous genes including *MUC2* and *MUC5B* (22), did not bind to *MUC4* 5'-UTR, and knocking down *DNMT1* by siRNA only led to a slight increase of *MUC4* mRNA expression, indicating that this enzyme does not play a major role in *MUC4* silencing.

It is known that DNA methylation and histone deacetylation are often tightly linked and cooperate to repress gene transcription (28). Our studies of the chromatin status in *MUC4* locus and especially histone modifications at the 5'-UTR covering the five CpG sites of interest indicated that complete or partial repression

of *MUC4* expression is associated with histone H3 deacetylation and methylation. On the contrary, *MUC4* expression is clearly associated with H3 acetylation and demethylation (Fig. 6).

The role of histone deacetylation was confirmed in our siRNA experiments in which we showed that the three class I HDACs were involved in *MUC4* regulation. Interestingly, ChIP assays revealed that control of *MUC4* expression involved different HDACs whether it was completely silenced or simply repressed. Indeed, in the nonexpressing cell line, our results showed HDAC1 binding to *MUC4* 5'-UTR, whereas in the low-expressing cell line, we found binding of HDAC3. It is known that class I HDACs may not be completely redundant and that HDAC3 may have some unique properties (40). In particular, they are associated with different multiprotein silencing complexes, HDAC1 and HDAC2 being associated with Mi2-NuRD and Sin3A complexes (41), while HDAC3 is associated with the nuclear receptor corepressor (N-CoR) complex (40). Here we show that complete or partial silencing of *MUC4* involves different mechanisms and thus may probably result in differences in the established histone code. This hypothesis is supported by our ChIP assays showing that *MUC4* 5'-UTR is associated with methylation of K9H3 in the nonexpressing cell line, whereas it is associated with trimethylation of K27H3 in the low-expressing cell line.

The effect of DNA methylation on promoter activation by Sp1, a transcription factor that binds GC-rich cis-elements, is rather controversial because some studies (42, 43) have shown that Sp1 binding was inhibited by CpG methylation, while others showed that Sp1 was able to bind and activate transcription even when the binding site was CpG methylated (44). We have already shown that activation of *MUC2* and *MUC5B* mucin genes by Sp1 was impaired by promoter methylation (21, 22). In this work, we found that methylation of two Sp1 cis-elements previously identified in the -461/-1 region (19) altered *MUC4* activation by Sp1, whereas methylation of a neighboring *mHpaII*-sensitive element had no effect. These findings are different from a previous study in which methylation of adjacent binding sites of *p21^{Cip1}* promoter but not methylation of the central CpG dinucleotides affected its activation by Sp1 (43). Hence, the effect of methylation on Sp1 transactivating and binding activities is gene specific and may depend both on the strength of the Sp1 interaction with DNA and on the sequence of the Sp1 binding site (consensus or containing mismatch) present in the promoter. Other binding sites for transcription factors involved in *MUC4* regulation and that could be influenced by methylation are present in the *MUC4* proximal promoter and 5'-UTR. Especially, two functional PEA3 cis-elements (45) could potentially be methylated (46) and consequently alter *MUC4* regulation by PEA3. Moreover, it has been suggested that CpG methylation regulates the cell specificity of transforming growth factor (TGF)- β -responsive genes (47). TGF- β was shown to be an activator of *MUC4* expression in the

CAPAN-2 pancreatic cancer cell line (20). The process implies binding of the Smad4 transcription factor to the *MUC4* proximal promoter. Hence, methylation of *MUC4* 5'-UTR could also be a limiting step in its regulation by TGF- β .

In conclusion, this work shows for the first time the important role of epigenetics in the regulation of *MUC4* mucin gene expression and indicates that both DNA methylation and histone modifications coexist and are responsible for *MUC4* silencing in epithelial cancer cell lines. *MUC4* may thus be considered as a new complementary biomarker, cancer specific, useful to establish methylation signatures. Mechanisms at the DNA level have been deciphered, and we have identified five key CpG sites located in the 5'-UTR, which methylation correlates with *MUC4* repression. These CpG sites may provide new biological tools to detect early reactivation of *MUC4* expression in cancers and easily screen epithelial tumors overexpressing *MUC4*. Histone deacetylation, mediated by HDAC1 or HDAC3, also plays an important role in silencing the gene in nonexpressing and low-expressing cell lines. Finally, an important finding was that the HDAC inhibitor TSA was able to strongly repress *MUC4* expression in a high-expressing cell line. This result thus provides a new way of repressing *MUC4* expression, the ligand of ErbB2 oncogene, by using HDAC inhibitors. It will certainly have important implications at the therapeutic level, because these two proteins form a complex in cancer cells, promote tumor proliferation, and can be inhibited by HDAC inhibitors. Targeting them in tumors in which they are overexpressed with TSA or other HDAC inhibitors appears thus as a very promising therapeutic strategy. FJ

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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES

1

1—Please define GC and CpG if possible.

2—Per journal style, key words must not repeat terms that appear in the title. Please add up to 5 additional key words.

3—“DNA demethylation and establishment of a derepressive histone code” correct as edited? Or was “DNA demethylation or methylation and establishment of a derepressive or repressive histone code” meant?
