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Genetic regulation of MUC1 expression by *Helicobacter pylori* in gastric cancer cells

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ABSTRACT

Helicobacter pylori infection of the stomach is associated with the development of gastritis, peptic ulcers, and gastric adenocarcinomas, but the mechanisms are unknown. MUC1 is aberrantly overexpressed by more than 50% of stomach cancers, but its role in carcinogenesis remains to be defined. The current studies were undertaken to identify the genetic mechanisms regulating *H. pylori*-dependent MUC1 expression by gastric epithelial cells. Treatment of AGS cells with *H. pylori* increased MUC1 mRNA and protein levels, and augmented *MUC1* gene promoter activity, compared with untreated cells. *H. pylori* increased binding of STAT3 and MUC1 itself to the *MUC1* gene promoter within a region containing a STAT3 binding site, and decreased CpG methylation of the *MUC1* promoter proximal to the STAT3 binding site, compared with untreated cells. These results suggest that *H. pylori* upregulates MUC1 expression in gastric cancer cells through STAT3 and CpG hypomethylation.

1. Introduction

Helicobacter pylori is a Gram-negative, motile, microaerophilic, helix-shaped bacillus that colonizes the human gastric mucosa [1]. Approximately 50% of the world's population is infected with *H. pylori*, which has been epidemiologically linked with the development of gastritis, stomach ulcers, and gastric cancer [2]. While the majority of patients with gastritis are colonized by *H. pylori*, only 10–20% of infected subjects develop ulcers and less than 2% progress to cancer [3]. The mechanisms through which a small subset of *H. pylori*-infected patients eventually develop stomach cancer are unknown.

MUC1 is a membrane-tethered mucin that is normally expressed on the apical surface of polarized epithelia [4]. MUC1 is a heterodimer consisting of a glycosylated ectodomain noncovalently associated with a cytoplasmic domain (MUC1-CD) which regulates intracellular signaling and gene transcription. In most epithelial-derived cancer cells, MUC1 is overexpressed and loses its apical polarity. Overexpression of MUC1-CD leads to dysregulated signaling cascades and altered patterns of gene transcription, likely mediated through phosphorylation of one or more of its 7 tyrosine residues [4,5]. In gastric cancer cells, MUC1 interacts with the *H. pylori* cytotoxin-associated gene A (cagA), a major bacterial virulence factor, upregulates Wnt-β-catenin signaling, and increases cyclin D1-dependent cell proliferation, all of which are known to contribute to gastric carcinogenesis [6]. While the *MUC1* promoter contains putative binding sites for a variety of transcription factors [7], the role of altered transcriptional regulation in MUC1 overexpression and gastric tumorigenesis remains to be determined.

Cytosine methylation of CpG dinucleotides regulates the expression of most eukaryotic genes [8]. Unmethylated CpGs are often grouped as CpG islands in gene promoters. In many cancers, CpG islands undergo hypo- or hypermethylation, resulting in transcriptional activation or silencing, respectively. CpG methylation inhibits gene expression by masking promoter binding sites for transcription factors, and/or by promoting the binding of transcriptional silencers [8]. Abnormal DNA methylation of multiple genes occurs in gastric cancer cells, as well as normal gastric epithelial cells following *H. pylori* infection, and restoration of the preinfection pattern of DNA methylation was documented following antibiotic therapy to eradicate the pathogen [9–11]. These results suggest that epigenetic alterations due to *H. pylori* infection might contribute, in part, to gastric carcinogenesis. However, relatively little is known about *H. pylori*-regulated methylation/

Abbreviations: cagA, cytotoxin-associated gene A; CD, cytoplasmic domain; ChIP, chromatin immunoprecipitation; MOI, multiplicity of infection; STAT, signal transducer and activator of transcription.

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demethylation of specific genes that may play a role in gastric cancer development, including MUC1.

Zrihan-Licht et al. [12] reported that the *MUC1* gene was hypomethylated in breast cancer cells expressing MUC1 protein, whereas the same DNA sequences were methylated in MUC1-nonexpressing cells. *MUC1* gene methylation in overexpressing breast cancer cells was low between nucleotides −100 and +100 relative to the transcription start site, whereas methylation of this region was high of MUC1-nonexpressing cells [13]. Further, MUC1 expression in MUC1-negative cells was restored by treatment with the DNA methylation inhibitor, 5-aza-2′-deoxycytidine (5-aza-dC). In contrast, no studies have described *MUC1* gene methylation in gastric cancer cells or how *MUC1* methylation might be regulated in these cells. Therefore, the current study was undertaken to determine the effects of *H. pylori* on MUC1 expression and *MUC1* gene methylation, and to identify transcription factors binding to the *MUC1* promoter, in AGS gastric cancer cells.

2. Materials and methods

2.1. AGS cells and H. pylori

AGS cells (ATCC, Manassas, VA) were cultured in DME/F12 medium containing 10% heat inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) as described [14]. *H. pylori* strain 26695 was maintained on Columbia blood agar containing 7% defibrinated horse blood (Cleveland Scientific, Bath, OH), 20 μg/ml bacitracin, 20 μg/ml trimethoprim, 16 μg/ml cefsulodin, 6.0 μg/ml vancomycin, and 2.5 μg/ml fungizone (Sigma, St. Louis, MO) under microaerophilic conditions as described [14]. AGS cells were untreated or treated for 1 or 4 h with *H. pylori* at a multiplicity of infection (MOI) = 100. In selected experiments, AGS cells were treated for 72 h with 1.0 μM of 5-aza-dC (Sigma) in the absence of *H. pylori*.

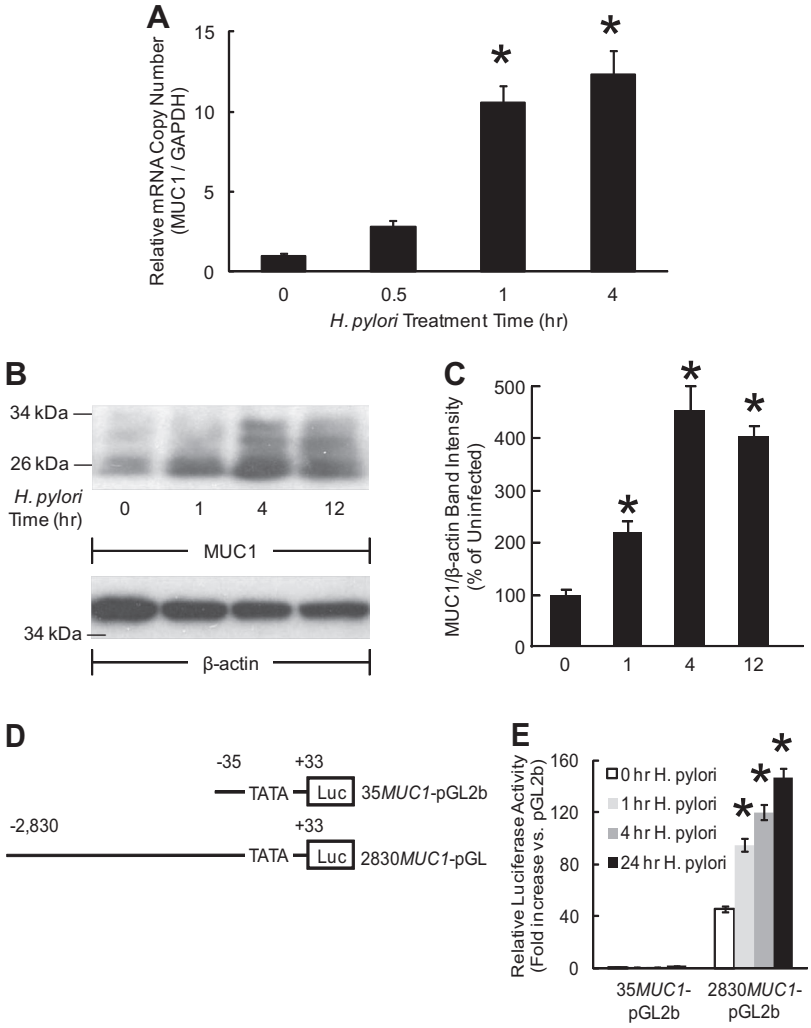

Fig. 1. *H. pylori* increases MUC1 expression. AGS cells were untreated (0 h) or treated with *H. pylori* strain 26695 (MOI = 100) for the indicated times. (A) Quantitative RT-PCR of MUC1 transcript levels normalized to GAPDH mRNA levels. Vertical bars represent mean ± SEM normalized mRNA levels ($n = 2$). (B) Equal protein aliquots of cell lysates were processed for MUC1 immunoblotting. To control for protein loading and transfer, the blots were stripped and reprobed for β-actin. The blots are representative of 3 experiments. (C) Quantitative densitometry of each MUC1 signal was normalized to the β-actin signal in the same lane in the same blot. Vertical bars represent mean ± SEM normalized densitometry values ($n = 3$). (D) Schematic illustration of firefly luciferase reporter constructs containing nucleotides −35 to +33 (35*MUC1*-pGL2b) or nucleotides −2830 to +33 (2830*MUC1*-pGL2b) of the *MUC1* promoter. The position of the TATA box (nucleotides −21 to −18) is indicated. (E) AGS cells were transfected with the pGL2b empty vector, 31*MUC1*-pGL2b, or 2830*MUC1*-pGL2b in the presence of a plasmid encoding *Renella* luciferase as an internal control. The cells were untreated or treated with *H. pylori* 26695 (MOI = 100) and luciferase activity was determined. Vertical bars represents the mean ± SEM normalized (firefly/*Renella*) luciferase activity relative to cells transfected with the pGL2b empty vector ($n = 3$). *Significantly increased MUC1 mRNA, protein, or luciferase activity of *H. pylori*-treated cells compared with untreated cells at $p < 0.05$.

2.2. Quantitative RT-PCR

Total RNA was isolated from AGS cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). One microgram of RNA was reverse transcribed into cDNA using the First Strand cDNA Synthesis Kit (Qiagen). PCR amplifications were performed using the primers listed in Table S1. PCR conditions were 94 °C for 2 min, 40 cycles at 94 °C for 20 s, 58 °C for 20 s, and 68 °C for 40 s, and final extension at 68 °C for 5 min as described [14].

2.3. Immunoblotting

AGS cells were lysed at 4 °C with PBS containing 1.0% NP-40, 1.0% sodium deoxycholate, and 1.0% protease inhibitor cocktail (Sigma). Equal protein aliquots were resolved by SDS–PAGE and processed for immunoblotting using a hamster monoclonal antibody against the COOH-terminal 17 amino acids of the MUC1-CD (Ab-5, ThermoFisher Scientific, Waltham, MA) as described [14]. Immunoreactive bands were quantified using ImageJ software [14].

2.4. Chromatin immunoprecipitation (ChIP)

AGS cells were treated for 10 min at 37 °C with 1% formaldehyde to cross-link DNA–protein complexes as described [15]. Cell lysates were sonicated to shear DNA, and immunoprecipitated with anti-MUC1-CD antibody, anti-STAT3 antibody, or nonimmune mouse IgG as a negative control (Cell Signaling Technology, Danvers, MA), followed by protein A/G-agarose (Invitrogen). DNA–protein complexes were eluted from the beads with 0.1 M $NaHCO_3$, 1.0% SDS, and 10 mM DTT, or with 10 mM DTT for reChIP assays. Protein was removed by digestion for 4 h at 65 °C with 1.0 μg/ml of proteinase K and the remaining DNA was purified using the GenElute PCR Clean-Up Kit (Sigma). PCR was performed using the amplification conditions described above with primers targeting the −648/−144 or −648/−455 nucleotide regions of the *MUC1* promoter, or the −762/−636 region of the *NF-κB* promoter as a negative control (Table S1). Amplicons were visualized on ethidium bromide-stained agarose gels.

2.5. Bisulfite sequencing PCR

One microgram of AGS cell genomic DNA was treated with sodium bisulfite ($NaHSO_3$) using the BisulFlash DNA Modification Kit (Epigentek, Farmingdale, NY) following the manufacturer's instructions. Two overlapping DNA fragments were amplified by PCR using the conditions described above with primers targeting the −508/−129 or −209/+138 nucleotide regions of the *MUC1* gene (Table S1). Amplicons were subcloned into the pSC-A-amp/kan plasmid using the StrataClone PCR Cloning Kit (Agilent Technologies, Santa Clara, CA), plasmid DNA was purified using the QuickLyse Miniprep Kit (Qiagen), and subjected to nucleotide sequence analysis. Sequence results were analyzed using bisulfite sequencing DNA methylation analysis software [16].

2.6. Methylation specific PCR

One microgram of AGS cell genomic DNA was treated with sodium bisulfite as described above. PCR was performed using the amplification conditions described above with methylation-specific and nonmethylation-specific primers targeting the −6/+108 nucleotide region of the *MUC1* gene (Table S1). Amplicons were visualized on ethidium bromide-stained agarose gels and band intensities quantified using ImageJ software.

2.7. Methylation specific electrophoresis

One microgram of AGS cell genomic DNA was treated with sodium bisulfite as described above. Nested PCR was performed using the amplification conditions described above with primary primers for the −209/+231 region of the *MUC1* gene and secondary primers for the −146/+138 region (Table S1). Amplicons were resolved at 60 °C for 14 h at 50 V by denaturing gradient gel electrophoresis on 8% polyacrylamide gels containing a linear 30–50% urea gradient as described [17]. As gel mobility standards, a fully unmethylated DNA fragment was prepared using a sodium bisulfite-treated *MUC1* promoter PCR amplicon, and a fully methylated DNA standard was prepared by treatment of the same amplicon with the CpG methyltransferase, M.SssI (New England BioLabs)

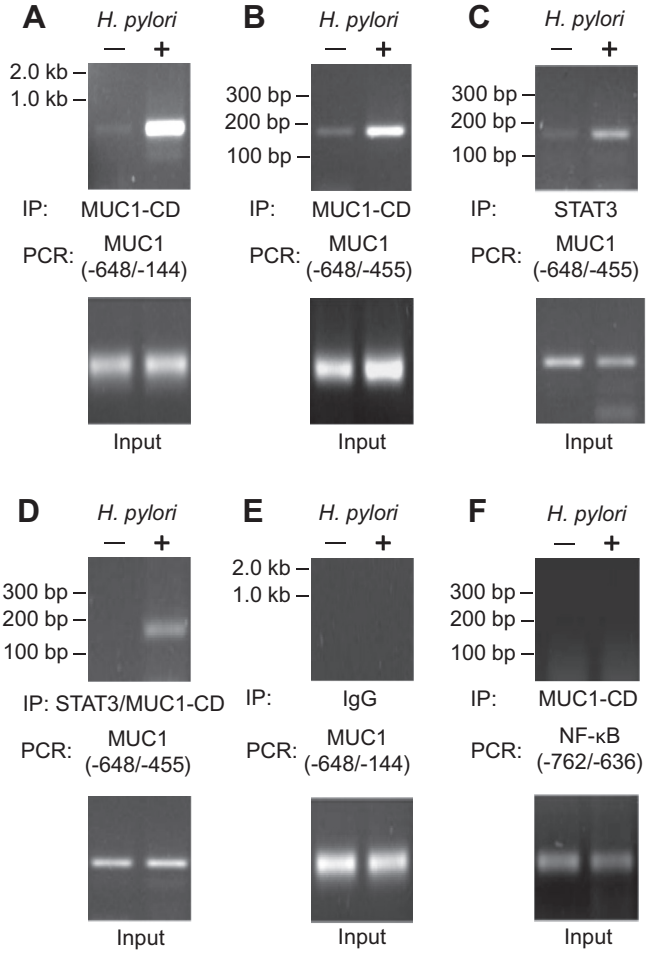

Fig. 2. *H. pylori* increases MUC1-CD and STAT3 binding to the *MUC1* promoter. AGS cells were untreated (−) or treated for 4 h with *H. pylori* 26695 (MOI = 100). DNA–protein complexes were cross-linked with formaldehyde and sheared. Sheared DNA was immunoprecipitated with anti-MUC1-CD antibody (A, B, F), anti-STAT3 antibody (C, D), or nonimmune IgG (E). Immunoprecipitates were amplified by PCR using primer pairs for the *MUC1* promoter between nucleotides −648/−144 (A, E), the *MUC1* promoter between nucleotides −648/−455 (B, C), or the *NF-κB* promoter between nucleotides −762/−636 (F). In (D), the anti-STAT3 antibody immunoprecipitates were reimmunoprecipitated with anti-MUC1-CD antibody prior to PCR for the *MUC1* promoter between nucleotides −648/−455. As input controls in all assays, PCR was performed using the relevant primer pairs without prior immunoprecipitation. The amplicons were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. The expected amplicon sizes were 505 bp for the *MUC1* promoter between nucleotides −648/−144, 194 bp for the *MUC1* promoter between nucleotides −648/−455, and 127 bp for the *NF-κB* promoter between nucleotides −762/−636. The positions of DNA ladder fragments in bp are indicated on the left. In (A)–(F), the results are representative of 3 experiments.

prior to bisulfite treatment. Amplicons were visualized by ethidium bromide staining.

2.8. Statistical analysis

Mean ± SEM values were calculated, and differences between means were assessed using the Student's *t*-test and considered significant at $p < 0.05$.

3. Results

Compared with normal gastric epithelia, MUC1 is overexpressed by greater than 50% of stomach cancers from *H. pylori*-infected individuals [18]. We asked whether *H. pylori* might upregulate MUC1 expression in human gastric cancer cells. Treatment of AGS cells with *H. pylori* increased MUC1 mRNA (Fig. 1A) and protein (Fig. 1B and C) levels in a time-dependent manner. *H. pylori* treatment of AGS cells expressing a *MUC1* promoter-luciferase plasmid encompassing nucleotides −2,830 to +33 relative to the transcription start site [19], increased *MUC1* transcriptional activity compared with untreated controls (Fig. 1D and E). However, *H. pylori* did not increase *MUC1*-driven luciferase activity in cells expressing a promoter deletion mutant (35*MUC1*-pGL2b) lacking its transcription factor binding sites but still retaining the TATA box. These results suggested that *H. pylori* increased MUC1 expression, in part, through transcriptional activation.

Multiple transcription factor binding sites have been identified in the *MUC1* promoter, notably signal transducer and activator of transcription 3 (STAT3) and NF-κB [7]. In breast cancer cells, the MUC1-CD was constitutively associated with STAT3, and both proteins interacted with and activated the *MUC1* promoter in an autoinductive loop [20]. In ChIP assays, we observed increased MUC1-CD binding to the *MUC1* promoter between nucleotides −648 and −144 relative to the transcription start site in *H. pylori*-treated AGS cells compared with untreated controls (Fig. 2A). Increased MUC1-CD binding to the *MUC1* promoter in response to *H. pylori* also was seen using another pair of PCR primers bordering nucleotides −648 and −455 (Fig. 2B). This −648/−455 DNA segment contains a consensus STAT3 binding site between nucleotides −503 and −495 (TTCCGGGAA) [20,21]. *H. pylori* increased STAT3 binding to the −648/−455 region of the *MUC1* promoter (Fig. 2C). Anti-MUC1-CD antibody immunoprecipitation of the

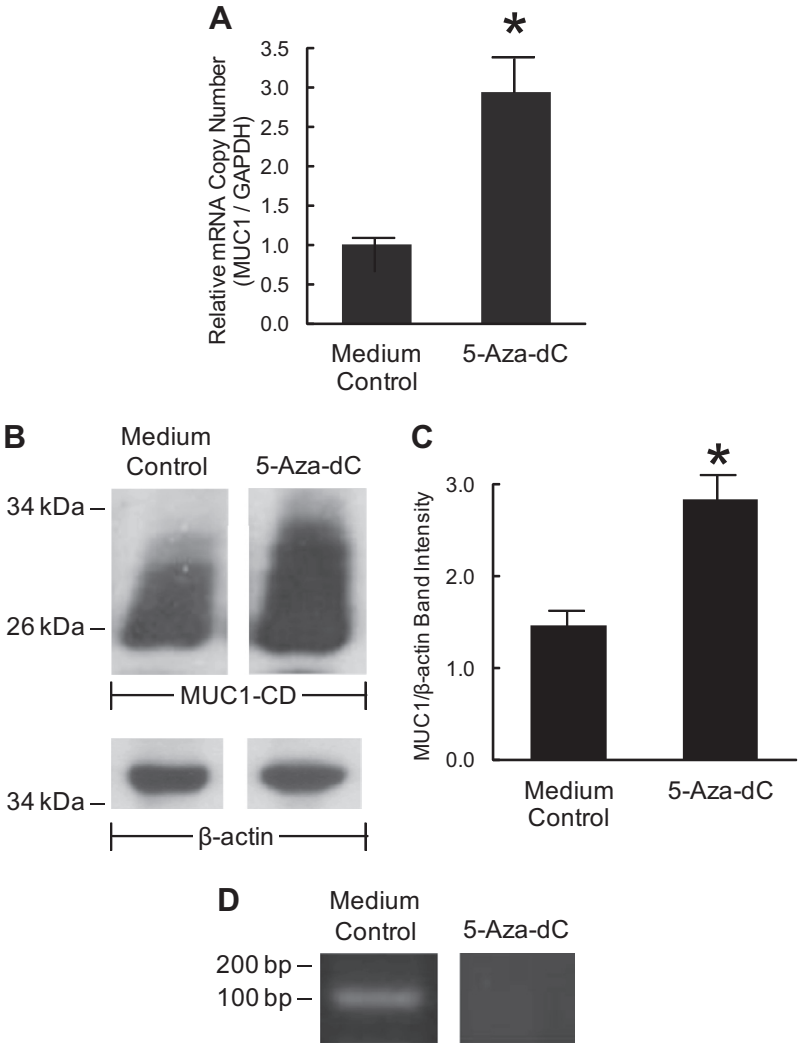

Fig. 3. 5-Aza-2′-deoxycytidine increases MUC1 expression. AGS cells were treated for 72 h with medium alone or 1.0 μM of 5-aza-dC. (A) Quantitative RT-PCR of MUC1 transcript levels normalized to GAPDH mRNA levels. Vertical bars represent mean ± SEM normalized mRNA levels ($n = 2$). (B) Equal protein aliquots of cell lysates were processed for MUC1 immunoblotting. To control for protein loading and transfer, blots were stripped and reprobed for β-actin. (C) Quantitative densitometry of each MUC1 signal was normalized to β-actin signal in the same lane in the same blot. Vertical bars represent mean ± SEM normalized densitometry values ($n = 3$). *Significantly increased MUC1 mRNA or protein levels of 5-aza-dC-treated cells compared with untreated cells at $p < 0.05$. (D) Methylation-specific primers were used to amplify the 114 bp region in the *MUC1* promoter between nucleotides −6 and +108 and containing a methylated + 80 cytosine. Unmethylated + 80 cytosines remained unamplified. The amplicons were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. The results are representative of 3 experiments.

anti-STAT3 antibody immunoprecipitates revealed that both proteins co-habited the *MUC1* promoter in the −648/−455 region (Fig. 2D). As negative controls, nonimmune IgG did not bind to the −648/−144 nucleotide region (Fig. 2E), and MUC1-CD binding was not observed to the irrelevant *NF-κB* promoter (Fig. 2F). Finally, ChIP assays using anti-MUC1-CD phosphotyrosine specific antibodies demonstrated increased binding of MUC1-CD which was phosphorylated at Tyr^{8}, Tyr^{29}, Tyr^{35}, and Tyr^{60}, but not Tyr^{20}, Tyr^{26}, or Tyr^{46}, to the −648/−455 region of the *MUC1* promoter in *H. pylori*-treated AGS cells compared with untreated controls (Supplementary Fig. S1).

Transcription factor binding to gene promoters is often increased by demethylation of cytosine residues in CpG dinucleotides [8]. 5-Aza-dC is a DNA methyltransferase inhibitor that acts as a global hypomethylating agent to increasing gene expression [18]. Treatment of AGS cells for 72 h with 1.0 μM of 5-aza-dC increased MUC1 mRNA (Fig. 3A) and protein levels (Fig. 3B and C) compared with medium alone controls. Methylation-specific PCR using primers targeting only methylated cytosine residues confirmed that treatment of AGS cells with 5-aza-dC inhibited methylation of the *MUC1* gene at the +80 cytosine residue (see Supplementary Fig. 2B) compared with medium controls (Fig. 3D).

We next asked whether *H. pylori* might decrease CpG methylation in the *MUC1* gene proximal to the −503/−495 STAT3 binding site. The *MUC1* gene between nucleotides −508 and +138 contains 21 CpG dinucleotides as potential methylation sites (Supplementary Fig. 2A). By bisulfite sequencing PCR, no differences in CpG methylation were seen in the distal half of this region (nucleotides −508/−129) in *H. pylori*-treated AGS cells compared with untreated controls (Supplementary Fig. 2B). In contrast, the average CpG methylation decreased from 15.4% in untreated cells to 8.3% following 1 h *H. pylori* treatment and to 5.2% following 4 h treatment within the proximal half of this region (nucleotides −209/+138) (Fig. 4A). Furthermore, the +80 cytosine that was identified as a site of methylation using 5-aza-dC (Fig. 3D), was confirmed to be hypermethylated in untreated and 1 h *H. pylori*-treated AGS cells, but was relatively hypomethylated in 4 h *H. pylori*-treated cells (Supplementary Fig. 2C). Thus, *H. pylori* decreases overall MUC1 CpG methylation within the −209/+138 segment at 1 and 4 h compared with untreated cells, with the +80 cytosine remaining methylated at 1 h but relatively unmethylated by 4 h.

Two approaches were used to verify decreased *MUC1* gene methylation in the −209/+138 nucleotide region and at the +80 cytosine following *H. pylori* treatment. First, by methylation specific electrophoresis, untreated AGS cells exhibited a high degree of CpG methylation in this segment (Fig. 4B, lane 1) that co-migrated with a fully methylated gel mobility standard, whereas following 4 h *H. pylori* treatment there was a tendency towards decreased CpG methylation (Fig. 4B, lanes 2 and 3 *vs.* 1). Second, by methylation specific PCR, while the +80 cytosine residue was methylated in untreated AGS cells (Fig. 4C, lanes 2 *vs.* 1), this site was unmethylated in cells treated for 4 h with *H. pylori* (Fig. 4C, lanes 3 *vs.* 4).

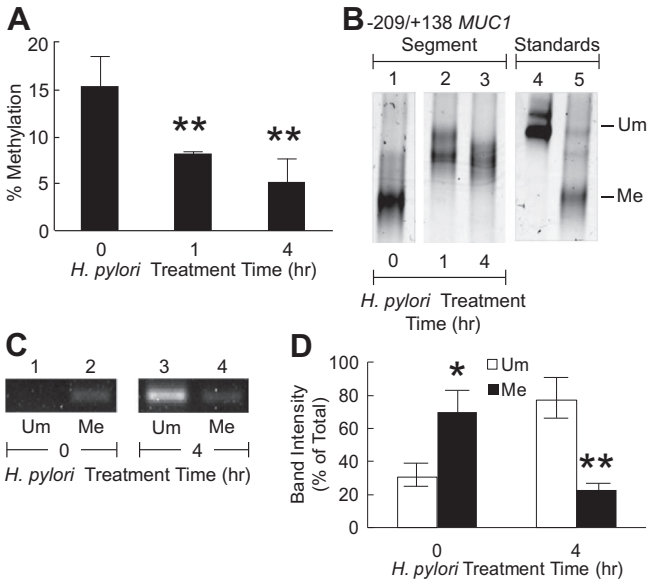

Fig. 4. *H. pylori* decreases methylation of the *MUC1* gene. AGS cells were untreated (0 h) or treated for 1 or 4 h with *H. pylori 26695* (MOI = 100), genomic DNA was isolated, and treated with sodium bisulfite. (A) For bisulfite sequencing PCR, primers were used to amplify the −209/+138 DNA segment, the amplicons were cloned, the clones were sequenced, and bisulfite sequencing DNA methylation analysis software [16] was used for statistical analysis. Vertical bars represent mean ± SEM percent methylation ($n = 3$). **Significantly decreased methylation in *H. pylori*-treated cells compared with untreated controls at $p < 0.05$. (B) For methylation specific electrophoresis, nested PCR was performed to amplify the −209/+138 segment of the *MUC1* gene. Amplicons were resolved by urea denaturing gradient gel electrophoresis and visualized by ethidium bromide staining. Fully unmethylated (Um) and fully methylated (Me) gel mobility standards are indicated (lanes 4, 5). (C) For methylation specific PCR, primers targeting the methylated (Me) or the unmethylated (Um) + 80 cytosine were used to amplify the −6/+108 segment of the *MUC1* gene, and amplicons were visualized on ethidium bromide-stained agarose gels. (D) Quantitative densitometry of each amplicon signal in (C) was normalized to the combined densitometry of both signals in the same gel. Vertical bars represent mean ± SEM normalized densitometry values ($n = 3$). *Significantly increased methylation compared with unmethylation in untreated cells at $p < 0.05$. **Significantly decreased methylation compared with unmethylation in *H. pylori*-treated cells at $p < 0.05$. The results are representative of 3 experiments.

4. Discussion

MUC1 is overexpressed by the majority of adencarcinomas, including those originating in the gastric epithelium [5,6]. Increased MUC1 expression in tumor cells is largely mediated by transcriptional activation of its gene [21–23]. Compared with other cancers, however, transcriptional regulation of MUC1 expression in gastric tumors is poorly understood. In breast cancer cells, the *MUC1* promoter between nucleotides −598 and −485 was shown to be important for its transcriptional regulation [22,23]. STAT3 and NF-κB binding sites located within this region regulated cytokine-mediated *MUC1* gene transcription in normal breast epithelia and breast cancer cells [21,24,25].

The mechanisms whereby overexpression of MUC1 contributes to epithelial carcinogenesis are relatively unknown. Most studies have focused on the role of MUC1-CD and its ability to quantitatively and qualitatively alter intracellular signaling pathways and transcription factor activity during tumor formation [5]. Among the MUC1-CD-regulated signaling cascades are the PI3K-Akt, ERK1/2, and Wnt-β-catenin pathways [4]. STAT3 and MUC1-CD were both identified at the STAT3 binding site (−503/−495) in the *MUC1* promoter in breast cancer cells, suggesting a feed-forward regulatory loop to transcriptionally activate *MUC1* gene expression [20]. STAT3 was constitutively phosphorylated in human gastric cancers [26–28], and STAT3 activation directly correlated with decreased survival of gastric cancer patients [29–31]. Other studies demonstrated not only increased STAT3 or phospho-STAT3 expression in gastric adenocarcinomas [29,32], but also that STAT3 contributed to the proliferation and invasion of gastric cancer cells [29,33]. Infection of humans or mice with cagA-expressing *H pylori* also activated STAT3 [34], although the *in vivo* mechanisms remain to be defined. In the context of these prior reports, the current results suggest that *H. pylori*-driven STAT3 activation promotes *MUC1* transcriptional activation, either alone or in concert with MUC1-CD, leading to increased *MUC1*

expression. Alternatively, *H. pylori*-dependent demethylation of the *MUC1* gene might increase STAT3 binding in the absence of direct STAT3 activation, thereby upregulating MUC1 expression. Further molecular and genetic studies are in progress to define the mechanistic details through which *H. pylori* increases MUC1 expression and the role of MUC1 in the development of stomach cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.142.

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