Crucial Roles of Sp1 and Epigenetic Modifications in the Regulation of the *CLDN4* Promoter in Ovarian Cancer Cells*

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Claudins form a large family of tight junction proteins that have essential roles in the control of paracellular ion flux and the maintenance of cell polarity. Many studies have shown that several claudin family members are abnormally expressed in various cancers. In particular, *CLDN4* (encoding claudin-4) is overexpressed in ovarian cancer. However, although *CLDN4* overexpression is well established, the mechanisms responsible for this abnormal regulation remain unknown. In the present study, we delineate a small region of the *CLDN4* promoter critical for its expression. This region contains two Sp1 sites, both of which are required for promoter activity. However, because of the ubiquitous expression of Sp1, these sites, although necessary, are not sufficient to explain the patterns of gene expression of *CLDN4* in various ovarian tissues.

We show that the *CLDN4* promoter is further controlled by epigenetic modifications of the Sp1-containing critical promoter region. Cells that overexpress *CLDN4* exhibit low DNA methylation and high histone H3 acetylation of the critical *CLDN4* promoter region, and the reverse is observed in cells that do not express *CLDN4*. Moreover, the *CLDN4*-negative cells can be induced to express *CLDN4* through treatment with demethylating and/or acetylating agents. Because *CLDN4* is elevated in a large fraction of ovarian cancer, the mechanism leading to deregulation may represent a general pathway in ovarian tumorigenesis and may lead to novel strategies for therapy and an overall better understanding of the biology of this disease.

Recently, several claudin genes have been shown to be abnormally expressed in human malignancies (4). In particular, *CLDN4* has now been shown to be significantly elevated in several malignancies such as breast, pancreas, prostate, and ovary (4). In ovarian cancer, we and several other groups have shown that the genes for *CLDN3* and *CLDN4*, as well as their corresponding proteins (claudin-3 and -4) are frequently up-regulated (5–10). Because many *CA125*-negative ovarian cancers overexpress these claudins, they may represent diagnostically useful biomarkers for this disease (11). Furthermore claudin-3 and claudin-4 are receptors for the cytotoxic *Clostridium perfringens* enterotoxin (12), and intra-abdominal administration of *Clostridium perfringens* enterotoxin might represent an interesting new avenue for ovarian cancer therapy (13). Although the exact roles of claudin overexpression in cancer remain unclear, recent data suggest that *CLDN3* and *CLDN4* overexpression may lead to increased invasion, motility, and cell survival in ovarian cells (14), suggesting important roles for these proteins in ovarian tumorigenesis. In addition, there is evidence that *CLDN4* may be elevated in cisplatin-resistant tumors (15). In trying to clarify the regulation of *CLDN4*, recent work has shown that in mouse Eph4 and CSG1 epithelial cells, Snail suppressed *Clnd4* expression (16). Nonsteroidal anti-inflammatory drugs have also been shown to induce *CLDN4* expression in a human gastric cancer cell line (17). However, the mechanisms leading to *CLDN4* overexpression in ovarian and other cancers remain unclear.

In the present study, we investigate the transcriptional regulation of *CLDN4* gene in ovarian cancer cells and report that Sp1 is crucial for *CLDN4* promoter activity. In addition, we find that epigenetic modifications such as DNA methylation and histone modification also play important roles in the regulation of this gene.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Tissue Samples—**Ovarian cancer lines OVCAR-4, OVCAR-5, UC1101, HEY, and BG-1 cells were cultured in McCoy’s 5A growth medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Ovarian cancer cell line A2780 was cultivated in RPMI1640 (Invitrogen) supplemented with 10% FBS, 4 μg/ml human recombinant FBS, and 10% fetal bovine serum.

**siRNA Transfection—**siRNA was electroporated into ovarian cancer cell lines OVCAR-4, OVCAR-5, A2780, HEY, and BG-1, each of which was transfected at 60–90% confluence with siRNA directed against specific cDNA pools (Dharmacon Research, Lafayette, CO). Transfected cells were selected on the basis of resistance to G418 (0.8 mg/ml) and maintained in RPMI1640 supplemented with 10% fetal bovine serum and G418.

**Western Blotting—**Whole-cell extracts were prepared and analyzed by Western blotting using specific antibodies. The following antibodies were used: 

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§ The abbreviations used are: FBS, fetal bovine serum; siRNA, small interfering RNA; MeCP2, methyl-CpG-binding protein 2; MBDD2, methyl-CpG-binding domain protein 2; MeCP1, methyl-CpG-binding protein 1; TSA, trichostatin A; 5-aza-dC, 5-aza-2′-deoxycytidine; RT, reverse transcription; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation.
insulin (Invitrogen), and antibiotics. HOSE-B, an ovarian surface epithelial cell line immortalized with E6 and E7 (18), was cultivated in RPMI1640 supplemented with 10% FBS and antibiotics. ML-3, an SV40-immortalized ovarian cystadenoma cell line (19), was cultivated in McCoy’s supplemented with 10% FBS and antibiotics. C32, a melanoma cell line, was cultivated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% nonessential amino acids, 2 mM glutamine, and antibiotics. Ten frozen primary ovarian cancers were obtained through the Collaborative Human Tissue Network Gynecologic Oncology Group (Children’s Hospital, Columbus, OH). In addition, four paraffin-embedded ovaries were obtained from the Department of Pathology of The Johns Hopkins Medical Institutions (Baltimore, MD). Primary T cells were kindly given by Dr. Vishwa Deep Dixit (NIA, National Institutes of Health, Baltimore, MD).

**Isolation of Promoter Fragments and Site-directed Mutagenesis**—The 5’ region (−1866 to +188, relative to the major transcriptional start site, as determined by ovarian expressed sequence tag analysis) of human CLDN4 were amplified by PCR and inserted into the pGL3 Basic vector (Promega, Madison, WI). The resulting plasmid was designated pGL3-C4P(−1866). Nested deletions of the CLDN4 promoter were made from pGL3-C4P(−1866) using an Exo III/mung bean deletion kit (Stratagene, La Jolla, CA). After narrowing down the critical promoter region, three putative transcription factor-binding sites (two sites for Sp1, designated as Sp1A and Sp1B, and one site for PPUR) were identified by using the Transcription Element Search System (TESS) (Computational Biology and Informatics Laboratory, School of Medicine, University of Pennsylvania, Philadelphia, PA) and TFSEARCH (Real World Computing Partnership, Tokyo, Japan). A mutant construct for each of the putative transcription factor-binding sites was made with the QuickChange site-directed mutagenesis kit (Stratagene).

**Cell Transfection and Reporter Assay**—Transfection experiments of BG-1 and A2780 cells were performed using FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s protocol. Briefly, the cells were grown in 12-well tissue culture plates so that the cell layer was 50–60% confluent on the day of transfection. For each well, 50 µl of medium containing 1.5 µl of FuGENE 6 was mixed with 500 ng of reporter plasmid and 50 ng of pCMV/β-gal vector (Clontech, Mountain View, CA). For OVCAR-5, transfection was done at 80% cell confluence using Lipofectamine 2000 (Invitrogen). For each well, 1.6 µg of each reporter plasmid and 160 ng of pCMV/β-gal mixture in 100 µl of OPTI-MEM I (Invitrogen) was transfected with 4 µl of Lipofectamine 2000 diluted with 100 µl of OPTI-MEM I. The cells were harvested 48 h after transfection, and the firefly luciferase activity was measured using a luciferase assay system (Promega). β-Galactosidase activity was used to normalize transfection efficiency, and each luciferase activity was calculated relative to that of the pGL3 Promoter vector (Promega). All of the experiments were repeated three times in duplicate.

5-Aza-2’-deoxycytidine (5-aza-dC) and Trichostatin A (TSA) Treatment—A2780 cells were treated with various concentrations (0, 0.1, 0.5, 1.0, 5.0, and 10 µM) of DNA methyltransferase inhibitor, 5-aza-dC (Sigma) for 48 h and histone deacetylase inhibitor, TSA (Sigma). TSA was added for the last 24 h at a final concentration of 300 nM. UCI101, HEY, HOSE-B, OVCAR-5, OVCAR-4, and BG-1 cells were treated with 10 µM of 5-aza-dC for 48 h either without TSA or with 300 nM of TSA for the last 24 h.

**Quantitative Analysis of CLDN4 mRNA Levels by Real-time RT-PCR**—Total RNA was isolated with TRIzol (Invitrogen) from cell lines (treated or untreated) and ovarian cancer tissue samples according to the manufacturer’s protocol. In tissues, cancer cells were manually microdissected using hematoxylin-stained frozen sections of these ovarian cancer samples under a microscope according to the NCI protocol (cgap-mf.nih.gov/Protocols/). One µg of total RNA was used to generate cDNA using TaqMan reverse transcription reagents (PE Applied Biosystems, Foster City, CA). The SYBR Green I assay and the 7300 real time PCR system (PE Applied Biosystems) were used for detecting the PCR products of CLDN4 as described (6). The comparative C_T method (PE Applied Biosystems) was used to determine the relative CLDN4 expression level of each sample using the C_T value observed in untreated A2780 cells as reference. Glyceraldehyde-3-phosphate dehydrogenase values were used for normalization. Relative CLDN4 expression levels of other cell lines, UCI101, HEY, HOSE-B, OVCAR-5, OVCAR-4, and BG-1, were calculated in similar fashion.

**Immunoblotting**—Ninety percent confluent cell cultures were washed with Hanks’ balanced salt solution (Invitrogen), and whole cell lysates were made using cell lysis buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, and 2% SDS). Protein concentration was determined using a BCA assay kit (Pierce). Twenty µg of total proteins were separated by 14% SDS-PAGE (Tris-glycine gels; Invitrogen) for claudin-4 immunoblotting and 10–20% SDS-PAGE for Sp1 and Sp3 immunoblotting and transferrered to polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked with 5% nonfat dry milk, washed in Tris-buffered saline with 0.05% Tween 20 (v/v), probed with the primary antibody (anti-claudin-4, 1:250 (Invitrogen); anti-Sp1, 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Sp3, 1:200 (Santa Cruz Biotechnology)), washed, and incubated in horseradish peroxidase-conjugated secondary antibody (anti-mouse or anti-rabbit IgG, 1:10,000; Amersham Biosciences). For detection, enhanced chemiluminescence was carried out using ECL (Amersham Biosciences).

**Bisulfite Sequencing of CLDN4 Promoter**—Ovarian cancer cells or normal ovarian surface epithelial cells were isolated using a PALM MicroLaser System (P.A.L.M. Mirolaser Technologies, Bernried, Germany) from frozen or paraffin-embedded sections, respectively. Genomic DNA extraction and treatment of genomic DNA with sodium bisulfite were performed according to available protocols (www.protocol-online.org/Protocols/). One µg of total DNA was used for bisulfite treatment with the EZ DNA Methylation Gold Kit (Zymo Research). One µg of bisulfite-treated DNA was used for PCR amplification. PCR amplifications of the relevant regions were performed using the following primers: for the 231-bp sequence, forward 5'-GAGATTGGATAGTTAGGGATT and reverse 5'-CAAACCAAAATATAAACAACCA, and for the 335-bp sequence, forward 5'-TGGA-
AGGAATTGTTTTGTATATTT and reverse 5’-CATAAA-C CCTCCCAATAATCTAC. The thermal cycle conditions were as follows: 95 °C for 2 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and elongation at 72 °C for 40 s and then 72 °C for 15 min. PCR fragments were ligated into the TOPO TA cloning vector (Invitrogen). Following transformation, plasmids from individual bacterial colonies were isolated, and the PCR fragments were sequenced. The DNA methylation profile of each sample was determined by the sequence results of at least five clones.

**Electromobility Shift Assay (EMSA)**—Nuclear extracts from BG-1 cells were prepared using a TransFactor extraction kit (Clontech) and quantified using the BCA assay kit (Pierce). The double-stranded oligonucleotide probes (sequences available from the authors) were labeled with [γ-32P]ATP (Amersham Biosciences) using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA). Radiolabeled probes (1 × 106 cpm) were incubated with 3 μg of nuclear extracts in a 20 μl of binding reaction (7.5% glycerol, 15 mM Tris-HCl, pH 7.5, 75 mM NaCl, 1.5 mM EDTA, pH 8.0, 1.5 mM dithiothreitol, 0.3% Nonidet P-40, 1 mg/ml bovine serum albumin, 1 μg of poly(dI-dC), and 500 ng of salmon sperm DNA) for 30 min at room temperature. For competition assays, a 100-fold excess amount of unlabeled competitor was premixed with radiolabeled probe before being added to the binding mixture. To demonstrate the presence of Sp1 protein in the DNA-protein complex, nuclear protein extracts were preincubated at room temperature with 2 μg of specific antibodies (Sp1 and Sp3; Santa Cruz Biotechnology) for 30 min before incubating with the radiolabeled wild type DNA probes. The reaction products were resolved on a 6% nondenaturing polyacrylamide gel at 200 V for 2.5 h. The gels were dried under vacuum and exposed overnight to a Phosphor Screen. The relevant protein-DNA probe complexes were quantified with a Storm 860 PhosphorImager (Molecular Dynamics, Piscataway, NJ) using ImageQuant software.

**In Vitro Methylation of the Critical CLDN4 Promoter Region**—Plasmids pGL3-C4P(−105) containing the critical CLDN4 promoter region and pGL3-C4P(−49), which lacks this region, were in vitro methylated by HhaI, HpaII, and SssI methylases (New England Biolabs) according to the manufacturer’s recommendations. The extent of methylation was verified by digesting the methylated DNA with restriction enzymes HhaI, HpaII, and BstUl (New England Biolabs), respectively. The unmethylated control (mock), consisted of pGL3-C4P(−105) and pGL3-C4P(−49) mixed with all of the components required for in vitro methylation except methylases.

**Nuclease Accessibility Assay**—The nuclei of BG-1, OVCAR-4, OVCAR-5, and A2780 cells were prepared as described (20) with minor modifications and digested with restriction enzyme Eco0109I (New England Biolabs). DNA from Eco0109I-digested nuclei was amplified by PCR with primer sets of CL4P-N-1 (5’-CTGAGACTGGATAGTGACTC-3’), CL4P-N-3 (5’-AGCCATATAAATGTCTCAACC-3’), and CL4P-N-5 (5’-TTGGACCCGTTAGTCAGGAG-3’) and the PCR products were separated by electrophoresis on a 6% nondenaturing polyacrylamide gel. The primer set of CL4P-N-1 and CL4P-N-5 and the set of CL4P-N-3 and CL4P-N-5 were designed to amplify a 272-bp fragment (−177 to +95) and a 129-bp fragment (−34 to +95), respectively.

**ChIP Assay**—A detailed protocol is available from the authors upon request. Briefly, BG-1 and A2780 cells (3.0 × 106 cells/cell line) were cross-linked with 1% formaldehyde at room temperature for 10 min. Cells were harvested with SDS lysis buffer, and DNA was sheared to 200–1000 bp by sonication. After preclaring the lysates, 4 μg of specific antibodies (anti-Sp1, anti-MBD2, and anti-acetylated histone H3; Upstate Biotechnology, Inc., Lake Placid, NY; anti-Sp3, Santa Cruz Biotechnology; anti-MeCP2, abcam, Cambridge, MA) were used to immunoprecipitate the protein-DNA complexes. DNA was quantified by real time PCR using CLDN4-specific primers. The primers for the CLDN4 promoter region were 5’-TGCCCTTTGTGTGGCTGG-3’ (forward) and 5’-ATGCGCAAGGCCACGAGTG-3’ (reverse). Each experiment was repeated three times.

**RNA Interference**—RNA interference was achieved by using siRNA pools of Sp1 and Sp3 (SMARTpool reagent; Dharmacon, Lafayette, CO). A functional, nontargeting siRNA (iControl™ nontargeting siRNA number 1; Dharmacon) was used as a control for potential nonspecific effects caused by siRNA transfection. BG-1 cells were transfected at 80% confluency with Lipofectamine 2000 (100 nM of each siRNA pool). The cells were harvested 72 h after transfection, and whole cell lysates were prepared using the lysis buffer described above.

**RESULTS**

**Identification of a Critical CLDN4 Promoter Region**—To delineate a critical region within the promoter of CLDN4, a nested series of reporter plasmids containing various lengths of the promoter region were constructed (Fig. 1A). These constructs were transfected into BG-1, OVCAR-5, and A2780 cell lines for the analysis of promoter activity. BG-1 and OVCAR-5 were chosen as representatives for CLDN4-expressing cells, and A2780, which has very little amount of CLDN4 mRNA, was chosen as nonexpressing cells (CLDN4 expression was measured by real time RT-PCR as described under “Experimental Procedures”). As shown in Fig. 1A, the luciferase activity of all three cell lines decreased significantly from clone pGL3-C4P(−105) to clone pGL3-C4P(−49), suggesting the presence of important promoter elements between bp −105 and −49. Although A2780 cells only express low levels of CLDN4 mRNA, the patterns of luciferase activity for the different constructs in this line were similar to the patterns measured in BG-1 and OVCAR-5, two cell lines expressing high levels of CLDN4. The same pattern of luciferase activity was also found in three additional ovarian cancer cell lines (OVCAR-4, HOSE-B, and UC1101), regardless of their CLDN4 expression status (data not shown), suggesting additional mechanisms of CLDN4 gene regulation in vivo.

**Site-directed Mutagenesis of the CLDN4 Promoter**—Using the TESS and TFSEARCH software to scan the minimal promoter region identified above, we found two putative binding sites for Sp1 (labeled Sp1A and Sp1B) and one PPUR putative binding site. Reporter constructs containing mutated sequences for each of these sites, as well as clones pGL3-C4P(−105) and pGL3-C4P(−49), were transfected into BG-1,
Mutating either of the Sp1 sites brought the promoter activity level down to basal level in all three lines, but mutation of the PPUR site did not significantly affect luciferase activity (Fig. 1B), clearly implicating the Sp1 sites as essential for the activity of the minimal CLDN4 promoter region. Again, the promoter activity patterns were similar in all three lines regardless of endogenous CLDN4 expression. These findings indicate that the basal transcription machinery, in which Sp1 is involved, is intact in A2780 cells, and that mechanism(s) are involved in silencing CLDN4 in low expressing cells such as the A2780 cell line.

Induction of CLDN4 mRNA and Protein Expression by 5-aza-dC and TSA—We next considered the possibility of epigenetic modification of the CLDN4 promoter as a mechanism of silencing in nonexpressing cells. A2780 cells were treated with 5-aza-dC and TSA to investigate the involvement of DNA methylation and histone deacetylation, respectively, in CLDN4 gene silencing. 5-aza-dC treatment of the A2780 cell line led to dose-dependent increases in CLDN4 mRNA 49.8-fold from basal level, and these increases were synergistically enhanced 252-fold by adding TSA to the cultures (Fig. 2A). TSA itself also induced CLDN4 mRNA 9.6-fold from the basal level (Fig. 2A). Interestingly, although the claudin-4 protein is not expressed in A2780, it was induced by treatment with 5-aza-dC and TSA. However, there was no direct correlation between mRNA and protein levels (see immunoblot in Fig. 2A), suggesting additional mechanisms of regulation.

To explore epigenetic modification of the CLDN4 promoter in other ovarian cell lines, UCI101, HEY, HOSE-B, OVCAR-5, OVCAR-4, and BG-1 cells were also treated with 5-aza-dC and TSA, and the levels of CLDN4 were measured by real time RT-PCR. CLDN4 mRNA was significantly induced with the treatment of 5-aza-dC in UCI101, HEY, OVCAR-5, and A2780 cells. Mutating either of the Sp1 sites brought the promoter activity level down to basal level in all three lines, but mutation of the PPUR site did not significantly affect luciferase activity (Fig. 1B), clearly implicating the Sp1 sites as essential for the activity of the minimal CLDN4 promoter region. Again, the promoter activity patterns were similar in all three lines regardless of endogenous CLDN4 expression. These findings indicate that the basal transcription machinery, in which Sp1 is involved, is intact in A2780 cells, and that mechanism(s) are involved in silencing CLDN4 in low expressing cells such as the A2780 cell line.

**FIGURE 1. Identification of the critical CLDN4 promoter element using luciferase assays.** A, nested deletion analysis. The constructs were transfected into BG-1 (CLDN4-expressing cells), OVCAR-5 (CLDN4-expressing), and A2780 (CLDN4-negative cells). The relative level of CLDN4 expression (relative to A2780) was measured by real time RT-PCR and is indicated for each cell line. Each luciferase activity shown represents the average of three independent experiments and is calculated relative to that of pGL3 promoter vector (SV40 promoter sequence). The luciferase activity of all three cell lines decreased significantly from clone pGL3-C4P(−105) to clone pGL3-C4P(−49), indicating the presence of important promoter elements between bp −105 and −49 (relative to transcriptional start site). B, luciferase assays of site-directed mutants of the pGL3-C4P(−105) vector. Two Sp1-binding sites (Sp1A and Sp1B) and one PPUR-binding site were mutated by site-directed mutagenesis and assayed for activity along with plasmids pGL3-C4P(−105) and pGL3-C4P(−49) as positive and negative controls, respectively. The Sp1A- and Sp1B-binding site mutations exhibited a significant decrease in luciferase activity in all three cell lines, suggesting that both Sp1-binding sites are crucial for CLDN4 promoter activity. In contrast, mutation of the PPUR-binding site did not significantly affect luciferase activity.
CLDN4 Promoter Regulation

no significant effects on CLDN4 promoter activity in HEY and HOSE-B cell lines. 5-aza-dC and TSA did not induce CLDN4 mRNA in OVCAR-5, OVCAR-4, and BG-1 cell lines, all of which already express high levels of CLDN4 mRNA (Fig. 2B). These findings suggest that epigenetic modifications such as DNA methylation and histone deacetylation are involved in CLDN4 gene silencing and suggest an absence of silencing by epigenetic modifications in cells that express high levels of CLDN4.

DNA Methylation Profile of the Critical CLDN4 Promoter Region—Bisulfite sequencing covering the region between Sp1-binding sites and half of the CpG island, which is located just downstream of the Sp1-binding sites (Fig. 3A), was performed to investigate the methylation profile of the critical CLDN4 promoter region. The DNA fragment amplified for bisulfite sequencing contains a total of 25 CpGs (regions I and II in Fig. 3A).

The cell lines and the tissues were divided into two groups: Group 1, which expresses high levels of CLDN4 mRNA (and detectable claudin-4 protein), and Group 2, which expresses lower levels of CLDN4 mRNA (and undetectable claudin-4 protein). Group 1 included four normal paraffin-embedded ovaries that were used to obtain ovarian surface epithelial cells by laser capture microdissection. Two of four normal ovarian surface epithelial cell samples were also used for immunohistochemistry of claudin-4, and its expression was weakly positive in both samples (data not shown). Although sparse DNA methylation was found in the Sp1-binding sites (the CpG site -48) of some cell lines and ovarian cancer tissues in Group 1, the maximum DNA methylation level observed was 20%. Therefore, the Sp1-binding sites can be considered to be hypomethylated in Group 1. The downstream region of Sp1 binding was more methylated, although the maximum DNA methylation level was still under 50%. In OVT-9, the Sp1-binding sites was completely unmethylated. By contrast, DNA methylation levels of the Sp1-binding sites in Group 2 were

and HOSE-B cell lines, which have low basal levels of CLDN4 mRNA (Fig. 2B). In the UCI101 cell line, TSA induced CLDN4 mRNA expression when used alone and synergistically enhanced induction of CLDN4 mRNA with 5-aza-dC, similar to what we observed in A2780 cells (Fig. 2A). However, TSA had
over 80% except UCI101, in which the DNA methylation level was 20% (Fig. 3C). However, the DNA methylation levels of the other sites in UCI101 were over 40%. Therefore, the Sp1-binding sites can be considered to be hypermethylated in Group 2. In addition to hypermethylation of Sp1-binding sites, the downstream region in Group 2 was also strongly hypermethylated except in ML-3, where the methylation level was low downstream of the Sp1-binding sites (the CpG sites +88, +112, and +121 had DNA methylation level of 20%). Interestingly, the C32 cell line (melanoma) and normal primary T cells, both of which had low levels of CLDN4, also exhibited hypermethylation of this critical region, suggesting that this mechanism of regulation may not be tissue-specific. Together, these results show that the DNA methylation of the Sp1-binding sites and the downstream region was strongly correlated with CLDN4 gene silencing.

**Nuclease Accessibility Assay of the CLDN4 Promoter**—The chromatin structure of the critical region delineated above was studied by nuclease accessibility assays. The 129:272 bp ratio reflects the amount of digested chromatin at the Eco0109I site in the critical CLDN4 promoter region (Fig. 4A). The ratio was significantly increased in BG-1, OVCAR-4, and OVCAR-5 cell
lines compared with mock control (Fig. 4B), indicating that the recognition site (−72) for Eco0109I is accessible, and therefore the chromatin is open in these three cell lines expressing moderate or high levels of CLDN4. However the ratio was not changed after Eco0109I digestion in A2780 cell line, suggesting a closed chromatin structure (Fig. 4B). After treatment of A2780 with either 300 nM TSA or 10 μM 5-aza-dC and 300 nM TSA, the 129:272 bp ratio was increased slightly, and the chromatin therefore adopted a more open configuration. Thus activation of the CLDN4 locus is associated with chromatin remodeling.

Inhibition of CLDN4 Promoter Activity by in Vitro DNA Methylation—The effect of in vitro methylation on the promoter activity of CLDN4 gene was investigated through transient transfection of CpG-methylated reporter plasmids into BG-1 cells. Reporter plasmids pGL3-C4P(−105) (which contain the critical CLDN4 promoter region) or pGL3-C4P(−49) (which lacks the critical region) were methylated in vitro by HhaI methylase, HpaII methylase, or SssI methylase (Fig. 5). The extent of methylation of the constructs was verified through digestion with the corresponding methylation-specific restriction enzymes (supplemental Fig. S1). The luciferase activity of CpG-methylated reporter plasmids were calculated relative to those of unmethylated plasmids (unmethylated controls). The luciferase activity of SssI-methylated pGL3-C4P(−105) decreased to 8.84%, whereas that of SssI-methylated pGL3-C4P(−49) decreased to 22.2%. There was almost no decrease in the luciferase activities of HhaI methylated plasmids compared with unmethylated controls (113% and 85.0%).

The decrease in luciferase activity of each HpaII-methylated plasmid compared with the unmethylated control was much smaller than that of SssI-methylated plasmids (59.3 and 50.4%). These results suggested that inhibition of the CLDN4 promoter activity is related to the density of methylated CpGs. This is consistent with the patterns of endogenous methylation we observed in cells. Indeed, the densely methylated CLDN4 promoter region observed in the cells of Group 2 (Fig. 3C) express low levels of CLDN4, whereas the sparsely methylated pattern observed in Group 1 (Fig. 3B) expresses higher levels.

Specific Binding of Sp1 and Sp3 Transcription Factors to the Critical CLDN4 Promoter Region—To directly demonstrate that Sp1 protein binds to its putative binding site within the critical CLDN4 promoter region, EMSA was performed. Three shifted complexes were observed α, β, and γ (Fig. 6A), all of which could be reduced by the addition of excess amount of unlabeled probe. These bands were not observed in the presence of labeled mutant probe, indicating that the DNA/protein interactions were specific. The addition of Sp1 antibody significantly reduced the amount of α complex without affecting the other complexes, whereas Sp3 antibody reduced the β complex. The α complex may contain some Sp3 because a mixture of Sp1/Sp3 antibody could reduce this band more efficiently than Sp1 alone. The γ complex was not affected by either Sp1 or Sp3 antibody. These results suggest that the α complex is mostly made up of Sp1 (and some Sp3), whereas β is constituted mostly of Sp3. The γ complex does not appear to include either Sp1 or Sp3; however, it may include proteins that bind in a methylation-specific manner, because competition with a methylated
probe was more efficient at reducing band γ than competition with an unmethylated probe.

EMSA was also performed on mutant probes to understand the contribution of each of the Sp1 sites to the binding patterns observed. Any mutation in the Sp1A or Sp1B or between the two sites resulted in some disruption of the γ complex (Fig. 6A). Interestingly, the β complex (mostly Sp3 binding) did not seem to require the Sp1A site. Similarly, Sp1A mutation did not affect the γ complex, but the other mutations did lead to destabilization of this complex. The CpG methylated DNA probe was used to investigate whether DNA methylation in the Sp1-binding sites interfere with Sp1 binding. Of the two Sp1-binding sites within the DNA probe, only the portion Sp1B has one CpG within its binding site. Therefore, if DNA methylation can interfere with Sp1 binding to the Sp1B-binding site, the formation of all three bands should be decreased when using the CpG methylated wild type DNA probe (H-Met-P) to an extent similar to what we observed using mutant probe 2. However, complex formation was only slightly decreased by using the H-Met-P compared with the unmethylated CpG wild DNA probe (H-Unmet-P), indicating that Sp1 and Sp3 can bind to their binding sites in the CLDN4 promoter region regardless of DNA methylation status. On the other hand, the γ complex was increased by using H-Met-P, indicating that the proteins forming this complex bind preferentially to their sites when Sp1B is methylated.

Recruitment of MBD2 (MeCP1 Complex) to the CLDN4 Promoter—ChIP assays were performed in A2780 (low expressor) and BG-1 (high expressor) to further elucidate the mechanisms of CLDN4 promoter regulation. Binding of both Sp1 and Sp3 to the CLDN4 promoter region was observed in both BG-1 and A2780 cell lines (Fig. 6B). Although the critical CLDN4 promoter region is hypermethylated in the A2780 cell line (Fig. 3C), Methylation did not prevent Sp1 and Sp3 binding. This finding is consistent with our observation of the same Sp1 and Sp3 binding efficiencies to the DNA probes regardless using the CpG methylated or unmethylated DNA probes in EMSA (Fig. 6A). Because we have shown that DNA methylation and histone acetylation of the CLDN4 promoter are involved in gene silencing, we used antibodies to acetylated histone H3, MeCP2, and MBD2 to investigate the chromatin structure in these cell lines. The histone H3 of CLDN4 promoter in the BG-1 cell line was much more acetylated than in A2780 cells (Fig. 6B). In contrast, MBD2 binding was elevated in A2780 compared with BG-1, suggesting that DNA methylation in A2780 cells recruits transcriptional repressors to CLDN4. There was no evidence of MeCP2 binding to the critical CLDN4 promoter region in either line. Therefore, MeCP1, which is a large protein complex including MBD2 and histone deacetylases, is likely responsible for CLDN4 silencing in A2780.

Inhibition of Claudin-4 Protein Expression by Sp1 siRNA—To confirm the role of Sp1/Sp3 in the regulation of CLDN4 expression in ovarian cancer cells, we used RNA interference to
knock down Sp1 and Sp3 expression in BG-1 cells, which express CLDN4 at high levels. Knockdown of Sp1 led to a significant reduction of the claudin-4 protein (Fig. 7). In contrast, Sp3 knockdown did not significantly affect claudin-4 expression. Mock transfection and transfection with a control siRNA did not affect the expression of any of the proteins.

DISCUSSION

Since the initial study that found CLDN4 among the most highly elevated genes in ovarian cancer (5), many studies have confirmed and extended this finding (6–10). CLDN4 as well as other CLDNs have also been found abnormally expressed in several human cancers (4). However, whereas CLDN4 overexpression is well established, the mechanisms responsible for this abnormal regulation remain unknown. Because CLDN4 may have a role in the aggressive behavior (14) and chemoresistance (15) of ovarian cancer, a better understanding of the mechanism controlling the expression of this gene may provide new opportunities for therapy.

In the present study, we first narrowed down the critical CLDN4 promoter region to a small area located between base pairs −105 and −49 that contains two Sp1-binding sites. Interestingly, we found that both Sp1 sites were crucial for the proper activity of the critical region (Fig. 1B) and that this activity was observed even in cells that did not endogenously express CLDN4. Because these transiently transfected constructs are unlikely to adopt the proper chromatin structure, this observation suggests that the transcription factors that bind to the Sp1-binding sites exist in the cells regardless of the CLDN4 expression status and that gene silencing of CLDN4 is likely caused by other mechanisms such as DNA methylation and/or histone modification. Indeed, EMSA confirmed that Sp1 and Sp3, two transcription factors that are ubiquitously expressed, could bind the putative sites identified in the minimal region. Similar mechanisms of Sp1-mediated regulation have been reported for the NF1 (neurofibromatosis type 1) and APC (adenomatous polyposis of the colon) promoters (21, 22).

To explore the involvement of DNA methylation and histone modification in CLDN4 gene silencing, we first treated the cells with 5-aza-dC and TSA. Synergistic derepression of the CLDN4 gene by 5-aza-dC and TSA or derepression with 5-aza-dC alone was observed in the A2780, UCI101, HEY, and HOSE-B cell lines, which have low endogenous levels of CLDN4 mRNA (Fig. 2). The effect of 5-aza-dC and TSA was reduced (or absent) in
cells that express high levels of CLDN4. In addition, nuclease accessibility assays demonstrated a significant difference in chromatin structure around the Sp1-binding sites between cells that express high levels of CLDN4 and cells that do not. Indeed, we found that the chromatin configuration was “closed” in the A2780 cell line, whereas it was “open” in the CLDN4 protein-expressing cell lines, including in A2780, in which expression had been restored with 5-aza-dC and TSA treatment (or with TSA alone) (Fig. 4B). This is consistent with our ChIP assay findings that acetylated histone H3 is much higher in cells expressing CLDN4. In addition, we investigated the DNA methylation profiles of the region covering the Sp1-binding sites and a portion of the CpG island shown in Fig. 3 and found that DNA methylation of the Sp1-binding sites and the down-stream region was strongly correlated with CLDN4 gene silencing in ovarian cell lines and other tissues. Furthermore, we confirmed a direct effect of DNA methylation on gene silencing of CLDN4 using the in vitro methylated constructs in a luciferase assay (Fig. 5). The constructs densely methylated by SssI methylase showed significantly decreased luciferase activity compared with unmethylated constructs. Conversely, the constructs sparsely methylated by the HhaI or HpaII methylase did not exhibit any significant changes in luciferase activity. Taken together, all of these findings strongly suggest the involvement of DNA methylation and histone modification in CLDN4 gene silencing. This is consistent with a large body of literature showing that DNA methylation and histone acetylation can work in concert in the regulation of gene transcription (23).

**FIGURE 6.** Binding of Sp1 and Sp3 transcription factors to the critical CLDN4 promoter region. A, EMSAs were performed using wild type DNA probes and mutant probes (shown schematically on the right) and nuclear extracts from BG-1 cells. The sequences of the probes are available from the authors. The labeled and unlabeled probes are indicated as H- and C-, respectively, and CpG methylated and unmethylated probes are indicated as Unmet and Met, respectively. Three shifted bands, α, β, and γ, were identified when using wild type DNA probes. The specificity of these bands was shown by using the unlabeled competitors (C-Unmet-P and C-Met-P) and mutant probes (M1–4). B, ChIP assays using antibodies to Sp1, Sp3, acetylated H3, MeCP2, and MBD2 were performed in A2780 (low CLDN4 expressor) and BG-1 (high CLDN4 expressor) cells. The results are expressed as percentages of immunoprecipitated (IP) DNA to total input DNA (Input).

**FIGURE 7.** Sp1 knockdown leads to a reduction in claudin-4 protein. Sp1 and Sp3 siRNAs were transfected into BG-1 cells and whole cell lysates were analyzed by immunoblotting for Sp1, Sp3, and claudin-4. A functional, nontargeting siRNA (control siRNA) was also transfected to exclude nonspecific effects caused by siRNA transfection. For Sp3, two specific bands, the full-length (100 kDa) and the truncated form (60 kDa) were seen. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) immunoblotting was performed as loading control.
Epigenetic modifications may be a common mechanism of CLDN regulation in cancer, since promoter methylation was previously shown to be correlated with CLDN7 silencing in breast cancer cells (24).

After demonstrating that epigenetic modifications on the CLDN4 promoter are critical in the silencing of this gene in nonexpressing cells, we investigated how these changes correlate with the transcription factor binding patterns in the critical CLDN4 promoter region. Interestingly, EMSA and ChIP assays showed Sp1 and Sp3 binding to the critical CLDN4 promoter region in A2780 cells and in BG-1 cells. Previous studies have shown that the binding of Sp1 to its site is not necessarily affected by DNA methylation, and the behavior appears to be promoter-dependent. In the promoters for p21Cop1, 11 β-hydroxysteroid dehydrogenase type 2, or GSTP1 (glutathione S-transferase p1), the Sp1 binding affinity to the respective promoter region was found to be diminished by DNA methylation (25–27). In contrast, Sp1 binding affinity to the luteinizing hormone receptor (LHR) promoter was not influenced by DNA methylation (28). In the present study, EMSA and ChIP assays clearly indicated that Sp1 and Sp3 binding to the critical CLDN4 promoter region was not inhibited by DNA methylation, similar to what was observed for the LHR promoter. We show that, among the proteins studied, the only difference between the A2780 and BG-1 cell lines was the increased binding of the corepressor MBD2 in A2780 cells. Because the MeCP1 complex binds to densely methylated CpGs via MBD2 and directly suppresses promoter activity in cooperation with histone deacetylase 1/2, our results suggest that MeCP1 plays a direct and important role in CLDN4 gene silencing in ovarian cancer cell lines and probably in other tissues as well. This is consistent with our observed derepression by 5-aza-dC and TSA. It has been reported that MeCP1 plays an important role in LHR gene silencing of JAR cell line without interfering with Sp1 and Sp3 binding (28).

Finally, we sought to confirm the roles of Sp1 and Sp3 in the CLDN4 promoter regulation using RNA interference. Consistent with our reporter assays demonstrating a crucial role for Sp1 sites in CLDN4 promoter activity, Sp1 knockdown significantly decreased Claudin-4 expression (29–31). Because we show that Sp3 complexes are present at much lower levels than Sp1 complexes in the CLDN4 promoter region (Fig. 6), we expected that Sp3 might have a lesser role in CLDN4 gene regulation. As expected, Sp3 knockdown showed little effect on CLDN4 protein levels in siRNA experiments (Fig. 7). Therefore, we surmise that Sp1, not Sp3, is the main regulatory factor for CLDN4 gene expression in addition to DNA methylation and histone modifications.

Although CLDN4 mRNA was induced 250-fold from its basal level by 5-aza-dC and TSA in A2780 cells, the final amounts of expression were still much below the levels expressed endogenously in BG-1 and OVCAR-4 cell lines or in ovarian cancer tissues. Therefore, it is probable that other regulatory factors are required to provide maximal expression of the CLDN4 gene. It is well known that Sp1 interacts with ERα directly in many genes (34, 35). In addition, it has been found that c-Myc repressed the p21 promoter by interacting with Sp1/Sp3 (36). With respect to CLDN4 gene regulation, it has been shown that it can be repressed directly by Snail in mouse Eph4 and CSG1 epithelial cells (16). It will be interesting to investigate whether these factors interact with Sp1 in ovarian cancer cells to affect the expression of CLDN4.

Normal ovarian surface epithelial cells, which are thought to be the tissue of origin for most ovarian cancers, express low levels of CLDN4 mRNA (6) and exhibit low methylation of the promoter (Fig. 3B). Therefore, to express CLDN4 at the levels observed in tumors, ovarian cancer cells likely need to activate or overexpress regulatory factors during progression. Because CLDN4 is elevated in a large fraction of ovarian cancers, the mechanism leading to its deregulation may represent a general pathway of ovarian tumorigenesis and therefore an ideal target for novel therapeutic strategies.

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