

Epigenetic Silencing of Occludin Promotes Tumorigenic and Metastatic Properties of Cancer Cells via Modulations of Unique Sets of Apoptosis-Associated Genes

Makoto Osanai,¹ Masaki Murata,¹ Nami Nishikiori,^{1,2} Hideki Chiba,¹ Takashi Kojima,¹ and Norimasa Sawada¹

Departments of ¹Pathology and ²Ophthalmology, Sapporo Medical University School of Medicine, Sapporo, Japan

Abstract

Occludin is the first identified integral protein for the tight junction (TJ), and its long COOH-terminal domain is considered to have functions in receiving and transmitting cell survival signals. Loss of TJ-associated molecules, such as occludin, has been correlated with tumor progression in carcinogenesis; however, the precise molecular mechanisms explaining its loss of expression and whether occludin expression has any effects on cancer phenotypes remain to be clarified. Here, we show that forced expression of occludin in cancer cells exhibits enhanced sensitivity to differently acting apoptogenic factors, and thus inhibits the tumorigenicity of transformed cells, via modulation of unique sets of apoptosis-associated genes. In addition, studies using deletion mutants of occludin constructs show that 44 amino acids at the COOH-terminal end play a critical role in modifying the cellular phenotypes. Interestingly, occludin decreases cellular invasiveness and motility, thereby abrogating metastatic potencies of cancer cells. We also found that occludin expression is silenced by CpG island hypermethylation on its promoter region. Synergy with a demethylator and histone deacetylase inhibitor or retinoids that stimulate retinoic acid receptor α induces endogenous occludin, which is sufficient for apoptotic sensitization. Our results show the functional diversity of occludin and suggest that methylator phenotype of occludin provides enhanced tumorigenic, invasive, and metastatic properties of cancer cells, identifying occludin as a likely candidate for a tumor-suppressor gene in certain types of cancer. (Cancer Res 2006; 66(18): 9125-33)

Introduction

Tight junctions (TJs) are the apical-most intercellular structures in epithelial and endothelial cells, playing central roles in the formation of cell polarity and functioning as major determinants of paracellular permeability (1, 2). One of the first identified TJ-associated molecules was occludin, which has been considered to be a requisite integral protein for TJ structure and function (3). Occludin (~65 kDa) is a membrane protein with four transmembrane domains forming two extracellular loops and a long cytoplasmic tail (4). Previous reports have shown that occludin localizes to the TJ, and occludin overexpression in a number of cell types induces the formation of TJ-like structures, supporting the

idea that occludin is a key player in the formation of TJ (5). However, occludin expression in cells that lack endogenous TJ does not result in generation of typical TJ strands, and occludin-deficient cells and animals have fully developed to form well-organized TJs structurally (6, 7). Thus, it is now well accepted that the claudin family, which has been shown to contain >20 members, is the main constituent for TJ, rather than occludin.

The long cytoplasmic domain of occludin is rich in serine, threonine, and tyrosine, and serine/threonine phosphorylation levels show a strong correlation with TJ formation (4). Occludin directly associates with cytoplasmic scaffolding proteins, such as zona occludens (ZO)-1, a submembrane component of TJs, thereby providing a direct linkage to the actin cytoskeleton. In addition, different types of signaling transduction molecules that have proliferative and differentiative capacities, such as transcription factors, lipid phosphatases, and cell cycle regulators, are associated with TJs. Indeed, many of the cytoplasmic junctional components are signaling proteins that have functions in receiving or transmitting signals, such as atypical protein kinase C (PKC), Rho proteins, PKC- ξ , c-Yes, and phosphoinositide-3-kinase (PI3K; ref. 8). Recently, Y-box transcription factor ZO-1-associated nucleic acid binding protein (ZONAB) has been proved to bind the SH-3 domain of ZO-1, which contains multiple protein-protein interaction domains, and ZONAB and ZO-1 functionally interact with the regulation of erbB-2 promoter activity in cells (9), indicating that TJ protein directly participates in the control of gene expression. Although another TJ protein, ZO-2, also associates with Fos, Jun, and CCAAT/enhancer binding protein, occludin directly interacts with nonreceptor tyrosine kinase c-Yes (10, 11), suggesting that TJs may have a direct functional association with signal transduction pathway(s). Consistent with this, our recent study has revealed that occludin is linked with apoptotic machinery involving mitogen-activated protein kinase (MAPK) and Akt signaling pathways in murine hepatocytes (12).

Accumulating evidence has shown that the disruption of TJ structure is associated with cancer development, which may be causally involved in malignant phenotypes, such as local tumor growth, invasion, and metastasis at distant sites. We and others have shown that loss of TJ-associated molecules is correlated with tumor development in carcinogenesis (13). In addition, decreased and/or impaired TJ formation has been reported for various types of cancer, and genes having an oncogenic character are known to disrupt TJs (14–16). Conversely, overexpression of TJ-related proteins, such as claudin-1 and claudin-4, in cancer cells has been reported to induce apoptosis and suppress the metastatic potencies of these cells (17–19). Although these observations suggest the plausible notion that the malignant progression of cancer is associated with decreased and/or impaired TJ phenotypes, the functional consequences of the reexpression of

Requests for reprints: Makoto Osanai, Department of Pathology, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku, 060-8556 Sapporo, Japan. Phone: 81-11-611-2111, ext. 2702; Fax: 81-11-613-5665; E-mail: osanaim@sapmed.ac.jp.
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specific TJ-associated proteins in cancer cells remain to be elucidated.

These evidences raise the hypothesis that occludin interacts with a signal transduction pathway that have functions in regulating cell survival in cancer cells, eventually acting as a signal transmitter for the cells at TJs. To evaluate this, we examined the possibility of whether reexpression of occludin in cancer cells had any effects on its phenotype. Here, we show that occludin expression exhibits enhanced cellular sensitivity to various apoptogens, and thus inhibits the tumorigenic potencies of the transformed cells. Our findings imply that occludin is a likely candidate for a tumor-suppressor gene in certain types of cancer.

Materials and Methods

Cell line. All cell lines were maintained in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Sanko Jyunkyaku, Tokyo, Japan), 10 mmol/L HEPES, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cell lines used in this study were as follows: HeLa, human cervical carcinoma cell; B16F10, murine melanoma cell; AC2M2, metastatic variant of murine breast cancer cell SP1 (SP1 was isolated from a mammary intraductal adenocarcinoma that arose spontaneously in an 18-month-old CBA/J female retired breeder; ref. 20); MCF-7, human breast cancer cell; U373MG, human glioma cell; and RLE, rat lung endothelial cell.

Construction of expression vectors and transfection. Full-length murine occludin cDNA [coding region from nucleotides 223 to 1,788 (occludin²²³⁻¹⁷⁸⁸); Genbank accession no. U49185] was amplified by reverse transcription-PCR (RT-PCR) from 0.1 µg of total RNA extracted from mouse kidney, using a *Xho*I-tagged sense primer and a *Hind*III-tagged antisense primer. We also amplified occludin mutants occludin²²³⁻¹⁶⁵⁶, occludin²²³⁻¹⁵⁴⁸, occludin²²³⁻¹⁴⁴⁰, and occludin²²³⁻¹³¹⁴, as well as occludin¹³¹⁵⁻¹⁷⁸⁸, having various degrees of deletion in the cytoplasmic domain. After subcloning into a TA-cloning vector (pCR2.1) using a TA cloning kit (Invitrogen, Tokyo, Japan), the ~1.6, 1.4, 1.3, 1.3, 1.0, and 0.5 kb digested *Xho*I-*Hind*III fragments were ligated into the response plasmid, pcDNA3.1(-) (Invitrogen), and designated pcDNA3.1(-)-occludin²²³⁻¹⁷⁸⁸ [encoded 522 amino acid (aa) polypeptide], pcDNA3.1(-)-occludin²²³⁻¹⁶⁵⁶ (478 aa), pcDNA3.1(-)-occludin²²³⁻¹⁵⁴⁸ (442 aa), pcDNA3.1(-)-occludin²²³⁻¹⁴⁴⁰ (406 aa), pcDNA3.1(-)-occludin²²³⁻¹³¹⁴ (364 aa), and pcDNA3.1(-)-occludin¹³¹⁵⁻¹⁷⁸⁸ (158 aa), respectively. We also cloned each cDNA fragment into a different expression plasmid, pEGFP-C1 (Clontech, Palo Alto, CA), to facilitate the fluorescent detection of transduced occludin in transfected cells. The integrity of the final constructs was confirmed by direct sequence analysis. All primers used to make these constructs are available upon request.

Five micrograms of each plasmid were transfected into cells using FuGENE 6 transfection reagent (Roche, Indianapolis, IN). G418 (Sigma, Tokyo, Japan)-resistant clones were expanded as a monoclonal population. Three weeks later, cell lines were selected to examine the strong expression of introduced gene by Northern blot analysis and Western blot analysis. Because our preliminary experiments showed that observed phenotypes of the cells in the event of occludin overexpression were similar but not same between at least three different independent clones, these cells were equally mixed to establish stably transfected cell lines to avoid possible clonal variation. Cells transfected with an empty vector were used as a control. Small interfering RNA (siRNA) targeted to occludin (Santa Cruz Biotechnologies, Santa Cruz, CA) was also transfected into the cells using FuGENE 6 reagent (Roche).

Cell death analyses. These procedures were described in detail previously (21). Apoptosis was stimulated with H₂O₂ (Sigma) at a dose range of 0 to 100 µmol/L for 1 to 24 hours. Cells were also treated with various death-inducing agents, including heat shock at 42°C for 30 minutes, etoposide (50 µmol/L), cisplatin (10 µg/mL), and γ-irradiation (50 or 100 Gy).

In some experiments, all-*trans* retinoic acid (atRA, 10 nmol/L-2.5 µmol/L; Sigma), which is an apoptotic sensitizer (21), MAPK inhibitor (PD98059, 50 µmol/L; Calbiochem-Novabiochem, San Diego, CA), p38 MAPK inhibitor

(SB203580, 20 µmol/L; Calbiochem-Novabiochem), or PI3K inhibitor (LY294002, 20 µmol/L, Calbiochem-Novabiochem) was added and maintained throughout the experiments. To examine the effect of a demethylating agent, the cells were treated with 5'-aza-2'-deoxycytidine (5'-Aza-dC, 0-5 µmol/L; Sigma) for 48 hours, after which a histone diacetylase inhibitor or trichostatin A (0-300 nmol/L, Sigma) was added, followed by incubation for an additional 24 hours at 37°C.

Terminal deoxyribonucleotide transferase-mediated nick-end labeling assay and immunohistochemistry. Cells cultured on collagen-coated glass coverslips were subjected to the terminal deoxyribonucleotide transferase-mediated nick-end labeling (TUNEL) assay. Apoptotic cells were visualized using an *In situ* Cell Death Detection kit (Roche). We also carried out a staining procedure other than for terminal deoxynucleotidyl transferase as a negative control. For the immunohistochemistry, the cells on a coverslip were reacted with a primary antibody against Ki67 (DakoCytomation, Tokyo, Japan). The positive cells stained by the specified procedures were scored using light microscopy by counting the number of cells under low magnification (×100) in 10 separate arbitrarily selected fields in each section.

Anoikis induction. Cells were prevented from adhering to the plastic of cell culture dishes to induce anoikis. This was achieved by coating each 6-cm dish with 4 mL of 1% agarose in water and this solidified agarose was equilibrated and overlaid with DMEM supplemented with 10% FBS overnight. After trypsinization, cells (2 × 10⁵) were added to these plates, which were then rotated horizontally several times to allow the cells to form spheroidal aggregations before being returned to the incubator. Cells were collected time dependently up to 7 days and subjected to the apoptotic assay.

Colony-forming assays in two- and three-dimensional cultures. Control and occludin-transfected cells (5 × 10⁵ per well) were plated on six-well plates 24 hours before exposure to a lethal dose of γ-irradiation (>100 Gy). A small fraction of cells could survive and these were cultured for 7 days to form separate colonies. Soft agar assay was carried out in 6-cm dishes to assess the colony formation in three dimensions. Cells (1 × 10⁴) uniformly suspended in 6 mL of 0.33% agarose gel with growth medium (DMEM supplemented with 5% FBS) were overlaid onto a base layer of 1% agarose gel equilibrated with growth medium overnight before use for this assay. These plates were incubated for at least 3 weeks. Colonies developed on cell culture dishes in soft agar suspension were scored using phase-contrast microscopy by counting colony numbers under low magnification (×100) in 10 separate arbitrary fields in each plate. Cell clusters of ≥100 µm in diameter were defined as positive results in the soft agar assay.

In vitro invasion assay and transmigration assay. After coating 24-well cell culture inserts with 8 µm pores (Becton Dickinson, Franklin Lakes, NJ) with Matrigel (Life Technologies), cell suspension (5 × 10⁵ in 0.5 mL of DMEM with 0.5% FBS) was added to the upper compartment, while DMEM containing 10% FBS was added to the bottom chamber to create a chemotactic gradient. Invasive cells were measured after 48-hour incubation at 37°C. The bottom surface of the top chamber was wiped with cotton swabs, and cells that passed through the filters into the lower surface of wells were quantitated. Similarly, transmigration assay through the endothelial monolayer was done as described previously (22). Briefly, we did this assay by using 24-well cell culture inserts with 8 µm pores in a coculture system using both various cancer cell lines and RLE:rtTA:hepatocyte nuclear factor (HNF)-4α L23, which is an endothelial cell line with inducible expression of HNF-4α after doxycycline treatment. The endothelial monolayers of RLE:rtTA:HNF-4α L23 cells on filters were treated with 1 µmol/L doxycycline before 24-hour assay. Doxycycline-mediated HNF-4α expression up-regulated endothelial Fas ligand, resulting in the induction of massive apoptosis of cancer cells when the cancer cells were overlaid on the endothelial monolayer. Transmigrated viable cells on the lower surface of the transwell were quantitated.

Wound-healing assay. Cells were grown to confluence on 10-cm dishes. A scratch was then made through the cell monolayer using a pipette tip. After washing twice with PBS, fresh tissue culture medium was added and we took photographs of wounded areas in a time-dependent manner up to 24 hours after making the wound.

Reverse transcription-PCR and Southern blot analysis. Total RNA (1 μ g) extracted using TRIzol (Life Technologies) was reverse-transcribed using M-MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). For analysis of gene expression, the gene of interest was amplified from dilutions of cDNA using specific sense and antisense primers for up to 40 cycles, and we examined various cycling variables for each PCR experiment to define optimal conditions for linearity to allow for semiquantitative analysis of signal intensity. Electrophoresed DNA from the agarose gel was transferred to a Hybond N+ nylon membrane overnight (Amersham Biosciences, Piscataway, NJ). Full-length cDNA of each gene was used as a probe labeled with [³²P]dCTP to a specific activity of $\sim 1 \times 10^9$ cpm/ μ g using a random priming-based cDNA labeling kit (Nippon Gene, Tokyo, Japan). rRNA was shown to use the same amounts of RNA for reverse transcription reactions. Triplicate independent PCR reactions were carried out to ensure the reproducibility of expression quantification. For the densitometric analysis, signals in Southern blot analysis were quantitated using Scion Image 1.62 (Scion Corporation, Frederick, MD).

Western blot analysis. Whole-cell lysates extracted from 1×10^7 cells, each with 20 μ g denatured proteins, were run on 12% to 15% polyacrylamide gels containing SDS and electroblotted onto nitrocellulose filters. Filters were then blocked with 5% nonfat dry milk and immunoblotted with antibodies against occludin (Zymed Laboratories, San Francisco, CA), phosphorylated MAPK (Cell Signaling, Beverly, MA), extracellular signal-regulated kinase 1/2 (ERK1/2; Promega, Madison, WI), and β -actin (Santa Cruz Biotechnologies) proteins. After extensive washing, the filters were reacted with peroxidase-labeled corresponding secondary antibodies in PBST and again washed; finally, the immunoreactions were visualized by using an enhanced chemiluminescence (ECL) system (Amersham Biosciences).

Gelatin zymography. The gelatinolytic activity of matrix metalloproteinases (MMP) was determined in the supernatants of B16F10-Vec and B16F10-Oc^{522aa} cells treated with or without 1 μ mol/L atRA for 24 hours. Aliquots of conditioned medium were subjected to electrophoresis without heating under nonreducing conditions in 0.1% gelatin-containing 9% polyacrylamide gels. After electrophoresis, the gels were washed with 2.5% Triton X-100 for 1 hour to remove the SDS, followed by incubation overnight at 37°C in Tris buffer [50 mmol/L Tris-HCl, 200 mmol/L NaCl, and 10 mmol/L CaCl₂ (pH 7.4)]. Gels were then stained for 30 minutes with 0.5% Coomassie brilliant blue in 30% methanol/10% acetic acid. Clear bands appeared on the blue background in the areas of gelatinolytic activity.

cDNA array analysis. These procedure was described in detail previously (21). cDNA microarray analysis was done to analyze the apoptosis-specific signaling pathway using GEArray and mRNA levels were quantitated by GEArray Analyzer software (SuperArray, Bethesda, MD).

Methylation-specific PCR. The methylation status of the promoter CpG islands of occludin was analyzed by methylation-specific PCR (MSP) on sodium bisulfite-converted DNA (23). Briefly, 2 μ g genomic DNA was denatured with 2 mol/L NaOH for 10 minutes, followed by incubation with 3 mol/L sodium bisulfite (pH 5.0) for 16 hours at 50°C. DNA was then precipitated with ethanol and resuspended in 20 μ L of water. Aliquots (2 μ L) were used as templates for PCR. The primers for MSP were as follows: 5'-GAGTATCGAACGTATTCGGGG-3' and 5'-GCGCTACCTAAC-GAACGCA-3' for the amplification of methylated alleles; 5'-AGTTTATGGT-GAATTGGTTATTGAG-3' and 5'-ACAACCTAATCTACCAAACCTAAA-3' for the amplification of unmethylated alleles.

Tumor growth *in vivo*. After acclimation for 7 days, we injected cells of wild-type (wt) B16F10 and its transfectant (2×10^5 per mouse) s.c. into the craniolateral thorax (axilla) of syngenic 6- to 7-week-old male mice (C57BL/6, CLEA Japan, Tokyo, Japan). For AC2M2 cells, a 100 μ L volume of cells was injected into a mammary fat pad in 6-week-old male mice (CBA/J, Charles River Japan, Yokohama, Japan). We examined the primary tumor and lung, subjecting for histologic evaluation for micrometastasis. The volume (V) of the primary tumors was calculated by the following equation: $V = (\pi / 6) \times [(L + W) / 2]^2$, where L is the length and W is the width. The maintenance and handling of animals were carried out using protocols approved by the Sapporo Medical University Animal Care Facility.

Statistical analysis. Unless otherwise specified, all data represent the mean \pm SD of at least three independent experiments, each in triplicate wells. Statistical differences were analyzed using the paired *t* test and were considered statistically significant when $P < 0.05$.

Results

Forced expression of occludin enhances the sensitivity to apoptogens. In the present study, we established a human cervical cancer cell line HeLa, constitutively overexpressing wt occludin (occludin²²³⁻¹⁷⁸⁸) that encodes 522 aa polypeptide, thus designated HeLa-Oc^{522aa}. HeLa cell transfected with an empty vector was used as a control (HeLa-Vec). We first determined the differential sensitivity of these cells to 24-hour exposure to oxidative stress mediated by H₂O₂, which is a common cell stressor. Before the apoptotic stimulation, HeLa-Vec and HeLa-Oc^{522aa} were changed with medium without FBS supplementation and were exposed to H₂O₂ in the dose range of 0 to 100 μ mol/L in the presence or absence of 1 μ mol/L atRA as an apoptotic sensitizer (21). Forced expression of wt occludin clearly enhanced the sensitivity to H₂O₂-induced cell death in a dose- and time-dependent manner in HeLa cells (Fig. 1A and data not shown). Although we have previously shown that the type of cell death induced by H₂O₂ is apoptosis (21), we next used the TUNEL method to confirm the type of cell death and that the assay used in this study was sensitive enough to quantitate the cell death. TUNEL assay also revealed that occludin increased the sensitivity to H₂O₂-induced cell death (Fig. 1A, right), which was coincident with the results when compared with our assay (Fig. 1A, left). To confirm the functional relationship between occludin expression and apoptotic sensitization, we next examined the effects of siRNA targeted to occludin. siRNA transfection could effectively silence the constitutively expressed occludin (Fig. 1B, left). siRNA-mediated silencing inhibited occludin-mediated enhancement of cell death in occludin-expressing cells, whereas the siRNA transfection did not affect HeLa-Vec cells (Fig. 1B, right). These results indicate that occludin expression modulates the apoptotic sensitivity of the cells.

COOH-terminal end is responsible for the occludin-mediated apoptotic induction. Previous reports have shown that the COOH terminus of occludin is important in its function (4). We hypothesized that the COOH terminus of occludin is responsible for the transmission of apoptosis-inducing signals inside the cells. To test this hypothesis, we overexpressed COOH-terminally truncated occludin in HeLa cells (Fig. 1C). However, the deletion mutants, which did not show membrane localization by determining fluorescent detection of the cells, had no effect on increasing cell sensitivity to the various apoptotic inducers (Fig. 1C, right), suggesting that the 44 aa at the COOH-terminal end is necessary for membrane homing and regulation of apoptotic sensitivity.

We next examined the signal transduction pathway(s) involved in the occludin-mediated apoptotic sensitization by using various specific inhibitors of kinase activities. A MAPK inhibitor (PD98059) partially but significantly abrogated the enhancement of apoptosis mediated by occludin overexpression, whereas other inhibitors, such as a p38 MAPK inhibitor (SB203580) and PI3K inhibitor (LY294002), had no effects (Fig. 1D, left). Western blot analysis also showed that protein levels of phosphorylated MAPK were decreased in HeLa-Oc^{522aa} cells challenged with or without atRA treatment (Fig. 1D, right); this down-regulation was significantly abrogated by the treatment with PD98059, but not with SB203580 and LY294002 (data not shown). The decrease of pMAPK initiated by synergistic effects of atRA and occludin (Fig. 1D) may explain

the data on the cell death sensitivities of occludin-expressing cells showing that the apoptogen-mediated cell death in HeLa-Oc^{522aa} cells was markedly enhanced in the presence of atRA (Fig. 1A). This observation suggests that occludin is associated with the MAPK signaling pathway, being consistent with our previous result (12).

Occludin overexpression abrogates tumorigenic potency *in vitro*. Our observations prompted us to investigate whether occludin expression affects the tumorigenic potency. Because anoikis (apoptosis resulting from the loss of cell matrix interaction) is a well-accepted model for determining cell survival independent of cell attachment, we next examined the effect of occludin on anoikis. DNA fragmentation analysis at 24 hours confirmed the time-dependent activation of apoptosis when cells were cultured on agarose-coated dishes (Fig. 2A). Pretreatment with siRNA targeted to occludin significantly inhibited this anoikis sensitization effect (Fig. 2B). Given the pleiotropic effects of cellular apoptosis and/or proliferation on the regulation of tumor development, we investigated the effect of occludin expression in HeLa cells on the rate of cellular proliferation. Manual cell counting every 24 hours up to day 6 following plating of equal numbers of cells did not show any significant difference in cellular proliferation or in the DNA synthesis rate of cells labeled with Ki67 (data not shown). On the other hand, colony formation assay in two- and three-dimensional cultures, a measure of cell transformation, showed a significant decrease in both the number and size of colonies in the event of occludin overexpression in a number of cell types (Fig. 2C and D). Interestingly, cellular proliferation rates assessed by Ki67 labeling indexes were not affected under any conditions (Fig. 2C and D). These results strongly suggest that occludin plays an important role

in the regulation of cellular transformation and thus potentially contributes to colony-forming capability.

Retinoic acid-induced endogenous occludin is sufficient for apoptotic sensitization. To exclude the possibility that the forced expression of occludin in the cells resulted in possible unidentified artifacts, we tried to induce endogenous occludin by treatment with retinoids, because our previous study showed that atRA could effectively induce occludin mRNA in F9 cells (24). The retinoic acid receptor α (RAR α)-selective compounds Am80 (4[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]benzoic acid) and Am580 (4(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthamido)benzoic acid), as well as atRA, could increase endogenous occludin in HeLa cells (Fig. 3A). This up-regulation of occludin mediated by these retinoids could increase the sensitivity to H₂O₂-induced apoptosis (Fig. 3B), which resulted in significant abrogation of colony formation in two- and three-dimensional cultures (Fig. 3C). This effect was not cell-type specific, because we obtained similar data from four different cell types. Importantly, atRA-induced increase of apoptotic sensitivity was partially but significantly suppressed by siRNA-mediated silencing of occludin (Fig. 3B), suggesting that the observed phenotypes (Fig. 3B and C) were due to endogenous occludin expression on the cells. RAR antagonists, retinoid X receptor (RXR) agonists, and RXR antagonists did not show significant differences compared with the control (data not shown), demonstrating that RAR α is preferentially involved in the occludin expression.

Occludin overexpression decreases invasiveness and cell motility *in vitro*. We next determined whether occludin expression in cancer cell had a causal role in its invasive and

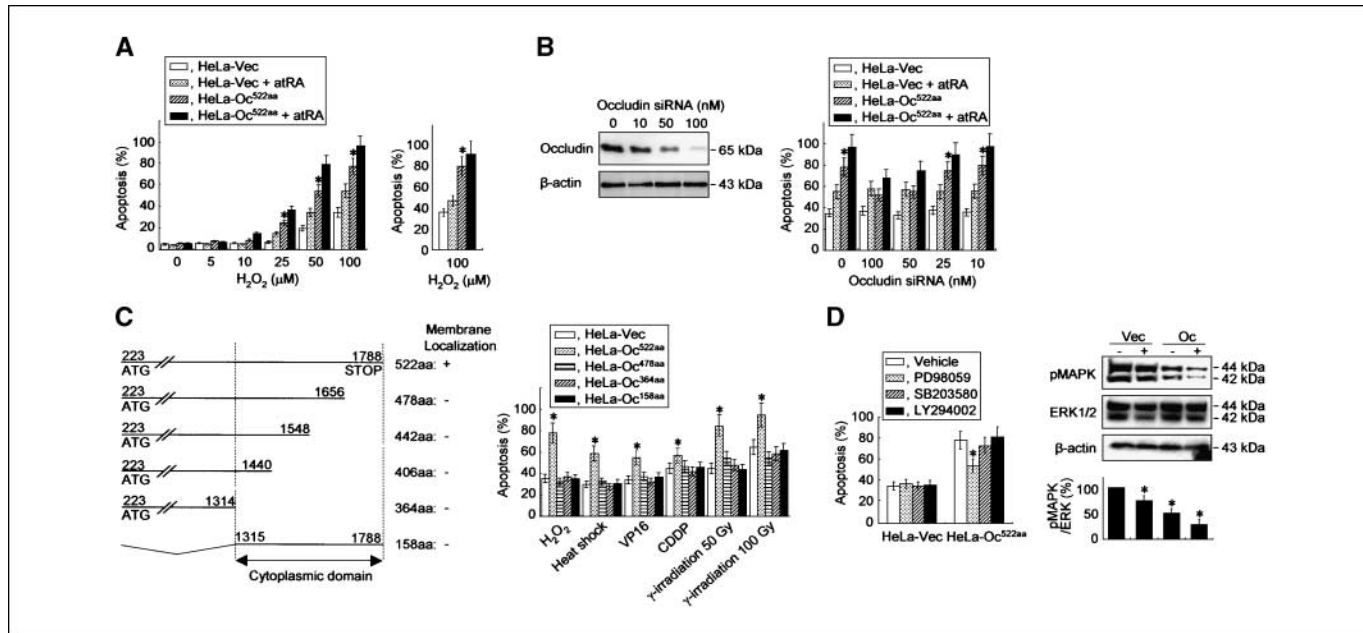
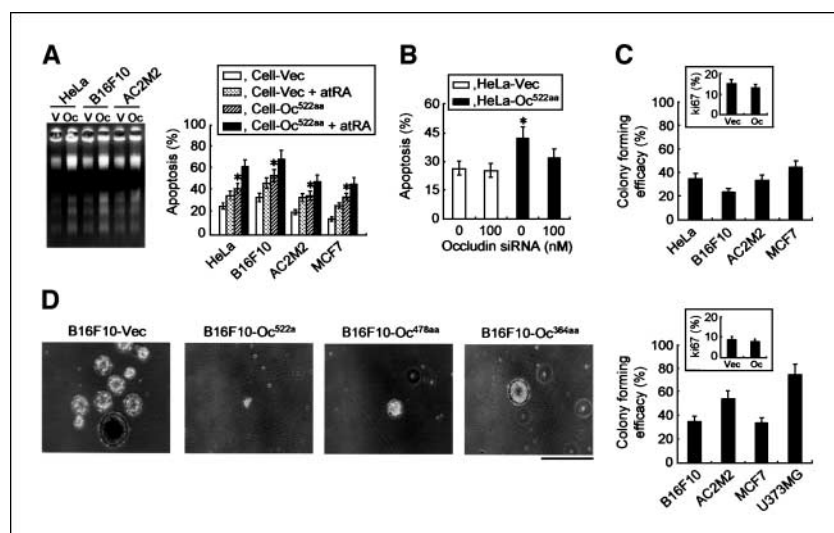


Figure 1. Forced expression of occludin promotes apoptosis. *A*, cell death of HeLa-Vec and HeLa-Oc^{522aa} cells was examined by two different assays (*left*, diphenylamine method used in this study; *right*, TUNEL assay) after 24 hours in the presence or absence of various concentrations of H₂O₂ with or without 1 μ mol/L atRA treatment. *B*, siRNA targeted to occludin abrogated the apoptotic sensitization effects of occludin with or without 1 μ mol/L atRA treatment in the presence of 100 μ mol/L H₂O₂ (*right*). Western blot analysis confirmed the silencing effects of siRNA (*left*). *C*, constructs of deletion mutants to identify the gene locus responsible for the apoptotic sensitization effect (*left*). Membrane localizations were determined by distribution of enhanced green fluorescent protein (EGFP) signals under fluorescent microscopy after transient transfection with each deletion mutant linked to EGFP. Cell death was examined with a number of apoptotic inducers in HeLa cells transfected with various deletion mutants (*right*). *D*, effects of three types of kinase inhibitors on apoptosis. The cells were treated with PD98059, SB203580, or LY294002 for 24 hours before the apoptotic stimulation, and apoptosis was stimulated with 100 μ mol/L H₂O₂ for 24 hours (*left*). Western blot analysis of pMAPK and ERK1/2 treated for 24 hours with (+) or without (-) 1 μ mol/L atRA (*right*) in HeLa-Vec (Vec) and HeLa-Oc^{522aa} (Oc) cells. The signals of pMAPK (p44) are normalized to the expression of ERK1/2 (p44) in each right panel and are defined as 100% in HeLa-Vec cells without the treatment. *, $P < 0.05$ versus control cells.

Figure 2. Occludin expression suppresses the tumorigenicity *in vitro*. **A** and **B**, anoikis was induced after adding cells to agarose-coated dishes to avoid cell attachment. DNA laddering analysis to confirm apoptotic cell death (**A**, left). Quantification of anoikis in the presence or absence of 1 $\mu\text{mol/L}$ atRA as an apoptotic sensitizer in wt occludin (Oc^{522aa})–expressing various cancer cells (**A**, right), and siRNA effect in HeLa cells (**B**). siRNA (100 nmol/L) was transfected 48 hours before anoikis induction. **C** and **D**, occludin expression suppresses colony formation in two-dimensional (**C**) or three-dimensional (**D**) cultures of indicated cells. Quantifications of colonies in occludin^{522aa} (Oc)–expressing cells and representative result of this assay (**D**, left). The number of colonies formed from control cells was defined as 100%. Insets, Ki67 labeling indexes in B16F10 cells. Bar, 250 μm . *, $P < 0.05$ versus control cells.



motile properties. Employing a Boyden chamber invasion assay using Matrigel and a transmigration assay through an endothelial monolayer, we observed significant decreases in the invasive capacity of occludin-expressing cells compared with the control cells (Fig. 4A and B). Interestingly, no deletion mutant showed any effect in this assay, supporting the notion that the COOH-terminal end is important for the occludin function. A gelatin zymogram for MMP activation showed a decrease in MMP activity caused by occludin overexpression with the synergistic effects of atRA (Fig. 4C). In addition, a monolayer wound-healing assay revealed that occludin expression significantly inhibited cellular migratory activities (Fig. 4D). These data suggest that occludin expression contributes to the abrogation of highly invasive and motile phenotypes of cancer cells.

Occludin expression inhibits tumor development and metastasis *in vivo*. Based on these *in vitro* observations, we postulated that occludin expression affected the tumorigenicity *in vivo*. We next investigated the tumor-forming capabilities of occludin-transfected cells in a syngenic mouse transplantation model. Occludin expression was sufficient to abrogate *in vivo* tumorigenicity, showing a markedly decelerated rate of tumor formation (Fig. 5A and B). We also assessed the effect of occludin on metastatic potency by screening the mice for lung lesions. No macroscopic metastatic lesion was observed in any setting; however, histologic examination showed that occludin expression showed significant inhibition of the metastatic lesion formation in the lung (Fig. 5C).

Occludin expression is epigenetically inactivated by methylation. Aberrant promoter region CpG island hypermethylation is associated with transcriptional silencing of many genes in a wide variety of cancers (25). In addition, CpG island of the occludin promoter is densely methylated in human hepatocellular carcinoma (26), and histone diacetylase inhibitors up-regulate the expression of TJ proteins, contributing to cellular differentiation (27). Indeed, CpG islands exist in the promoter regions both of human and murine occludin.³ We then did MSP analysis and found that CpG islands in the occludin promoter region were densely methylated in B16F10 cells (Fig. 6A). AC2M2 was partially

methyated in this region and endogenous occludin expression was detected (Fig. 6A). Treatment with 5'-Aza-dC and trichostatin A (28) could induce reexpression of occludin (Fig. 6A, bottom). This synergistic effect was sufficient for apoptotic sensitization (Fig. 6B) and suppression of colony-forming capabilities in two- and three-dimensional cultures (Fig. 6C). We next used siRNA-mediated silencing of occludin on the cells to examine whether the observed phenotypes induced by 5'-Aza-dC/trichostatin A treatment was due to reexpression of endogenous occludin, because 5-Aza-dC/trichostatin A treatment leads to the derepression of a large number of genes (28). Transfection with occludin-specific siRNA partially but significantly abrogated the 5'-Aza-dC/trichostatin A-mediated increase of apoptosis (Fig. 6B), making it possible to link the induction of endogenous occludin with the observed phenotypes (Fig. 6C). These data suggest that epigenetic silencing of occludin is, at least in part, involved in the tumor development.

Occludin overexpression affects multiple signaling pathways. To explore the effects of occludin expression, we screened gene expression profiles with the GEArray system (an apoptosis pathway-specific gene array). Occludin expression clearly affected a wide variety of genes associated with the components of the apoptotic machinery (Fig. 6D). Changes in patterns of gene expression were consistent with those of B16F10 and AC2M2 cells (data not shown). Although occludin overexpression induced up-regulation of apoptosis-inducing genes, such as *bax*, and, conversely, down-regulation of apoptosis-inhibitory genes, such as *bcl-2* and *survivin*, we observed more marked changes in the set of caspase family genes, which are involved in apoptosis-executing pathways. Occludin expression was also associated with a number of tumor necrosis factor (TNF) receptor family members, TNF receptor-associated factor family members, and *p53* and *ATM* pathway genes. These gene expression signatures may be sufficient to explain the altered phenotypes mediated by occludin expression.

Discussion

Here, we show that occludin overexpression in cancer cells significantly enhanced the sensitivity to differently triggered apoptotic stimuli and thus suppressed tumor development. Apoptosis is an evolutionarily conserved process in the regulation of tissue homeostasis, and numerous reports have documented

³ <http://cpgislands.usc.edu>.

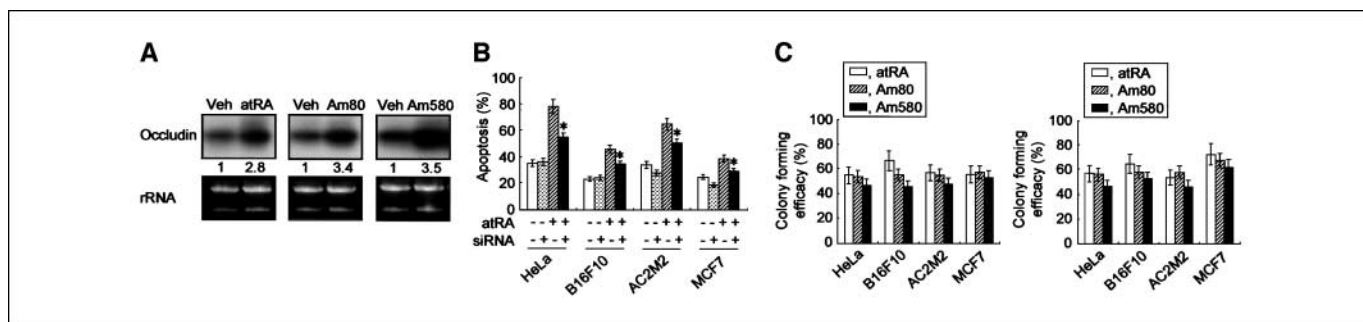


Figure 3. RAR α agonists preferentially induce endogenous occludin. *A*, endogenous occludin expression is preferentially induced by RAR α stimulation such as 1 μ M atRA, 100 nmol/L Am80, or 100 nmol/L Am580 for 24 hours. Quantification of expression levels is shown below the gel image. *B*, cells were pretreated with (+) or without (-) 1 μ M atRA 24 hours before apoptotic stimulation. The cell deaths were quantitated after 24 hours in the presence of 100 μ M H₂O₂ in combination with (+) or without (-) 100 nmol/L occludin-specific siRNA transfection. *, $P < 0.05$ versus cells without siRNA transfection. *C*, colony forming efficacy was evaluated after 24 hours treatment with 1 μ M atRA, 100 nmol/L Am80, or 100 nmol/L Am580 in two-dimensional (*left*) and three-dimensional (*right*) cultures. The cells pretreated with DMSO (vehicle) were used as a control and defined as 100% for colony forming efficacy.

that loss of responsiveness to apoptosis is a contributing factor for a malignant phenotype, because it is widely believed that impaired or decreased susceptibility to respond to various apoptotic signals is associated with oncogenic transformation (29–32). Therefore, it is clear that loss of occludin expression contributes to the acquisition of apoptotic resistance of cells and thus plays a key role in the carcinogenic process. Occludin also suppressed highly progressive cancer phenotypes, such as invasive and motile properties, resulting in an important contribution to the metastatic suppression, because the process of metastasis involves an intricate interplay between altered cell adhesion, survival, proteolysis, migration, and homing on target organs (33). Our results thus show that loss of occludin expression favors multiple steps known to be important in tumorigenesis and metastasis. We could not exclude the possibility that forced expression of occludin in cancer cells might have some interesting but as yet unidentified effects on the cells. However, our results show that RAR α stimulants preferentially up-regulate endogenous occludin, which is sufficient for the acquisition of appreciable apoptotic sensitivity. This mechanism is partially explained by our preliminary study of the occludin promoter using ~2.5 kb upstream from the transcription initiation codon, suggesting that RAR α agonists stimulate occludin expression at the transcriptional level.⁴

Gene expression profiles show that a large number of signal transduction molecules are associated with the promotion of apoptotic cell death due to occludin overexpression. Given the extensive range of physiologic activities of TJs, it is not surprising that occludin affects a vast array of signaling pathways in a given cell and tissue (34). Our study using deletion mutants of occludin shows evidence that 44 aa at the COOH-terminal end have an important role in receiving and transmitting signals to the cell interior to modulate cell behavior. This observation is consistent with a number of reports showing that the components of lipid signaling pathways, such as MAPK, PI3K, and Akt, interact with the COOH-terminal region of occludin and mediate cell growth and differentiation (35–37). Indeed, we predicted four putative phosphorylation sites (two tyrosines and two serines) by MAPK in this region, whereas further mutation study will be required for the identification of the responsible residue(s). According to our observations, it is possible to accept that occludin is not a simple

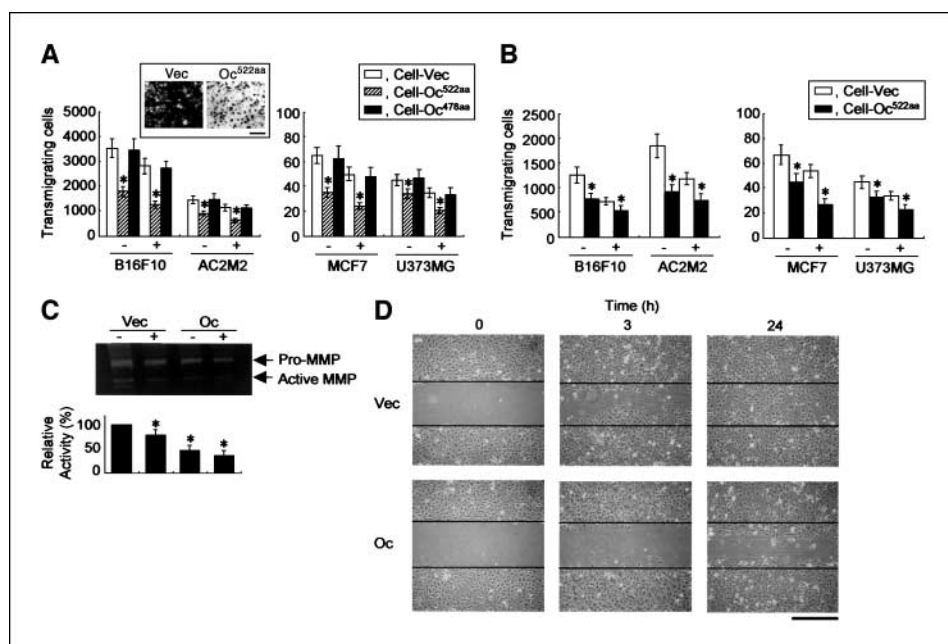
static constituent of TJ, but is involved in the signal transduction pathway(s) regulating cell survival, eventually acting as a signal transmitter for the cells through the TJs. This notion is also supported by recent evidence showing that occludin is associated with the transforming growth factor- β signaling (38) and apoptotic machinery in murine hepatocytes (12). On the other hand, one group has documented that reexpression of *claudin-1*, a TJ-associated gene, induces apoptosis in breast tumor spheroids (19). Based on the observation that breast cancer spheroids transfected with *claudin-1* grown in suspension showed massive apoptosis but no difference in cell death characteristics in two-dimensional culture, it was considered that TJ protein claudin-1 in cancer cells formed diffusion barriers and restricted the access of nutrients and growth factors inside the spheroids. Because we observed significantly different cell death sensitivities even in adherent cells, we can exclude their proposed mechanism for why occludin expression suppresses tumorigenicity.

It has been shown that occludin has a potent inhibitory effect on Raf1-induced tumorigenesis and the second extracellular domain of occludin appears to be critical for this function in Pa4-Raf1, a normal rat parotid acinar cell line transfected with oncogenic *raf-1* (16). We cannot explain these discrepant observations; however, one possible explanation is based on the different cell type used in that study. For example, Pa4 cells, parental cells of Pa4-Raf1, have a highly differentiated phenotype to form tight colonies of polarized epithelial cells, and in Pa4-Raf1 only one oncogene was introduced. On the other hand, the cells in this study were characterized by their tumorigenicity and metastatic potency. Thus, it is reasonable to believe that these cells have multiple genetic alterations because fully transformed phenotypes of cancer require multiple oncogenic hits. In accordance with our explanation of cell differentiation, our cells did not show epithelial polarization and did not form an epithelial monolayer without satisfactory levels of endogenous occludin expression. This cellular phenotype enables us to perform a permeability study after occludin transfection because occludin was first identified as an essential constituent for TJ (3), and this morphologic property is not sufficient to achieve the functions of extracellular domains of occludin due to the lack of cell-cell contacts.

Promoter CpG island methylation is an important means to transcriptionally silence a particular gene associated with a carcinogenic step. Our results show that occludin expression was epigenetically inactivated by DNA methylation. Occludin is located in the chromosome 5q13 locus in the human, and cytologic

⁴ Unpublished result.

Figure 4. Occludin suppresses cellular invasion and motility *in vitro*. **A**, occludin expression suppresses invasive capacity in cancer cells, as assessed by Boyden chamber invasion assay using 24-well cell culture inserts precoated with Matrigel matrix. Invading cells were quantitated in various cell lines with (+) or without (-) 1 $\mu\text{mol/L}$ aTRA treatment. *Insets*, representative result in B16F10 cells. **B**, transmigration assay of wt occludin ($\text{Oc}^{522\text{aa}}$)-expressing cancer cells. Quantification of cells that transmigrated through the endothelial monolayer pretreated with (+) or without (-) doxycycline. **C**, gelatin zymography for determination of MMP activity with (+) or without (-) 1 $\mu\text{mol/L}$ aTRA treatment in B16F10-Vec (*Vec*) and B16F10- $\text{Oc}^{522\text{aa}}$ (*Oc*) cells. Quantification of MMP activity shows that occludin and aTRA synergistically decrease MMP activity. **D**, cell migration by monolayer wound healing assay in B16F10-Vec and B16F10- $\text{Oc}^{522\text{aa}}$ cells. Photographs were obtained 0, 3, and 24 hours after standard scrape wounding. *Dotted lines*, front of the initial wound. Bars, 200 μm (**A**); 1 mm (**D**). *, $P < 0.05$ versus control cells.



abnormalities, such as loss of heterozygosity and deletion, have been observed in the neighboring chromosomal regions in various cancers, including hematopoietic malignancies, cancer arising in the lung, colon, thyroid, brain, bone, and nasopharyngeal organs (39–41). Although this evidence implies that there are other mechanisms silencing occludin in certain types of cancer, our observations provide strong evidence for our previous finding that loss of occludin expression correlates with cancer progression (13).

Because of the strong differentiation-inducing effects on cancer cells, retinoids show promise in the treatment and chemoprevention of epithelial carcinogenesis and in cancer differentiation therapy (42, 43). Their strong chemopreventative effects are thought to be achieved by complex mechanisms, including antiproliferative activity, apoptosis-sensitization effect, antiangiogenic activity, and inhibition of metastasis (21). In this study, we clearly verified the contribution of occludin to the antitumor activities of retinoids

in vitro. If natural or synthetic retinoids satisfactorily up-regulate occludin expression *in vivo*, it is reasonable to propose that retinoid-induced chemoprevention is partially mediated by the induction of endogenous occludin in the target cells. Other TJ-associated genes, such as *claudin-1* and *claudin-4*, are also reported to be involved in the carcinogenic process (17–19). In addition, we found that either the synergy with a demethylator and histone diacetylase inhibitor or a retinoid that stimulated RAR α could cause expression of endogenous occludin, resulting in the acquisition of higher apoptotic sensitivity. These results suggest the possible use of TJ-associated genes as potential biomarkers for cancer progression and may pave the way to new therapeutic modalities.

Our present study could not provide a significant insight for explaining the complex pathogenesis observed in occludin-deficient animals (7). However, we clearly show that many of the genes involved in apoptotic pathways are modulated to favor cell death

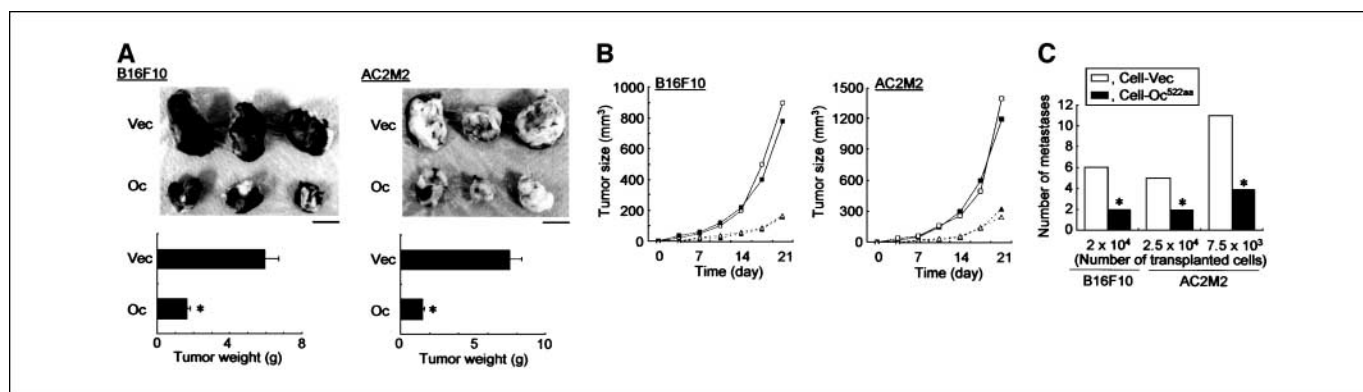


Figure 5. Occludin expression suppresses tumorigenicity and lung metastasis *in vivo*. **A** and **B**, B16F10 and AC2M2 cells transfected with occludin^{522aa} (*Oc*) grow more slowly than controls (*Vec*) in allograft transplantation models. **A**, representative developed tumors and their weights. Tumor volume was calculated in two dimensions in a time-dependent manner after inoculation of the vector (*square symbol* and *solid line*) or occludin^{522aa}-transfected (*triangle symbol* and *dotted line*) cells in the first (*filled*) and second (*open*) lines of experiments (**B**). Bar, 1 cm. **C**, quantitative analysis of lung metastases in mice following transplantation of B16F10 and AC2M2 cells transfected with vector (*Vec*) and wt occludin [$\text{Oc}^{522\text{aa}}$, B16F10 (2×10^4 cells per animal): Vec, $n = 8$; Oc, $n = 10$; AC2M2 (2.5×10^4 cells per animal): Vec, $n = 16$; Oc, $n = 15$; AC2M2 (7.5×10^3 cells per animal): Vec, $n = 8$; Oc, $n = 12$]. Mice were sacrificed after 21 days in B16F10 cells, 34 ± 2.1 days in AC2M2-Vec, and 37 ± 2.8 days in AC2M2- $\text{Oc}^{522\text{aa}}$ cells postinoculation. *, $P < 0.05$ versus control group.

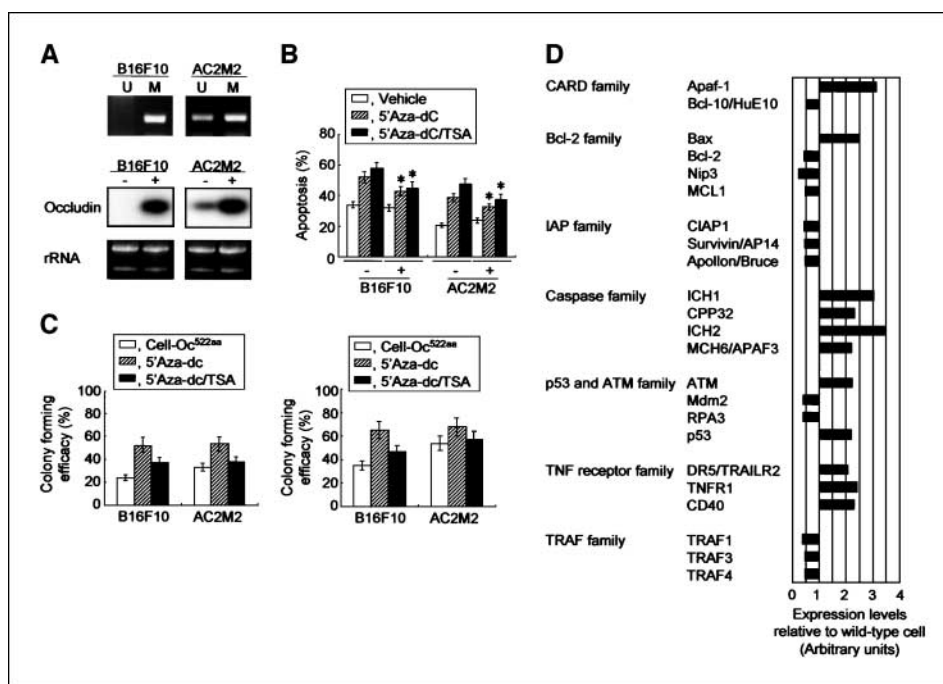


Figure 6. Occludin expression is epigenetically silenced by CpG island promoter methylation and affects multifaceted signaling pathways. *A* and *B*, MSP analysis to detect the unmethylated (*U*) and methylated (*M*) alleles on CpG islands of the occludin promoter (*A*, top). Synergy with (+) or without (–) 1 μ mol/L 5'-Aza-dC and 100 nmol/L trichostatin A (TSA) induces endogenous occludin (*A*, bottom), which is sufficient for apoptotic sensitization in the presence (+) or absence (–) of 100 nmol/L occludin-specific siRNA (*B*). *C*, colony-forming efficacy after either 5'-Aza-dC (1 μ mol/L) alone or 5'-Aza-dC (1 μ mol/L) and trichostatin A (100 nmol/L) treatment in two-dimensional (*left*) and three-dimensional (*right*) cultures. Occludin^{522aa}-overexpressing cells (*Cell-Oc^{522aa}*) were used as a control. *D*, gene expression profile in occludin-overexpressing HeLa cells in an apoptosis pathway-specific array. We identified a number of genes that increased or decreased by an average change of $\geq 100\%$ or $\leq -100\%$ ($n = 2$), which are shown in the graph in functional gene groupings.

when occludin is overexpressed, and thus contribute to the significant suppression of tumorigenic and metastatic potencies. Our observation suggests that occludin is a likely candidate for a tumor-suppressor gene in certain types of cancer. Further studies along this line will address the detailed molecular mechanism by which occludin abrogates the aggressiveness of cancer cells. We believe that strategies that reexpress endogenous occludin in cancer may be an important means to increase the sensitivity of cancer cells to apoptosis-inducing therapies.

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Epigenetic Silencing of Occludin Promotes Tumorigenic and Metastatic Properties of Cancer Cells via Modulations of Unique Sets of Apoptosis-Associated Genes

Makoto Osanai, Masaki Murata, Nami Nishikiori, et al.

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