Epigenetic silencing of claudin-6 promotes anchorage-independent growth of breast carcinoma cells

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Abstract

Cancer cells often exhibit loss of functional tight junctions (TJs), and disruption of the TJ structure is associated with cancer development. However, whether loss of a certain type of claudin, an integral membrane protein of TJs, is involved in malignant phenotypes remains to be clarified. Based on the report that claudin-6 functions as a tumor suppressor for breast cancer, we show here that suppression of claudin-6 expression results in increased resistance to various apoptogens, and causally enhances anchorage-independent growth properties. Since claudin-6 expression is partially silenced by promoter CpG island hypermethylation in MCF7 breast carcinoma cells, a synergistic effect of a demethylator and histone deacetylase inhibitor upregulates the expression of endogenous claudin-6, which is sufficient for apoptotic sensitization and abrogation of colony-forming efficacy. In addition, decreased expression of claudin-6 promotes cellular invasiveness and transendothelial migration, accompanied by an increase in matrix metalloproteinase activity. These data suggest that the methylator phenotype of claudin-6 may at least partially contribute to enhanced tumorigenic and invasive properties of breast carcinoma cells.

Introduction

Tight junctions (TJs) are intercellular structures in epithelial and endothelial cells, primarily playing a critical role in cell-cell adhesion.^(1,2) Recent evidence has revealed that TJs are directly involved in the regulation of cellular functions such as proliferation, differentiation, and apoptosis, due to the ability of TJ proteins to recruit various signaling molecules that have proliferative and differentiative capacities, including transcription factors, lipid phosphatases, and cell cycle regulators.⁽¹⁻³⁾ While one of the first identified TJ-associated molecules was occludin,^(4,5) claudins, of which there are at least 24, have been shown to be the main constituents as integral membrane proteins of TJs.^(1,2)

A growing body of evidence has demonstrated that cancer cells, particularly in those tumors that manifest high metastatic potential, often exhibit loss of functional TJs.⁽⁶⁾ Disruption of the TJ structure has been shown to be associated with cancer development, which may be causally involved in malignant phenotypes such as local tumor growth, invasion, and metastasis at distant sites.⁽⁶⁻⁸⁾ We have also demonstrated that epigenetic silencing of occludin contributes to enhanced tumorigenicity of cancer cells.^(9,10) In parallel, 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.^(11,12) In addition, a number of immunohistochemical studies showed reduced expression of claudins in a variety of cancer tissues.^(11,13,14) Many members of the claudin family show a distinct organ-specific distribution of such expression and are abnormally regulated in several human cancers; however, the functional roles of claudins in cancer cells have not been clearly defined.

Based on the finding that claudin-6 is preferentially expressed in mammary epithelial cells and functions as a tumor suppressor for breast cancer,⁽¹⁵⁾ we examined whether loss of

claudin-6 had any effect on MCF7 breast carcinoma cells. Herein, we clearly show that the knockdown of endogenous claudin-6 in MCF7 cells resulted in increased resistance to a number of differently acting apoptogens, and causally enhanced anchorage-independent growth. These findings support our plausible hypothesis that the decreased expression of claudin-6 may at least partially contribute to enhanced tumorigenicity of breast carcinoma cells.

Materials and Methods

Cell line and transfection. MCF7, a human breast carcinoma cell line, was maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Sanko Jyunyaku, Tokyo, Japan), 10 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin. Claudin-6-specific small interfering RNA (siRNA, 10 nM as a default concentration in the dose range of 10-100 nM; QIAGEN, Tokyo, Japan) was transfected into the cells 48 h before apoptotic stimulation, using FuGENE 6 (Roche, Indianapolis, IN). Negative control siRNA (Santa Cruz Biotechnologies, Santa Cruz, CA) was also transfected. siRNA-mediated suppression of claudin-6 expression was confirmed by quantitative reverse transcription (RT)-PCR and western blot analysis.

Cell death analyses. Apoptosis was stimulated with oxidative stress using H₂O₂ (Sigma, Tokyo, Japan) in the dose range from 0 to 100 µM with cells treated for 1 h to 24 h, and various death-inducing agents, including heat shock at 42°C for 30 min, etoposide (50 µM), cisplatin (10 µg/ml), and γ-irradiation (100 Gy). Anoikis was induced by preventing cells from adhering to the cell culture dishes.⁽⁹⁾ In some experiments, cells were transfected with or without claudin-6-specific siRNA in the presence or absence of all-*trans* retinoic acid (atRA, 100 nM, Sigma), which is an apoptotic sensitizer.⁽¹⁶⁾ To examine the effect of a demethylating agent, the cells were treated with 5'-aza-2'-deoxycytidine (5'Aza-dC, 1 µM as a default concentration in the dose range from 0-5 µM, Sigma) for 48 h, after which a histone deacetylase inhibitor (HDAI) or trichostatin A (TSA, 100 nM as a default concentration in the dose range from 0-300 nM, Sigma) was added, followed by incubation for an additional 24 h at 37°C. Apoptotic cell death was evaluated as described.^(9,16) Briefly, total cells in the

tissue culture wells were harvested, and resuspended in lysis buffer containing 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5% Triton X-100 for 15 min at 4°C. The cells were then centrifuged at 12,000 xg for 20 min at 4°C to separate intact chromatin in the pellet from DNA fragments in the supernatant that preferentially contained low molecular weight cellular DNA.⁽⁹⁾ The DNA amounts in each pellet and supernatant were determined using a diphenylamine reagent and the fragmentation rate (%) was expressed as (fragmented DNA / total DNA) x 100. DNA samples were also electrophoresed on 2.5% agarose gels to show the ladder formation due to nuclear fragmentation. To confirm the quantitiveness of cell death, we also performed the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay using an In Situ Cell Death Detection Kit (Roche). The positive cells were scored using light microscopy by counting the number of cells under low magnification (x 100) in 10 separate arbitrarily selected fields in each section.

Quantitative RT-PCR analysis. Total RNA (1 µg) extracted by TRIzol (Invitrogen) 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 parameters for each PCR experiment to define optimal conditions for linearity to allow for quantitative analysis of signal intensity. Triplicate independent PCR reactions were carried out to ensure the reproducibility of expression quantification. For the densitometric analysis, signals were quantitated using Scion Image 1.62 (Scion Corporation, Frederick, MD). Oligonucleotide sequences of all primers used for the PCR reactions are available on request.

Colony forming assay in two- and three-dimensional cultures. To assess a colony forming efficacy, cells (5×10^5 cells/well) transfected with or without claudin-6-specific siRNA were plated on 6-well plates 24 h prior to exposure to a lethal dose of γ -irradiation (>100 Gy). A small fraction of cells could survive and these were cultured for at least 7 days to form separate colonies (two-dimensional cultures). For a soft agar assay (three-dimensional cultures), cells (1×10^4 in 6 cm dishes) 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. After incubation for at least 5 weeks, colonies developed in soft agar suspension were scored using phase-contrast microscopy by counting colony numbers under low magnification (x 100) in 10 separate arbitrary fields in each plate. Cell clusters of more than approximately 100 μm in diameter were defined as positive results.

***In vitro* invasion assay and transmigration assay.** A cell suspension (5×10^5 in 0.5 ml of DMEM with 0.5% FBS) was added to 24-well cell culture inserts with 8 μm pores (Becton Dickinson, Franklin Lakes, NJ) precoated with Matrigel (Invitrogen), while DMEM containing 10% FBS was added to the bottom chamber to create a chemotactic gradient. Invasive cells were measured after 48 h 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 the well were quantitated. Similarly, transmigration assay through a rat lung endothelial cell (RLE) monolayer was done as described.⁽¹⁷⁾ Transmigrated viable cells on the lower surface of the transwell (8 μm pores) were quantitated after harvesting the cancer cells on the endothelial monolayer for 24 h.

***In vitro* adhesion assay of the endothelial monolayer.** We performed adhesion assay of cancer cells in the media supplemented with 10% FBS with or without claudin-6-specific siRNA in the presence or absence of 1 μ M atRA. MCF7 cells were transfected with a pEGFP-C1 vector (Invitrogen) to facilitate the fluorescent detection of adherent cells 48 h before the assay, and were overlaid onto an RLE endothelial monolayer. After washing with PBS to remove nonadherent cells, cells with green signals were counted by fluorescent microscopy.

Gelatin zymography. The gelatinolytic activity of matrix metalloproteinases (MMPs) was determined in the supernatants of MCF7 cells treated with or without 1 μ M atRA for 24 h in the presence or absence of claudin-6 siRNA, using a standard protocol. Clear bands appeared on the background in the areas of gelatinolytic activity.

Methylation-specific PCR (MSP). The methylation status of the promoter CpG islands of claudin-6 was analyzed by MSP on sodium bisulfite-converted DNA.^(9,10,18) Briefly, 2 μ g of genomic DNA was denatured with 2 M NaOH for 10 min, followed by incubation with 3 M sodium bisulfite (pH 5.0) for 16 h 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' - AGGTGAGTTCCCCATGTCAC -3' and 5' - CCGAAGGACCCTATCACCTC -3' for the amplification of methylated alleles; 5' - TGTGAAGGTGGTTGGTGTGT -3' and 5' - CCCACCCCATATTCATCCTA -3' for the amplification of unmethylated alleles.

Statistical analysis. Unless otherwise specified, all data represent the mean $\pm$ SD of at least 3 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

Claudin-6 expression is partially suppressed by CpG island hypermethylation. Aberrant promoter region CpG island hypermethylation is associated with transcriptional silencing of many genes in a wide variety of cancers.⁽¹⁹⁾ Our previous studies have demonstrated that the synergistic effect of a demethylator (5'Aza-dC) and HDAC inhibitor (TSA) upregulates the expression of endogenous occludin, which eventually enhances the apoptotic sensitivity to various apoptogens.^(9,10) Therefore, we first examined whether treatment with the demethylator and HDAC inhibitor altered the expression of other TJ-associated genes. We found that treatment with 5'Aza-dC and TSA could induce upregulation of a number of TJ-associated genes such as those of occludin, claudin-1, -2, and -6 (Fig. 1a). The most striking effect by these agents was observed in the gene-expression alterations in claudin-6, in which claudin-6 mRNA was increased approximately 3-fold by treatment with 5'Aza-dC alone and 5-fold by the synergistic effect with 5'Aza-dC and TSA (Fig. 1b). These agents did not change the expression of claudin-3, -4, or -7 (Fig. 1a), and the expression of claudin-5 and -8 was not observed in MCF7 cells (data not shown). These findings suggested that claudin-6 expression was preferentially suppressed by CpG island hypermethylation in MCF7 cells.

According to a search of a genome database, CpG islands exist in the promoter region of human claudin-6. We then performed MSP analysis of MCF7 cells and found that CpG islands in the claudin-6 promoter were strongly methylated (Fig. 1c). However, MCF7 cells were partially unmethylated in this region (Fig. 1c) and, as a result, an appreciable amount of endogenous claudin-6 expression was detected (Fig. 1a, 2a).

Decreased expression of claudin-6 provides apoptotic resistance in MCF7 cells. It has been documented that claudin-6 is preferentially expressed in mammary epithelial cells and that the expression of claudin-6 is decreased in mammary cancer cell lines and breast cancer sample compared to normal breast tissue.⁽¹⁵⁾ We also found that the promoter region of claudin-6 was densely methylated in MCF7 cells (Fig. 1c). To examine the functional role of claudin-6 in MCF7 cells, we determined the differential sensitivity of apoptosis to 24 h exposure to H₂O₂, which is a common cell stressor used to induce apoptosis.^(9,16) Prior to the apoptotic stimulation, the cell medium was changed to one without FBS supplementation and cells were exposed to the apoptogens. Preliminary experiments confirmed that a wide range of concentrations of siRNA could effectively suppress endogenous claudin-6 expression (Fig. 2a, 2b). Treatment with atRA clearly enhanced the sensitivity to H₂O₂-induced cell death in dose- and time-dependent manners, whereas knockdown of claudin-6 expression provided significant resistance to apoptosis (Fig. 2c, left panel, and Fig. 2d). We further employed the TUNEL assay and showed that with decreased expression of claudin-6 the sensitivity to H₂O₂-induced cell death was suppressed (Fig. 2c, right panel), which was coincident with the results of our assay (Fig. 2c, left panel). On the other hand, manual cell counting every 24 h up to day 6 after plating equal numbers of cells showed no significant differences in the cell proliferation and DNA synthesis rates (Fig. 2e). We next determined whether the suppressive role due to loss of claudin-6 was applicable to other apoptogenic stimuli, including oxidative stress, heat shock, genotoxic agents, and γ -irradiation. Decreased expression of claudin-6 also resulted in appreciable inhibition of apoptotic cell death against these stimuli (Fig. 2f), suggesting that the protective role of claudin-6 against apoptosis could be observed following exposure to a broad range of cell stressors. These observations suggested that claudin-6 expression modulated the apoptotic sensitivity of the cells.

We could not examine the function of TJs of MCF7 cells because this cell line did not form a uniformly flat epithelial monolayer sheet on the semipermeable membrane support. Nor did we observe any morphological alterations in the course of the experiment when claudin-6 expression was induced or suppressed in the MCF7 cells (data not shown).

Decreased expression of claudin-6 inhibits anoikis. Evasion of and resistance to apoptosis are characteristic of cancer, and breakdown of anoikis may be a predominant contributor to oncogenic progression.^(20,21) We therefore examined whether siRNA-mediated knockdown of claudin-6 expression inhibited anoikis in MCF7 cells. DNA fragmentation analysis demonstrated that decreased expression of claudin-6 clearly showed the limited sensitivity to anoikis (Fig. 3a). TUNEL assay also revealed that atRA increased the sensitivity to anoikis in MCF7 cells, whereas siRNA-mediated knockdown of claudin-6 expression resulted in resistance to anoikis even in the presence of atRA (Fig. 3b). Suppression of endogenous claudin-6 expression in MCF7 cells provided significant resistance to H₂O₂-induced apoptosis and anoikis, causing significant promotion of colony formation in two- and three-dimensional cultures (Fig. 3c). Since claudin-6 siRNA was transfected transiently in this experiment, it was clear that endogenous expression of claudin-6 was increased with cell division. We should note here that MCF7 cells required 24 h for maximum reduction of claudin-6 after being transfected with claudin-6-specific siRNA, and significant suppression of claudin-6 was observed for 5 days (Fig. 3d). This suggested that the colony formation was primarily determined by the sensitivity to apoptosis within a few days after plating for cell culture, in which significant suppression of claudin-6 expression was observed by siRNA transfection.

Methylator phenotype of claudin-6 promotes anchorage-independent growth. We next examined whether the observed phenotypes induced by 5'Aza-dC/TSA treatment were due to enhanced expression of endogenous claudin-6, because 5-Aza-dC/TSA treatment leads to the derepression of a large number of genes.^(18,19) In MCF7 cells, either 5'Aza-dC alone and a synergistic effect with 5'Aza-dC and TSA was sufficient for apoptotic sensitization (Fig. 4a) and for suppression of colony forming capabilities in two- (Fig. 4b, left panel) and three-dimensional cultures (Fig. 4b, right panel). Importantly, claudin-6-specific siRNA significantly abrogated these effects, being possible to link the induction of endogenous claudin-6 with the observed phenotypes mediated by 5'Aza-dC/TSA. In addition, double knockdown of claudin-6 and occludin had marked effects on apoptosis (Fig. 4a) and colony-forming efficacy (Fig. 4b), when compared with the cells transfected only with claudin-6-specific siRNA.^(9,10) Negative control siRNA had no effect on the cells during the course of the experiment (data not shown). These observations suggested that epigenetic silencing of claudin-6 may partially but significantly contribute to enhanced anchorage-independent growth of cancer cells.

Claudin-6 decreases invasiveness and transmigration through endothelial monolayer.

To determine whether claudin-6 expression in cancer cells had a causal role in its invasive and transmigrative properties, we performed a Boyden chamber invasion assay and a transmigration assay through an endothelial monolayer. We found that with decreased expression of claudin-6 invasive and transmigrative capacities of MCF7 cells were significantly increased, when compared with the control cells (Fig. 5a, 5b). A gelatin zymogram for MMP activation also demonstrated that claudin-6 knockdown resulted in an increase in MMP activity and efficiently abrogated atRA-mediated downregulation of MMP

activity (Fig. 5c). In addition, transfection with claudin-6-specific siRNA increased adhesion to the endothelial cells, which is known to be critical step for cancer cells to form a distant metastasis (Fig. 5d). These data suggested that loss of claudin-6 expression was potentially associated with the invasive and transmigrative phenotypes of cancer cells.

Discussion

We have demonstrated that the methylator phenotype of occludin favors multiple steps known to be important in tumorigenesis, identifying occludin as a likely tumor suppressor gene in certain types of cancer.^(9,10) Similarly, this study was undertaken to understand the biological significance of altered claudin-6 expression in breast cancer. We found that epigenetic silencing of claudin-6 expression potentially contributed to the highly tumorigenic and invasive properties of MCF7 breast carcinoma cells.

Promoter CpG island hypermethylation is an important means to epigenetically silence a particular gene associated with a carcinogenic step at the transcriptional level.⁽¹⁹⁾ Our results showed that claudin-6 expression was partially but significantly inactivated by aberrant CpG island DNA hypermethylation in its promoter region. The impact of our findings on breast cancer might be limited since the silencing of claudin-6 by DNA methylation was only shown in one breast cancer cell line, MCF7, and the loss of claudin-6 expression has not been demonstrated to occur during human breast cancer progression. However, the expression of claudin-6 in mammary epithelial cells has been shown to be decreased in a different number of mammary cancer cell lines and breast cancer sample compared to normal breast tissue.⁽¹⁵⁾ Consistent with this finding, our observations indicate the importance of claudin-6 as at least a partial regulator of breast carcinogenesis.

It is now clear that claudin is a main constituent of TJs, whereas it has been shown that certain TJ-associated proteins directly associate with different signal transduction molecules that have functions in receiving or transmitting signals such as atypical protein kinase C and Rho proteins, and participate in the control of gene expression via the modulation of transcription machinery in the nucleus, implying that TJ-associated molecules

are signal transmitters located at TJs.⁽¹⁻³⁾ Other TJ proteins also associate with Fos, Jun, CCAAT/enhancer binding protein, and activator protein-1, demonstrating that TJs have a direct functional association with a variety of signal transduction pathways.⁽¹⁻³⁾ Considering this view, the possible mechanisms of observed phenotypes mediated by the decreased expression of claudin-6 in breast cancers might be primarily explained by signal transduction from membrane-anchored claudin-6 to the nucleus, and eventually claudin-6 might have direct associations with a different type of signaling molecule in the cells. It is thus reasonable to believe that the certain TJ-associated genes such as claudin-6 and occludin may be committed to identical signaling pathways and have functional links under the control of common signaling components in the cells, since we observed similar effects on the cancer phenotype when the expression of either occludin and claudin-6 was suppressed. This explanation is also supported by the evidence that tyrosine residues located at the carboxyl-terminal are conserved among at least 8 claudin family members, claudin-1-8, and occludin. Although claudin-4 is, for example, a substrate for EphA2 receptor tyrosine kinase,⁽²²⁾ other claudins have the potential to be phosphorylated by EphA2 due to structural homology between the TJ-associated molecules. Since EphA2 is frequently overexpressed in a variety of cancers, including breast cancers, and is a direct transcriptional target of the Ras-Raf-mitogen-activated protein kinase pathway,^(23,24) it is thus possible that the cellular transformation crucial for the progression of cancer is associated with the phosphorylation of different types of claudins, which are regulated by indistinguishable signals. The tyrosine phosphorylation of TJ-associated proteins dissociates submembrane components of TJs, and causally decreases integration of claudins into sites of cell-cell contact, consistent with the well-accepted concept that oncogenic transformation during carcinogenesis is accompanied by a disruption of TJs.

Our previous studies have demonstrated that epigenetic silencing of occludin results in cellular resistance to apoptogenic stimuli and is involved in the cellular senescence-escape program, consequently contributing to the enhanced tumorigenic, invasive, and metastatic properties of cancer cells.^(9,10) However, it is clear that apoptotic resistance, increased cell migration, and cell adhesion to endothelial cells are basically different and functionally separate cellular events. Given the extensive range of physiological activities of TJs and the functional diversity of TJ-associated genes, it is not surprising that TJ-associated molecules such as claudin-6 affect various cellular functions in a given cell. Alternatively, there may be other as yet unidentified signaling in the cells that is activated and modulated by claudin-6. No direct proof exists to support this possibility, and we cannot presently determine whether claudin-6 has specific effects on the cells when compared with the other types of claudins.

On the other hand, several studies have confirmed the paradoxical upregulation of claudins in various cancers derived from the breast, stomach, pancreas, and ovary.^(25,26) In addition, certain types of claudins have been shown to modify tumor invasion by regulation of MMP activity, suggesting the potential involvement of claudins in invasion and metastasis of cancer cells.^(27,28) Although the functional importance of claudins in cancers has been suggested by a number of studies, future studies should address the underlying mechanisms by which certain TJ proteins determine the progressiveness of cancer cells.

It has been postulated that the formation of TJs reduces the highly tumorigenic phenotype of cancer cells. A number of reports have shown that the overexpression of TJ-associated genes confers less tumorigenic and invasive phenotypes *in vivo*, accompanied by an increase in TJs between cell boundaries in certain claudin-overexpressing cells.^(7,8) These observations lead to the conclusion that TJ protein in cancer cells forms diffusion barriers and restricts the access of nutrients and growth factors inside the cell aggregates, and that an

increase in TJ-based cell-cell adhesion may inhibit the dissociation of cancer cells from the primary tumor. We can exclude the former proposed mechanism because we observed significantly different cell death sensitivities even in adherent cells. Interestingly, it is interesting to propose the novel strategy that cell-cell adhesion formed by TJs may suppress the invasiveness into cancer-surrounding tissue and the formation of distant metastases.

Our present study raises the possibility of developing claudin-6 as a potential biomarker and as a promising candidate for therapy that would abrogate the highly tumorigenic phenotype of breast cancer. Because of the high specificity of claudin expression patterns in cancer, claudins may act as useful molecular markers and prognostic indicators for many different cancers. We believe that certain TJ proteins may not only represent promising targets for tumor detection and diagnosis, but also provide new opportunities for therapeutic intervention.

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Figure legends

Figure 1. Claudin-6 expression is epigenetically silenced by CpG island promoter hypermethylation. (a) Quantitative RT-PCR analysis to examine the changes of TJ-associated genes in MCF7 cells after culture with vehicle (lane 1), 5'Aza-dC (lane 2), and 5'Aza-dC/TSA (lane 3). (b) Quantitative analysis of the gene expression of occludin and claudin-6 for independent triplicate experiments, as examined in (a). The cells treated with vehicle were used as a control, and gene expression in these cells was defined as 100%. (c) MSP analysis to detect the unmethylated (UM) and methylated (M) alleles on CpG islands of the claudin-6 promoter. *, $p < 0.05$, vs. cells with vehicle treatment.

Figure 2. Knockdown of claudin-6 provides cellular resistance to apoptosis in MCF7 cells. (a) Gene-expression alteration of claudin-6 after transfection with various concentrations of claudin-6-specific siRNA. Densitometric analyses for independent triplicate experiments are shown below the representative image. The signal of claudin-6 without the transfection was defined as 100%. (b) Western blot analysis confirmed the silencing effect of siRNA targeted to claudin-6 (+: 25 nM). (c) Left panel, cell death mediated by various concentrations of H_2O_2 after 24 h in the presence or absence of atRA with or without claudin-6-specific siRNA (Cl-6) transfection. Right panel, TUNEL assay to confirm the type of cell death and cell death rates after 24 h with or without claudin-6-specific siRNA transfection in the presence of 100 μM H_2O_2 . Negative control siRNA (Neg) had no effect on the cells. (d) Cell death in the presence (open symbol and dotted line) or absence (closed symbol and solid line) of atRA at the indicated time points up to 24 h in the presence of 100 μM H_2O_2 in combination with (circle) or without (square) claudin-6-specific siRNA transfection. (e) Cell proliferation

assay with (open symbol) or without (closed symbol) claudin-6-specific siRNA transfection for up to 6 days. Insets show the Ki67 labelling indices at day 6. (f) Cell death was quantitated after 24 h in the presence or absence of claudin-6-specific (Cl-6) or negative control (Neg) siRNA transfection with various death stimuli. Lane 1, H₂O₂ (100 μ M); lane 2, cisplatin (10 μ g/ml); lane 3, etoposide (50 μ M); lane 4, heat shock at 42°C for 30 min; lane 5, γ -irradiation (50 Gy); lane 6, γ -irradiation (100 Gy). *, $p < 0.05$, vs. cells without atRA treatment; #, $p < 0.05$, vs. cells without claudin-6 siRNA transfection.

Figure 3. Decreased expression of claudin-6 enhances colony-forming efficacy in MCF7 cells. (a, b) Anoikis was induced after adding cells to agarose-coated dishes to avoid cell attachment. DNA laddering analysis to confirm apoptotic cell death (a). Quantification of anoikis in the presence or absence of atRA with or without claudin-6-specific (Cl-6) or negative control (Neg) siRNA transfection (b). (c) Knockdown of claudin-6 expression promotes colony formation in two- (left panel) or three-dimensional (right panel) cultures. The number of colonies formed from control cells was defined as 100%. (d) Quantitative RT-PCR analysis confirmed the silencing effect of claudin-6-specific siRNA during the course of the experiment. Densitometric analyses for independent triplicate experiments are shown below the representative image. The signal of claudin-6 was defined as 100% in MCF7 cells without the transfection. *, $p < 0.05$, vs. cells without atRA treatment; #, $p < 0.05$, vs. cells without claudin-6 siRNA transfection.

Figure 4. Epigenetic silencing of claudin-6 provides resistance to apoptosis and promotes anchorage-independent growth in MCF7 cells. (a) Endogenous expression of claudin-6 induced by 5'Aza-dC alone or synergy with 5'Aza-dC and TSA is sufficient for apoptotic

sensitization. siRNA-mediated knockdown of claudin-6 (Cl-6) expression decreases apoptotic sensitivity, and double knockdown of claudin-6 (Cl-6) and occludin (Oc) synergistically enhances the anti-apoptotic effect. (b) Colony forming efficacy after either 5'Aza-dC alone or 5'Aza-dC and TSA treatment in two-dimensional (left panel) and three-dimensional (right panel) cultures. The number of colonies formed from control cells without the treatment was defined as 100%. *, $p < 0.05$, vs. cells without either 5'Aza-dC alone or 5'Aza-dC/TSA treatment; #, $p < 0.05$, vs. cells without either claudin-6 siRNA alone or claudin-6 and occludin siRNA transfection.

Figure 5. Suppression of claudin-6 expression increases cellular invasion and transmigration through an endothelial monolayer. (a) Decreased expression of claudin-6 enhances invasive capacity in MCF7 cells, as assessed by Boyden chamber invasion assay using Matrigel matrix (left panel), and its quantitative analysis of invading cells transfected with (+) or without (-) claudin-6-specific (Cl-6) or negative control (Neg) siRNA (right panel). Scale bar, 200 μm . (b) Transmigration assay in MCF7 cells that transmigrated through an endothelial monolayer, transfected with (+) or without (-) claudin-6-specific siRNA. (c) Gelatin zymography for determination of MMP activity in the presence or absence of atRA with or without claudin-6 siRNA transfection. The signals of MMP-2 (lower band) are quantitated for MMP activities and are defined as 100% in MCF7 cells without the treatment and transfection. (d) Adhesion assay of cancer cells with (filled square and solid line) or without (filled circle and dotted line) claudin-6-specific siRNA transfection to a monolayer of endothelial cells in the presence (right panel) or absence (left panel) of 1 μM atRA. *, $p < 0.05$, vs. cells without atRA treatment; #, $p < 0.05$, vs. cells without claudin-6 siRNA transfection.

a **b** **c**

a **b** **c** **d**

a **b**