

Claudin-3 and Claudin-4 Expression in Ovarian Epithelial Cells Enhances Invasion and Is Associated with Increased Matrix Metalloproteinase-2 Activity

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Abstract

Claudin proteins form a large family of integral membrane proteins crucial for tight junction formation and function. Our previous studies have revealed that claudin-3 and claudin-4 proteins are highly overexpressed in ovarian cancer. To clarify the roles of claudins in ovarian tumorigenesis, we have generated human ovarian surface epithelial (HOSE) cells constitutively expressing wild-type claudin-3 and claudin-4. Expression of these claudins in HOSE cells increased cell invasion and motility as measured by Boyden chamber assays and wound-healing experiments. Conversely, small interfering RNA (siRNA)-mediated knockdown of claudin-3 and claudin-4 expression in ovarian cancer cell lines reduced invasion. Claudin expression also increased cell survival in HOSE cells but did not significantly affect cell proliferation. Moreover, the claudin-expressing ovarian epithelial cells were found to have increased matrix metalloproteinase-2 (MMP-2) activity indicating that claudin-mediated increased invasion might be mediated through the activation of MMP proteins. However, siRNA inactivation of claudins in ovarian cancer cell lines did not have a significant effect on the high endogenous MMP-2 activity present in these cells, showing that malignant cells have alternative or additional pathways to fully activate MMP-2. Taken together, our results suggest that claudin overexpression may promote ovarian tumorigenesis and metastasis through increased invasion and survival of tumor cells. (Cancer Res 2005; 65(16): 7378-85)

Introduction

Tight junctions, the apical-most component of the intercellular junctional complex, form the primary barrier to paracellular transport of solutes across the cells. Tight junctions also play a critical role in establishing and maintaining epithelial cell polarity by acting as diffusion barrier to movement of proteins and lipids within the plasma membrane (1–3).

Claudins are the major integral membrane proteins forming the backbone of tight junctions (1, 4, 5). The claudin family consists of 23 transmembrane proteins exhibiting distinct tissue- and development-specific distribution patterns (4, 6). These proteins can form homodimers or heterodimers to produce paired strands between adjacent cells, thereby determining the characteristic permeability properties of different epithelial tissues (7). Disruption of tight junction barrier function and changes in permeability

properties have been shown to be associated with a number of pathologic conditions such as kidney disorders, inflammatory bowel disease, pulmonary edema, diarrhea, and jaundice (8–12). In addition, mutations in claudin genes are also linked to genetic disorders. For example, mutation in *CLDN14* leads to non-syndromic recessive deafness (13) and the mutated *CLDN16* gene has been associated with hereditary hypomagnesemia (14).

Modulations in tight junction structure and function has also been shown in epithelial tumorigenesis (15, 16). A SAGE study by our group previously showed that *CLDN3* and *CLDN4* are among the most highly up-regulated genes in ovarian cancer (17). Other studies have also identified claudin-3 or claudin-4 as highly up-regulated in ovarian cancer (18–21). A detailed immunohistochemical analysis of claudin-3 and claudin-4 protein expression using an ovarian tissue array recently revealed high levels of expression of these two proteins in the majority of primary ovarian tumors (22). In addition, several other studies have reported aberrant claudin expression in various cancers. Some examples include increased expression of claudin-3 and claudin-4 in prostate and uterine cancers (23, 24), high claudin-4 expression in pancreatic cancer (25, 26), down-regulation of claudin-7 in head and neck cancer (27) and metastatic breast cancer (28), and increase in claudin-3 and claudin-4 in breast cancer (29). However, the exact role of claudin overexpression and the functional importance of these proteins in development of ovarian cancer remain unclear.

The COOH-terminal region of claudin proteins contains a PDZ domain-binding motif that can potentially interact with a number of PDZ domain-containing proteins such as ZO proteins (ZO-1, ZO-2, and ZO-3; refs. 30, 31). These interactions can also serve as adapters for other proteins involved in cell signaling. A number of other cytosolic and nuclear proteins which includes regulatory proteins Rab3b, Rab13, tumor suppressors like PTEN, transcription factors like ZONAB, and HuASH1 have also been shown to interact directly or indirectly with tight junction complex (32–36). These interactions suggest that tight junctions, in addition to acting as barriers to paracellular flow of solutes, may play an important role in regulating other cell functions, such as proliferation and tumor suppression.

In the present study, we overexpressed claudin-3 and claudin-4 in immortalized normal human ovarian surface epithelial (HOSE; which lack endogenous claudin-3 and claudin-4 expression) and studied their effects on tight junction function, cell proliferation, and cell invasion. We also used small interfering RNA (siRNA) technology to knockdown claudin-3 and claudin-4 in ovarian cancer cell lines. We show for the first time that overexpression of claudin-3 and claudin-4 proteins in ovarian cells is associated with phenotypic changes, increasing survival and enhancing invasion and motility of ovarian epithelial cells. Conversely, claudin-3 and claudin-4 reduction in ovarian cancer cell lines leads to the opposite effect with respect to cell invasion. We also show that

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overexpression of claudin-3 and claudin-4 in ovarian cells also leads to activation of matrix metalloproteinase-2 (MMP-2) thus suggesting a mechanism for the increased invasive potential of claudin-expressing ovarian cells. However, siRNA inhibition of claudin in fully malignant cells did not decrease the high endogenous MMP activity present in those cells. Overall, our results suggest that claudin expression is involved in invasion and metastasis of ovarian tumors.

Materials and Methods

Cell lines. HOSE-B, a normal human ovarian surface epithelial cell line immortalized with E6 and E7 (37) was cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin), and 300 µg/mL G418 and 5 ng/mL epidermal growth factor. IOSE 80, also immortalized normal ovarian surface epithelial cells were obtained from Dr. N. Auersperg (University of British Columbia, Vancouver, Canada; ref. 38). These cells were maintained in Medium 199 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and antibiotics. The ovarian cancer cell lines HEY, OVCAR-5, and OV167 were maintained in McCoy's 5A medium supplemented with 10% FBS and antibiotics.

Expression constructs. Full-length cDNAs for claudin-3 and claudin-4 were PCR amplified from ovarian cancer cell line OVCAR-5 following RNA isolation and reverse transcription. For amplification of *CLDN3*, the primers used were AGAGAATTCACCATGTCCATGGGCTGGAGATC and GAGGTCTAGCTAGCTAGTTCCTTGGCGGTC. The primers used for *CLDN4* amplification were AGAGAATTCACCATGGCTCCATGGGGCTAC and GAGGTCTAGCTAGCTAGTTCCTTGGCGGTC. The resulting PCR fragments of claudin-3 and claudin-4 were cloned into *EcoRI* and *Sall* restriction sites of the mammalian expression vector pCIneo (Promega, Madison, WI) and the sequence of all the constructs was verified by sequencing.

Generation of cell lines stably expressing claudin-3 and claudin-4. Cells were cultured to 80% confluency and transfections were done using Fugene following the manufacturer's instructions (Roche, Indianapolis, IN). HEY, IOSE-80, and HOSE-B cells were transfected with pCIneo-CLDN4, pCIneo-CLDN3, and pCIneo-EV (empty vector) plasmids. For HOSE-B transfections, each of the vector was cotransfected with pCMV-zeo vector (Invitrogen) and transfected cells were selected with Zeocin (350 µg/mL; Invitrogen). IOSE-80 and HEY clones were selected and maintained in medium supplemented with G418 (500 µg/mL). Colonies were screened by immunoblotting analysis to identify stable clones expressing claudin-3 and claudin-4. To rule out that the effects observed were due to clonal variations, stable cell pools of HOSE and IOSE-80 cells were also established.

Small interfering RNA knockdown experiments. Claudin-3 and claudin-4 specific siRNA oligos were purchased from Ambion, Inc. (Austin, TX). OVCAR-5 and OV167 ovarian cancer cell lines were selected to study effects of claudin knockdown on invasion of ovarian cancer cells. OVCAR-5 is both claudin-3 and claudin-4 positive (22), whereas OV167 is positive for claudin-3.¹ Cells cultured in 6-well plates were transfected with siRNA duplexes using LipofectAMINE 2000 (Invitrogen) following manufacturer's instructions. Mock transfections and nonspecific siRNA duplexes were used as the negative controls. Cells were treated for 48 to 72 hours to allow maximum knockdown, after which they were either harvested for Western blot analysis or used for invasion assays. In addition, culture media collected from the siRNA-transfected cells were analyzed for MMP activity as described below.

Immunoblotting. Confluent cell cultures were washed with HBSS (Invitrogen) and whole cell lysates were made using lysis buffer: 62.5 mmol/L Tris-HCl (pH 6.8), 10% glycerol, and 2% SDS. Protein concentration was

determined using the bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL). Twenty micrograms of total proteins were separated by 10% to 20% SDS-PAGE on Tris-Glycine gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). The membranes were blocked with 5% nonfat dry milk, washed in TBST buffer, and probed with the primary antibody at the following dilutions: anti-claudin-3, 1:200; anti-claudin-4, 1:250 (Zymed, San Francisco, CA). The blots were then washed and incubated in horseradish peroxidase-conjugated secondary antibody (anti-mouse or anti-rabbit IgG: 1:10,000; Amersham Pharmacia Biotech, Piscataway, NJ). For detection, enhanced chemiluminescence was carried out using the enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

Transepithelial electric resistance measurements. Cells were plated at a density of 1×10^5 per well in 12-well Transwell filters with a membrane pore size of 0.4 µm (Corning Costar, Cambridge, MA). Transepithelial electric resistance (TER) was measured using a Millicell-ERS epithelial volt ohmmeter (World Precision Instruments, New Haven, CT). Readings were taken daily for 7 days. The TER values were calculated by subtracting the blank values from the sample values and normalized to the growth area of the monolayer. High-resistance Madin-Darby canine kidney cells were plated as positive controls.

Cell proliferation and clonogenic assays. Single-cell suspensions of claudin-expressing and control HOSE-B cells in complete medium were seeded at equal densities in 12-well plates. Cell count was assayed using an 5-(3-carboxymethoxy-phenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium, inner salt (MTS) assay by Cell Titer 96 Aqueous One Solution Cell Proliferation Assay kit according to manufacturer's instructions (Promega). The resulting formazan product was quantitated by a multiwell spectrophotometer by measuring absorbance at 490 nm. These experiments were done in triplicates and repeated at least twice.

For clonogenic assays, HOSE-B cells were plated at equal densities in 6-well plates. After 24 hours, cells were transfected with pCIneo-CLDN4, pCIneo-CLDN3, or pCIneo-EV vector (cotransfected with pCMV-zeo vector) using Fugene following the manufacturer's recommendations (Roche). Two independently purified plasmids of each construct were used for transfections. Transfected cells were selected with Zeocin (350 µg/mL) until distinct colonies had formed (10-14 days). The dishes were then rinsed with HBSS before the colonies were stained with 0.25% Crystal violet/20% ethanol for 5 minutes and counted manually.

Invasion and cell migration assay. The cell invasion capabilities of the claudin-expressing clones and the siRNA-transfected cells were determined using a modified Boyden chamber invasion assay (39). Fluoroblok inserts (BD Biosciences, San Jose, CA) of 8-µm pore size were coated with 150 µL of 80 mg/mL of Matrigel (BD Biosciences). Cells were cultured to about 80% confluency and serum starved overnight. On the day of the assay, cells were treated with 5 µmol/L Calcein-AM (Molecular Probes, Eugene, OR) for 1 hour at 37°C. After the treatment, cells were trypsinized and viable cell count taken. About 50,000 cells were plated onto the top of each of the coated filters in serum-free medium. An equal volume of the same medium containing 20% FCS was placed in the lower chamber (i.e., the well beneath the filter) to act as a chemoattractant. The assay plate was incubated at 37°C and the total number of invasive cells were measured by taking readings every hour for about 6 hours with an initial 0 hour reading using a Cytofluor 4000 set for bottom-read fluorescence. Experiments were repeated at least four times, with triplicates in each experiment. For assessing cell migration, the assay was carried out essentially as above, except that the cells were plated on top of uncoated fluoroblok inserts.

Wound-healing experiment. Cells were grown to confluency on 60-mm cell culture dishes coated with fibronectin, collagen (BD Biosciences), or uncoated plastic dishes. A scratch was made through the cell monolayer using a pipette tip. After washing with HBSS, fresh culture medium was added. Photographs of the wounded area were taken immediately after making the scratch (0 hour time point) and after 24 hours to monitor the invasion of cells into the wounded area.

Analysis of matrix metalloproteinase activity by gelatin zymography. Activities of MMP in the culture medium of cells were assessed using gelatin zymography. HOSE-B clones or siRNA-transfected cells were plated

¹ Unpublished data.

at equal density in T-25 flasks and allowed to grow to 80% confluency. Cells were serum starved for 24 hours after which the cell conditioned medium was collected and concentrated using centricon-10 concentrator (Millipore). The protein concentrations in the concentrated medium were determined using the BCA protein assay kit (Pierce). Equal amount of proteins were then separated under nonreducing conditions on a 10% zymogram gel containing 0.1% gelatin (Invitrogen). After electrophoresis, gels were incubated in 2.5% Triton X-100 for 60 minutes to remove the SDS followed by an overnight incubation at 37°C in the zymogram developing buffer (Invitrogen). Gels were then stained for 3 hours in 30% methanol, 10% glacial acetic acid, and 0.5% Coomassie blue G-250 (Bio-Rad Laboratories, Hercules, CA) and destained for 1 hour in 30% methanol/10% glacial acetic acid. Clear bands appear on the Coomassie stained blue background in the areas of gelatinolytic activity. Experiments were carried out with at least two independent lots of culture medium and repeated twice with each lot.

Results

Claudin expression in HOSE cells. To study the possible roles of claudin protein overexpression in ovarian cells, human ovarian cell line HOSE-B was transfected with mammalian expression vector pCI-neo encoding wild-type claudin-3 (pCIneo-CLDN3) or claudin-4 (pCIneo-CLDN4) cDNAs. Cells were also transfected with the empty vector as control. Immunoblot analysis of the selected stable clones using claudin-3- and claudin-4-specific antibodies showed that clone HOSE-CLDN3 expressed high levels of claudin-3 and clones HOSE-CLDN4-1 and CLDN4-2 both expressed claudin-4. The control cells (HOSE-EV) transfected with the empty vector did not express these proteins (Fig. 1).

Claudin expression is known to be important for tight junction formation. To determine whether overexpression of claudin-3 and claudin-4 in ovarian cells results in generation of functional tight junctions, the different clones of HOSE-B cells were grown to confluency and TER was measured. None of the claudin-expressing clones developed TER above background levels (data not shown), indicating that in this ovarian epithelial cell line, expression of claudin proteins does not result in tight junction formation.

Morphologic changes associated with overexpression of claudin-3 and claudin-4. Interestingly, overexpression of both

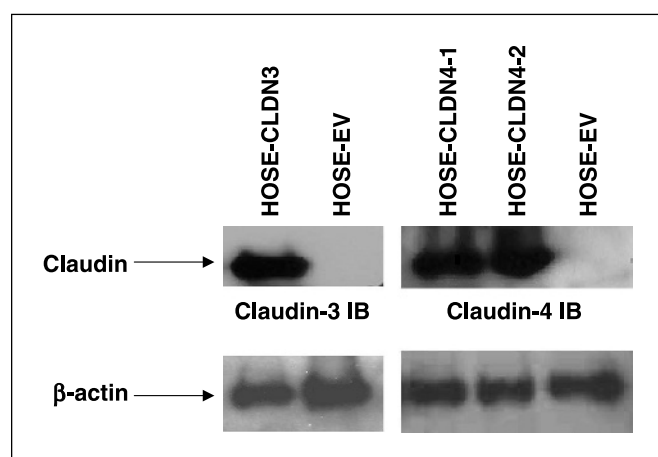


Figure 1. Immunoblot analysis of claudin expression in stably transfected HOSE-B cells using anti-claudin-3 and anti-claudin-4 antibodies. HOSE-CLDN3, cells stably transfected with full-length wild-type claudin-3, expressed high levels of claudin-3. HOSE-CLDN4-1 and CLDN4-2 are stable clones selected for overexpression of claudin-4 protein. Empty vector control cells (HOSE-EV) do not express any detectable claudin-3 and claudin-4 proteins. β -Actin was used as loading control.

claudin-3 and claudin-4 was associated with morphology changes in HOSE-B cells. Indeed, whereas the control cells exhibited compact growth with cobblestone appearance, the claudin-4-expressing cells were more fibroblastic in appearance, with irregular morphology and elongated processes (Fig. 2). Claudin-3-expressing cells were also found to exhibit irregular patterns of growth compared with the control cells (Fig. 2).

Claudins expression does not affect cell proliferation but increases cell survival in clonogenic assays. To determine whether claudin overexpression can influence the proliferation rate of HOSE cells, MTS assays were carried out on the different clones. HOSE-B cells expressing claudin-3 or claudin-4 did not differ significantly in their proliferation rates compared with that of the empty vector control cells (Fig. 3A). Manual counting of growing cells was also done and confirmed the MTS findings (data not shown).

We next investigated the possibility that claudin expression might confer a survival advantage when expressed in epithelial ovarian cells. Clonogenic assays clearly showed that claudin-expressing HOSE-B cells yielded increased colony formation compared with the parental cells (Fig. 3B-C). However, the size of the colonies was not significantly different between claudin-expressing and -nonexpressing cells, an observation consistent with our above results showing that claudin do not affect cell proliferation.

Claudin-3 and claudin-4 expression enhances invasiveness and cell migration of ovarian cells. Differences in invasion between claudin-expressing cells and control HOSE cells were evaluated using a modified Boyden chamber invasion assay. Calcein-labeled cells were placed on matrigel-coated fluoroblok inserts and the cells invading through matrigel were analyzed every hour for up to 6 hours using a fluorimeter. The results show that both claudin-3- and claudin-4-expressing HOSE-B cells were significantly more invasive than the empty vector controls (Fig. 4A). In addition, the rate of cell migration between these cells was also compared using the two-chamber assay with uncoated inserts (instead of matrigel-coated filters). Interestingly, HOSE-B cells expressing claudin-3 as well as claudin-4 showed increased migration compared with the empty vector control (Fig. 4B). In addition, to extend these findings to an additional nontransformed ovarian surface epithelium cell line, IOSE80 cells were transfected with claudin expression vectors and tested for invasion. Similar to the results described above, IOSE80 expressing claudins exhibited increased invasion compared with IOSE80 cells that did not express these proteins (data not shown).

To determine whether these observations could be extended to cancer cells, the ovarian cancer cell line HEY, which does not express claudin-3 or claudin-4, was used. HEY transfectants stably expressing claudin-3 were obtained and tested for invasion. Similar to what we observed with HOSE cells, claudin expression led to an increase in invasion (Fig. 4C). This result shows that the claudin-mediated cell invasion increase also occurs in cancer cells, which may explain why the vast majority of advanced ovarian cancers express these proteins (22).

We also compared the cell motility between the different HOSE clones using a wound-healing/scratch assay, a widely used qualitative method of studying cell migration. The extent of wound closure can be taken as a direct measure of cell motility. Twenty-four hours after generating the wound in the monolayer of cells grown on collagen, the closure was almost complete in claudin-3- and claudin-4-expressing HOSE-B cells, whereas a distinct gap still

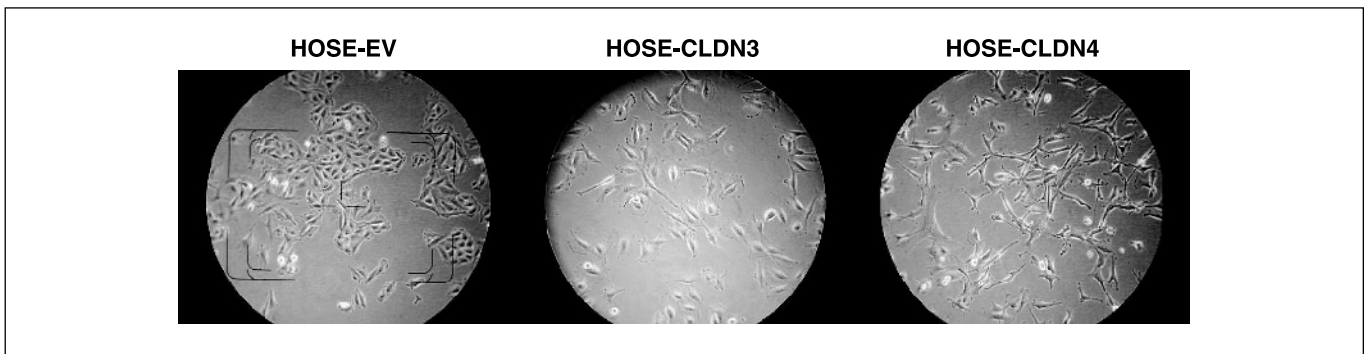


Figure 2. Morphologic changes observed in claudin-expressing HOSE cells. HOSE-EV (transfected with empty vector), HOSE-CLDN3 (expressing claudin-3), and HOSE-CLDN4-1 (expressing claudin-4) cells exhibited marked difference in morphology. HOSE-CLDN3 cells are more irregular compared with the empty vector control HOSE-EV. HOSE-CLDN4-1 cells also showed more irregular shapes with numerous extended processes and a fibroblastic appearance. The vector control cells are more compact and cobblestone-like in appearance. Magnification, 20 \times .

existed in the control cells, showing that claudin-expressing cells migrated much faster than the control cells (Fig. 5). Qualitatively identical results were obtained using fibronectin-coated dishes as well as uncoated dishes, showing that this effect is not substrate dependent (data not shown).

Knockdown of claudin expression is associated with reduced invasion of ovarian cancer cells. To further confirm the role of claudin overexpression on invasive properties of ovarian cancer cells, we carried out siRNA-mediated knockdown of *CLDN3* and *CLDN4* expression in ovarian cancer cell line OVCAR-5. Transient siRNA transfections were carried using *CLDN3* and *CLDN4* specific siRNA duplexes. Transfection with claudin-specific siRNA duplexes could efficiently repress the expression of claudin-3 and claudin-4 by >90%, whereas their expression remained unchanged in mock- or control siRNA-treated cells (Fig. 6A).

Interestingly, as shown in Fig. 6B, inhibition of claudin-3 and claudin-4 expression in OVCAR-5 cells significantly reduced the invasive potential of these cells. Similar results were obtained upon knockdown of claudin-3 in OV167 (data not shown), another ovarian cancer cell line, which express claudin-3 but not claudin-4.

Effects of claudin overexpression on matrix metalloproteinase-2 activity. A recent study has indicated modulatory effects of claudins on MMP-2 activation (40). To understand the mechanisms underlying claudin-induced increased invasion of HOSE cells, changes in MMP-2 activity were examined by gelatin zymography. Whereas the pro-MMP-2 band was present in culture-conditioned media from all the clones of HOSE-B cells, including the control, the active form of MMP-2 was elevated in the claudin-expressing cells (Fig. 7). On the other hand, siRNA knockdown of claudin-3 and claudin-4 in OVCAR-5 cells, a fully malignant ovarian cancer cell line, did not lead to a decrease in the large amount of activity present in these cells (data not shown). Fully malignant ovarian cells may therefore have additional or alternative pathways to activate the high levels of MMP activity observed in these cells.

Discussion

Changes in permeability properties and loss of cell polarity are hallmarks of epithelial cell tumorigenesis (15). Tight junctions, the structures critical for maintaining these functions in epithelial

cells, have been shown to be modulated in a number of epithelial cancers such as colon cancer (16), liver cancer (41), and thyroid cancer (42). Recent studies by our group and several others have shown changes in tight junction-associated claudin protein expression in various epithelial cancers. For example, overexpression of claudin-3 and claudin-4 in ovarian cancer at both the mRNA and protein levels has been reported in many studies (17–24, 29). Additional recent reports from studies on various cancers have revealed that whereas claudin protein expression is up-regulated in certain cancers like prostate cancer (23) and pancreatic cancer (25, 26), these proteins can also be down-regulated in other cancers such as head and neck cancer (27) and breast cancer (28). These results have led to the suggestion that claudin proteins, either alone or in combination with other proteins, may represent useful biomarkers for detection and diagnosis of certain cancers (18).

Although these various studies show that changes in claudin expression are associated with various cancers, the functional significance of these changes in epithelial tumorigenesis has remained unclear. The focus of the present study was to understand the possible functional consequences of claudin overexpression in ovarian tumorigenesis. We found that overexpression of claudin proteins in HOSE cells does not result in the formation of functional tight junctions. This is consistent with our previous finding that claudin-3 and claudin-4 overexpression in various ovarian cancer cell lines was not associated with the ability of these cells to form tight junctions (22). Importantly, we show that claudin-3 and claudin-4 expression in nontransformed ovarian epithelial cells and in ovarian cancer cells leads to increased invasion, suggesting a possible molecular basis for the observed increased invasiveness of ovarian tumors. This may explain our previous observation that the majority of ovarian cancers overexpress these proteins, whereas ovarian cystadenomas do not (22). Consistent with this hypothesis, siRNA-mediated knockdown of claudin-3 and claudin-4 in ovarian cancer cell lines results in a decrease in invasion by these cells. From a mechanistic standpoint, however, this observation is somewhat surprising. Increased expression of tight junction proteins would be expected to increase cell adhesion and consequently decrease invasion and motility. Indeed, claudin-7 down-regulation in breast cancer has been associated with increased cellular discohesion and the ability of breast cancer cells to disseminate (28). In addition, experiments in pancreatic cell lines have recently shown that expression of claudin-4 leads to reduced invasiveness, tumorigenicity, and

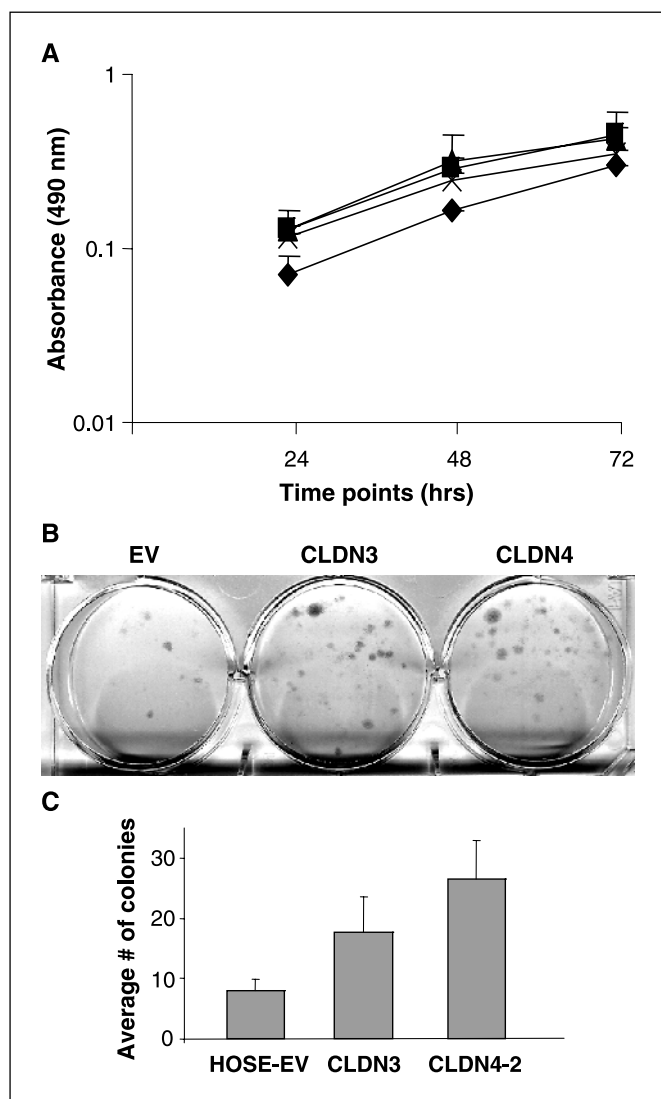


Figure 3. Effects of claudin expression on cell proliferation and survival. *A*, cell proliferation. An equal number of cells from the indicated clones were plated and the MTS assay was done after 24, 48, and 72 hours. Points, means of at least two independent experiments. Each experiment done in triplicates. HOSE-EV (◆), CLDN3 (■), CLDN4-1 (▲), and CLDN4-2 (×). Claudin-3- and claudin-4-expressing HOSE-B cells did not differ significantly in proliferation rate compared with the vector transfected cells. *B*, clonogenic assays. Various clones were plated and selected in the presence of zeocin. HOSE-B cells expressing claudin-3 (CLDN3) or claudin-4 (CLDN4) had an increased number of surviving colonies compared with EV-transfected cells. *C*, quantitation of the clonogenic assay. Colonies from experiment shown in (*B*) were counted and plotted.

metastatic potential of these cells (43). The reasons for this discrepancy are unclear at the present but may be related to tissue-specific differences in claudin function or even to variations in response in different cell lines. Clearly, members of the claudin family are known to be expressed in a tissue-specific manner and may have different function depending on the exact molecular circuitry of the cells (6).

Our observation of increased invasion induced by claudin overexpression in ovarian cancer suggests that these proteins may have unexpected functions in ovarian cancer. In one scenario, the claudin proteins may act in a dominant-negative fashion, inhibiting cell adhesion and promoting invasion. Alternatively, claudins may have intrinsic activities promoting invasion. Our

finding that claudin expression leads to increased MMP-2 activity suggests that the second alternative is more likely. There is a precedent for such a mechanism, as it was recently shown that claudin-1 expression could activate MMP-2 activity in kidney cells (40). Considering that MMP-2 has been shown to be important in

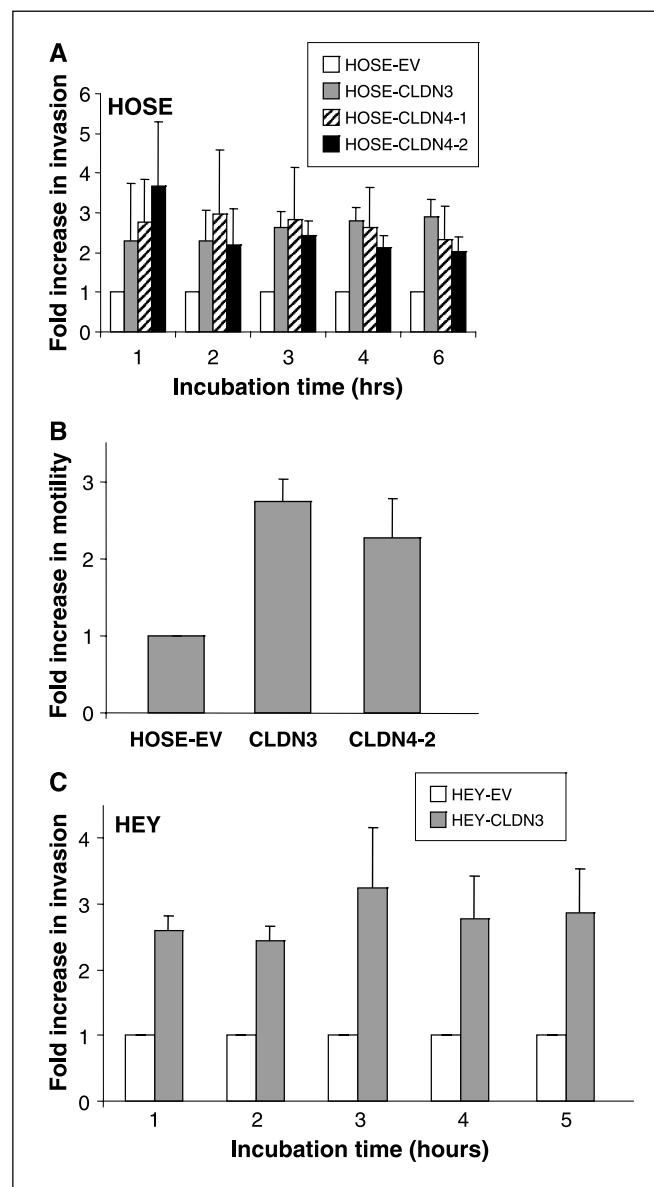
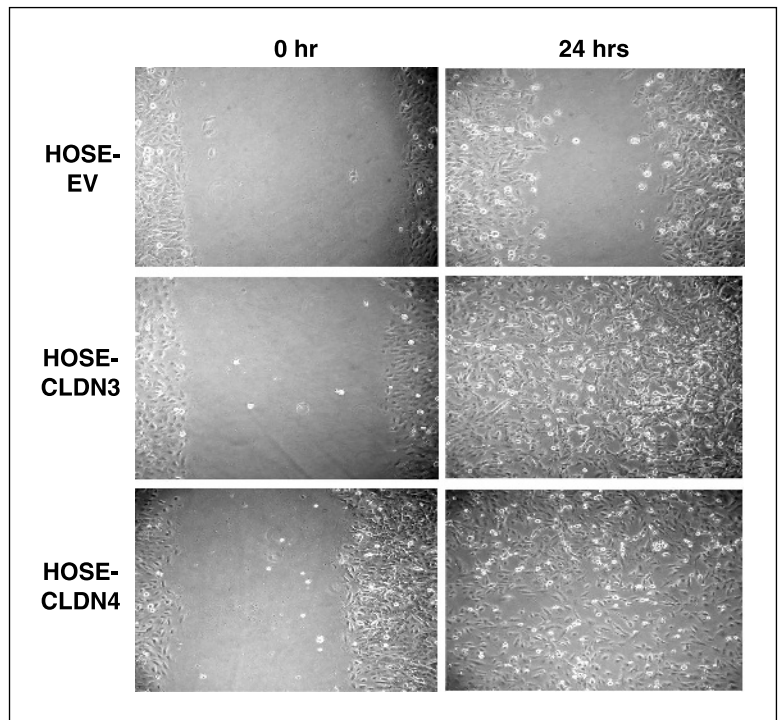


Figure 4. Effects of claudin expression on cell invasion and migration of ovarian cells. *A*, modified Boyden chamber invasion assay was done on HOSE-B stable transfectants. The number of invading cells were measured every hour for up to 6 hours. Columns, mean fold increase in invasion of claudin-expressing HOSE cells compared with the empty vector transfected cells; bar, SD. Empty columns, HOSE-EV; gray columns, HOSE-CLDN3 cells; cross-hatched columns, HOSE-CLDN4-1; black columns, HOSE-CLDN4-2. From four independent experiments, with each experiment done in triplicates. Both claudin-3- and claudin-4-expressing cells were more invasive compared with the vector control cells. *B*, two-chamber cell migration assay on HOSE-B stable clones. The effects of claudin overexpression on migration of the indicated clones were compared by measuring the number of cells migrating through the uncoated filters (instead of matrigel-coated filters). Columns, mean fold increase in migration of claudin-expressing HOSE cells compared with the empty vector control cells (6-hour time point); bars, SD. *C*, modified Boyden chamber invasion assay was done on HEY stable transfectants. HEY cells transfected with empty vector (open columns) or stably transfected for claudin-3 expression (gray columns) were compared for invasiveness.

Figure 5. Enhanced invasion by cells expressing claudin in wound-healing assays. Uniform scratches were made in confluent cultures of different claudin-expressing and vector control HOSE-B cells grown on collagen-coated dishes. Following the wound, the extent of closure was visually monitored. Photographs captured using a camera fixed to the Zeiss microscope indicate relative wound closure between different cell lines. By 24 hours, the scratch is completely sealed in the cultures of both claudin-3- and claudin-4-expressing cells, whereas a gap still exists in vector control cells.



ovarian cancer progression (44), our results suggest the possibility that claudin proteins may be involved in the control of motility and invasion through MMP-2, at least early in ovarian tumor progression. Interestingly, siRNA knockdown of claudins do not lead to a decrease in MMP-2 activity in fully malignant cells (data not shown), suggesting that the fully activated state rely on multiple pathways and that inhibition of claudin is not sufficient to reduce MMP-2 activity in these cells. It is interesting that claudin inhibition still leads to a reduction in invasion (Fig. 6), suggesting

that other claudin activities (e.g., motility) are still crucial in fully malignant cells.

Metastasis is a complex phenomenon that requires a number of specific steps such as decreased adhesion, increased motility and invasion, proteolysis, and resistance to apoptosis (45). Whereas claudin expression did not significantly alter the cell adhesion properties of the cell (data not shown), it did increase the migration/motility as shown by both Boyden chamber and wound-healing assays. In addition, examination of claudin-3- and

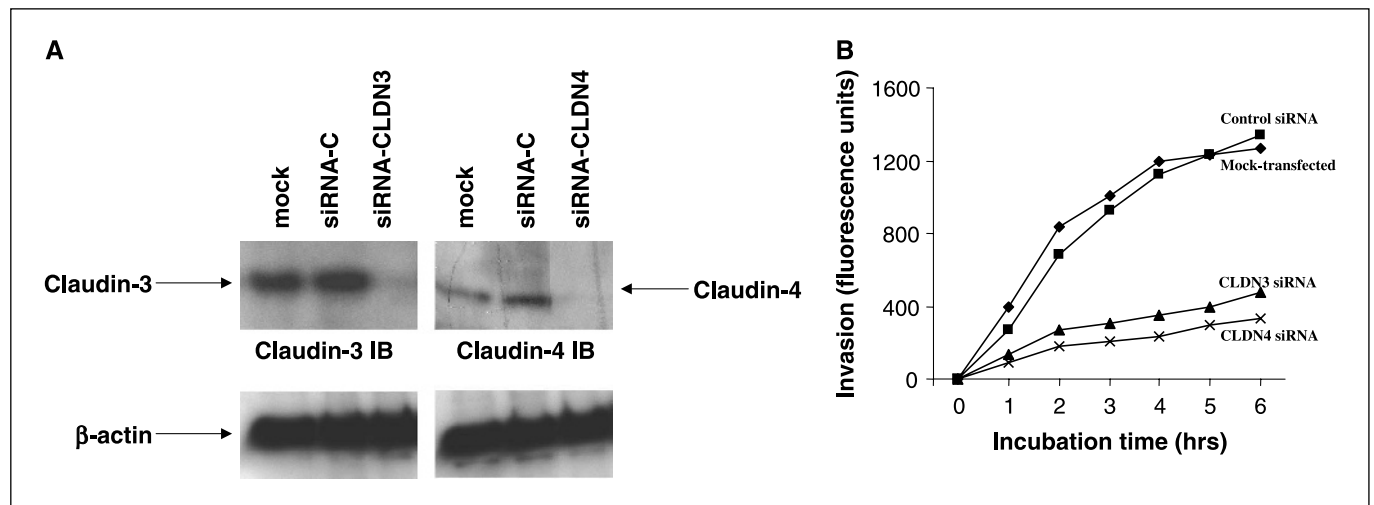


Figure 6. siRNA-mediated silencing of claudin expression reduces invasion of ovarian cancer cells (OVCAR-5). *A*, Western blot analysis. OVCAR5 cells were transfected with claudin-3- or claudin-4-specific and control siRNA duplexes, as described in Materials and Methods. After 48 to 72 hours, total cell lysates were prepared and analyzed for claudin expression. Transfection of claudin-specific siRNA oligos resulted in >90% reduction in expression of the protein, whereas the levels of claudin protein in the control and mock-transfected cells were not significantly altered. *B*, Boyden chamber invasion assay. The number of OVCAR-5 invading cells was measured every hour for up to 6 hours. Mock-transfected cells (◆), control siRNA oligo (■), CLDN3 siRNA (▲), and CLDN4 siRNA (×). Points, averages of triplicates from one experiment. The experiment was repeated at least three times. Inhibition of either claudin-3 or claudin-4 expression resulted in reduction of invasion of OVCAR-5 cells.

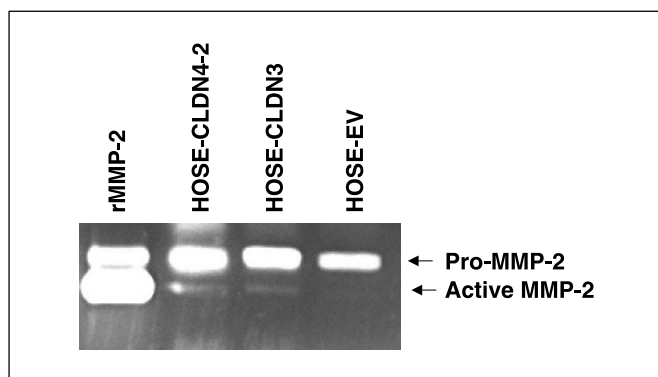


Figure 7. Gelatin zymography of MMP-2 activity. Conditioned media collected from different HOSE transfectants (HOSE-CLDN4 expressing claudin-4, HOSE-CLDN3 expressing claudin-3, and HOSE-EV, which does not express either protein) were concentrated and protein concentration quantitated. Equal amounts of protein were run for carrying out SDS-PAGE-gelatin zymography. Gelatin lysis bands appear as clear areas against the blue (dark) background. The arrow indicates the gelatinolytic activity of MMP-2. Representative of two independent experiments. Recombinant MMP-2 (rMMP-2) was used as control.

claudin-4-expressing cells also showed that these proteins, while not having a direct effect on cell proliferation, seemed to affect survival as measured by clonogenic assays. Our results thus show that claudin expression favor three steps known to be important in metastasis (migration, invasion, and survival), suggesting that claudin expression in ovarian and other cancers may be important for metastasis. This is consistent with our previous findings that claudin overexpression is observed in ovarian carcinomas but not in nonmalignant ovarian cystadenomas (22). From a tumor progression standpoint, our current finding that claudin can promote

multiple steps known to be important for metastasis is particularly interesting. It has been suggested that the concept of metastasis genes was problematic, as alterations of these genes would not be expected to confer a growth advantage in the primary tumor (46). The discovery that certain genes, such as those encoding claudins, can simultaneously affect processes relevant to both the primary tumor (survival) and to metastasis development (invasion, motility, and survival) may help explain how the metastatic phenotype can be selected for in the primary tumor.

The results presented here suggest for the first time that claudin overexpression may favor cell growth and metastasis in ovarian cancer. Whereas the mechanisms for this phenomenon are still under investigation, it is clear that inhibition of claudin expression or function may represent an interesting strategy for cancer therapy (47), especially in light of our siRNA experiment results. Indeed, preliminary studies with the *Clostridium perfringens* enterotoxin (CPE), a toxin that specifically binds claudin-3 and claudin-4 have been promising. Experiments have shown that CPE has cytotoxic effects towards breast cancer cells (29), prostate cancer cells (23), and pancreatic cancer cells (25, 48), as long as these cells express claudin-3 or claudin-4. These observations are consistent with the findings in this report suggesting a role for claudin in cell survival. In addition, our present work suggests that therapeutic inhibition of claudins may also reduce cell motility and invasion of claudin-expressing cells, making these protein excellent new targets for cancer therapy.

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