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Membrane-Type 1 Matrix Metalloproteinase Expression Is Regulated by Zonula Occludens-1 in Human Breast Cancer Cells

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Abstract

The acquisition of a migratory/invasive phenotype by tumor cells is characterized by the loss of cell-cell adhesion contacts and the expression of degradative properties. In this study, we examined the effect of the disorganization of occludin/zonula occludens (ZO)-1 tight junction (TJ) complexes on the expression of membrane-type 1 matrix metalloproteinase (MT1-MMP). We first compared the expression of MT1-MMP and the localization of occludin/ZO-1 complexes in breast tumor cell lines displaying various degrees of invasiveness. We showed that the expression of MT1-MMP in invasive breast tumor cell lines correlates with the absence of occludin and with a cytoplasmic localization of ZO-1. In contrast, noninvasive cell lines displayed a membrane staining for both ZO-1 and occludin and did not express MT1-MMP. *In vivo,* cytoplasmic ZO-1 and MT1-MMP could be detected in invasive tumor clusters of human breast carcinomas. We then used RNA interference strategy to inhibit ZO-1 expression in invasive BT549 cells and to evaluate the effect of ZO-1 down-regulation on MT1-MMP expression. We observed that ZO-1 small interfering RNA transfection down-regulates MT1-MMP mRNAs and proteins and subsequently decreases the ability of tumor cells to invade a reconstituted basement membrane in a Boyden chamber assay. Inversely, transfection of expression vectors encoding wild-type ZO-1 or the NH_2-terminal fragment of ZO-1 comprising the PSD95/DLG/ZO-1 domains in BT549 activated a human MT1-MMP promoter luciferase reporter construct and increased cell invasiveness. Such transfections concomitantly activated the β-catenin/TCF/LEF pathway. Our results therefore show that ZO-1, besides its structural role in TJ assembly, can intervene in signaling events promoting tumor cell invasion.

INTRODUCTION

The acquisition of an invasive phenotype by epithelial tumor cells requires the disruption of intercellular adhesion. Although the reorganization of E-cadherin/β-catenin complexes of adherens junctions during tumor cell migration and invasion has been largely documented, little is known about the implication of tight junction (TJ) molecules in tumor progression (1). In normal epithelial cells, TJs are composed of transmembrane molecules (occludin, claudin, and junctional adhesion molecules) linked to the actin cytoskeleton through cytoplasmic submembranous components, including zonula occludens (ZO)-l, ZO-2, and ZO-3 (2, 3). ZO-1 (220 kDa) interacts directly with the others TJ proteins through its different structural conserved domains, three PSD95/ DLG/ZO-1 (PDZ) domains, one Src homology 3 domain, and one guanylate kinase homologous (GK) domain. ZO-1 is thus able to bind occludin via the GK domain, some claudins via the first PDZ domain, ZO-2 or ZO-3 via the second PDZ domain, and actin via the COOH-terminal end (2). ZO-1 also binds other TJ proteins, such as AF-6 and cingulin (2). Because of its interactions with many TJ components, ZO-1 acts as a crucial central regulator of the structural organization of the TJs at the plasma membrane. In carcinomas, ZO-1 is generally considered a tumor suppressor (4). Indeed, several studies have reported that a reduction of ZO-1 expression correlates with tumor dedifferentiation and progression in breast and colorectal carcinomas (5-7). It has also been shown that ZO-1 displayed a cytoplasmic distribution in breast carcinomas (7). In addition, ZO-1 has been found overexpressed in primary and metastatic pancreatic cancers (8). Generating different ZO-1 deletion constructs, it has been shown that a form of ZO-1, which lacks the GK domain and thus does not bind occludin and fails to be recruited to the plasma membrane, is able to confer tumorigenicity to transfected Madin-Darby canine kidney I (MDCK I) cells injected into nude mice (9). In addition, migrating epithelial MDCK I

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cells have been reported to display a ZO-1 nuclear localization (10). It has also been reported previously that ZO-1 contains several nuclear sorting signals, suggesting that ZO-1 shuttles between the plasma membrane and the nucleus (11). Based on these results, the concept has emerged that, in addition to its structural role in TJ organization, ZO-1, once delocalized from TJs, may be involved in signaling pathways favoring epithelial cell migration and invasion.

Besides the loss of cell-cell contacts, tumor invasion is also characterized by the expression of degradative properties by tumor cells. Among the proteases implicated in this process, matrix metalloproteinases (MMP) occupy a central role. MMPs are a family of zinc endopeptidases comprising 25 members that degrade many extracellular matrix (ECM) components. Although the majority of MMPs are secreted as soluble enzymes, six membrane-bound MMPs have also been identified (12). Among the six membrane-type MMPs, membrane-type 1 MMP (MT1-MMP), which possesses a transmembrane domain, has been particularly implicated in pericellular proteolysis associated with cell migration (13). In addition to the degradation of structural ECM components, such as fibronectin, vitronectin, laminin-1, laminin-5, fibrin, and collagen type I, II, and III, MT1-MMP can also degrade cell adhesion molecules, such as CD44 and α_v, integrin, as well as some cytokines, including MCP-3, stromal cell-derived factor-1α, and pro-tumor necrosis factor-α (14). MT1-MMP is also known to be an activator of other MMPs, particularly of MMP-2. Owning these properties, MT1-MMP regulates various cell functions that contribute to enhanced metastatic abilities. MT1-MMP has indeed been shown to be associated with tumor progression, particularly in cell invasion, angiogenesis, and metastasis. Accordingly, several studies have described an over-expression of MT1-MMP in many types of human carcinomas (14). Moreover, numerous reports have indicated that MT1-MMP is produced by invasive tumor cells *in vitro* as well as *in vivo* (15). Interestingly, MT1-MMP expression in epithelial tumor cells has been shown to correlate with and be regulated by a reorganization of E-cadherin/β-catenin complexes (16, 17). These data emphasize a role of cell-cell contact molecules in the regulation of tumorpromoting genes, such as *MMP*.

In the present study, we investigated the potential role of ZO-1 in the regulation of MT1-MMP in invasive tumor cells. We document an association between the reorganization of occludin/ZO-1 complexes and the expression of MT1-MMP in invasive breast tumor cell lines. We also report that ZO-1 small interfering RNA (siRNA) transfection in invasive cells decreases MT1-MMP expression and invasive properties. In addition, transfection of ZO-1 cDNA was found to concomitantly activate the MT1-MMP promoter and the β-catenin/TCF/LEF pathway.

MATERIALS AND METHODS

Cell culture

All human mammary epithelial cells were obtained from the American Type Culture Collection (Rockville, MD). MCF-7, BT20, MDA-435, BT549, and Hs578T cells were cultured in DMEM containing 10% FCS.

Transfection of small interfering RNA

Three 19-nucleotide specific sequences were selected in the coding sequence of ZO-1 (Genbank accession no. L14837) to generate 21-nucleotide sense and 21-nucleotide antisense strands of the type (19N) TT (N, any nucleotide). The sense and antisense strands were then annealed to obtain duplexes with identical 3' overhangs. The sequences were submitted to a BLAST search against the human genome to ensure the specificity of the siRNA to the targeted sequence. Three corresponding scramble duplexes, which do not recognize any sequence in the human genome, were used as controls. The 19-nucleotide specific sequences for the three ZO-1 siRNAs are as follows: ZO-1 Sil, 5'-GUUAUACGAGCGAUCUCAU-3'; ZO-1 Si2, 5'-GGAGGAAACAG-CUAUAUGG-3'; and ZO-1 Si3, 5'-GACGAGAUAAUCCUCAUUU-3'. For transfection of the siRNA duplexes, 75,000 cells were plated in six-well plates in 2 mL/well of culture medium. Twenty-four hours after plating, the cells were transfected by phosphate calcium precipitation by adding in each well 200 μL of a mixture containing the siRNA duplexes (50, 20, or 2 nmol/L), 140 mmol/L NaCl, 0.75 mmol/L Na_2HPO_4, 6 mmol/L glucose, 5 mmol/L KC1, 25 mmol/L HEPES, and 125 mmol/L $CaCl_2$. Twenty-four hours after transfection, the cells were extensively washed with PBS and incubated for 48 hours in culture medium before they were harvested for reverse transcription-PCR (RT-PCR) analyses, Western blotting analyses, or Boyden chamber assays.

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Transient transfections of zonula occludens-1 expression vectors and reporter assay

The expression vectors encoding wild-type ZO-1 (ZO-1-WT) or the NH_2-terminal fragment of ZO-1 comprising the PDZ domains, ZO-1-PDZ (1-3), have been characterized previously (9). The MT1-MMP promoter luciferase reporter vector was constructed by subcloning a 1.2-kb fragment of the 5' promoter region of MT1-MMP (18) into the firefly luciferase reporter plasmid pGL-3 (Promega, Madison, WI). The TOP-FLASH and FOP-FLASH plasmids containing three wild-type (TOP-FLASH) or mutated (FOP-FLASH) copies of the β-catenin/TCF binding sites upstream of a minimal herpesvirus thymidine kinase promoter driving the firefly luciferase expression were a kind gift from H.C. Clevers (University Hospital, Utrecht, The Netherlands; ref. 19).

Transient transfections were done with Fugene transfection reagent (Roche, Indianapolis, IN). For immunofluorescence analyses, Boyden chamber assays, or preparation of cytosolic versus nuclear extracts, cells were plated in six-well plates or 10 cm Petri dishes 24 hours before the addition of the DNA/Fugene mixture. The cells were transfected according to the manufacturer's instructions with a mixture (100 μL for six-well plates and 400 μL for 10 cm Petri dish) containing 30 μL Fugene per 1 mL serum-free DMEM and 20 μg ZO-l-WT or ZO-l-PDZ expression vector (or the corresponding pLNCX empty vector). The cells were collected 24 or 48 hours after transfection. For promoter luciferase assays, 50,000 cells were plated in 24-well plates 30 minutes before the addition of the DNA/Fugene mixture. Each well was incubated with a mixture containing 20 μL serum-free DMEM, 0.6 μL Fugene, 0.2 μg firefly promoter luciferase reporter plasmid (either the MT1-MMP promoter, the TOP-FLASH or the FOP-FLASH reporter construct), 0.2 μg ZO-l-WT or ZO-1-PDZ expression vector (or the corresponding pLNCX empty vector), and 0.8 ng *Renilla* luciferase expression vector phRG-TK (Promega). Twenty-four hours after transfection, the cells were lysed in 50 μL passive lysis buffer and the luciferase activity was determined with a luminometer using the Dual Luciferase Assay System (Promega) on 20 μL lysate. For each experiment, the firefly luciferase activity was normalized to the activity of the *Renilla* luciferase used as internal control. The results were expressed as fold induction determined by normalizing each firefly luciferase value to the *Renilla* luciferase internal control value and by dividing these normalized values with the mean normalized value of the corresponding reporter construct transfected with the empty expression vectors. To assess the β-catenin/ TCF/LEF activities, the normalized values obtained with the FOP-FLASH reporter plasmid were subtracted from the normalized values obtained with the TOP-FLASH reporter plasmid. The resulting values were then divided with the mean normalized value of the condition transfected with the empty expression vector. Each experiment was done at least thrice in triplicate. Data are expressed as means ± SD. A nonparametric test (Mann-Whitney *U* test) was done and $P < 0.05$ was considered significant.

Immunofluorescence

Confluent monolayers of cells cultured on glass coverslips were fixed with methanol for 10 minutes at -20°C. For confocal microscopy analyses of ZO-1, cells were fixed in 2.5% paraformaldehyde in PBS for 30 minutes and then permeabilized with 0.2% Triton X-100 for 15 minutes at room temperature. The coverslips were then saturated for 10 minutes with 3% bovine serum albumin (BSA) in PBS. Monolayers were next (after intermediate washes in PBS) incubated for 1 hour with a monoclonal antibody to ZO-1 (Z01-1A12, Zymed Laboratories, San Francisco, CA) or occludin (OC-3F10, Zymed Laboratories) and then with a biotinylated anti-mouse IgG (Amersham, Arlington Heights, IL) for 1 hour. Coverslips were exposed to Alexa Fluor 492-coupled streptavidin for 30 minutes (Molecular Probes, Eugene, OR). The coverslips were then mounted with Aquapolymount antifading solution (Agar, Essex, United Kingdom) onto glass slides and observed under a Zeiss fluorescence microscope or with a MRC-600 confocal laser scanning microscope (Bio-Rad, Richmond, CA).

Western blotting analyses

For ZO-1, occludin, and MT1-MMP detection, cells were rinsed twice in PBS and extracted in lysis buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% (v/v) Igepal, 1% (w/v) sodium deoxycholate, 5 mmol/L iodoacetamide, 0.1% (w/v) SDS] containing Complete protease inhibitor cocktail (Roche). To examine the subcellular distribution of β-catenin, cytosolic and nuclear extracts of BT549 cells transfected with the ZO-1-WT or ZO-l-PDZ expression vectors (or the corresponding pLNCX empty vector) were done using the ProteoExtract Subcellular Proteome Extraction kit (Calbiochem, La Jolla, CA). Protein concentration was determined with the detergent-compatible protein assay (Bio-Rad). Total protein (10 μg) was separated on 7.5% and 12% SDS-PAGE gels for ZO-1 and for occludin and MT1-MMP analyses, respectively. Cytosolic extracts (4 μg) or nuclear extracts (2 μg) were separated on 10% SDS-PAGE to analyze the expression of β-catenin. The proteins were transferred to polyvinylidene difluoride membranes (NEN, Boston, MA), which were then blocked with 5% milk (w/v), 0.1% Tween 20 (w/v) in PBS for 2 hours before exposure to the primary antibody overnight at 4°C: a monoclonal antibody to ZO-1 (ZO1-1A12), a monoclonal antibody to occludin (OC-3F10), a monoclonal

antibody directed against the hemopexin-like domain of MT1-MMP (clone 2D7, kindly provided by Dr. Rio, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France), and a monoclonal antibody directed against the unphosphorylated form of β-catenin (clone 8E4, A.G. Scientific, San Diego, CA). The filters were then incubated with either a horseradish peroxidase-conjugated goat anti-rabbit or swine anti-mouse antibody (DAKO, Glostrup, Denmark). Signals were detected with an Enhanced Chemiluminescence Plus kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Subsequent detection of actin (using a rabbit antibody to actin, clone A2066, Sigma-Aldrich, St. Louis, MO) or c-*myc* (using a monoclonal antibody to c-*myc,* clone 9E10, Sigma-Aldrich) was done on the same filters as a control. The values obtained for MT1-MMP were normalized to values obtained for actin in three independent transfection experiments. Results were expressed as fold induction determined by dividing the normalized value with the normalized value of the corresponding scrambled siRNA control. Data are expressed as means ± SD. A nonparametric test (Mann-Whitney *U* test) was done and $P < 0.05$ was considered significant.

Reverse transcription-PCR analyses

Total RNA extraction was done with the RNA isolation kit (Roche). RT-PCR was done using 10 ng total RNA the GeneAmp Thermostable RNA PCR kit (Perkin-Elmer, Foster City, CA), and pairs of primers for human MT1-MMP and for 28S rRNA (Eurogentec, Seraing, Belgium). Forward and reverse primers for human MT1-MMP and 28S were designed as follows: MT1-MMP primers (forward 5'-GGATACCCAATGCCCATTGGCCA-3' and reverse 5'-CCATTGGGCATCCAGAAGAGAGC-3') and 28S primers (forward 5'-GTTCACCCACTAATAGGGAACGTGA-3' and reverse 5'-GGATTCTGACTTAGAGGCGTTCAGT-3'). Reverse transcription was done at 70°C for 15 minutes. Amplification cycles were as follows: 15 seconds at 94°C, 20 seconds at 68°C, and 10 seconds at 72°C. Twenty cycles were allowed for MT1-MMP amplification and 15 cycles for 28S amplification. Products were separated on acrylamide gels, stained with SYBR Gold (Molecular Probes), and quantified by fluorimetric scanning (LAS-1000, Fuji, Stamford, CT). The values obtained for MT1-MMP amplification were normalized to the values obtained for 28S amplification in three independent transfection experiments. Results were expressed as fold induction determined by dividing the normalized value of a given siRNA with the normalized value of the corresponding scrambled siRNA control. Data are expressed as means ± SD. A nonparametric test (Mann-Whitney *U* test) was done and $P < 0.05$ was considered significant.

Modified Boyden chamber invasion assay

The *in vitro* invasive properties of transfected cells were assessed using a modified Boyden chamber assay. Cells (10^4) were suspended in 30 μL serum-free medium supplemented with 0.1% BSA and placed in the upper compartment of a 48-well invasion chamber (Neuroprobe, Pleasanton, CA). The lower compartment of the chamber was filled with 30 μL medium containing 10% FCS and 1% BSA The two compartments were separated by a porous filter (8 μm pore, Nucleopore, Pleasanton, CA) coated with Matrigel (1 mg/10 mL used to coat the filter). After 24 hours of incubation at 37°C, the filters were fixed in methanol for 10 minutes and stained with hematoxylin for 5 minutes. The cells at the upper surface of the filters were wiped away with a cotton swab. Quantification of the invasion assay was done by counting the number of cells at the lower surface of the filters (10 fields at 400-fold magnification). Experiments were done at least thrice in triplicate. Data are expressed as means ± SD. A nonparametric test (Mann-Whitney *U* test) was done and $P < 0.05$ was considered significant.

Immunohistochemistry on breast tissues

Human tissues were obtained from biopsies of 15 invasive breast ductal carcinomas. The samples were fixed in formalin and embedded in paraffin. Immunohistochemistry for ZO-1 and MT1-MMP was done on 5 μm serial sections. The sections were deparaffinized, rehydrated, and treated with 0.3% H_2O_2 for 5 minutes to quench endogenous peroxidase activity. Slides were then incubated for 30 minutes with a monoclonal antibody to either ZO-1 (ZO1-1A12) or MT1-MMP (clone 113-B7, Oncogene Science, Cambridge, MA). After several washes, the slides were successively incubated with biotin-coupled anti-mouse antibody (DAKO) and then with a horseradish peroxidase-conjugated streptavidin (DAKO). The peroxidase activity was revealed using the LSAB kit (DAKO). The slides were briefly counterstained with Mayer's hematoxylin, mounted, and observed under a Zeiss Axiophot microscope.

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RESULTS

Membrane-type 1 matrix metalloproteinase is expressed by invasive, occludin-negative breast tumor cell lines

To examine the relationship between the presence or localization of occludin/ ZO-1 complexes and MT1-MMP expression, we first examined the expression of these three molecules in two noninvasive (MCF-7 and BT20) and three invasive (MDA-435, BT549, and Hs578T) epithelial breast cancer cell lines. The invasive phenotype of these breast cancer cells, assessed by a modified Boyden chamber assay, has been characterized in previous studies and was confirmed in our present study (data not shown; refs. 20-22). By Western blotting, we observed an inverse correlation between occludin and MT1-MMP expression. Occludin was only detected in noninvasive cell lines, whereas MT1-MMP was only found in invasive cell lines. In contrast, ZO-1 was expressed in all the cell lines analyzed regardless of their invasive properties (Fig. 1).

The cytoplasmic localization of zonula occludens-1 correlates with invasiveness of tumor cells

We then examined more closely the subcellular localization of occludin and ZO-1 in the different tumor cell lines. By immunofluorescence microscopy, we found that occludin was present at the cell membrane of noninvasive tumor cells (as illustrated in MCF-7 in Fig. *2A*) and was absent in invasive tumor cells (as illustrated in BT549 in Fig. *2A*). Regarding ZO-1, we observed that ZO-1 was mainly localized at the membrane of noninvasive cells, whereas it showed a predominantly diffuse cytoplasmic staining in invasive tumor cells (Fig. *2A* and *B*).

ZO-1 and MT1-MMP expression was also observed in several cases of human ductal breast carcinomas. Staining of serial sections of breast carcinomas with ZO-1 and MT1-MMP antibodies revealed the concomitant expression of MT1-MMP and ZO-1 in invasive tumor clusters, where ZO-1 showed a heterogenous cytoplasmic localization (Fig. 3). Although in all cases ZO-1 was expressed in tumor cells staining for MT1-MMP, not all MT1-MMP-positive cells showed ZO-1 labeling.

Figure 1. *Expression of occludin, ZO-1, and MT1-MMP in human breast cancer cell lines. Western blot analyses of ZO-1, occludin, and MT1-MMP were done on five human tumor cell lines (MCF-7, BT20, MDA-435, BT549, and Hs578T). Actin was used as a loading control.*

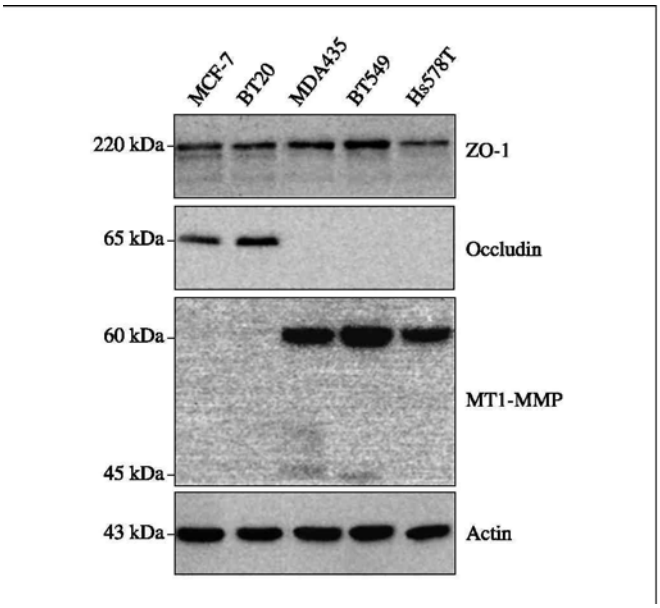

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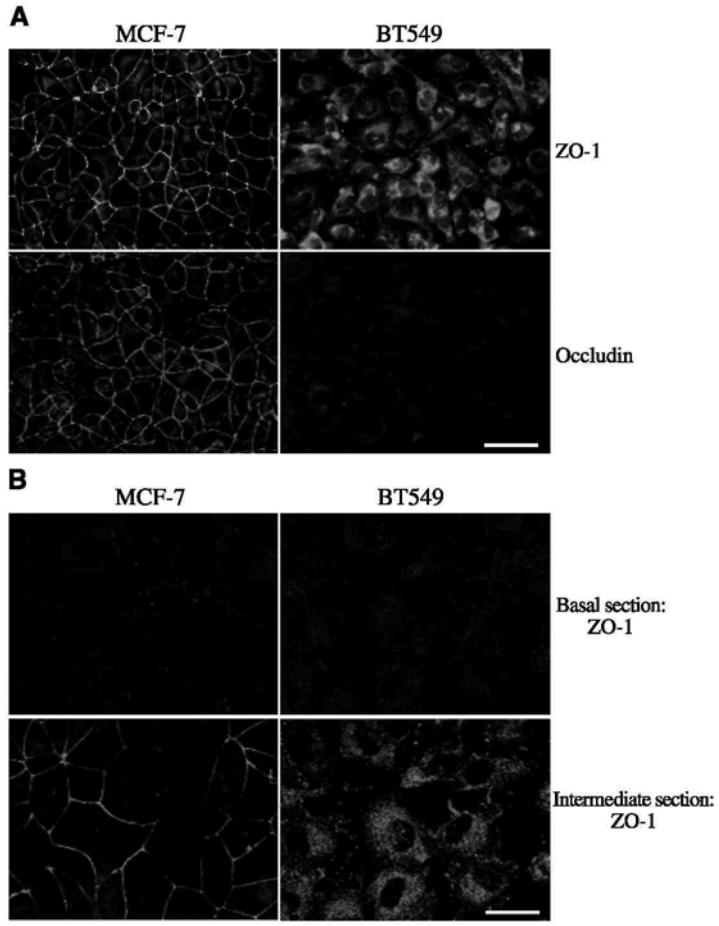
Figure 2. *Localization of occludin/ZO-1 in human tumor cell lines. A, ZO-1 and occludin immunolabeling in MCF-7 and BT549 monolayers. Bar, 50 µm. B, confocal microscopy analyses of ZO-1 localization in MCF-7 and BT549 cell monolayers. Optical sections were taken in the basal or the intermediate region of the cells. Bar, 16 µm.*



Regulation of membrane-type 1 matrix metalloproteinase expression by zonula occludens-1

To evaluate the potential implication of ZO-1 in the regulation of MT1-MMP expression, we used a RNA silencing strategy to down-regulate the endogenous ZO-1 levels in three invasive tumor cell lines. Transfection of a siRNA against ZO-1 down-regulated ZO-1 expression in the three cell lines at the protein level (Fig. 4). We concomitantly observed a clear diminution of MT1-MMP protein in tumor cells treated with the ZO-1 siRNA compared with cells treated with scrambled control siRNA (Fig. 4). Because the siRNA-mediated down-regulation of ZO-1 worked best in BT549 cells, we used this cell line to further investigate the regulation of MT1-MMP expression by ZO-1. To exclude a potential nonspecific effect of the ZO-1 siRNA used above, we first confirmed our results using two different siRNAs against ZO-1. As revealed by RT-PCR analyses, all three ZO-1 siRNAs significantly reduced MT1-MMP mRNA expression in BT549 tumor cells compared with scrambled control siRNAs (0.46 ± 0.06, 0.52 ± 0.05, and 0.71 ± 0.04 for Si-1, Si-2, and Si-3, respectively; $P < 0.05$; Fig. *5B* and C). Accordingly, Western blotting also revealed a significant decrease of the 60-kDa active form of MT1-MMP (0.5 ± 0.05, 0.28 ± 0.05, and 0.22 ± 0.05 for Sil, Si-2, and Si-3, respectively; $P < 0.001$; Fig. *5D* and *E*). Concomitantly, we also observed a down-regulation of the 45-kDa form of MT1-MMP (Fig. *5E*). As shown in Fig. 6, the inhibiting effect of ZO-1 Si3 siRNA on MT1-MMP expression is observed at various concentrations of siRNA. We also tested the effect of ZO-1 silencing on cell invasion in the Boyden chamber assay and observed that ZO-1 Si3 siRNA at 2, 20, and 50 nmol/L significantly reduced the invasive properties of the BT549 cells (435 ± 40 versus 150 ± 15, 468 ± 69 versus 70 ± 9, and 453 ± 43 versus 55 ± 2.9 cells per 10 fields for 2, 20, and 50 nmol/L concentrations of scrambled Si3 siRNA versus ZO-1 Si3 siRNA, respectively; $P < 0.001$; Fig. *6B).*

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Figure 3. *Cytoplasmic ZO-1 and MT1-MMP expression in ductal breast carcinomas. ZO-1 (A) and MT1-MMP (B) immunolabelings are detected on invasive tumor cells (T) in ductal breast carcinomas.*

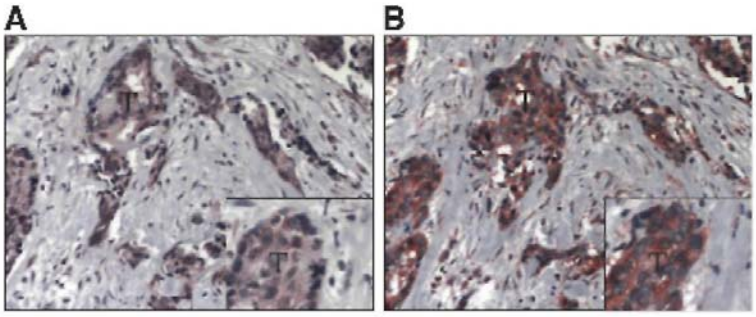
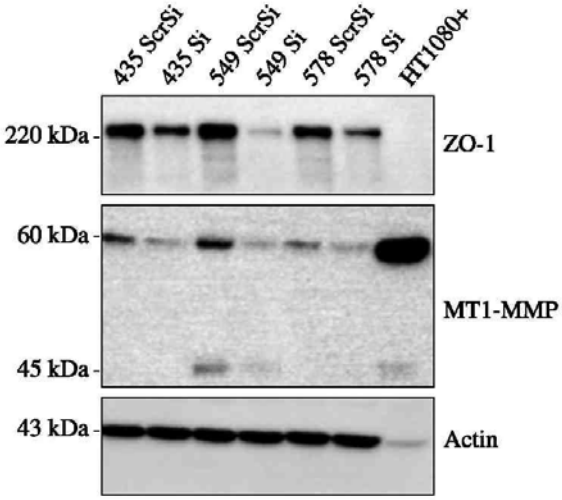


Figure 4. *Down-regulation of MT1-MMP expression in invasive tumor cell lines by ZO-1 siRNA.* Western blot analyses of ZO-1, MT1-MMP, and actin expression were done on total extracts of MDA-435, BT549, and Hs578T transfected with the ZO-1 Si3 siRNA or its corresponding scrambled control siRNA at a 50 nmol/L concentration. HT1080 cells treated with concavalin A (HT1080+) were used as a control to reveal the two forms of MT1-MMP at 60 and 45 kDa.



Zonula occludens-1 regulates the membrane-type 1 matrix metalloproteinase promoter and activates the β-catenin/TCF/ LEF transcriptional activity

It has been shown previously that the NH_2-terminal part of ZO-1 encompassing the three PDZ domains is able to activate the β-catenin/TCF pathway in MDCK I cells (9). β-catenin, once delocalized from the adherens junction, can indeed accumulate in the cytoplasm, translocate to the nucleus, and, through binding to TCF/LEF transcription factors, directly regulate the transcription of genes implicated in tumor invasion (1). We thus examined the possibility that the regulation of MT1-MMP by ZO-1 could be associated with the activation of this pathway. For this purpose, BT549 tumor cells were transiently cotransfected with expression vectors encoding the NH_2-terminal portion of ZO-1, including the three PDZ domains (ZO-1-PDZ) or ZO-l-WT and a MT1-MMP promoter reporter plasmid or the TOP-FLASH/FOP-FLASH reporters. First, we observed that transient transfection of ZO-1-WT and ZO-1-PDZ expression vectors increased the cytoplasmic labeling of ZO-1 in BT549 cells and their invasiveness in Boyden chamber assays (1.97 + 0.25- and 1.89 + 0.16-fold induction for ZO-l-WT and ZO-1-PDZ transfection, respectively; Fig. *1A* and *B*). We also found that both ZO-1-WT and ZO-1-PDZ expression vectors significantly increased the activity of the MT1-MMP promoter in BT549 tumor cells (3.11 + 0.3- and 2.48 + 0.3-fold induction for ZO-l-WT and ZO-l-PDZ transfection, respectively; $P < 0.001$; Fig. 7C). This was accompanied by an increased activity of the TOP-FLASH reporter system (9.12 + 3.6- and 5.85 + 1.96-fold induction for ZO-l-WT and ZO-l-PDZ transfection, respectively), attesting of the activation of the β-catenin/TCF/LEF pathway following ZO-l-WT and ZO-l-PDZ transfection ($P < 0.001$; Fig. *7D*). It has been shown that the NH_2-terminal unphosphorylated form of β-catenin is involved in the transcriptional activity of β-catenin (23). Using an antibody specific for the unphosphorylated form of β-catenin, we therefore examined by

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Western blotting the presence of this particular form of β-catenin in cytosolic or nuclear extracts of cells transfected with ZO-1-WT and ZO-1-PDZ expression vectors. Consistent with the results obtained with the TOP-FLASH reporter assays, we found an accumulation of the unphosphorylated form of β-catenin in the nucleus of ZO-1-WT or ZO-1-PDZ-transfected cells compared with control vector transfected cells (Fig. *7E*).

Figure 5. Down-regulation of MT1-MMP expression by ZO-1 siRNAs. BT549 tumor cells were transfected with three different ZO-1 siRNAs (Si1-Si3) or their corresponding scrambled control siRNAs (ScrSi1-ScrSi3) at 50 nmol/L. A, Western blot analyses of ZO-1 expression in BT549 cells transfected with the siRNAs. B, RT-PCR analyses of MT1-MMP and 28S expression in BT549 cells transfected with the siRNAs. C, quantification of RT-PCR analyses of MT1-MMP mRNA normalized with 28S values. Data are fold induction relative to the scrambled siRNA controls ($P < 0.05$). D, Western blot analyses of MT1-MMP and actin expression in BT549 cells transfected with the ZO-1 siRNAs. HT1080 cells treated with concavalin A (HT1080+) were used as a control to reveal the two forms of MT1-MMP at 60 and 45 kDa. E, quantification of the level of the 60 kDa activated MT1-MMP protein normalized to actin levels. Data are fold induction relative to the scrambled siRNA controls ($P < 0.001$).

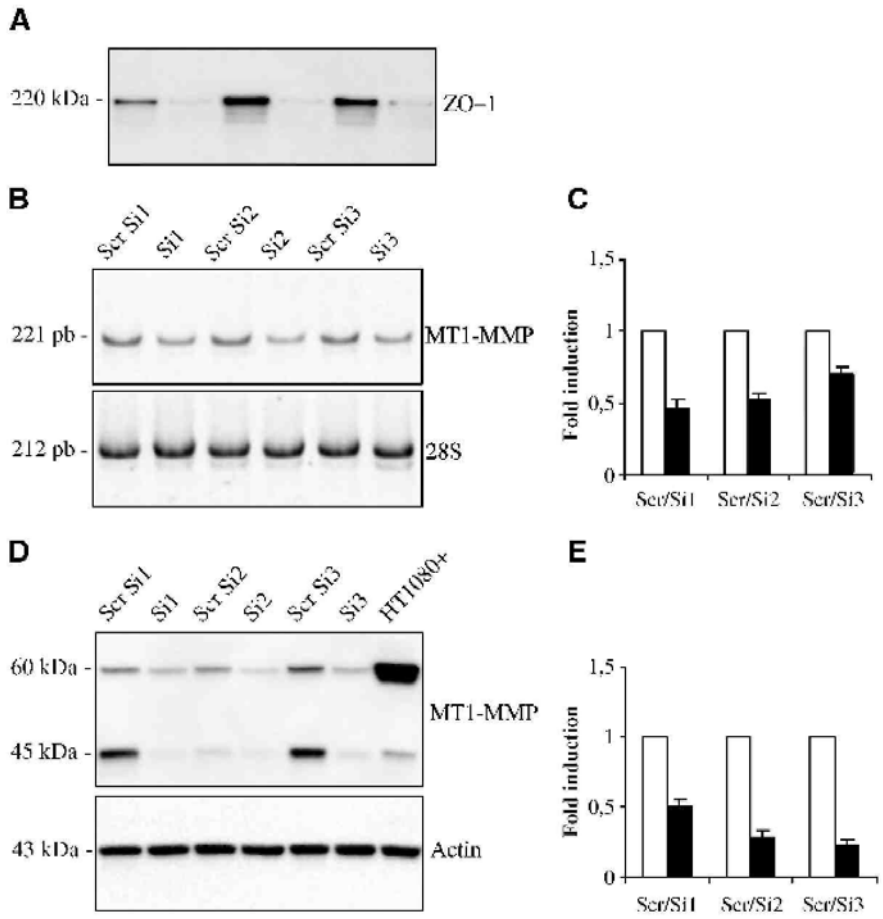

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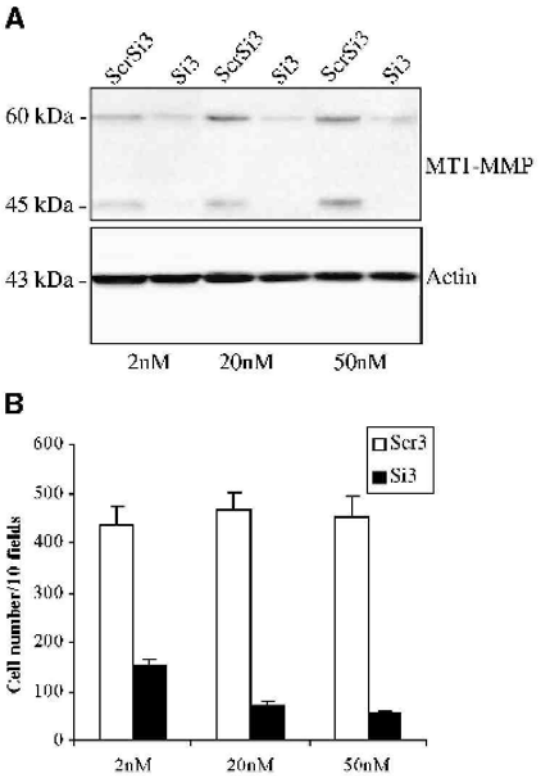
Figure 6. *Effect of ZO-1 siRNAs on in vitro cell invasion. BT549 tumor cells were transfected with either ZO-1 Si3 siRNA or the scrambled Si3 control siRNA at 2, 20, or 50 nmol/L.* *A,* Western blot analyses of MT1-MMP expression in the transfected cells. Actin was used as a loading control. *B,* analysis of the invasive capacity of ZO-1 siRNA-transfected BT549 cells compared with scrambled control siRNA transfectants in a modified Boyden chamber assay ($P < 0.001$).



DISCUSSION

In the present study, we have shown that (*a*) the lack of occludin expression and the cytoplasmic localization of ZO-1 correlates with the invasive phenotype of tumor cells, (*b*) MT1-MMP is expressed only in the occludin-negative invasive tumor cell lines, (*c*) ZO-1 and MT1-MMP can be detected in invasive tumor clusters in breast carcinomas, (*d*) the reduction of ZO-1 in invasive tumor cells decreases the expression of MT1-MMP and their invasive properties, and (*e*) the activation of the MT1-MMP promoter by ZO-1 is associated with the activation of the β-catenin/TCF/LEF signaling pathway.

We first showed that occludin was detected only in noninvasive tumor cells as a membrane staining. This agrees with several reports showing that the loss of occludin expression correlates with tumor progression in human endometrial and prostate carcinomas (24, 25). Similarly, *in vitro,* occludin was also observed in well-differentiated epithelial cell lines but not detected in invasive tumor cells and in dedifferentiated epithelial cell lines (9, 26, 27). These findings strengthened the hypothesis that occludin expression could be a hallmark of a noninvasive status. Interestingly, we also found that, although ZO-1 was present in all cell lines, it was present in the cytoplasm of invasive tumor cells, whereas in noninvasive cells it localized to the plasma membrane. Accordingly, Gottardi et al. have described that ZO-1 delocalizes from the plasma membrane in migrating epithelial cells at the edge of wounded monolayers, correlating with dedifferentiation of these cells and suggesting that ZO-1 may have a role in signaling events (10). *In vivo,* we found a diffuse cytoplasmic distribution of ZO-1 in breast tumors as also reported by Martin et al. (7). Taken together, these data show that a reorganization of membrane-associated occludin/ZO-1 complexes is associated with enhanced migratory and invasive properties of epithelial tumor cells and agree with the general concept describing that the acquisition of an effective invasive phenotype is related to a dedifferentiation of the epithelial tumor cells.

The redistribution of ZO-1 in invasive tumor cells suggests that, besides its role in cell-cell adhesion, ZO-1 is implicated in the regulation of invasive genes. Accordingly, we show that the accumulation of ZO-1 in the

cytoplasm is associated with the expression of MT1-MMP in invasive tumor cell lines, a correlation that can also be observed in invasive tumor clusters of breast carcinomas. In the literature, it has been repeatedly reported that MT1-MMP is associated with the migratory and invasive phenotype of tumor cells; in particular, MT1-MMP has been directly implicated in the migration/invasion of several dedifferentiated cell lines (15, 28-31). More than a correlation, we now show that ZO-1 can modulate MT1-MMP levels. Indeed, the down-regulation of ZO-1 levels by siRNAs clearly decreased MT1-MMP expression in invasive tumor cells as well as their *in vitro* invasive capacity. Inversely, the transfection of ZO-1 into BT549 cells increased the MT1-MMP promoter activity and their invasive properties. Only a few studies have implicated ZO-1 in the regulation of gene expression. The *Drosophilia* orthologue of ZO-1, tamou, has been linked to the regulation of the *extramacrochaetae* gene that participates in neural development (32). Balda and Matter have shown that ZO-1 via its interaction with a Y-box transcription factor, ZO-1-associated nucleic acid-binding protein, regulates ErbB-2 expression, thereby modulating epithelial cell differentiation and morphogenesis (33). In addition, the NH_2-terminal three PDZ domains of ZO-1, which by themselves are unable to localize to the plasma membrane and accumulate in the cytoplasm, promote a phenotypic conversion characterized by the repression of epithelial markers and the induction of mesenchymal features in transfected MDCK I and corneal epithelial cells (9, 34). In these studies, transfection of full-length ZO-1 led to its recruitment to the plasma membrane in MDCK I cells and did not induce any phenotypic conversion. This emphasized that ZO-1, once delocalized from the cell membrane, could play a role in the regulation of genes controlling cell differentiation (9, 34). Interestingly, in our experiments, transient transfection of either the PDZ domains or the full-length ZO-1 construct activated the human MT1-MMP promoter in invasive BT549 tumor cells. Our cells do not express occludin, which is required to maintain ZO-1 to the TJ plasma membrane, likely explaining why, in contrast to MDCK I cells, full-length ZO-1 also mediates an effect. Taken together, our results provide evidence that ZO-1 can regulate MT1-MMP expression and could thereby modulate the invasive properties of epithelial tumor cells.

The mechanisms by which ZO-1 affects the expression of tumor-promoting genes, such as *MT1-MMP,* remain unclear. Because of its delocalization in dedifferentiated epithelial cells, it would be pertinent to believe that ZO-1, once delocalized from the cell membrane, could modulate specific transcriptional pathways that control the expression of invasion-associated genes.

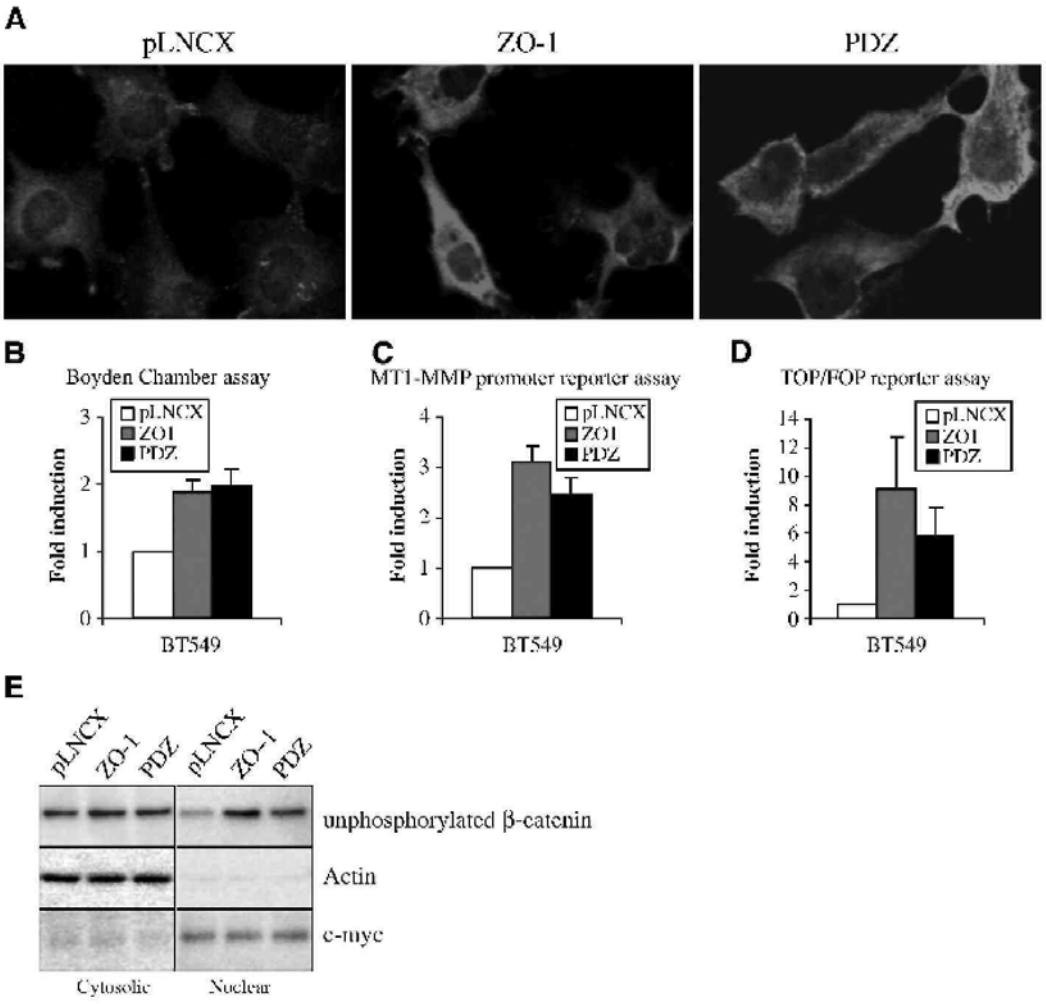
An interaction between ZO-1 and the β-catenin/TCF/LEF pathway has accordingly been documented. Reichert et al. have indeed reported the activation of the β-catenin/TCF/LEF transcription pathway following the transfection of MDCK I cells with the ZO-1-PDZ construct (9). In agreement with the results in MDCK I cells, transfection of BT549 cells with the Z0-1-PDZ and the Z0-1-WT construct activated the β-catenin/TCF/LEF pathway concomitantly with the MT1-MMP promoter. Consistent with the activation of β-catenin/TCF/LEF transcriptional activity, the unphosphorylated form of β-catenin accumulated in the nucleus of BT549 cells transfected with Z0-1-WT and ZO-l-PDZ compared with vector transfected cells. This agrees with the literature data showing that the unphosphorylated form of β-catenin is implicated in the transcriptional activity of β-catenin in the nucleus (23), although the selective binding of β-catenin to E-cadherin or TCF/LEF factors does only rely on the NH_2-terminal unphosphorylated status of β-catenin (35). Thus, although the implication of β-catenin-independent regulatory mechanisms cannot be excluded, our data suggest that the delocalization of ZO-1 could activate the β-catenin/TCF/LEF pathway, which could in turn induce MT1-MMP expression. Indeed, *MT1-MMP* has recently been reported to be a target gene of the β-catenin/TCF/ LEF pathway and β-catenin was shown to activate MT1-MMP transcription via its interaction with a TCF-binding element present in the MT1-MMP promoter (16, 17). The mechanism by which ZO-1 affects the β-catenin pathway is still unknown and a direct link between ZO-1 and β-catenin has not been established. Nevertheless, ZO-1 could interact with other molecules of the Wnt signaling pathway or of adherens junctions, thereby modulating the β-catenin signaling functions. For instance, in cells lacking TJs or in early stages of cell-cell adhesion of epithelial cells, ZO-1 has been reported to directly bind to α-catenin, a molecule that links the actin cytoskeleton to the E-cadherin/β-catenin complex, suggesting, that under certain conditions, α-catenin acts as a linker between ZO-1 and β-catenin (36, 37). More recently, ZO-1 and ZO-2 have been implicated in localizing ARVCF, which, like β-catenin, is an armadillo repeat protein and also binds to E-cadherin, to the plasma membrane or the nucleus (38).

Irrespective of the precise mechanism involved, our data are consistent with a scenario where ZO-1, if delocalized from TJ, can activate the β-catenin/TCF/LEF pathway, which in turn could regulate the expression of MT1-MMP.

In conclusion, our data establish a correlation between MT1-MMP expression and a cytoplasmic deloalization of ZO-1 in invasive occludin-negative tumor cells. Furthermore, we show that ZO-1 contributes to tumor cell invasion by modulating MT1-MMP expression likely via activation of β-catenin/TCF/LEF transcriptional

activity. These findings emphasize the dual role of adhesion molecules, such as ZO-1, as structural components of cell-cell junctions in noninvasive epithelial cells and as contributing factors to tumor progression through their ability to induce the expression of tumor-promoting genes in invasive cells.

Figure 7. Activation of β-catenin/TCF/LEF pathway and MT1-MMP promoter by ZO-1. *A,* immunofluorescence analyses of the localization of ZO-1 in BT549 tumor cells transfected with the empty expression vector (pLNCX), the ZO-1-WT expression vector (ZO-1), or the NH_2-terminal ZO-1-PDZ expression vector (PDZ). *B,* Boyden chamber analyses of the invasive capacity of BT549 cells transfected with ZO-1 -WT (ZO-1) or ZO-1 -PDZ (PDZ) or the control vector (pLNCX). Data are fold induction relative to the transfection of the empty expression vector ($P < 0.001$). *C,* MT1-MMP promoter reporter assay. BT549 tumor cells were cotransfected with a human MT1-MMP promoter luciferase reporter plasmid and either the empty expression vector (pLNCX), the ZO-1-WT expression vector (ZO-1), or the NH_2-terminal ZO-1-PDZ expression vector (PDZ). Data are fold induction relative to the cotransfection of the MT1-MMP reporter plasmid with the empty expression vector ($P < 0.001$). *D,* TOP-FLASH/FOP-FLASH reporter assay. The TOP-FLASH or FOP-FLASH reporter plasmids were cotransfected with the empty expression vector (pLNCX), the ZO-1 -WT expression vector (ZO-1), or the ZO-1 -PDZ expression vector (PDZ) in BT549 tumor cells. The normalized FOP values were subtracted from the normalized TOP values. Data are fold induction relative to the values obtained in the controls transfected with the empty expression vector ($P < 0.001$). *E,* Western blot analyses of the unphosphorylated form of β-catenin in cytosolic and nuclear fractions of BT549 cells transfected with either the empty expression vector (pLNCX), the ZO-1-WT expression vector (ZO-1), or the NH_2-terminal ZO-1-PDZ expression vector (PDZ). Actin and c-*myc* expression were analyzed as controls.



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References

1. Conacci-Sorrell M, Zhurinsky J, Ben Ze'ev A. The cadherin-catenin adhesion system in signaling and cancer. J Clin Invest 2002;109:987-91.

2. Gonzalez-Mariscal L, Betanzos A, Nava P, Jaramillo BE. Tight junction proteins. Prog Biophys Mol Biol 2003;81: 1-44.

3. Itoh M, Nagafuchi A, Yonemura S, Kitani-Yasuda T, Tsukita S, Tsukita S. The 220-kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy. J Cell Biol 1993;121:491-502.

4. Tsukita S, Itoh M, Nagafuchi A, Yonemura S, Tsukita S. Submembranous junctional plaque proteins include potential tumor suppressor molecules. J Cell Biol 1993; 123:1049-53.

5. Hoover KB, Liao SY, Bryant PJ. Loss of the tight junction MAGUK ZO-1 in breast cancer: relationship to glandular differentiation and loss of heterozygosity. Am J Pathol 1998;153:1767-73.

6. Kaihara T, Kusaka T, Nishi M, et al. Dedifferent-iation and decreased expression of adhesion molecules, E-cadherin and ZO-1, in colorectal cancer are closely related to liver metastasis. J Exp Clin Cancer Res 2003; 22:117-23.

7. Martin TA, Watkins G, Mansel RE, Jiang WG. Loss of tight junction plaque molecules in breast cancer tissues is associated with a poor prognosis in patients with breast cancer. Eur J Cancer 2004;40:2717-25.

8. Kleeff J, Shi X, Bode HP, et al. Altered expression and localization of the tight junction protein ZO-1 in primary and metastatic pancreatic cancer. Pancreas 2001;23:259-65.

9. Reichert M, Muller T, Hunziker W. The PDZ domains of zonula occludens-1 induce an epithelial to mesenchymal transition of Madin-Darby canine kidney I cells. Evidence for a role of β-catenin/Tcf/Lef signaling. J Biol Chem 2000;275:9492-500.

10. Gottardi CJ, Arpin M, Fanning AS, Louvard D. The junction-associated protein, zonula occludens-1, localizes to the nucleus before the maturation and during the remodeling of cell-cell contacts. Proc Natl Acad Sci USA 1996;93:10779-84.

11. Gonzalez-Mariscal L, Islas S, Contreras RG, et al. Molecular characterization of the tight junction protein ZO-1 in MDCK cells. Exp Cell Res 1999;248:97-109.

12. Polette M, Nawrocki-Raby B, Gilles C, Clavel C, Birembaut P. Tumour invasion and matrix metalloproteinases. Crit Rev Oncol Hematol 2004;49:179-86.

13. Seiki M, Yana I. Roles of pericellular proteolysis by membrane type-1 matrix metalloproteinase in cancer invasion and angiogenesis. Cancer Sci 2003;94:569-74.

14. Seiki M. Membrane-type 1 matrix metalloproteinase: a key enzyme for tumor invasion. Cancer Lett 2003;194: 1-11.

15. Gilles C, Newgreen D, Sato H, Thompson EW. Matrix metallop roteinases and epithelia-to-mesenchymal transition: implications for carcinoma metastasis. In: Savagner P, editor. Rise and fall of epithelial phenotype. Eurekah.com and New York: Kluwer Academic/Plenum Publishers.; 2004. Chap. 22.

16. KQubek F, Spaderna S, Jung A, Kirchner T, Brabletz T. β-Catenin activates a coordinated expression of the proinvasive factors laminin-5 72 chain and MTl-MMP in colorectal carcinomas. Int J Cancer 2004;108:321-6.

17. Takahashi M, Tsunoda T, Seiki M, Nakamura Y, Furukawa Y. Identification of membrane-type matrix metalloproteinase-1 as a target of the β-catenin/Tcf4 complex in human colorectal cancers. Oncogene 2002; 21:5861-7.

18. Lohi J, Lehti K, Valtanen H, Parks WC, Keski-Oja J. Structural analysis and promoter characterization of the human membrane-type matrix metalloproteinase-1 {MTl-MMP) gene. Gene 2000;242:75-86.

19. Korinek V, Barker N, Morin PJ, et al. Constitutive transcriptional activation by a β-catenin-Tcf complex in APC-/- colon carcinoma. Science 1997;275: 1784-7.

20. Sommers CL, Byers SW, Thompson EW, Torri JA, Gelmann EP. Differentiation state and invasiveness of human breast cancer cell lines. Breast Cancer Res Treat 1994;31:325-35.

21. Thompson EW, Paik S, Brunner N, et al. Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. J Cell Physiol 1992; 150:534-44.

22. Nawrocki RB, Polette M, Gilles C, et al. Quantitative cell dispersion analysis: new test to measure tumor cell aggressiveness. Int J Cancer 2001;93:644-52.

23. Staal FJ, Noort MM, Strous GJ, Clevers HC. Wnt signals are transmitted through N-terminally dephos-phorylated β-catenin. EMBO Rep 2002;3:63-8.

24. Busch C, Hanssen TA, Wagener C, OBrink B. Down-regulation of CEACAMl in human prostate cancer: correlation with loss of cell polarity, increased proliferation rate, and Gleason grade 3 to 4 transition. Hum Pathol 2002;33:290-8.

25. Tobioka H, Isomura H, Kokai Y, Tokunaga Y, Yamaguchi J, Sawada N. Occludin expression decreases with the progression of human endometrial carcinoma. Hum Pathol 2004;35:159-64.

26. Hoevel T, Macek R, Mundigl O, Swisshelm K, Kubbies M. Expression and targeting of the tight junction protein CLDN1 in CLDN1-negative human breast tumor cells. J Cell Physiol 2002;191:60-8.

27. Wan H, Winton HL, Soeller C, et al. Tight junction properties of the immortalized human bronchial epithelial cell lines Calu-3 and 16HBE14o-. Eur Respir J 2000;15:1058-68.

28. Gilles C, Polette M, Coraux C, et al. Contribution of MT1-MMP and of human laminin-5 γ*2* chain degradation to mammary epithelial cell migration. J Cell Sci 2001;114:2967-76.

29. Ueda J, Kajita M, Suenaga N, Fujii K, Seiki M. Sequence-specific silencing of MTl-MMP expression suppresses tumor cell migration and invasion: importance of MTl-MMP as a therapeutic target for invasive tumors. Oncogene 2003;22:8716-22.

30. Sounni NE, Devy L, Hajitou A, et al. MTl-MMP expression promotes tumor growth and angiogenesis through an up-regulation of vascular endothelial growth factor expression. FASEB J 2002;16:555-64.

31. Sato H, Takino T, Okada Y, et al. A matrix metalloproteinase expressed on the surface of invasive tumour cells [see comments]. Nature 1994;370:61-5.

32. Takahisa M, Togashi S, Suzuki T, et al. The *Drosophila* tamou gene, a component of the activating pathway of extramacrochaetae expression, encodes a protein homologous to mammalian cell-cell junction-associated protein ZO-1. Genes Dev 1996;10:1783-95.

33. Balda MS, Matter K. The tight junction protein ZO-1 and an interacting transcription factor regulate ErbB-2 expression. EMBO J 2000;19:2024-33.

34. Ryeom SW, Paul D, Goodenough DA. Truncation mutants of the tight junction protein ZO-1 disrupt corneal epithelial cell morphology. Mol Biol Cell 2000; 11:1687-96.

35. Gottardi CJ, Gumbiner BM. Distinct molecular forms of β-catenin are targeted to adhesive or transcriptional complexes. J Cell Biol 2004;167:339-49.

36. Imamura Y, Itoh M, Maeno Y, Tsukita S, Nagafuchi A. Functional domains of α-catenin required for the strong state of cadherin-based cell adhesion. J Cell Biol 1999; 144:1311-22.

37. Itoh M, Nagafuchi A, Moroi S, Tsukita S. Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to α catenin and actin filaments. J Cell Biol 1997;138:181-92.

38. Kausalya PJ, Phua DC, Hunziker W. Association of ARVCF with ZO-1 and ZO-2: binding to PDZ-domain proteins and cell-cell adhesion regulate plasma membrane and nuclear localization of ARVCF. Mol Biol Cell 2004;15:5503-15.