Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail

Junichi Ikenouchi*, Miho Matsuda*, Mikio Furuse and Shoichiro Tsukita‡

Department of Cell Biology, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

*These authors contributed equally to this work

‡Author for correspondence (e-mail: htsukita@mfour.med.kyoto-u.ac.jp)

Accepted 27 January 2003

Journal of Cell Science 116, 1959-1967 © 2003 The Company of Biologists Ltd

doi:10.1242/jcs.00389

Summary

Snail is a transcription repressor that plays a central role in the epithelium-mesenchyme transition (EMT), by which epithelial cells lose their polarity. Claudins and occludin are integral membrane proteins localized at tight junctions, which are responsible for establishing and maintaining epithelial cell polarity. We examined the relationship between Snail and the promoter activity of claudins and occludin. When Snail was overexpressed in cultured mouse epithelial cells, EMT was induced with concomitant repression of the expression of claudins and occludin not only at the protein but also at the mRNA level. We then isolated the promoters of genes encoding claudins and occludin, in which multiple E-boxes were identified. Transfection experiments with various promoter constructs as well as electrophoretic mobility assays revealed that Snail binds directly to the E-boxes of the promoters of claudin/occludin genes, resulting in complete repression of their promoter activity. Because the gene encoding E-cadherin was also reported to be repressed by Snail, we concluded that EMT was associated with the simultaneous repression of the genes encoding E-cadherin and claudins/occludin (i.e. the expression of adherens and tight junction adhesion molecules, respectively).

Key words: Snail, Claudin, Occludin, Tight junction, Epithelium-mesenchyme transition

Introduction

The epithelium-mesenchyme transition (EMT) has attracted increasing interest from both developmental cell biologists and cancer researchers (Hay, 1995). EMT occurs in various steps in normal development, including mesoderm and neural-crest formation. Furthermore, the process of acquisition of an invasive phenotype by tumors of epithelial origin can be regarded as a pathological version of EMT. At the cellular level, EMT includes two distinct steps: decreased intercellular adhesion (to dissociate from the epithelial cellular sheets) and increased cell motility (to migrate into connective tissues). These steps are always associated with the total loss of epithelial cell polarity.

The tight junction (TJ) is an important structure that determines epithelial cell polarity and disappears during EMT. TJs constitute the epithelial junctional complex, together with adherens junctions (AJs) and desmosomes, and are located at the most apical part of the complex (Farquhar and Palade, 1963). TJs create the primary barrier to the diffusion of solutes through the paracellular pathway and maintain cell polarity as a boundary between the apical and basolateral plasma membrane domains (Schneeberger and Lynch, 1992; Gumbiner, 1993; Anderson and van Itallie, 1995; Tsukita et al., 2001). On ultrathin section electron microscopy, TJs appear as a series of discrete sites of apparent fusion, involving the outer leaflet of the plasma membranes of adjacent cells (Farquhar and Palade, 1963). On freeze-fracture replica electron microscopy, TJs appear as a set of continuous, anastomosing intramembranous particle strands (TJ strands) (Staehelin, 1974).

The molecular architecture of TJs has been unraveled rapidly in recent years. Three closely related PDZ-domain-containing proteins (ZO-1, ZO-2 and ZO-3) constitute the undercoat structure of TJs together with other peripheral membrane proteins such as cinglin, 7H6 antigen and symplekin (Stevenson et al., 1986; Gumbiner et al., 1991; Balda et al., 1993; Citi et al., 1988; Zhong et al., 1993; Keon et al., 1996; Mitic and Anderson, 1998; Tsukita and Furuse, 1999a). As constituents of TJ strands themselves, two distinct types of integral membrane proteins have been identified: occludin and claudins (Furuse et al., 1993; Furuse et al., 1998a; Furuse et al., 1998b). Both occludin and claudins bear four transmembrane domains but do not show any sequence similarity with each other. Claudins and occludin are thought to constitute the backbone of TJ strands and to modulate some functions of TJs, respectively (Tsukita et al., 2001). Claudins compose a multi-gene family consisting of more than 20 members (Morita et al., 1999; Tsukita and Furuse, 1999b).

The question has naturally arisen of how TJs with such a complicated molecular architecture disappear during EMT. Recently, a zinc-finger transcription factor, Snail, has been implicated in the switching mechanism for EMT (Nieto, 2002). This gene was initially identified in *Drosophila* to be responsible for gastrulation (Grau et al., 1984; Nusslein-Volhard et al., 1984; Alberga et al., 1991). When chick embryos were treated with antisense oligonucleotides against *Slug*, the functional homolog

of *Snail* in chicks, their mesoderm formation was affected (Nieto et al., 1994). Furthermore, *Snail*-deficient mice died at the gastrulation stage because of incomplete EMT (Carver et al., 2001). These mice developed a mesodermal layer that expressed some mesoderm-specific genes, but the mesoderm layer possessed some epithelial characteristics such as apical-basal polarity, retaining the expression of E-cadherin, a major cell adhesion molecule at AJs. This was consistent with our previous observation that a *Drosophila Snail* mutant failed to downregulate *E-cadherin* expression at the ectoderm prior to gastrulation (Oda et al., 1998).

Recently, Snail has been shown to bind directly to E-boxes in the *E-cadherin* promoter and to repress *E-cadherin* expression directly, resulting in the destruction of AJs (Cano et al., 2000; Batlle et al., 2000). In this study, from the viewpoint of epithelial cell polarity, we examined the molecular mechanism for the destruction of TJs during EMT. We first established an in vitro mouse Snail-induced EMT using mouse cultured epithelial cells, and found that Snail directly suppressed the gene expression of claudins and occludin. Furthermore, we showed that E-boxes in the *claudin* and *occludin* promoters were responsible for this Snail-induced repression of their promoter activities. We believe that these findings provide a new insight into the molecular mechanism of EMT.

Materials and Methods

Antibodies and cells

Rat anti-mouse occludin monoclonal antibody (mAb) (MOC37) (Saitou et al., 1997) and mouse anti-rat ZO-1 mAb (T8-754) (Itoh et al., 1993) were developed and have been characterized previously. Mouse anti-p120 mAb, mouse anti-cytokeratin 18 mAb (clone 18.04) and rabbit anti-claudin-3 pAb were purchased from Transduction Laboratories, Progen Biotechnik and Zymed Laboratories, respectively. Rat anti-mouse E-cadherin mAb (ECCD-2) was a gift from M. Takeichi (Kyoto University, Kyoto, Japan). Mouse Eph4 epithelial cells, mouse CSG1 epithelial cells, mouse NIH/3T3 fibroblasts, mouse L fibroblasts and human 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

SDS-PAGE and immunoblotting

The whole cell lysates of cultured cells were subjected to one-dimensional SDS-PAGE (12.5%), according to the method of Laemmli (Laemmli, 1970), and gels were stained with Coomassie brilliant blue R-250. For immunoblotting, proteins were electrophoretically transferred from gels onto nitrocellulose membranes, which were then incubated with the first antibody. Bound antibodies were detected with biotinylated second antibodies and streptavidin-conjugated alkaline phosphatase (Amersham, Arlington Heights, IL). Nitroblue tetrazolium and bromochloroindolyl phosphate were used as substrates for the detection of alkaline phosphatase.

Immunofluorescence microscopy

Cells cultured on cover slips were rinsed twice with PBS and fixed with ice-cooled methanol for 10 minutes. After rinsing in PBS, the fixed cells were blocked with 1% bovine serum albumin (BSA) in PBS for 30 minutes and then incubated with primary antibodies for 30 minutes. They were then rinsed three times with PBS and incubated with appropriate secondary antibodies for 30 minutes. The secondary antibodies used were FITC-conjugated donkey anti-rabbit IgG polyclonal antibody (pAb), FITC-conjugated donkey anti-mouse IgG pAb and FITC-conjugated donkey anti-rat IgG pAb (Jackson Immunoresearch, West Grove, PA). After rinsing with PBS, the samples were embedded in 90% glycerol-PBS containing 0.1% *para*-phenylendiamine and 1% *n*-propylgalate.

Northern blotting

Aliquots of total RNA (10 μg) were separated by 1.0% agarose-formaldehyde gel electrophoresis. Hybridization with digoxigenin (DIG)-labeled RNA probes was performed according to the manufacturer's protocol (Roche). Briefly, RNA was transferred onto positively-charged nylon membranes, followed by ultraviolet (UV) cross-linking. Nylon membranes were then hybridized with DIG-labeled RNA probes at 65°C in a buffer solution containing 50% formamide. After thorough washing and blocking, the membranes were incubated with alkaline-phosphatase-conjugated anti-DIG antibodies for 1 hour. After extensive rinsing, the membranes were incubated with the 1,2-dioxetane substrate CSPD (Tropix, Bedford, MA) and exposed to X-ray film. To obtain DIG-labeled probes, reverse-transcription PCR was performed. Total RNA was isolated according to the method developed previously (Chomczynski and Sacchi, 1987).

Snail expression vector and transfection

Using a total cDNA population obtained from NIH/3T3 cells as a template, the full-length cDNA of mouse *Snail* was amplified by PCR and cloned into the pCAG vector (pCAG-mSnail). Similarly, a human Snail expression vector (pCAG-hSnail) was constructed after its full-length cDNA was amplified by PCR using a total cDNA library of SW480 cells as a template. A mouse *Snail* mutant lacking the SNAG (Snail/Gfi) domain was created according to the method described previously (Cano et al., 2000).

The cultured cells were transfected with one of the above expression vectors or an empty vector in serum-free DMEM containing 50 μM $CaCl_2$ using LipofectAmine Plus (Gibco BRL). After a 2-week selection in growth medium containing 400 μg ml^{-1} of G418, resistant colonies were separated and then screened. Ten and six independent clones were established for Snail-expressing Eph4 and CSG1 cells, respectively.

Isolation of promoter fragments, mutagenesis and reporter assays

Mouse *claudin-3* (–1536 to +141), mouse *claudin-4* (–1669 to +105), mouse *claudin-7* (–2336 to +195) and human *occludin* (–135 to +145) promoter fragments were cloned by screening the mouse and human genomic library with respective cDNA fragments, and were then inserted into the pGL3 vector (Promega). These reporter constructs (0.5 μg DNA $well^{-1}$) were transfected into cells cultured on 12-well dishes as described above and, 24 hours after transfection, firefly luciferase (Luc) and *Renilla* luciferase (RLluc) activities were measured using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. RLluc activity was used to normalize Luc activity. In all experiments, the total amount of transfected DNA was standardized with an empty pCAG vector.

To mutate the E-box sequence in the mouse *claudin-7* promoter, a Quickchange Site Directed Mutagenesis Kit (Stratagene) was used. The core sequence, 5′-CA(G/C)(G/C)TG-3′, was mutated to 5′-AA(G/C)(G/C)TA-3′.

Electrophoretic mobility shift assay

The double-stranded oligonucleotides corresponding to the following E-box sequences were synthesized: the mouse *claudin-7* E-box (+88 to +112), 5′-GGTGCGCCGCACCTGCTCG-CCCGCA-3′, and the human *occludin* E-box (+14 to +42), 5′-CATCCGAGTTTCAGGT-

GAATTGGTCACC-3′. They were then end-labeled with ^{32}P-α-CTP using the Klenow enzyme. In mutated double-stranded oligonucleotides, the core sequence 5′-CA(G/C)(G/C)TG-3′ was mutated to 5′-AA(G/C)(G/C)TA-3′. The mRNA of hemagglutinin (HA)-tagged Snail was synthesized in vitro with T7 RNA polymerase using pCITE-HAmSnail as a template and then translated in rabbit reticulocyte lysate (Promega).

An electrophoretic mobility shift assay was performed essentially according to the method described previously (Kasai et al., 1992). Briefly, in vitro translated HA-Snail protein (or luciferase as a control) (1 μg protein) was incubated with ^{32}P-labeled oligonucleotides in 50 ml gel retardation buffer consisting of 12 mM HEPES (pH 7.8), 100 mM KCl, 15 mM $ZnCl_2$, 1 mM DTT, 12% (v/v) glycerol, 0.05% NP-40, 20 μg ml^{-1} BSA, and 700 mg ml^{-1} poly (dI-dC) for 30 minutes at room temperature. For the competition experiments, unlabeled oligonucleotides were added 10 minutes before the labeled ones. To detect the super-shift of the band, the solution was incubated with anti-HA mAb [or anti-green-fluorescent-protein (anti-GFP) mAb as a control] for 15 minutes at room temperature. Samples were then electrophoresed on 4% acrylamide gel with 0.5 M Tris-borate EDTA, and the gels were dried, followed by autoradiography.

Biotinylated oligonucleotide precipitation assay

DNA precipitations were carried out essentially according to the method described previously (Hata, 2000). Briefly, human 293 cells transiently expressing mouse HA-Snail were lysed, and the lysate was pre-absorbed using ImmunoPure streptavidin-agarose beads (Pierce) for 1 hour. The sample was then incubated with 1 μg of biotinylated double-stranded oligonucleotides corresponding to the E4 E-box sequence of the *claudin-7* promoter, an E-box sequence of the *occludin* promoter, or their mutated sequences (see above), together with 10 μg of poly(dI-dC) for 16 hours. Biotinylated DNA-protein complexes were recovered using streptavidin-agarose beads for 1 hour, rinsed with HKMG buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT and 0.5% NP-40) and separated on SDS-polyacrylamide gels. Bound HA-Snail was detected by immunoblotting with anti-HA mAb.

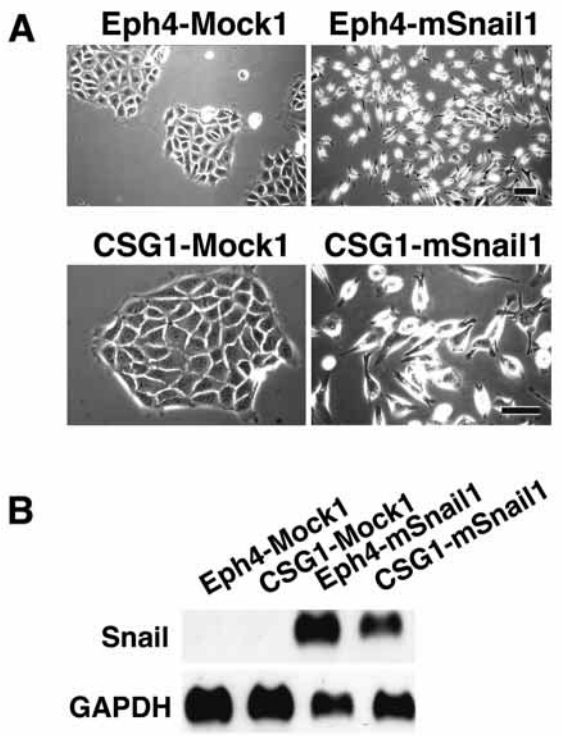


Fig. 1. Establishment of Eph4 and CSG1 stable transfectants expressing mouse *Snail*. Mouse *Snail* cDNA was isolated and introduced into mouse epithelial cell lines Eph4 and CSG1. Phase contrast images revealed the in vitro Snail-induced EMT in Eph4 and CSG1 cells (A). Both Eph4-Mock and CSG1-Mock cells exhibited a typical cobblestone-like appearance. By contrast, when *Snail* was overexpressed, these cells acquired a more fibroblastic phenotype (Eph4- and CSG1-mSnail). Northern blotting confirmed the absence and presence of *Snail* mRNA in parental and transfectants, respectively (B). As a control, the mRNA levels of GAPDH are shown. Bars, 60 μm.

Results

Exogenous expression of *Snail* induces EMT in cultured mouse epithelial cells

To examine the effects of the Snail expression on epithelial morphology in cultured cells, dog MDCK II cells or human HT29M6 cells have previously been used (Cano et al., 2000; Batlle et al., 2000). In this study, we attempted to establish in vitro Snail-induced EMT using cultured mouse epithelial cells. For this purpose, we chose two mouse epithelial cell lines, Eph4 and CSG1, that show a typical cobblestone-like appearance under confluent culture conditions. When mouse *Snail* cDNA was introduced into these cells, they acquired a more fibroblastic phenotype (Fig. 1): they were rounded with many cellular protrusions, and their cell-cell adhesion appeared to be downregulated. We isolated several independent stable clones for each cell line. Because all of the independent clones obtained showed the same phenotype, we mainly used one clone of the Eph4 transfectants expressing mouse *Snail* (Eph4-mSnail) for further analyses.

Snail alters the expression levels of AJ and TJ integral membrane proteins

We examined the expression of AJ and TJ components in Eph4 and Eph4-mSnail cells by immunoblotting (Fig. 2A). Consistent with previous observations in dog and human epithelial cells (Cano et al., 2000; Batlle et al., 2000), the expression of E-cadherin in Eph4-mSnail cells was completely repressed. Furthermore, TJ integral membrane proteins such as claudin-3 and occludin became undetectable at the protein level. However, the undercoat proteins such as p120 (a cadherin-binding protein) and ZO-1 (an occludin/claudin-binding protein) did not alter their expression levels compared with the wild-type cells. This expression pattern of AJ and TJ components in Eph4-mSnail cells is very similar to that in NIH/3T3 fibroblasts. We then compared the subcellular distribution of these proteins between parental Eph4 and Eph4-mSnail cells by immunofluorescence microscopy (Fig. 2B). In Eph4-mSnail cells, E-cadherin became undetectable not only at cell-cell contact regions but also in the cytoplasm. In these cells, an AJ undercoat component, p120, showed no concentration at the cell-cell contact regions but was diffusely distributed in the cytoplasm. Interestingly, claudin-3 and occludin, which were concentrated at TJs in parental Eph4 cells, completely disappeared in Eph4-mSnail cells, whereas ZO-1 (a TJ undercoat protein) changed its localization from TJs to the cytoplasm. Non-junctional epithelial markers such

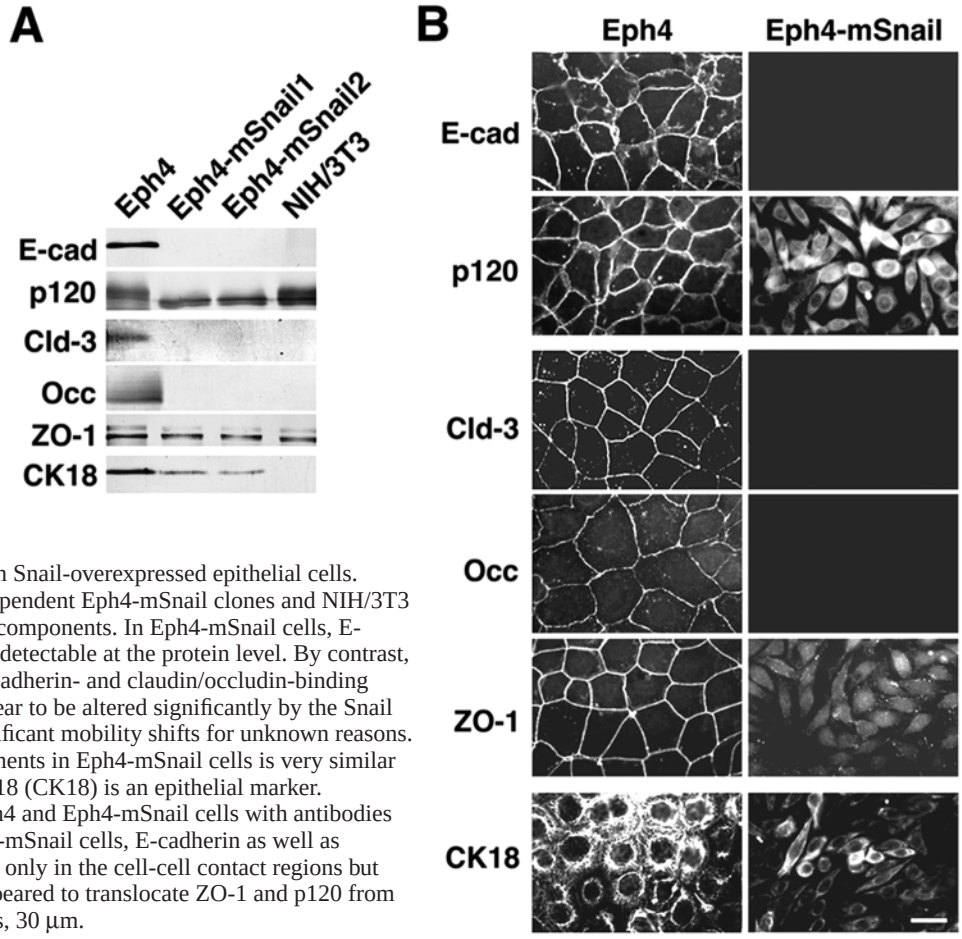
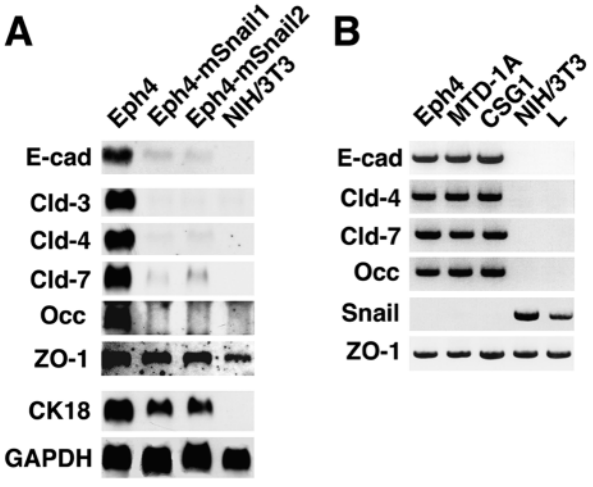


Fig. 2. Behavior of AJ and TJ constituents in Snail-overexpressed epithelial cells. (A) Immunoblotting of Eph4 cells, two independent Eph4-mSnail clones and NIH/3T3 cells with antibodies specific for AJ and TJ components. In Eph4-mSnail cells, E-cadherin, claudin-3 and occludin became undetectable at the protein level. By contrast, the expression levels of p120 and ZO-1 (E-cadherin- and claudin/occludin-binding undercoat protein, respectively) did not appear to be altered significantly by the Snail overexpression, although p120 showed significant mobility shifts for unknown reasons. The expression pattern of AJ and TJ components in Eph4-mSnail cells is very similar to that in NIH/3T3 fibroblasts. Cytokeratin-18 (CK18) is an epithelial marker. (B) Immunofluorescence microscopy of Eph4 and Eph4-mSnail cells with antibodies specific for AJ and TJ components. In Eph4-mSnail cells, E-cadherin as well as claudin-3/occludin became undetectable not only in the cell-cell contact regions but also in the cytoplasm. By contrast, Snail appeared to translocate ZO-1 and p120 from the junctional regions to the cytoplasm. Bars, 30 μm.

as cytokeratin-18 appeared to be downregulated by the Snail overexpression, but not completely.

The next question was whether the disappearance of occludin/claudins in Eph4-mSnail cells is due to direct repression of their transcription by Snail or to some indirect mechanism that facilitates their degradation. To address this question, we performed northern blotting (Fig. 3A). In Eph4-mSnail cells, the transcription of *claudin-3*, *claudin-4*, *claudin-7* and *occludin*, all of which were expressed abundantly in parental Eph4 cells, completely shut down together with the *E-cadherin* transcription. Consistent with the immunoblotting data, the mRNA levels of *ZO-1* (and also p120; data not shown) did not alter significantly. Furthermore, the expression patterns of these mRNAs in Eph4-mSnail cells were very similar to those in NIH/3T3 fibroblasts and L fibroblasts (Fig. 3A,B). Taken together, we concluded that Snail directly and simultaneously represses the transcription of occludin and distinct species of claudins.



Snail represses the promoter activities of the claudin and occludin genes

Random selection and transfection experiments identified a core of six bases [CA(G/C)(G/C)TG] as the consensus binding site for Snail (Muhin et al., 1993; Fuse et al., 1994; Inukai et al., 1999; Kataoka et al., 2000). This motif is identical to the

Fig. 3. The mRNA levels of AJ and TJ constituents in epithelial cells overexpressing Snail. (A) Northern blotting of Eph4, Eph4-mSnail and NIH/3T3 cells. Snail completely shut down the transcription of *E-cadherin*, *claudin* genes and *occludin*, but not of *ZO-1* or *cytokeratin-18*. As a control, the GAPDH gene was detected in equal amounts in all samples. (B) Comparison of the mRNA levels of *E-cadherin*, *claudin-4*, *claudin-7*, *occludin*, *Snail* and *ZO-1* between mouse epithelial cells (Eph4, MTD-1A and CSG1) and fibroblasts (NIH/3T3 and L).

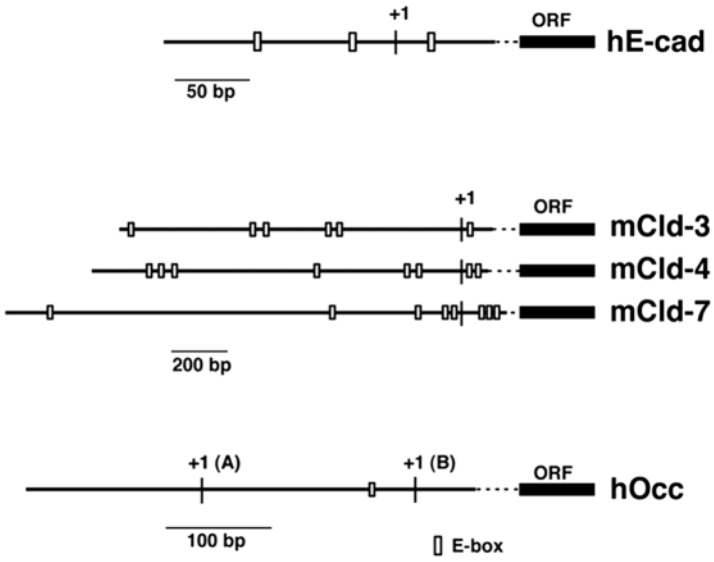


Fig. 4. Schematic representation of the promoter region of human *E-cadherin*, mouse *claudin-3*, *claudin-4* and *claudin-7*, and human *occludin*. The putative transcription start point for each claudin promoter was estimated according to the expressed sequence tag database. Open box, E-box; +1, putative transcription start point; ORF, open reading frame. For the *occludin* gene, two possible transcription start points have been suggested (Mankertz et al., 2000).

E-box. Indeed, the human *E-cadherin* promoter contains three E-boxes (Fig. 4) and Snail was reported to directly bind to these E-boxes to repress *E-cadherin* transcription (Cano et al., 2000; Batlle et al., 2000). Therefore, we next examined whether the gene transcription of TJ integral membrane proteins, claudins and occludin is also directly regulated by Snail. Then, we isolated the promoters of mouse *claudin-3*, *claudin-4* and *claudin-7*. The putative transcription start point for each promoter was estimated according to the expressed sequence tag database (Fig. 4). Interestingly, these promoters contained six, eight and eight E-boxes, respectively. The human *occludin* promoter was isolated previously (Mankertz et al., 2000) and also contained one E-box (Fig. 4).

We then inserted the isolated fragments of the *claudin-3*, *claudin-4* and *claudin-7* promoters into the pGL3 plasmid upstream of the luciferase reporter gene, and transfected these reporter constructs into Eph4 epithelial cells and NIH/3T3 fibroblasts (Fig. 5A). In Eph4 cells, the *claudin* promoters induced a three- to tenfold increase in relative luciferase activity above that observed in NIH/3T3 cells, indicating that these promoter regions were sufficient to show the epithelium-specific activity. As previously reported (Mankertz et al., 2000), the isolated *occludin* promoter also showed similar epithelium-specific activity (data not shown). We next examined the ability of Snail to repress the *claudin* promoter activities. When the Snail expression vector and the

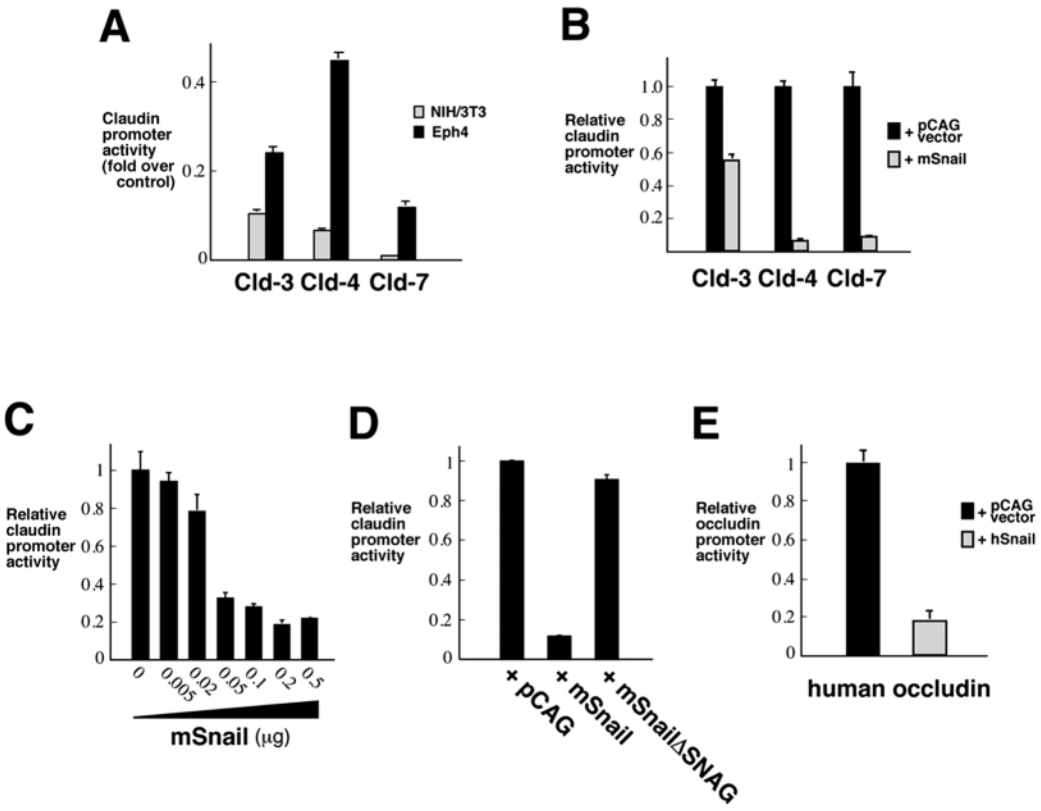


Fig. 5. Snail-induced repression of the promoter activities of *claudin* genes and *occludin*. Luciferase reporter constructs carrying mouse *claudin-3*, *claudin-4* or *claudin-7* promoter were transfected into Eph4 epithelial cells or NIH/3T3 fibroblasts singly or together with the *Snail* expression vector. In Eph4 cells, the *claudin* promoters induced a three- to tenfold increase in relative luciferase activity above that observed in NIH/3T3 cells, indicating that the *claudin* promoters are activated in an epithelium-specific manner (A). When mouse Snail was co-expressed in Eph4 cells, the activities of *claudin* promoters were remarkably repressed (B), and the repression of *claudin-7* promoter was depended on the dose of Snail (C). The *Snail* mutant lacking its N-terminal SNAG domain showed no repressor activity for the *claudin-7* promoter (D). Similarly, luciferase reporter construct carrying human *occludin* promoter was transfected into human HT29 epithelial cells (or Eph4 cells) singly or together with the *Snail* expression vector. The human occludin promoter was repressed by Snail in both HT29 cells (E) and Eph4 cells (data not shown). All results correspond to the average of three independent experiments.

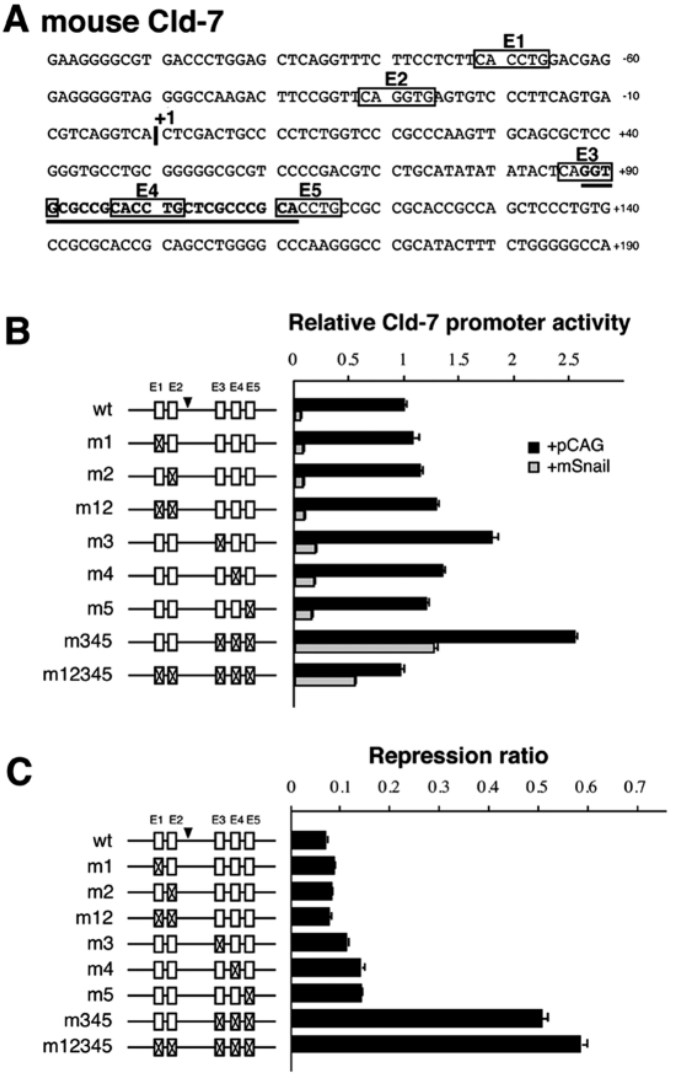


Fig. 6. Impairment of Snail-induced repression of the *claudin-7* promoter by mutations of the E-boxes. (A) A short fragment of the mouse *claudin-7* promoter region (–110 to +190; Fig. 4). This short fragment includes five E-boxes (E1-E5) and showed the epithelium-specific promoter activity (data not shown). Double-stranded oligonucleotides corresponding to the E4-containing sequence (underlined sequence) were used in the electrophoretic mobility shift/oligonucleotide precipitation assays in Fig. 7. (B,C) Mutational analyses. The core sequence, 5′-CA(G/C)(G/C)TG-3′, of E-boxes (E1~E5) was mutated to 5′-AA(G/C)(G/C)TA-3′ in various combinations (shadowed boxes). Luciferase reporter constructs carrying wild-type or these mutated *claudin-7* promoters were transfected into Eph4 cells together with a mouse *Snail* expression vector (mSnail) or an empty vector (pCAG). (B) Luciferase activity found in cells co-transfected with a wild-type reporter construct and pCAG empty vector was defined as 1.0. (C) The same set of data expressed as the Snail-induced repression ratio (+mSnail/+pCAG in B) for individual reporter constructs. As the number of mutated E-boxes increased, the *claudin-7* promoter became less sensitive to Snail. For no known reason, even when all five E-boxes were mutagenized, the Snail-induced repression ratio did not reach 1.0. The arrowhead shows the putative transcription start point. All results correspond to the average of three independent experiments.

claudin reporter constructs were co-transfected into Eph4 cells, the promoter activities of *claudin-3*, *claudin-4* and *claudin-7* were remarkably repressed (Fig. 5B), and this repression depended on the dose of *Snail* (Fig. 5C). Furthermore, when co-transfected with a *Snail* mutant lacking the N-terminal SNAG domain, which is required for the repressor activity of Snail in general (Grimes et al., 1996; Nakayama et al., 1998), the *claudin-7* promoter activity was not repressed (Fig. 5D). Similar Snail-induced repression was also observed for the *occludin* promoter in human epithelial cells (HT29) (Fig. 5E). These findings indicated that the transcription of *claudins* and *occludin* was directly regulated by Snail by modulating the activities of their promoters.

Snail directly binds to E-boxes in the claudin-7 promoter

To clarify further the molecular mechanism behind the Snail-induced repression of the claudin transcription, we examined a shorter fragment of the *claudin-7* promoter in more detail (Fig. 6A). This short fragment, with five E-boxes, also showed epithelium-specific promoter activity (data not shown). We generated a series of reporter constructs that carried various combinations of mutated E-boxes and introduced them into Eph4 cells together with the Snail expression vector or an empty vector (Fig. 6B). Interestingly, when a single E-box was mutated, no significant impairment of Snail-induced repression was observed. However, as the number of mutated E-boxes was increased, the *claudin-7* promoter became less sensitive to Snail (Fig. 6B,C), suggesting that E-boxes in the *claudin-7* promoter are responsible for the Snail-induced repression.

We next performed an electrophoretic mobility shift assay. When the in vitro translated HA-Snail was incubated with ^{32}P-labeled double-stranded oligonucleotides corresponding to the sequence containing one of the E-boxes (E4) of the *claudin-7* promoter (Fig. 6A), a single DNA-protein complex was observed (Fig. 7A). This complex was not formed with mutated oligonucleotides. Unlabeled wild-type oligonucleotides, but not mutated oligonucleotides, competed with the formation of the complex. Furthermore, anti-HA mAb, but not anti-GFP mAb, shifted the band of the complex upward. The same results were obtained when double-stranded oligonucleotides corresponding to the E-box in the *occludin* promoter were used (Fig. 7B). These findings indicated that Snail directly binds to the E-box in the *claudin-7* and *occludin* promoters, at least in vitro.

Finally, to confirm the binding of Snail to the E-box (E4) of the *claudin-7* promoters in the nuclear extract, we performed a biotinylated oligonucleotide precipitation assay (Fig. 7C, lanes 1-3). Biotin-labeled double-stranded wild-type or mutated oligonucleotides of the *claudin-7* E4 sequence (Fig. 6A) were incubated with a nuclear extract prepared from 293 cells transfected with the HA-Snail expression vector or an empty vector. The biotinylated oligonucleotides were then recovered using streptavidin-conjugated agarose beads, and bound HA-Snail was detected by immunoblotting with anti-HA mAb. The HA-Snail in the nuclear extracts bound specifically to the wild-type, but not to the mutated, oligonucleotides (Fig. 7C). Similar specific binding was detected when the oligonucleotides of the *occludin* E-box sequence were used (Fig. 7C, lanes 4-6). Taken together, we concluded that Snail binds directly to E-boxes in the *claudin*

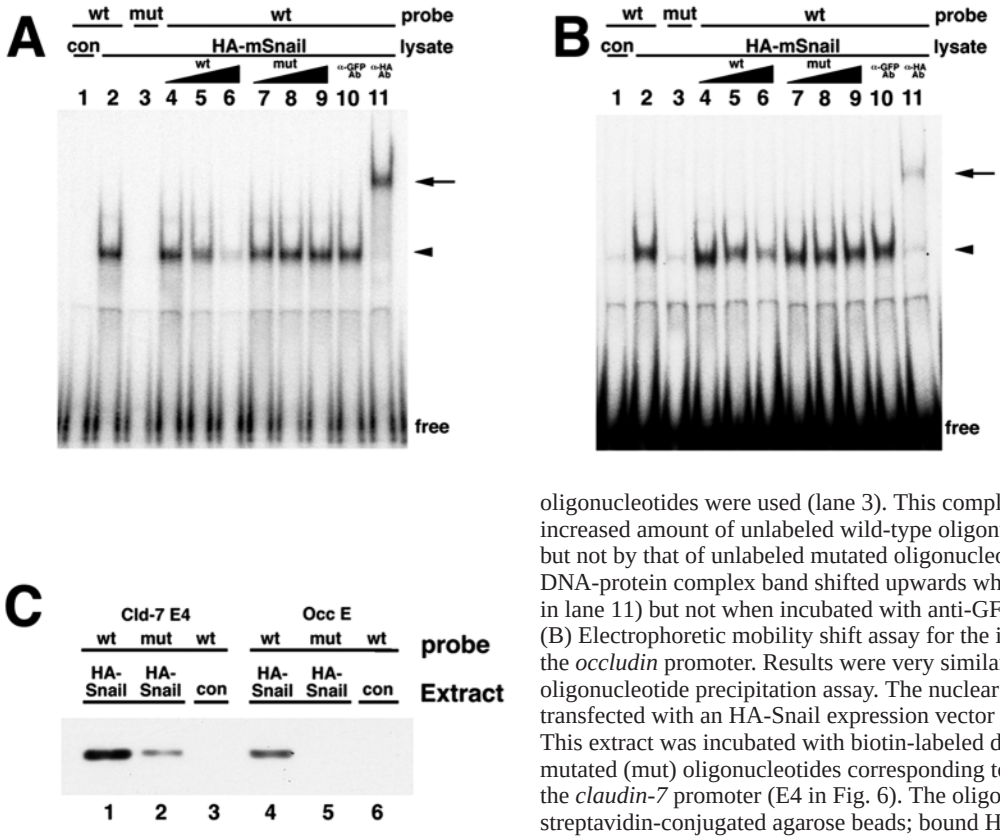


Fig. 7. Direct binding of Snail to the E-box in *claudin-7* and *occludin* promoters. (A) Electrophoretic mobility shift assay for the interaction of Snail with an E-box in the *claudin-7* promoter. ^{32}P-labeled double-stranded oligonucleotides corresponding to the sequence containing one of the E-boxes of the *claudin-7* promoter (E4 in Fig. 6) formed a DNA-protein complex with the in vitro translated/HA-tagged mouse Snail (HA-mSnail) (arrowhead in lane 2), but not with the in vitro translated luciferase (control; lane 1). This complex formation was not observed when mutated oligonucleotides were used (lane 3). This complex formation was affected by an increased amount of unlabeled wild-type oligonucleotides (arrowhead in lanes 4-6), but not by that of unlabeled mutated oligonucleotides (arrowhead in lanes 7-9). The DNA-protein complex band shifted upwards when incubated with anti-HA pAb (arrow in lane 11) but not when incubated with anti-GFP pAb (control, lane 10). (B) Electrophoretic mobility shift assay for the interaction of Snail with an E-box in the *occludin* promoter. Results were very similar to those shown in A. (C) Biotinylated oligonucleotide precipitation assay. The nuclear extract was prepared from 293 cells transfected with an HA-Snail expression vector (HA-Snail) or an empty vector (con). This extract was incubated with biotin-labeled double-stranded wild-type (wt) or mutated (mut) oligonucleotides corresponding to the sequence containing the E-box of the *claudin-7* promoter (E4 in Fig. 6). The oligonucleotides were then recovered using streptavidin-conjugated agarose beads; bound HA-Snail was detected by immunoblotting with anti-HA mAb. HA-Snail bound specifically to the wild type, not mutated, oligonucleotides (lanes 1-3). A similar specific binding was detected when the oligonucleotides of the E-box sequence in the *occludin* promoter were used (lanes 4-6).

and *occludin* promoters, and that Snail directly represses their activities.

Discussion

The EMT is an important mechanism for the development of multicellular organisms, by which, for example, the mesoderm and neural crest are generated (Hay, 1995). Thus, a detailed description of EMT in molecular terms is an important issue in developmental and cellular biology. However, EMT occurs in spatially restricted regions in a transient manner during embryogenesis, which has made it technically difficult to examine EMT at the molecular level. Set against this situation, forward genetics in *Drosophila* has brought a breakthrough: *Snail*, a transcription factor, plays a key role in triggering EMT (Grau et al., 1984; Nusslein-Volhard et al., 1984; Alberga et al., 1991). Now, we can induce EMT in cultured epithelial cells simply by exogenously expressing Snail (Cano et al., 2000; Batlle et al., 2000). This in vitro system prompted a direct investigation into two questions. First, what types of signaling events upregulate the *Snail* expression to trigger EMT in a spatially and temporally regulated manner? Second, what genes are direct targets for the transcription factor, Snail?

The promoter of *E-cadherin* has been identified as a direct target for Snail. Snail directly represses *E-cadherin* promoter activity (Cano et al., 2000; Batlle et al., 2000). During EMT, the epithelial phenotypes of cells are converted into mesenchymal phenotypes: various gene products involved in the epithelial phenotypes must alter in their distribution and expression. Many of these alterations could be explained as secondary events induced by the downregulation of E-cadherin. Indeed, several lines of evidence have shown that the disappearance or dysfunction of E-cadherin results in the loss of epithelial cell polarity (Takeichi, 1991; Rodriguez-Boulan and Nelson, 1989). For example, when epithelial cells were cultured at low Ca^{2+} concentration (to cause E-cadherin dysfunction), the junctional complex, including TJs, was destroyed and epithelial cell polarity was lost. By contrast, it was reported that the Snail-induced phenotypic changes could not simply be attributed to the loss of E-cadherin. When E-cadherin was exogenously expressed in Snail-expressing epithelial cultured cells, which lost the expression of endogenous E-cadherin showing mesenchymal phenotypes, the epithelial phenotypes were not completely restored (Cano et al., 2000). Therefore, we must search for other direct targets for Snail for a better understanding of the molecular mechanism behind EMT.

In this study, we examined the behavior of claudins and occludin, major constituents of TJ strands, using the in vitro Snail-induced EMT system. Because TJs are the key structures responsible for establishing and maintaining epithelial cell polarity, claudins and occludin were expected to show a remarkable change in their distribution during EMT. Surprisingly, however, their expression completely shut down at the transcription level. Furthermore, we found that, similar to the Snail-based regulation of *E-cadherin* transcription (Cano et al., 2000; Batlle et al., 2000), Snail completely repressed the *claudin* and *occludin* promoter activities through its direct

binding to E-boxes in these promoters. These findings unraveled the very sophisticated mechanism by which Snail caused EMT. Snail directly and simultaneously represses the expression of two distinct groups of important intercellular adhesion molecules, E-cadherin and claudins/occludin, which function at AJs and TJs, respectively.

The regulatory mechanism of the formation and destruction of TJs has been examined extensively, but promoter analyses of claudins and occludin are only just beginning. The expression of occludin was shown to be regulated by several factors at the transcription level (Mankertz et al., 2000; Chen et al., 2000; Li and Mrsny, 2000), and several transcription factors (such as the β-catenin/Tcf complex and Cdx homeodomain proteins/hepatocyte nuclear factor 1) were reported to bind directly to *claudin-1* and *claudin-2* promoters, respectively (Miwa et al., 2001; Sakaguchi et al., 2002). These findings indicated that the expression of claudins and occludin is finely controlled depending on the physiological and pathological conditions, but our data revealed that Snail eliminates these fine regulations to shut off the expression of distinct claudin species and occludin completely and simultaneously. It remains unclear why the expression of claudins and occludin must be repressed so completely in mesenchymal cells but, conversely, these data favored the notion that claudins and occludin, as well as E-cadherin, are key determinants of the epithelial phenotype, including epithelial cell polarity.

Finally, we should briefly discuss the relationship between Snail and TJs, in particular the expression of claudins, in malignant tumors. In some types of tumors of epithelial origin, Snail expression has been reported, when cancer cells acquired an invasive phenotype (Cano et al., 2000; Batlle et al., 2000). However, the expression patterns of claudins varied significantly depending on the type of tumor. In some breast cancers and squamous adenocarcinomas, claudins were frequently downregulated (Kramer et al., 2000; Al Moustafa et al., 2002). It is thus possible that this repression is due to the upregulation of *Snail* expression, although this has not yet been examined in detail. Recently, E12/47 and SIP1 were also found to downregulate *E-cadherin* expression through direct binding to single or paired E-boxes (Perez-Moreno et al., 2001; Comijn et al., 2001; Bolos et al., 2003). Therefore, the possibility could not be excluded that, in tumors, downregulation of claudins occur via upregulation of these non-Snail-related silencers. In future studies, we must further clarify the pathological relevance of downregulated *claudin* expression as well as the possible involvement of Snail in the alteration of *claudin* expression in malignant tumors.

We thank Shu Narumiya for allowing us to use the luminometer. This study was supported in part by a Grant-in-Aid for Cancer Research and a Grant-in-Aid for Scientific Research (A) from the Ministry of Education, Science and Culture of Japan to S.T., and by JSPS Research for the Future Program to M.F.

References

Al Moustafa, A. E., Alaoui-Jamali, M. A., Batist, G., Hernandez-Perez, M., Serruya, C., Alpert, L., Black, M. J., Sladek, R. and Foulkes, W. D. (2002). Identification of genes associated with head and neck carcinogenesis by cDNA microarray comparison between matched primary normal epithelial and squamous carcinoma cells. *Oncogene* **21**, 2634-2640.

Alberga, A., Boulay, J. L., Kempe, E., Dennefield, C. and Haenlin, M. (1991). The *snail* gene required for mesoderm formation in *Drosophila* is expressed dynamically in derivatives of all three germ layers. *Development* **111**, 983-992.

Anderson, J. M. and van Itallie, C. M. (1995). Tight junctions and the molecular basis for regulation of paracellular permeability. *Am. J. Physiol.* **269**, G467-G475.

Balda, M. S., González-Mariscal, L., Matter, K., Cereijido, M. and Anderson, J. M. (1993). Assembly of the tight junction: the role of diacylglycerol. *J. Cell Biol.* **123**, 293-302.

Batlle, E., Sancho, E., Franci, C., Dominguez, D., Monfar, M., Baulida, J. and Garcia de Herreros, A. (2000). The transcription factor Snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat. Cell Biol.* **2**, 84-89.

Bolos, V., Peinado, H., Perez-Moreno, M. A., Fraga, M. F., Esteller, M. and Cano, A. (2003). The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. *J. Cell Sci.* **116**, 499-511.

Cano, A., Perez-Moreno, M. A., Rodrigo, I., Locascio, A., Blanco, M. J., del Barrio, M. G., Portillo, F. and Nieto, M. A. (2000). The transcription factor Snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat. Cell Biol.* **2**, 76-83.

Carver, E. A., Jiang, R., Lan, Y., Oram, K. F. and Gridley, T. (2001). The mouse *Snail* gene encodes a key regulator of the epithelial-mesenchymal transition. *Mol. Cell. Biol.* **21**, 8184-8188.

Chen, Y., Lu, Q., Schneeberger, E. E. and Goodenough, D. A. (2000). Restoration of tight junction structure and barrier function by down-regulation of the mitogen-activated protein kinase pathway in *ras*-transformed Madin-Darby canine kidney cells. *Mol Biol Cell* **11**, 849-862

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Citi, S., Sabanay, H., Jakes, R., Geiger, B. and Kendrick-Jones, J. (1988). Cingulin, a new peripheral component of tight junctions. *Nature* **333**, 272-276.

Comijn, J., Berx, G., Vermassen, P., Verschueren, K., van Grunsven, L., Bruyneel, E., Mareel, M., Huylebroeck, D. and van Roy, F. (2001). The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. *Mol. Cell* **7**, 1267-1278.

Farquhar, M. G. and Palade, G. E. (1963). Junctional complexes in various epithelia. *J. Cell Biol.* **17**, 375-412.

Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., Tsukita, S. and Tsukita, S. (1993). Occludin: a novel integral membrane protein localizing at tight junctions. *J. Cell Biol.* **123**, 1777-1788.

Furuse, M., Fujita, K., Hiiragi, T., Fujimoto, K. and Tsukita, S. (1998a). Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J. Cell Biol.* **141**, 1539-1550.

Furuse, M., Sasaki, H., Fujimoto, K. and Tsukita, S. (1998b). A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. *J. Cell Biol.* **143**, 391-401.

Fuse, N., Hirose, S. and Hayashi, S. (1994). Diploidy of *Drosophila* imaginal cells is maintained by a transcriptional repressor encoded by *escargot*. *Genes Dev.* **8**, 2270-2281.

Grau, Y., Carteret, C. and Simpson, P. (1984). Mutations and chromosomal rearrangements affecting the expression of *snail*, a gene involved in embryonic patterning in *Drosophila melanogaster*. *Genetics*, **108**, 347-360.

Grimes, H. L., Chan, T. O., Zweidler-McKay, P. A., Tong, B. and Tsichlis, P. N. (1996). The Gfi1 proto-oncoprotein contains a novel transcriptional repressor domain, SNAG, and inhibits G1 arrest induced by interleukin-2 withdrawal. *Mol. Cell. Biol.* **16**, 6263-6272.

Gumbiner, B. (1993). Breaking through the tight junction barrier. *J. Cell Biol.* **123**, 1631-1633.

Gumbiner, B., Lowenkopf, T. and Apatira, D. (1991). Identification of a 160-kDa polypeptide that binds to the tight junction protein ZO-1. *Proc. Natl. Acad. Sci. USA* **88**, 3460-3464.

Hata, A., Seoane, J., Lagna, G., Montalvo, E., Hemmati-Brivanlou, A. and Massague, J. (2000). OAZ uses distinct DNA- and protein-binding zinc fingers in separate BMP-Smad and Olf signaling pathways. *Cell* **100**, 229-240.

Hay, E. D. (1995). An overview of epithelio-mesenchymal transformation. *Acta Anat.* **154**, 8-20.

Inukai, T., Inoue, A., Kurosawa, H., Goi, K., Shinjyo, T., Ozawa, K., Mao, M., Inaba, T. and Look, A. T. (1999). *SLUG*, a *ces-1*-related zinc finger transcription factor gene with antiapoptotic activity is a downstream target of the E2A-HLF oncoprotein. *Mol. Cell* **4**, 343-352.

Itoh, M., Nagafuchi, A., Yonemura, S., Kitani-Yasuda, T., Tsukita, S. and Tsukita, S. (1993). 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.* **121**, 491-502.

Kasai, Y., Nambum, J. R., Lieberman, P. M. and Crews, S. T. (1992). Dorsal-ventral patterning in *Drosophila*: DNA binding of snail protein to the *single-minded* gene. *Proc. Natl. Acad. Sci. USA* **89**, 3414-3418.

Kataoka, H., Maruyama, T., Yokode, M., Mori, S., Sano, H., Ozaki, H., Yokota, Y., Nishikawa, S. and Kita, T. (2000). A novel Snail-related transcription factor Smuc regulates basic helix-loop-helix transcription factor activities via specific E-box motifs. *Nucleic Acids Res.* **28**, 626-633.

Keon, B. H., Schäfer, S., Kuhn, C., Grund, C. and Franke, W. W. (1996). Symplekin, a novel type of tight junction plaque protein. *J. Cell Biol.* **134**, 1003-1018.

Kramer, F., White, K., Kubbies, M., Swisshelm, K. and Weber, B. H. (2000). Genomic organization of *claudin-1* and its assessment in hereditary and sporadic breast cancer. *Hum. Genet.* **107**, 249-256.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Li, D. and Mrsny, R. J. (2000). Oncogenic Raf-1 disrupts epithelial tight junctions via downregulation of occludin. *J. Cell Biol.* **148**, 791-800.

Mankertz, J., Tavalali, S., Schmitz, H., Mankertz, A., Riecken, E. O., Fromm, M. and Schulzke, J. D. (2000). Expression from the human occludin promoter is affected by tumor necrosis factor α and interferon γ. *J. Cell Sci.* **113**, 2085-2090.

Mitic, L. L. and Anderson, J. M. (1998). Molecular architecture of tight junctions. *Annu Rev Physiol* **60**, 121-142.

Miwa, N., Furuse, M., Tsukita, S., Niikawa, N., Nakamura, Y. and Furukawa, Y. (2001). Involvement of claudin-1 in the β-catenin/Tcf signaling pathway and its frequent upregulation in human colorectal cancers. *Oncol. Res.* **12**, 469-476.

Morita, K., Furuse, M., Fujimoto, K. and Tsukita, S. (1999). Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. *Proc. Natl. Acad. Sci. USA* **96**, 511-516.

Muhin, V., Luts, Y., Dennefeld, C. and Alberga, A. (1993). Definition of the DNA-binding site repertoire for the *Drosophila* transcription factor SNAIL. *Nucleic Acids Res.* **21**, 3951-3957.

Nakayama, H., Scott, I. C. and Cross, J. C. (1998). The transition to endoreduplication in trophoblast giant cells is regulated by the mSna zinc finger transcription factor. *Dev. Biol.* **199**, 150-163.

Nieto, M. A. (2002). The snail superfamily of zinc-finger transcription factors. *Nat. Rev. Mol. Cell. Biol.* **3**, 155-166.

Nieto, M. A., Sargent, M. G., Wilkinson, D. G. and Cooke, J. (1994). Control of cell behaviour during vertebrate development by *Slug*, a zing finger gene. *Science* **264**, 835-839.

Nusslein-Volhard, C., Weischaus, E. and Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. I. Zygotic loci on the second chromosome. *Wilhelm Roux's Arch. Dev. Biol.* **193**, 267-282.

Oda, H., Tsukita, S. and Takeichi, M. (1998). Dynamic behavior of the cadherin-based cell-cell adhesion system during *Drosophila* gastrulation. *Dev. Biol.* **203**, 435-450.

Perez-Moreno, M. A., Locascio, A., Rodrigo, I., Dhondt, G., Portillo, F., Nieto, M. A. and Cano, A. (2001). A new role for E12/E47 in the repression of E-cadherin expression and epithelial-mesenchymal transitions. *J. Biol. Chem.* **276**, 27424-27431.

Rodriguez-Boulan, E. and Nelson, W. J. (1989). Morphogenesis of the polarized epithelial cell phenotype. *Science* **245**, 718-725.

Saitou, M., Ando-Akatsuka, Y., Itoh, M., Furuse, M., Inazawa, J., Fujimoto, K. and Tsukita, S. (1997). Mammalian occludin in epithelial cells: its expression and subcellular distribution. *Eur. J. Cell Biol.* **73**, 222-231.

Sakaguchi, T., Gu, X., Golden, H. M., Suh, E. R., Rhoads, D. B. and Reinecker, H.-C. (2002). Cloning of the human claudin-2 5′-flanking region revealed a TATA-less promoter with conserved binding sites in mouse and human for caudal-related homeodomain proteins and hepatocyte nuclear factor-1α. *J. Biol. Chem.* **277**, 21361-21370.

Schneeberger, E. E. and Lynch, R. D. (1992). Structure, function, and regulation of cellular tight junctions. *Am. J. Physiol.* **262**, L647-L661.

Staehelin, L. A. (1974). Structure and function of intercellular junctions. *Int. Rev. Cytol.* **39**, 191-283.

Stevenson, B. R., Siliciano, J. D., Mooseker, M. S. and Goodenough, D. A. (1986). Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J. Cell Biol.* **103**, 755-766.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* **251**, 1451-1455.

Tsukita, S. and Furuse, M. (1999a). Structural and signaling molecules come together at tight junctions. *Curr. Opin. Cell Biol.* **11**, 628-633.

Tsukita, S. and Furuse, M. (1999b). Occludin and claudins in tight-junction strands: leading or supporting players? *Trend Cell Biol.* **9**, 268-273.

Tsukita, S., Furuse, M. and Itoh, M. (2001). Multifunctional strands in tight junctions. *Nat. Rev. Mol. Cell. Biol.* **2**, 285-293.

Zhong, Y., Saitoh, T., Minase, T., Sawada, N., Enomoto, K. and Mori, M. (1993). Monoclonal antibody 7H6 reacts with a novel tight junction-associated protein distinct from ZO-1, cingulin, and ZO-2. *J. Cell Biol.* **120**, 477-483.