ARTICLE
Journal of Cellular Biochemistry 113:499–507 (2012)

Journal of Cellular Biochemistry

Enhancement of Cell–Cell Contact by Claudin-4 in Renal Epithelial Madin-Darby Canine Kidney Cells

Akira Ikari,[1*] Kosuke Atomi,[1] Ayumi Takiguchi,[1] Yasuhiro Yamazaki,[1] Hisayoshi Hayashi,[2] Jotaro Hirakawa,[1] and Junko Sugatani[1,3]

[1] *Department of Pharmaco-Biochemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan*

[2] *Laboratory of Physiology, School of Food and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan*

[3] *Global Center of Excellence for Innovation in Human Health Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan*

ABSTRACT

Claudin-4 regulates ion permeability via a paracellular pathway in renal epithelial cells, but its other physiological functions have not been examined. We found that hyperosmotic stress increases claudin-4 expression in Madin-Darby canine kidney cells. Here, we examined whether claudin-4 affects cell motility, cell association, and the intracellular distribution of endogenous junctional proteins. Doxycycline-inducible expression of claudin-4 did not change endogenous levels of claudin-1, claudin-2, claudin-3, occludin, E-cadherin, and ZO-1. Claudin-4 overexpression increased cell association and decreased cell migration without affecting cell proliferation. Doxycycline did not change cell junctional protein levels, cell association or cell migration in mock-transfected cells. The insolubility of claudin-1 and -3 in Triton X-100 was increased by claudin-4 overexpression, but that of claudin-2, occludin, ZO-1, and E-cadherin was unchanged. Immunocytochemistry showed that claudin-4 overexpression increases the accumulation of claudin-1 and -3 in tight junctions (TJs). Furthermore, claudin-4 overexpression increased the association of claudin-4 with claudin-1 and -3. These results suggest that claudin-4 accumulates claudin-1 and -3 in TJs to enhance cell–cell contact in renal tubular epithelial cells. J. Cell. Biochem. 113: 499–507, 2012.

KEY WORDS: CLAUDIN-4; KIDNEY; CELL–CELL CONTACT; MIGRATION

Epithelial cells express junction complex proteins and form cell junctions such as tight junctions (TJs), adherens junctions, and desmosomes in the cell–cell contact area. E-cadherin establishes intimate cell–cell contact in adherence junction [Takeichi, 1995; Adams and Nelson, 1998]. In epithelial cellular sheets, adherens junctions and desmosomes are mechanically linked with adjacent cells, whereas TJs are responsible for intercellular sealing. TJs are located in the most apical region of lateral membranes and have roles in cell adhesion and paracellular barrier functions as well as cell polarization [Rodriguez-Boulan and Nelson, 1989; Gumbiner, 1993].

TJs are composed of transmembrane and peripheral proteins. The transmembrane proteins including claudins [Furuse et al., 1998; Morita et al., 1999; Tsukita et al., 2001] and occludin [Furuse et al., 1993] come into contact and make ion-selective pores. Claudins bear four transmembrane domains with cytoplasmic amino and carboxy termini. The carboxy terminus of most claudins has a PDZ-binding motif that can interact with the PDZ domains of scaffolding proteins including ZO-1, ZO-2, and ZO-3. ZO proteins link claudins to the actin-based cytoskeleton and make a belt-like TJ [Tsukita et al., 2009].

Claudin proteins are major components of TJ strands. To date 27 members of the claudin family have been identified [Turksen and Troy, 2004; Mineta et al., 2011]. The expression of each claudin varies among tissues and developmental conditions. Claudin-4 is expressed in high-resistance segments including the thin ascending

Abbreviations: MDCK, Madin-Darby canine kidney; TER, transepithelial electrical resistance; TJ, tight junction.

Additional supporting information may be found in the online version of this article.

Grant sponsor: Research Foundation for Pharmaceutical Sciences (KAKENHI); Grant number: 23590263; Grant sponsor: SRI Academic Research.

*Correspondence to: Dr. Akira Ikari, PhD, Department of Pharmaco-Biochemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan. E-mail: ikari@u-shizuoka-ken.ac.jp

Received 8 September 2011; Accepted 12 September 2011 • DOI 10.1002/jcb.23373 • © 2011 Wiley Periodicals, Inc.
Published online 21 September 2011 in Wiley Online Library (wileyonlinelibrary.com).

limb and collecting ducts in the kidney [Kiuchi-Saishin et al., 2002]. The knockdown of claudin-4 by siRNA increased permeability to sodium and enhanced the selectivity of TJs for cations in Madin-Darby canine kidney (MDCK) cells [Hou et al., 2006]. In contrast, the knockdown of claudin-4 decreased permeability to chloride in LLC-PK_1 [Hou et al., 2006] and collecting duct cells [Hou et al., 2010]. Claudin-4 may create charge-selective channels in the paracellular space. Furthermore, claudin-4 is involved in the regulation of invasive and metastatic potential in pancreatic cancer [Michl et al., 2003] and colonic cancer cells [Takehara et al., 2009].

The nephrons of the mammalian kidney can excrete concentrated urine by increasing the tonicity of the medullary interstitium. The osmotic concentration of urine can exceed 1,000 mOsm/kg H_2O. Hyperosmolarity causes damage to tubular epithelial cells. Integral cell–cell contact is necessary to maintain the morphology and function of renal tubule. The physiological and biochemical features of TJs are well characterized in MDCK cells. In the present study, we used MDCK cells as a model to examine the effects of claudin expression on cell–cell contact. We found that hyperosmotic stress increases claudin-4 expression without affecting the expression of claudin-1, claudin-3, and E-cadherin. Claudin-4 overexpression increased the accumulation of claudin-1 and -3 in TJs, the association of claudins with ZO-1, and the extent of cell association. In contrast, claudin-4 overexpression decreased cell migration. Our results indicate that the increase in claudin-4 expression enhances cell–cell contact in concert with claudin-1 and -3 in MDCK cells.

MATERIALS AND METHODS

MATERIALS

Anti-claudin-1, -2, -3, and -4, and ZO-1 antibodies were obtained from Zymed Laboratories (South San Francisco, CA), anti-E-cadherin antibody and doxycycline from BD Biosciences Clontech (Mountain View, CA), anti-FLAG antibody from Sigma–Aldrich (St. Louis, MO), and anti-β-actin and occludin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA). All other reagents were of the highest grade of purity available.

CELL CULTURE AND TRANSFECTION

The MDCK (type II) Tet-OFF cell line was obtained from BD Biosciences Clontech. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich) supplemented with 5% fetal calf serum (HyClone, Logan, UT), 0.07 mg/ml penicillin-G potassium,

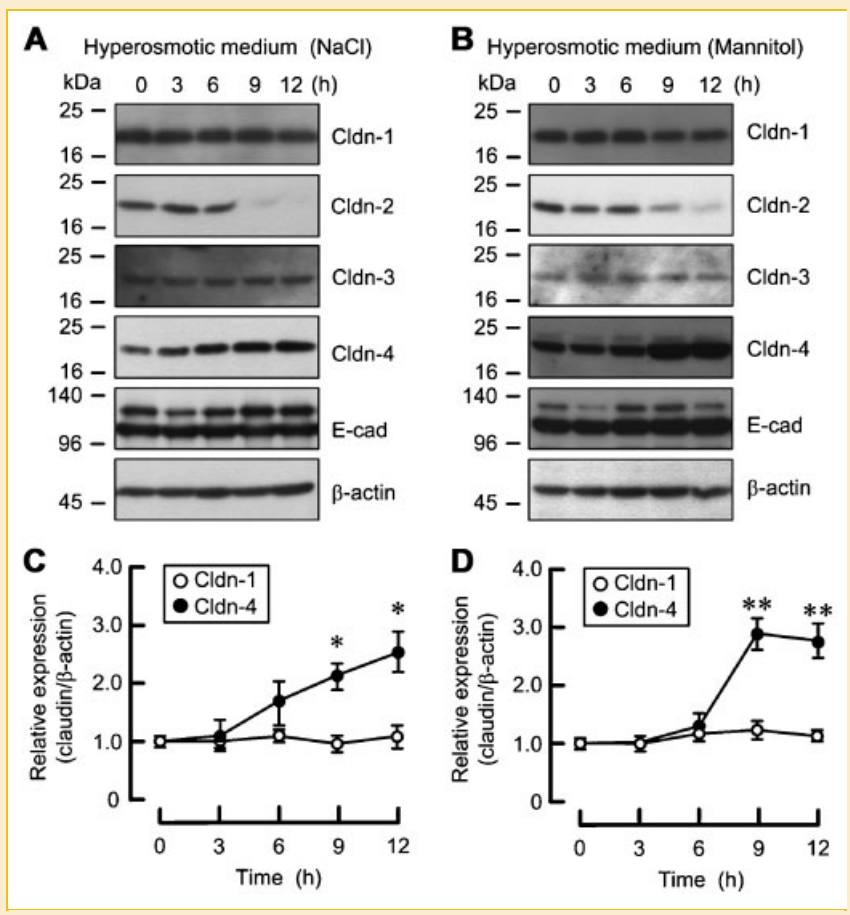

Fig. 1. Increase in claudin-4 protein caused by hyperosmotic stress. A, B: MDCK cells were incubated with NaCl or mannitol hyperosmotic medium for the periods indicated. The cell lysate was separated by SDS–PAGE, followed by immunoblotting with anti-claudin (cldn)-1, cldn-2, cldn-3, cldn-4, E-cadherin (E-cad), or β-actin antibody. C, D: The band densities of cldn-1 and -4 were expressed relative to the value in 0 h. n = 4–5. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with 0 h.

0.14 mg/ml streptomycin sulfate, and 0.1 mg/ml G418 in a 5% CO_2 atmosphere at 37°C. Hyperosmotic medium (550 mOsmol/kg H_2O) was prepared by adding NaCl or mannitol to normal medium (330 mOsm/kg H_2O). The osmolarity was measured with an Osmostat OM-6020 (Kyoto Daiichi Kagaku, Kyoto, Japan). Human claudin-4 cDNA was amplified by RT-PCR and subcloned into the vector pCMV-Tag2A. The FLAG-tagged claudin-4 cDNA was amplified by PCR and subcloned into the vector pTRE2hyg (BD Biosciences Clontech). The sequence analysis was consigned to Bio Matrix Research (Chiba, Japan). Cells were transfected with FLAG-tagged claudin-4/pTRE2hyg or mock vector using Lipofectamine 2000 as recommended by the manufacturer. Stable transfectants were selected with 0.2 mg/ml hygromycin B and maintained in the presence of 0.1 mg/ml hygromycin B and 0.1 μg/ml doxycycline. The cell line expressing FLAG-tagged claudin-4 was screened by immunoblotting. Three stable cell lines were generated (Supplementary Fig. 1) and we mainly used clone 5.

PREPARATION OF THE CELL LYSATE, DETERGENT SOLUBLE FRACTION AND IMMUNOPRECIPITANT

Confluent MDCK cells were scraped into cold PBS and precipitated by centrifugation. The cells were lysed in a RIPA buffer containing 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 0.1% SDS, 50 mM Tris–HCl (pH 8.0), a protease inhibitor cocktail (Sigma–Aldrich), 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate, and sonicated for 20 s. After centrifugation at 6,000*g* for 5 min, the supernatant was collected (cell lysate). Detergent soluble and insoluble fractions were prepared as described previously [Ikari et al., 2005]. Protein concentrations were measured using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard. For the immunoprecipitation assay, the cell lysate (500 μg) was incubated with anti-claudin-4 or ZO-1 antibody and Protein G-Sepharose beads (GE Healthcare UK Ltd., Amersham Place, England) at 4°C for 16 h with gentle rocking. After centrifugation at 6,000*g* for 1 min, the pellet was washed three times with the RIPA buffer. The immunoprecipitate was solubilized in a sample buffer for SDS–PAGE.

SDS–PAGE AND IMMUNOBLOTTING

SDS–PAGE was carried out as described previously [Ikari et al., 2001]. In brief, the sample (30 μg) was applied to the SDS–PAGE. Proteins were blotted onto a PVDF membrane and incubated with each primary antibody (1:1,000 dilution) for 16 h at 4°C, followed by a peroxidase-conjugated secondary antibody (1:5,000 dilution) for 1 h at room temperature. Finally, the blots were stained with a Pierce Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA).

CONFOCAL MICROSCOPY

MDCK cells were plated at a confluent density on cover glasses. The cells were fixed with methanol for 10 min at −20°C, then permeabilized with 0.2% Triton X-100 for 15 min. After blocking with 4% Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) for 30 min, the cells were incubated with anti-claudin-1, claudin-3, and ZO-1 antibodies for 16 h at 4°C. They were then incubated with Alexa Fluor 488 and Alexa Fluor 568-conjugated antibodies for 1 h at room temperature. Immunolabeled cells were visualized on an LSM 510 confocal microscope (Carl Zeiss, Germany) set with a filter appropriate for Alexa Fluor 488 (488 nm excitation, 530 nm emission) and Alexa Fluor 568 (543 nm excitation, 585–615 nm emission). Images were collected at 1.0-μm increments (vertical direction). The length of fluorescence distribution along the lateral membrane was measured by ImageJ (http://rsbweb.nih.gov/ij/).

MEASUREMENT OF TRANSEPITHELIAL ELECTRICAL RESISTANCE (TER) AND PARACELLULAR TRACER PERMEABILITY

MDCK cells were plated at a confluent density on Transwells with polyester membrane inserts (12 mm diameter, 0.4-μm pore size; Corning Incorporated-Life Sciences, Acton, MA). TER was measured using a Millicell-ERS epithelial volt-ohmmeter (Millipore, Billerica, MA). TER values (ohms × cm^2) were normalized based on the area of the monolayer and were calculated by subtracting the blank values from the filter and the bathing medium. The paracellular permeability to FITC-dextran (4,000 Da) for 1 h from the apical-to-basal compartments was measured with a Multilabel Counter 1420 ARVOsx (Perkin Elmer, Wellesley, MA) as described previously [Ikari et al., 2006].

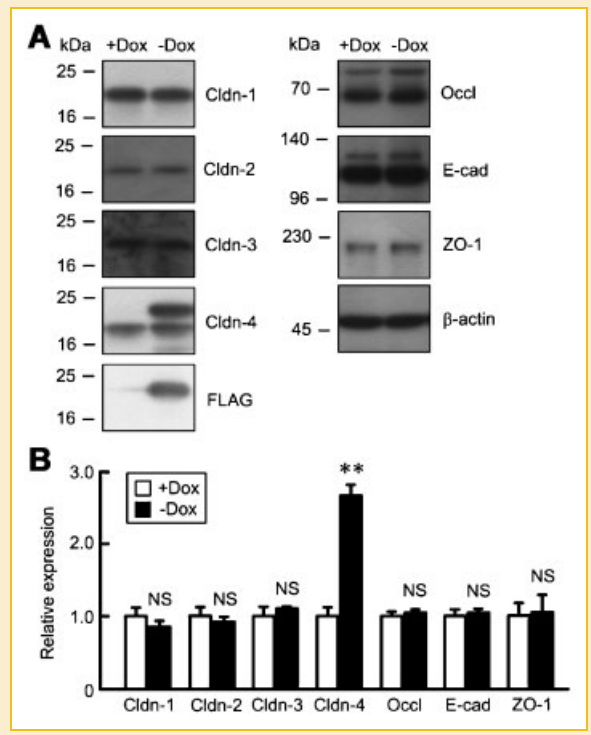

Fig. 2. Effect of claudin (cldn)-4 overexpression on the expression of endogenous junctional proteins. A: Cells expressing FLAG-tagged cldn-4/pTRE2hyg were incubated in the presence and absence of doxycycline (1 μg/ml) for 72 h. The cell lysate was separated by SDS–PAGE, followed by immunoblotting with anti-cldn-1, cldn-2, cldn-3, cldn-4, FLAG, occludin (occl), E-cad, ZO-1, or β-actin antibody. B: The band densities of junctional proteins were expressed relative to the value in the presence of doxycycline. n = 3. $^{**}P < 0.01$ compared with +Dox. NS, $P > 0.05$ compared with +Dox.

WOUND-HEALING MIGRATION ASSAY

Before wounding, cells were allowed to form a confluent monolayer in a six-well plate. Cells were wounded with a 1,000 μl pipette tip and incubated in new DMEM without FCS for the periods indicated. Photographs were taken using an Olympus IX70 microscope with acquisition software, QCapture Pro. The cell-free area was measured using ImageJ.

CELL ASSOCIATION ASSAY

The cell association assay was performed as described by Nagafuchi et al. [1994] with minor modifications. Confluent cells were treated with 0.01% trypsin in a solution containing 140 mM NaCl, 5.8 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 25 mM glucose, and 20 mM Hepes (pH 7.4) for 10 min at 37°C. The cells were scraped by cell scraper and dissociated through 30 rounds of pipetting. Photographs were taken using an Olympus IX70 microscope with QCapture Pro. The extent of cell association was represented by the index N_L/N_T, where N_L and N_T are the large particle (consisting of more than two cells) number and total particle number, respectively.

STATISTICS

Results are presented as means ± SEM. Comparisons between two groups were made using Student's *t*-test. Differences were assumed significant at $P < 0.05$.

RESULTS

INCREASE IN CLAUDIN-4 PROTEIN BY HYPEROSMOTIC STRESS

The level of claudin-4 protein was increased by NaCl and mannitol hyperosmotic stress in a time-dependent manner (Fig. 1). Similar results were reported in inner medullary collecting duct cells [Lanaspa et al., 2008]. Moreover, the hyperosmotic stress decreased claudin-2 level. Claudin-1, -3, and E-cadherin levels were unchanged. These results indicate that claudin-2 and -4 are more

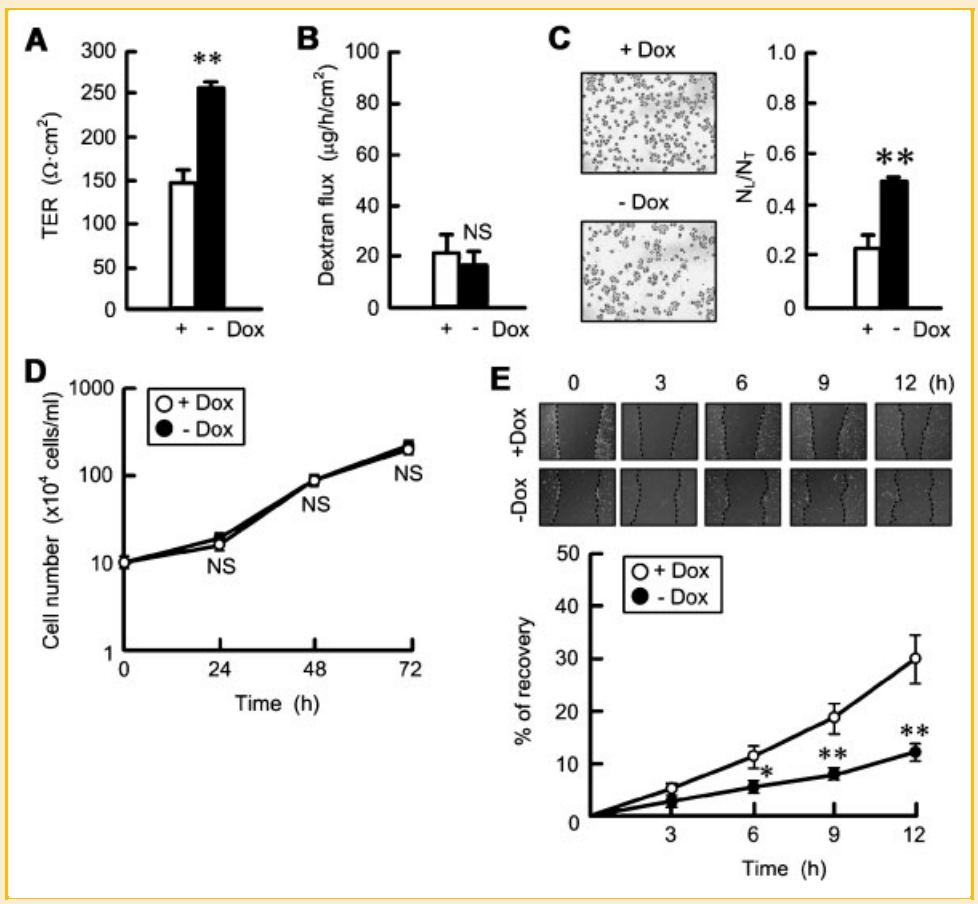

Fig. 3. Effect of claudin-4 overexpression on tight junctional permeability and cell motility. A,B: Cells expressing FLAG-tagged claudin-4/pTRE2hyg were cultured on a transwell in the presence and absence of doxycycline (1 μg/ml) for 72 h. TER was measured using a volt-ohmmeter (A). FITC-dextran (4,000 Da, 0.5 mg/ml) was applied to the apical compartment. After 1 h, the buffer in the opposite compartment was collected and fluorescent intensity was measured (B). C: Cells were incubated in the presence and absence of doxycycline for 72 h. After being collected with a scraper, the cells were treated with 0.01% trypsin for 10 min. The extent of cell association was represented by the index N_L/N_T. D: Cells were cultured in the presence and absence of doxycycline for 24, 48, and 72 h. After being detached with trypsin, viable cells were counted. E: The cells were cultured in the presence and absence of doxycycline for 72 h. Then, the confluent monolayer was scraped to create a mechanical injury and incubated for the periods indicated. The wounded area was quantified by measuring the cell-free area. Cell migration into the wounded area was represented as % of recovery relative to 0 h. n = 3–6. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with +Dox. NS, $P > 0.05$ compared with +Dox.

sensitive to hyperosmotic stress than claudin-1, -3, and E-cadherin. We recently reported that the knockdown of claudin-2 increases TER and cell migration [Ikari et al., 2010]. Here, we examined the effect of claudin-4 on the integrity and function of TJs.

EFFECT OF CLAUDIN-4 OVEREXPRESSION ON THE EXPRESSION OF ENDOGENOUS JUNCTIONAL PROTEINS AND CELL MOTILITY

The level of claudin-4 protein was increased by the removal of doxycycline in the MDCK cells expressing the FLAG-tagged claudin-4/pTRE2hyg vector (Fig. 2). Claudin-4 overexpression did not affect the expression of claudin-1, -2, and -3, occludin, E-cadherin, and ZO-1. The level of claudin-4 in the cells expressing FLAG-tagged claudin-4 was similar to that in the cells treated with hyperosmotic stress (Supplementary Fig. 1). Claudin-4 overexpression caused by the removal of doxycycline increased TER without affecting dextran flux (Fig. 3A,B). Van Itallie et al. [2001] reported that claudin-4 overexpression decreases permeability to sodium and increases TER in MDCK cells. Next, we examined the effect of claudin-4 overexpression on cell association in the absence of Ca^{2+} to avoid the involvement of E-cadherin. Claudin-4 overexpression increased the cell association index (N_L/N_T), which indicates resistance to digestion by trypsin (Fig. 3C), indicating that claudin-4 increases cell–cell contact in MDCK cells. Claudin-4 overexpression did not affect cell number (Fig. 3D), indicating that claudin-4 did not affect cell proliferation and cell death. In the wound healing assay, the injured area recovered in a time-dependent manner with 30% recovery after 12 h in the control cells (Fig. 3E). Claudin-4 overexpression decreased this to 12%. These results indicate that claudin-4 overexpression enhances cell–cell contact and decreases migration in MDCK cells.

EFFECT OF DOXYCYCLIN ON THE EXPRESSION OF ENDOGENOUS JUNCTIONAL PROTEINS AND CELL MOTILITY

As described above, the overexpression of claudin-4 caused by the removal of doxycycline increased TER and the cell association index, whereas it decreased cell migration. To avoid the possibility that doxycycline affects the expression of endogenous junctional proteins, the function of TJs and cell motility, we used cells expressing only the pTRE2hyg vector. The removal of doxycycline did not affect the levels of claudin-1, -2, -3, and -4, occludin, E-cadherin, and ZO-1 (Fig. 4). TER, dextran flux, the cell association index, cell proliferation, and cell migration were similar in the presence and absence of doxycycline (Fig. 5). These results indicate that doxycycline did not affect the function or morphology of TJs.

EFFECT OF CLAUDIN-4 KNOCKDOWN ON THE EXPRESSION OF ENDOGENOUS JUNCTIONAL PROTEINS AND CELL MOTILITY

The level of claudin-4 protein was decreased by the addition of doxycycline in the MDCK cells expressing the claudin-4/pSingle-tTS-shRNA vector (Supplementary Fig. 2). In the presence of doxycycline, the protein level of claudin-4 decreased by 67%. The expression of claudin-1, -2, and -3, occludin, E-cadherin, and ZO-1 was unaffected by the knockdown. In contrast to its overexpression, claudin-4 knockdown decreased TER and the cell association index (Supplementary Fig. 3A,C). Furthermore, the knockdown increased recovery by 37% after 12 h in the wound-healing assay

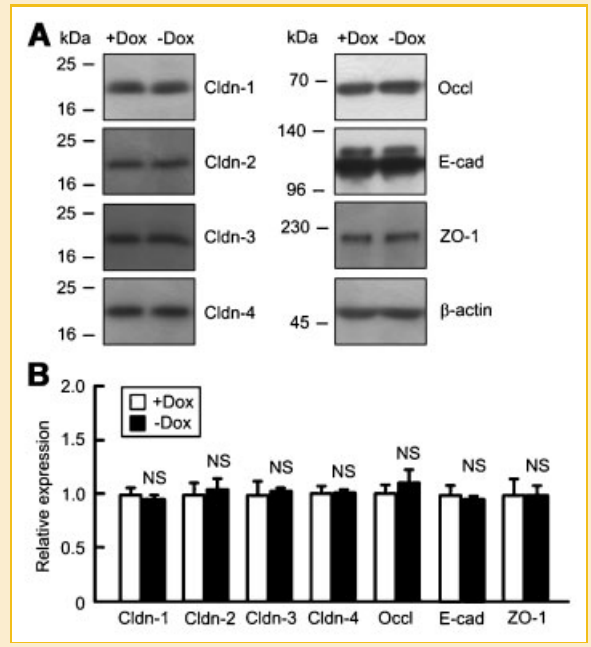

Fig. 4. Effect of doxycycline on the expression of endogenous junctional proteins in mock-transfected cells. A: Cells expressing only the pTRE2hyg vector were incubated in the presence and absence of doxycycline (1 μg/ml) for 72 h. The cell lysate was separated by SDS–PAGE, followed by immunoblotting with anti-claudin (cldn)-1, cldn-2, cldn-3, cldn-4, occl, E-cad, ZO-1, or β-actin antibody. B: The band densities of junctional proteins were expressed relative to the value in the presence of doxycycline. n = 3. NS, $P > 0.05$ compared with +Dox.

(Supplementary Fig. 3E). Similar to its overexpression, claudin-4 knockdown did not affect dextran flux or cell proliferation (Supplementary Fig. 3B,D). These results indicate that the knockdown of claudin-4 decreases cell–cell contact and increases migration in MDCK cells, as opposed to the overexpression of claudin-4.

EFFECT OF CLAUDIN-4 OVEREXPRESSION ON THE SOLUBILITY OF JUNCTIONAL PROTEINS IN TRITON X-100

The solubility of junctional proteins is known to reflect their incorporation into intercellular junctions; detergent-soluble proteins are cytosolic or loosely associated with the plasma membrane, whereas detergent-insoluble proteins are incorporated into intercellular junctions. Claudin-2, occludin, and ZO-1 were mainly distributed in the Triton X-100-insoluble fraction (Fig. 6). About 50% of all of the claudin-1, -3, and -4 was distributed in the Triton X-100-insoluble fraction. Claudin-4 overexpression significantly increased the insolubility of claudin-1 and -3. In contrast, the solubility of claudin-2, occludin, E-cadherin, and ZO-1 was unchanged by the overexpression.

EFFECT OF CLAUDIN-4 OVEREXPRESSION ON THE DISTRIBUTION OF JUNCTIONAL PROTEINS

The solubility of claudins may affect their intracellular distribution. Therefore, we examined the distribution of claudin-1, claudin-3, and ZO-1 by immunofluorescence microscopy. Claudin-1, -3, and

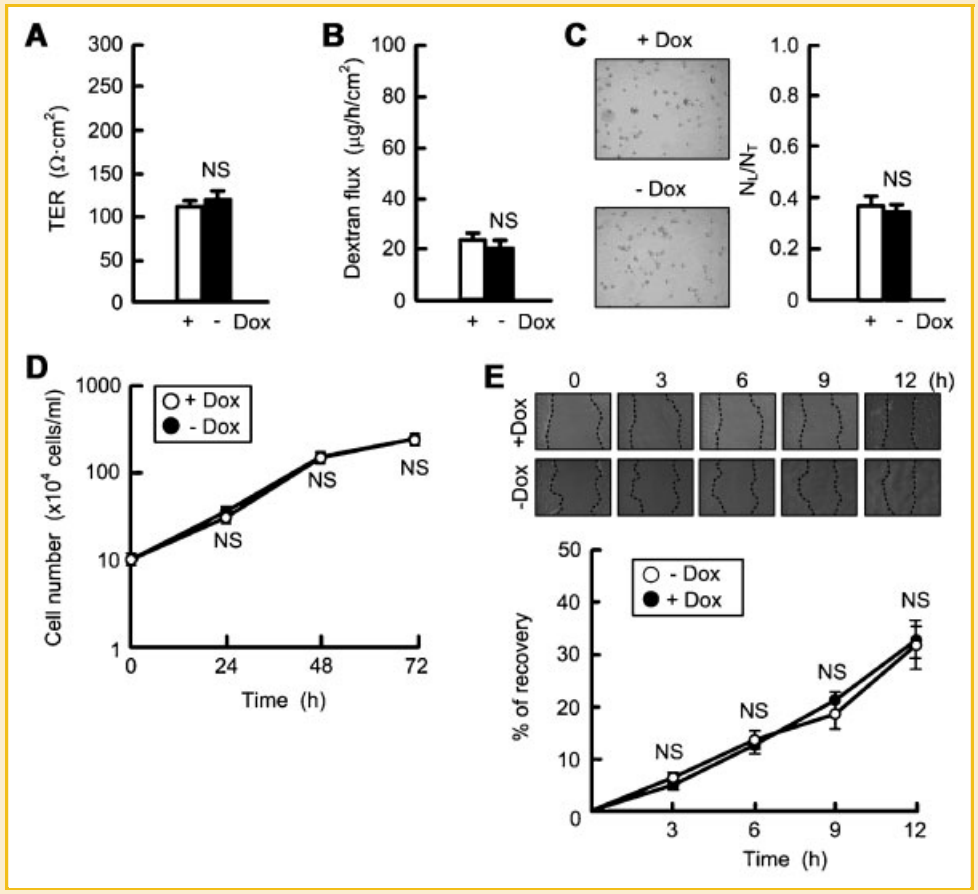

Fig. 5. Effect of doxycycline on tight junctional permeability and cell motility. A,B: Cells expressing pTRE2hyg were cultured on a transwell in the presence and absence of doxycycline (1 μg/ml) for 72 h. TER was measured using a volt-ohmmeter (A). FITC-dextran (4,000 Da, 0.5 mg/ml) was applied to the apical compartment. After 1 h, the buffer in the opposite compartment was collected and fluorescent intensity was measured (B). C: Cells were incubated in the presence and absence of doxycycline for 72 h. After being collected with a scraper, the cells were treated with 0.01% trypsin for 10 min. The extent of cell association was represented by the index N_L/N_T. D: Cells were cultured in the presence and absence of doxycycline for 24, 48, and 72 h. After being detached with trypsin, viable cells were counted. E: Cells were cultured in the presence and absence of doxycycline for 72 h. Then, the confluent monolayer was scraped and incubated for the periods indicated. The damaged area was quantified by measuring the cell-free area. Cell migration into the wound was represented as % of recovery relative to 0 h. n = 3–4. NS, $P > 0.05$ compared with +Dox.

ZO-1 were mainly distributed in the cell–cell contact area in the presence of doxycycline (Fig. 7). Claudin-4 overexpression did not affect the distribution of these proteins in the xy section. However, the distribution of the fluorescence of claudin-1 and -3 along the lateral membrane was decreased by claudin-4 overexpression. These results suggest that claudin-1 and -3 accumulated in TJs on the overexpression of claudin-4. In contrast, that of ZO-1 was unchanged by the overexpression.

INCREASE IN THE ASSOCIATION OF CLAUDINS WITH ZO-1 ON CLAUDIN-4 OVEREXPRESSION

To clarify the association of claudin-4 with claudin-1, -3, and ZO-1, we performed immunoprecipitation assays. Claudin-1, -3, and -4 were co-immunoprecipitated with ZO-1 in cells cultured in the presence of doxycycline (Fig. 8A). The amount of immunoprecipitant was increased by the removal of doxycycline, indicating that the association of ZO-1 with claudin-1, -3, and -4 was increased by claudin-4 overexpression. Similarly, the association of claudin-4 with claudin-1 and -3 was increased by its overexpression (Fig. 8B).

DISCUSSION

Renal tubules are often exposed to stress including hyperosmolarity, heavy metals and drugs. The renal failure caused by such stress is accompanied by damage to tubular epithelial cells. The injured cells are eliminated from the tubular surface. To maintain the morphology and function of renal tubules, epithelial cells must enhance cell–cell contact or the remaining cells must proliferate into free spaces and re-construct intercellular junctions. We recently reported that hyperosmotic stress causes cellular injury and decreases claudin-2 protein level within 6 h in MDCK cells [Ikari et al., 2010]. The knockdown of claudin-2 expression by siRNA increased cell migration. We suggested that claudin-2 is involved in the recovery from renal tubular injury. In the present study, we found that hyperosmotic stress increases claudin-4 protein level after 6 h (Fig. 1). After hyperosmotic stress, some cells had detached from the culture dish (data not shown). In the remaining cells, claudin-4 continued to be co-localized with ZO-1 in cell–cell border areas (Supplementary Fig. 4). These results suggest that

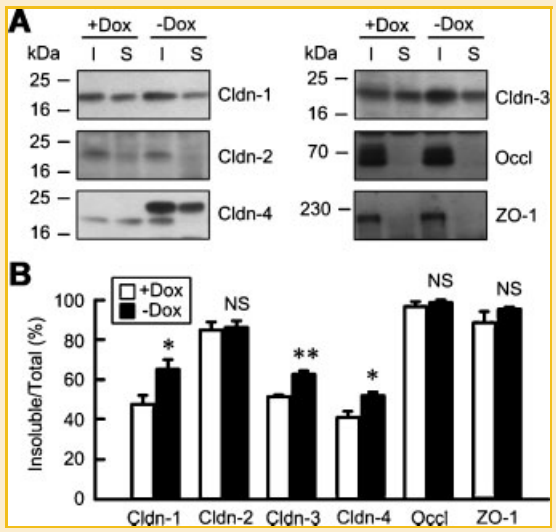

Fig. 6. Effect of claudin (cldn)-4 overexpression on the Triton X-100-insolubility of other junctional claudins. A: Cells expressing FLAG tagged claudin-4/pTRE2hyg were incubated in the presence and absence of doxycycline (1 μg/ml) for 72 h. Triton X-100-soluble (S) and insoluble (I) fractions were prepared and aliquots were separated by SDS–PAGE, followed by immunoblotting with anti-cldn-1, cldn-2, cldn-3, cldn-4, occl, or ZO-1 antibody. B: The band densities of junctional proteins were expressed as insoluble/total fraction. n = 3. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with +Dox. NS, $P > 0.05$ compared with +Dox.

hyperosmotic stress partially injures epithelial sheets, but the remaining cells form TJs with high level of claudin-4.

Claudin-4 overexpression decreased cell migration in MDCK cells (Fig. 3E). Similar results were reported for human gastric carcinoma AGS cells [Mima et al., 2005]. In contrast, cell migration was increased by claudin-4 overexpression in colonic cancer [Takehara et al., 2009] and ovarian epithelial cells [Agarwal et al., 2005]. Cell migration is regulated by integrins, matrix-degrading enzymes, cell–cell adhesion molecules, and cell–cell communication [Friedl and Wolf, 2003]. At present, we do not know how claudin-4 regulates cell migration and why claudin-4 overexpression has a different effect on cell migration in each cell, but the combination of claudins may affect cell migration.

E-cadherin mediates cell–cell adhesion through Ca^{2+}-dependent homophilic interactions [Adams and Nelson, 1998; Takeichi, 1995]. The loss of E-cadherin promotes the disruption of cell–cell contact and progression of cell migration. Re-expression of E-cadherin results in a recovery of cell–cell contact. Interestingly, the expression of E-cadherin was unaffected by claudin-4 overexpression in our experimental conditions. Nevertheless, the recovery rate for the wounded area was decreased by claudin-4 overexpression. We suggest that claudin-4 overexpression enhances cell–cell contact without affecting E-cadherin expression, resulting in a decrease in cell migration. Claudin-1, -2, and -3 were reported to enhance Ca^{2+}-independent cell–cell adhesion in mouse L fibroblasts [Kubota et al., 1999]. We found that claudin-4 overexpression increases the cell

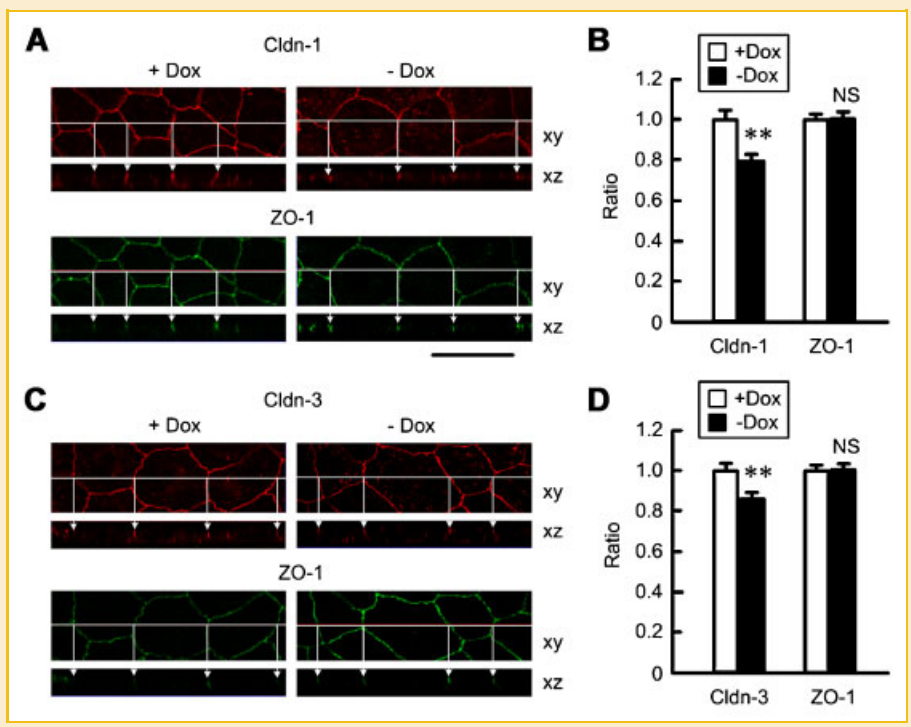

Fig. 7. Effect of claudin (cldn)-4 overexpression on the intracellular distribution of cldn-1 and cldn-3. A,C: Cells expressing FLAG-tagged claudin-4/pTRE2hyg were cultured in the presence and absence of doxycycline (1 μg/ml) for 72 h. After being fixed with methanol, the cells were double-stained with ZO-1 (green) plus cldn-1 (red) or cldn-3 (red). The images show xy and xz sections. Scale bars indicate 10 μm. B,D: The length of fluorescence distribution along the lateral membrane is expressed relative to that in the presence of doxycycline. n = 33–55. The scale bar represents 10 μm. $^{**}P < 0.01$ compared with +Dox. NS, $P > 0.05$ compared with +Dox. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

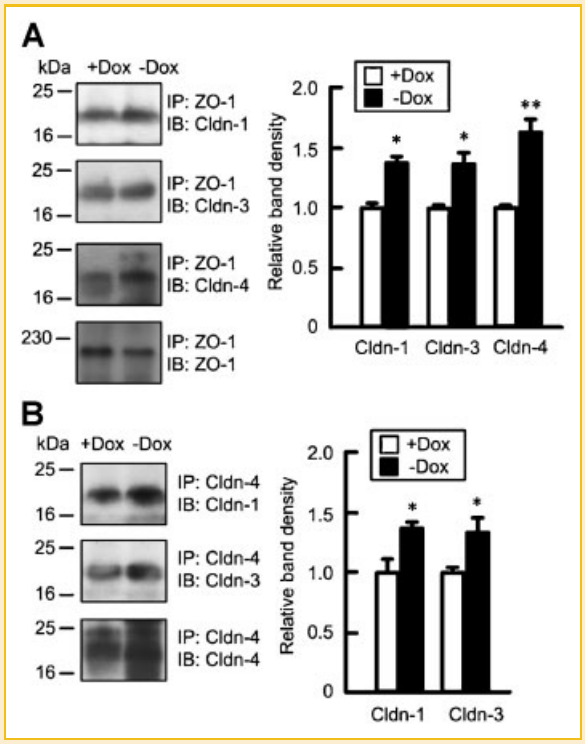

Fig. 8. Increase in the association of claudin (cldn)-4 with claudin-1, claudin-3, and ZO-1 on cldn-4 overexpression. Cells expressing FLAG-tagged cldn-4/pTRE2hyg were incubated in the presence and absence of doxycycline (1 μg/ml) for 72 h. The cell lysate was immunoprecipitated with anti-ZO-1 (A) or cldn-4 (B) antibody and the immune pellets were immunoblotted with anti-cldn-1, -3, -4, or ZO-1 antibody. The band densities of cldn-1, -3 and -4 were expressed relative to the value in the absence of doxycycline or isotonic medium. n = 3. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with +Dox.

association index in the absence of Ca^{2+} (Fig. 3C), indicating that claudin-4 enhances cell–cell contact. Our results suggest that claudin-4 overexpression enhances the integrity of the renal tubular epithelial sheet.

The solubility of TJ proteins reflects the integrity of TJs [Lambert et al., 2005; Basuroy et al., 2006]. Claudin-4 overexpression significantly increased the insolubility of claudin-1 and -3 in Triton X-100 (Fig. 6). In contrast, the insolubility of claudin-2, occludin, E-cadherin, and ZO-1 was unchanged by claudin-4 overexpression. These results indicate that the insolubility of claudin-1 and -3 is increased by claudin-4 overexpression. Immunofluorescence measurements showed that the distribution of claudin-1 and -3 narrowed with claudin-4 overexpression (Fig. 7). Furthermore, immunoprecipitation assays showed that the interaction of ZO-1 with claudin-1, -3, and -4 is increased by claudin-4 overexpression (Fig. 8). These results indicate that the increase in claudin-4 enhances the interaction of claudin-1 and -3 with ZO-1 and the accumulation of these claudins in TJs. Claudin-4 does not undergo heterotypic head-to-head interaction with claudin-1 and -3, while claudin-1 and -3 interact with each other [Furuse et al., 2001; Daugherty et al., 2007]. Notably, claudin-4 and -3 form a heteromeric complex when both are expressed in the same cell. We found that the interaction of claudin-4 with claudin-1 and -3 is increased by its overexpression (Fig. 8). The enhancement of claudin–claudin interaction may stabilize the TJ localization of claudins. At present, we do not know whether claudin-4 enhances cell–cell contact by itself. Claudin-4 may enhance cell association and cell migration in combination with other endogenous claudins (Fig. 9).

In summary, hyperosmotic stress increased claudin-4 expression after 6 h in MDCK cells. Claudin-4 overexpression had no effect on cell proliferation or the endogenous expression of claudin-1, -2, -3,

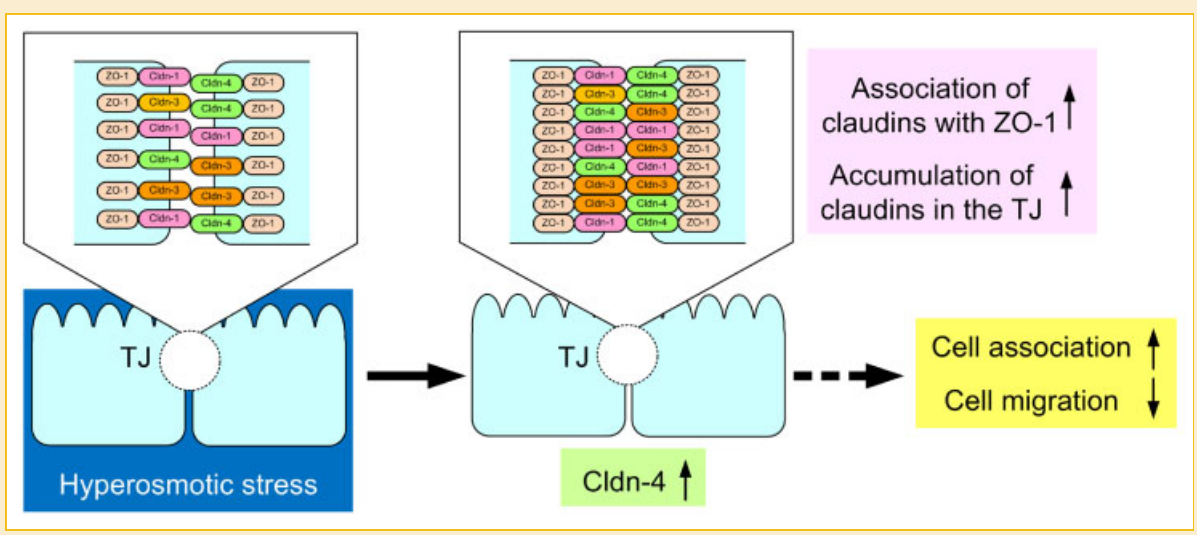

Fig. 9. A putative scheme for the enhancement of cell–cell contact by claudin-4. Hyperosmotic stress increases claudin-4 expression. The elevation of claudin-4 expression increases the accumulation of claudin-1 and -3 in TJs, the association of claudins with ZO-1, and the extent of cell association, whereas it decreases cell migration. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

occludin, E-cadherin, and ZO-1, while it increased TER and the insolubility of claudin-1 and -3 in Triton X-100. Furthermore, the extent of cell association was increased by claudin-4 overexpression. We suggest that the short exposure of cells to hyperosmotic stress decreases claudin-2 level to enhance cell migration, whereas a long exposure to hyperosmotic stress increases claudin-4 level to enhance cell–cell contact in renal tubules.

ACKNOWLEDGMENTS

This work was supported in part by KAKENHI (23590263), grants from the Research Foundation for Pharmaceutical Sciences and an SRI Academic Research Grant (to A.I.).

REFERENCES

Adams CL, Nelson WJ. 1998. Cytomechanics of cadherin-mediated cell–cell adhesion. Curr Opin Cell Biol 10(5):572–577.

Agarwal R, D'Souza T, Morin PJ. 2005. Claudin-3 and claudin-4 expression in ovarian epithelial cells enhances invasion and is associated with increased matrix metalloproteinase-2 activity. Cancer Res 65(16):7378–7385.

Basuroy S, Seth A, Elias B, Naren AP, Rao R. 2006. MAPK interacts with occludin and mediates EGF-induced prevention of tight junction disruption by hydrogen peroxide. Biochem J 393(Pt 1):69–77.

Daugherty BL, Ward C, Smith T, Ritzenthaler JD, Koval M. 2007. Regulation of heterotypic claudin compatibility. J Biol Chem 282(41):30005–30013.

Friedl P, Wolf K. 2003. Tumour-cell invasion and migration: Diversity and escape mechanisms. Nat Rev Cancer 3(5):362–374.

Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S. 1993. Occludin: A novel integral membrane protein localizing at tight junctions. J Cell Biol 123(6 Pt 2):1777–1788.

Furuse M, Fujita K, Hiiragi T, Fujimoto K, Tsukita S. 1998. Claudin-1 and -2: Novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. J Cell Biol 141(7):1539–1550.

Furuse M, Furuse K, Sasaki H, Tsukita S. 2001. Conversion of zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. J Cell Biol 153(2):263–272.

Gumbiner BM. 1993. Breaking through the tight junction barrier. J Cell Biol 123(6 Pt 2):1631–1633.

Hou J, Gomes AS, Paul DL, Goodenough DA. 2006. Study of claudin function by RNA interference. J Biol Chem 281(47):36117–36123.

Hou J, Renigunta A, Yang J, Waldegger S. 2010. Claudin-4 forms paracellular chloride channel in the kidney and requires claudin-8 for tight junction localization. Proc Natl Acad Sci USA 107(42):18010–18015.

Ikari A, Nakajima K, Kawano K, Suketa Y. 2001. Polyvalent cation-sensing mechanism increased Na^+-independent Mg^{2+} transport in renal epithelial cells. Biochem Biophys Res Commun 287(3):671–674.

Ikari A, Nakano M, Suketa Y, Harada H, Takagi K. 2005. Reorganization of ZO-1 by sodium-dependent glucose transporter activation after heat stress in LLC-PK_1 cells. J Cell Physiol 203(3):471–478.

Ikari A, Matsumoto S, Harada H, Takagi K, Hayashi H, Suzuki Y, Degawa M, Miwa M. 2006. Phosphorylation of paracellin-1 at Ser217 by protein kinase A is essential for localization in tight junctions. J Cell Sci 119(Pt 9):1781–1789.

Ikari A, Takiguchi A, Atomi K, Sato T, Sugatani J. 2010. Decrease in claudin-2 expression enhances cell migration in renal epithelial Madin-Darby canine kidney cells. J Cell Physiol 226(6):1471–1478. : DOI: 10.1002/jcp.22386.

Kiuchi-Saishin Y, Gotoh S, Furuse M, Takasuga A, Tano Y, Tsukita S. 2002. Differential expression patterns of claudins, tight junction membrane proteins, in mouse nephron segments. J Am Soc Nephrol 13(4):875–886.

Kubota K, Furuse M, Sasaki H, Sonoda N, Fujita K, Nagafuchi A, Tsukita S. 1999. Ca^{2+}-independent cell-adhesion activity of claudins, a family of integral membrane proteins localized at tight junctions. Curr Biol 9(18): 1035–1038.

Lambert D, O'Neill CA, Padfield PJ. 2005. Depletion of Caco-2 cell cholesterol disrupts barrier function by altering the detergent solubility and distribution of specific tight-junction proteins. Biochem J 387(Pt 2):553–560.

Lanaspa MA, Andres-Hernando A, Rivard CJ, Dai Y, Berl T. 2008. Hypertonic stress increases claudin-4 expression and tight junction integrity in association with MUPP1 in IMCD3cells. Proc Natl Acad Sci USA 105(41):15797–15802.

Michl P, Barth C, Buchholz M, Lerch MM, Rolke M, Holzmann KH, Menke A, Fensterer H, Giehl K, Lohr M, Leder G, Iwamura T, Adler G, Gress TM. 2003. Claudin-4 expression decreases invasiveness and metastatic potential of pancreatic cancer. Cancer Res 63(19):6265–6271.

Mima S, Tsutsumi S, Ushijima H, Takeda M, Fukuda I, Yokomizo K, Suzuki K, Sano K, Nakanishi T, Tomisato W, Tsuchiya T, Mizushima T. 2005. Induction of claudin-4 by nonsteroidal anti-inflammatory drugs and its contribution to their chemopreventive effect. Cancer Res 65(5):1868–1876.

Mineta K, Yamamoto Y, Yamazaki Y, Tanaka H, Tada Y, Saito K, Tamura A, Igarashi M, Endo T, Takeuchi K, Tsukita S. 2011. Predicted expansion of the claudin multigene family. FEBS Lett 585(4):606–612.

Morita K, Furuse M, Fujimoto K, Tsukita S. 1999. Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. Proc Natl Acad Sci USA 96(2):511–516.

Nagafuchi A, Ishihara S, Tsukita S. 1994. The roles of catenins in the cadherin-mediated cell adhesion: Functional analysis of E-cadherin-alpha catenin fusion molecules. J Cell Biol 127(1):235–245.

Rodriguez-Boulan E, Nelson WJ. 1989. Morphogenesis of the polarized epithelial cell phenotype. Science 245(4919):718–725.

Takehara M, Nishimura T, Mima S, Hoshino T, Mizushima T. 2009. Effect of claudin expression on paracellular permeability, migration and invasion of colonic cancer cells. Biol Pharm Bull 32(5):825–831.

Takeichi M. 1995. Morphogenetic roles of classic cadherins. Curr Opin Cell Biol 7(5):619–627.

Tsukita S, Furuse M, Itoh M. 2001. Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol 2(4):285–293.

Tsukita S, Katsuno T, Yamazaki Y, Umeda K, Tamura A. 2009. Roles of ZO-1 and ZO-2 in establishment of the belt-like adherens and tight junctions with paracellular permselective barrier function. Ann N Y Acad Sci 1165:44–52.

Turksen K, Troy TC. 2004. Barriers built on claudins. J Cell Sci 117(Pt 12): 2435–2447.

Van Itallie C, Rahner C, Anderson JM. 2001. Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability. J Clin Invest 107(10):1319–1327.