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Claudin-8 Expression in Renal Epithelial Cells Augments the Paracellular Barrier by Replacing Endogenous Claudin-2

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Abstract Claudins are transmembrane proteins of the tight junction that determine and regulate paracellular ion permeability. We previously reported that claudin-8 reduces paracellular cation permeability when expressed in low-resistance Madin-Darby canine kidney (MDCK) II cells. Here, we address how the interaction of heterologously expressed claudin-8 with endogenous claudin isoforms impacts epithelial barrier properties. In MDCK II cells, barrier improvement by claudin-8 is accompanied by a reduction of endogenous claudin-2 protein at the tight junction. Here, we show that this is not because of relocalization of claudin-2 into the cytosolic pool but primarily due to a decrease in gene expression. Claudin-8 also affects the trafficking of claudin-2, which was displaced specifically from the junctions at which claudin-8 was inserted. To test whether replacement of cation-permeable claudin-2 mediates the effect of claudin-8 on the electrophysiological phenotype of the host cell line, we expressed claudin-8 in high-resistance MDCK I cells, which lack endogenous claudin-2. Unlike in MDCK II cells, induction of claudin-8 in MDCK I cells (which did not affect levels of endogenous claudins) did not alter paracellular ion permeability. Furthermore, when endogenous claudin-2 in MDCK II cells was downregulated by epidermal growth factor to create a cell model with low transepithelial resistance and low levels of claudin-2, the permeability effects of claudin-8 were also abolished. Our findings demonstrate that claudin overexpression studies measure the combined effect of alterations in both endogenous and exogenous claudins, thus explaining the dependence of the phenotype on the host cell line.

Keywords Tight junction · Claudin · Paracellular transport · Renal epithelia

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Introduction

Tight junctions of polarized epithelial cells form a continuous network of cell-cell contacts which limits and controls passive paracellular transport of water, ions and macromolecules between two fluid compartments of different chemical composition. The diffusion barrier established by tight junctions and its size and charge selectivity can vary markedly between epithelia of different tissues or even within the same tissue. The renal tubules, for example contain both leaky and tight segments. In the leaky proximal segments, isotonic salt and water reabsorption occurs both by transcellular transport and by electrochemical gradient-driven passive paracellular transport. In contrast, the aldosterone-sensitive distal nephron is lined by a tight epithelium, which restricts paracellular diffusion and thus allows the establishment of high transtubular concentration gradients (Fanning, Mitic & Anderson, 1999; Van Itallie & Anderson, 2006).

The distinct paracellular permeability characteristics observed in different epithelia are thought to be determined by claudins, ~20-27 kDa transmembrane proteins of the tight junctions. Claudins form a multigene family, including at least 24 members in mammals, which are distinctively expressed in different tissues. Specific permeability

characteristics of cellular barriers are explained by a model in which claudins interact in the intercellular cleft in a homotypic or heterotypic manner and form channel-like selective pores in the paracellular pathway (Tsukita, Furuse & Itoh, 2001; Yu, 2003). Several studies have linked abnormalities in paracellular transport observed in different human hereditary diseases to mutations in claudins (Simon et al., 1999; Wilcox et al., 2001). The role of claudins at the tight junctions is further supported by investigations on transgenic mice in which knockout of claudin genes alters paracellular permeability (Ben-Yosef et al., 2003; Furuse et al., 2002; Miyamoto et al., 2005; Nitta et al., 2003).

A common approach to studying the specific functions of different claudin isoforms is heterologous overexpression in epithelial cell lines (i.e., transfection of exogenous DNA encoding a claudin isoform of interest into host cells) and analysis of paracellular transport characteristics. For most claudins, including claudins 1, 4, 5, 7, 8 and 14, the transepithelial electrical resistance (TER) of the host cell lines is increased, suggesting that these claudins strengthen the epithelial barrier to ions (Alexandre, Lu & Chen, 2005; McCarthy et al., 2000; Van Itallie, Rahner & Anderson, 2001; Wen et al., 2004; Yu et al., 2003). Most of these isoforms do so by selectively decreasing Na^+ permeability. By contrast, claudin-2 decreases TER by increasing Na^+ permeability, indicating that this claudin adds cation-selective pores to the paracellular pathway (Amasheh et al., 2002; Furuse et al., 2001).

Yet the determination of claudin functions by heterogeneous expression has significant limitations. Van Itallie, Fanning & Anderson (2003) recently demonstrated that the effects of heterologously expressed claudins on electrophysiological characteristics vary with the host cell line. For example, when claudin-2 and claudin-15 are expressed in LLC-PK1 cells (which are normally anion-selective), TER decreases and the cells become cation-selective. By contrast, when the same claudins are expressed in Madin-Darby canine kidney (MDCK) II cells, TER is slightly increased and ion selectivity is not changed. Of course, each host cell line already expresses its own set of endogenous claudins. Thus, the physiological properties of the paracellular pathway probably depend on the specific combination of endogenously and heterologously expressed claudins in that host cell.

There are at least two ways in which this might occur. First, endogenous claudins could combine with the heterologously expressed claudin to form heteromultimeric pores with novel and presumably unpredictable permeability properties. Second, the heterologously expressed claudin could add to or replace endogenous claudins, in which case paracellular permeability would change in a predictable manner. In fact, paracellular permeability does change in different host cell lines in a fairly predictable manner. Claudins that are predominantly cation-selective (like claudin-2) or anion-impermeable (like claudin-7) have the greatest effect when heterologously expressed in anion-selective cell lines, and the reverse is true for cation-impermeable claudins (Alexandre et al., 2005; Hou, Paul & Goodenough, 2005; Van Itallie et al., 2003). Claudin-2, which has high ionic permeability, has the greatest effect when heterologously expressed in tight cell lines (Amasheh et al., 2002; Furuse et al., 2001), while leaky cell lines have proven to be the best hosts to assay claudins with low ionic permeability (e.g., Van Itallie et al., 2001; Wen et al., 2005; Yu et al., 2003). However, the role of any specific endogenous claudin in the effect of a heterologously expressed claudin has never been tested before.

We recently generated MDCK II TetOff cells stably transfected with claudin-8 (Yu et al., 2003). Claudin-8 is a tight junction protein that associates with multi-PDZ domain protein 1 (Jeansonne et al., 2003). In the kidney, it has been localized at the tight junctions toward the distal end of the renal tubule (Gonzalez-Mariscal et al., 2006; Kiuchi-Saishin et al., 2002). We have previously demonstrated that claudin-8 is expressed along the entire aldosterone-sensitive distal nephron (Li, Huey & Yu, 2004). Its expression in these tubule segments of low paracellular permeability suggests a role of claudin-8 in barrier establishment. Consistent with this, induction of claudin-8 in MDCK II caused an increase in TER (Jeansonne et al., 2003; Yu et al., 2003) and a decrease in cation permeability, while no change in the permeability of neutral solutes was observed (Yu et al., 2003). Remarkably, the improvement of the epithelial barrier by expression of claudin-8 was accompanied by a significant downregulation of endogenous claudin-2. Thus, claudin-8 provides an interesting model to test the influence of a single endogenous claudin on the effect of a heterologously expressed claudin. In this study, we investigated the mechanism involved in the downregulation of claudin-2 in MDCK II cells heterologously expressing claudin-8. We further tested the hypothesis that the presence of endogenous claudin-2 in the host cell line is required to demonstrate the barrier properties of claudin-8, using host cells in which endogenous claudin-2 was missing or strongly downregulated. Our data show that claudin-2 replacement at the tight junctions plays a critical role in the effects manifested by claudin-8 expression.

Methods

Cell Culture and Transfection

MDCK II TetOff cells expressing an inducible N-terminal FLAG epitope-tagged mouse claudin-8 cDNA have been described before. MDCK I TetOff cells were generated by

transduction of MDCK I cells with the retroviral vector pRevTet-Off-IN (Clontech, Palo Alto, CA), using methods reported previously (Yu et al., 2003). Stable clones were selected in G418. One MDCK I TetOff cell clone was then further retrovirally transduced with a Tet-responsive N-terminal FLAG-tagged mouse claudin-8 construct and selected in hygromycin. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml G418 and 300 μg/ml hygromycin. Claudin-8 expression was suppressed by addition of 20 ng/ml doxycycline (Dox) to the culture medium (Dox^+) and induced by omitting Dox from the medium (Dox^-).

Immunoblotting and Immunohistochemistry

For analysis of the expression of claudin-8 and endogenous tight junction proteins by immunoblotting, confluent cells, previously seeded on Petri dishes in 33% confluent density, were homogenized in buffer (pH 7) containing 0.25 M sucrose, 30 mM histidine, 1 mM ethylenediaminetetraacetic acid (EDTA) and protease inhibitors (Complete Mini; Roche Diagnostics, Indianapolis, IN). For further fractionation, the homogenate was centrifuged briefly to spin down cell debris, followed by centrifugation at 100,000 x *g* for 25 min, separating the membrane-bound proteins (pellet) from the cytosolic fraction (supernatant). The 100,000 x *g* membrane pellet was resuspended in sucrose-histidine buffer. After boiling the samples in reducing loading buffer containing 2% sodium dodecyl sulfate (SDS), the proteins were loaded on a 10% denaturing polyacrylamide gel in aliquots of 20 μg and separated by electrophoresis. To minimize background staining in claudin-8 detection, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors [pH 8]) and claudin-8 was purified by immunoprecipitation. Insoluble material was sedimented by centrifugation at 14,000 x *g* for 15 min. The supernatant was precleared by incubation with Protein G-agarose beads (Invitrogen, Carlsbad, CA), then immunoprecipitated by incubation with mouse M2 anti-FLAG immunoglobulin G (IgG, 1:1,000; Zymed, San Francisco, CA) and Protein G-agarose (1 h each at 4°C). The beads were washed twice with RIPA buffer and once with buffer containing 1 M NaCl and 1 M Tris/HCl (pH 7.4) and boiled in loading buffer to release bound proteins. After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Richmond, CA) and visualized using the enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL). Claudin-8 was detected using an affinity-purified polyclonal antibody, 933 (Yu et al., 2003), diluted 1:500, while antibodies raised against occludin and claudins 1, 2, 3, 4 and 7 were obtained from Zymed and applied according to the manufacturer's protocol.

Protein expression and localization were further studied by indirect immunofluorescence staining as described previously (Yu et al., 2003), using paraformaldehyde-fixed cells grown on Transwell filters (0.4 μm pore size; Costar, Cambridge, MA). Primary antibodies were diluted 1:200 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), 5% goat serum and 0.3% Triton X-100. For immunodetection, secondary antibodies (Molecular Probes, Eugene, OR) coupled to either Alexa Fluor 488 (green) or 555 (red) at 1:1,000 dilution were used. Filters were mounted on glass slides using Vectashield antifade mounting medium (Vector Laboratories, Burlingame, CA) and analyzed either with a conventional epifluorescence microscope or a Nikon (Tokyo, Japan) PCM confocal microscope.

Metabolic Labeling and Pulse Chase

Cells were seeded at 33% confluent density onto 10-cm tissue culture plates. Three days later, they were washed twice in methionine- and cysteine-free DMEM containing 5% dialyzed fetal bovine serum (MFD) and incubated in MFD for 15 min at 37°C to deplete intracellular methionine pools. The medium was then replaced with 2.5 ml of MFD containing 200 μCi/ml of a ^{35}S-methionine/^{35}S-cysteine mix (Express Protein Labeling Mix; Perkin-Elmer Life and Analytical Sciences, Boston, MA) and incubated at 37°C for 1 h. The radioactive labeling mix was then removed, and the cells were washed twice in regular medium and incubated in regular medium at 37°C for 0, 3 or 6 h.

At each time point, the plates were placed on ice. The cells were washed once with ice-cold PBS, then lysed in RIPA buffer. Claudin-2 was immunoprecipitated as described above using polyclonal rabbit claudin-2 IgG (1:200, Zymed). Samples were boiled in electrophoresis sample buffer containing 2% SDS and loaded on a 10% denaturing polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie blue, fixed in 5% methanol/7% acetic acid and impregnated with liquid scintillant (Autofluor; National Diagnostics, Atlanta, GA). The gel was vacuum-dried for autoradiography (BioMax MS film; Kodak, Rochester, NY), and bands were quantified by densitometry.

Northern Blotting

The expression of claudin-2 in Dox^+ compared to Dox^- treated MDCK II TetOff claudin-8 cells was studied by Northern blot analysis. Cells were lysed with 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl and 0.7% β-mercaptoethanol. RNA was isolated by acid-

phenol extraction (Chomczynski & Sacchi, 1987), precipitated with isopropanol and loaded on a denaturing agarose-formaldehyde gel. After electrophoresis, RNA was transferred to a nylon membrane (Roche Diagnostics) by capillary transfer and hybridized overnight at 50°C with a ^{32}P-radiolabeled probe targeting the canine claudin-2 sequence, which was synthesized using the Rediprime II Random Prime Labeling kit (Amersham). The blot was washed under high-stringency conditions and exposed to light-sensitive film, and bands were quantified by densitometry.

Freeze Fracture

For freeze fracture, cells were fixed with 2% glutaraldehyde at 4°C for 30 min, scraped from the substrate and infiltrated with 25% glycerol in 0.1 M cacodylate buffer for 60 min at 4°C. After freezing in liquid nitrogen slush, cells were freeze-fractured at –115°C in a Balzers 400-freeze fracture unit. Replicas were cleaned with sodium hypochlorite, washed in distilled water, placed on Formvar-coated grids and examined under a Philips (Hamburg, Germany) 301 electron microscope. The number of parallel strands in the tight junctions was determined on micrographs at a final magnification of 62,500. The Wilcoxon ranked sum test was used to assess statistical significance in the difference of strand numbers.

TER Monitoring

The time course of TER of cells, plated on Transwell filters at confluent density (approximately 2 x 10^5 cells/cm^2), was monitored using a Millicell-ERS voltohmeter (Millipore, Bedford, MA). Chopstick-style Ag/AgCl-covered electrodes were immersed in the culture medium of the apical and basolateral compartments, and a 20-μA AC square-wave current was applied across the cell monolayer. In parallel, TER of blank filters was determined and subtracted from the resistance measured for cell-covered filters. Data are presented as means ± standard error (SE).

Diffusion Potential Measurements

The permeability of MDCK monolayers to Na^+ and Cl^- was determined by diffusion potential measurements using Ussing chambers (Cereijido et al., 1978). Cells were seeded on Snapwell filters (Costar) at confluent density and cultured under the indicated conditions. The filters were detached from the insert and mounted in Ussing chambers, which were filled with Ringer's buffer (150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], [pH 7.4]). The chambers were heated to 37°C and continuously bubbled with 95% O_2 and 5% CO_2. Two sets of electrodes, voltage-sensing electrodes made of Ag/AgCl pellets and current-passing electrodes of Ag wire, were immersed into the apical and basolateral medium via agar bridges containing 3 M KCl. The electrodes were connected via head stage amplifiers to a microcomputer-controlled voltage/current clamp (VCC-MC6; Physiologic Instruments, San Diego, CA). Before starting the experiment, asymmetry voltage between the voltage-measuring electrodes was compensated by adjustment of the offset potentiometer. All studies were performed in K^+-free solutions to inactivate the Na,K-ATPase; under these conditions, the spontaneous transepithelial potential in all the cell lines used was negligible. The TER and the monolayers' conductance, G (TER^{-1}), were calculated from the voltage evoked by a 90-μA current pulse. To determine the 2:1 NaCl dilution potential, the solution of the basolateral chamber was replaced by a modified Ringer's solution containing 75 mM NaCl (osmolarity adjusted with mannitol) and the voltage was read immediately under open-circuit conditions (values indicated refer to the apical side). The TER and NaCl dilution potentials of cell monolayers were corrected by subtracting the values determined for blank filters mounted in Ussing chambers.

Assuming a constant electrostatic field across the monolayer, the relative ionic permeability ratio, P_{Na}/P_{Cl}, was calculated from the NaCl dilution potential using the Goldman-Hodgkin-Katz equation, which has been previously shown to fit the behavior of MDCK II cells fairly well (Yu et al., 2003). In all calculations, activities rather than concentrations were used. The absolute permeabilities, P_{Na} and P_{Cl}, were derived by the method of Kimizuka & Koketsu (1964) using the equation

$$P_{Na} = \frac{RT}{F^2} \bullet \frac{G_M}{\alpha(1+\beta)}$$

where G_M is the monolayer's conductance per unit surface area, α is the ratio of NaCl activity in the apical and basolateral compartments ($a_{NaCl,ap}/a_{NaCl,bl}$) and β is the ratio of the permeability of Cl^- and Na^+ (P_{Cl}/P_{Na}). The data are presented as means ± SE. Statistical significance was determined using the unpaired two-tailed Student's t-test.

Results

Replacement of Endogenous Claudin-2 by Claudin-8 in MDCK II TetOff Cells

We previously observed that claudin-8 overexpression in low-resistance MDCK II cells leads to downregulation of endogenous claudin-2 in membrane fractions of cell lysates

(Yu et al., 2003). In the present study, we investigated the mechanisms which underlie the replacement of claudin-2 by claudin-8. First, the time course of claudin-8 expression and downregulation of claudin-2 in MDCK II TetOff cells was determined by Western blot analysis (Fig. 1a). While high levels of claudin-8 were detected within 1 day in culture, expression of claudin-2 occurred later and peaked after 3–4 days. A significant downregulation of claudin-2 in cells induced to express claudin-8 (Dox^-) was observed within the period of time when Dox^+ cells showed strong claudin-2 expression. Subsequent experiments were conducted at 3–4 days of culture to maximize differences in claudin-2 expression under Dox^+ and Dox^- conditions.

To test whether the induced expression of claudin-8 causes redistribution of claudin-2 from the membrane fraction into the cytoplasm, we compared the amount of claudin-2 in the whole-cell lysate as well as the membrane and cytosolic fractions by Western blot (Fig. 1b). Induction of claudin-8 decreased claudin-2 in the whole-cell lysate, showing true regulation of claudin-2 expression at the protein level. Furthermore, while membrane-bound claudin-2 was decreased under Dox^- conditions, the protein was not detectable in the cytosolic fraction, excluding the possibility that the protein redistributed into the cytoplasm.

Since redistribution of claudin-2 can be excluded, we tested whether the lower levels of claudin-2 in claudin-8-expressing MDCK II cells are due to decreased protein synthesis or increased protein degradation. The degradation rate of claudin-2 was determined by pulse chase metabolic labeling of newly synthesized proteins and immunoprecipitation of claudin-2 from the cell lysate. As shown in (Fig. 1c), the degradation rate of claudin-2 was similar for cells grown under Dox^+ and Dox^- conditions (no difference in slope of linear regression). However, the level of newly synthesized claudin-2 (as determined by the amount of radiolabeled protein at time zero) was significantly less in Dox^-treated cells. The result suggests that claudin-8 reduces the rate of synthesis of claudin-2 protein.

We next addressed whether this is due to regulation of claudin-2 mRNA abundance, using Northern blot analysis. Studying claudin-2 expression over a time course of several days, we found that induction of claudin-8 in MDCK II cells reduced levels of claudin-2 mRNA (Fig. 1d). The effect was greatest 3 days after plating the cells (57% less claudin-2 mRNA in Dox^- cells compared to Dox^+ cells, $p < 0.01$), correlating with the time course of regulation of claudin-2 at the protein level (Fig. 1a). Claudin-2 mRNA was not regulated by Dox in MDCK II TetOff cells which were not transfected with claudin-8 (*not shown*), proving that our observations are not an artifact of Dox but instead depend on claudin-8 expression.

We recently found that in cocultures of MDCK II TetOff claudin-8 cells (claudin-8-positive) and MDCK II TetOff cells (not transfected with claudin-8, claudin-8-negative), claudin-8 is strongly expressed at the borders between positive cells. In contrast, the levels of claudin-8 at cell-cell contacts formed by positive and negative cells are very low (*manuscript in preparation*). We exploited this coculture model to investigate whether claudin-2 is downregulated throughout the cell by regulation of overall level of expression or downregulated only at sites of claudin-8 expression. The latter case would indicate that claudin-8 also alters claudin-2 trafficking. Cocultures of MDCK II TetOff claudin-8 cells and MDCK II TetOff cells were stained by double immunofluorescence against claudin-2 and claudin-8 (Fig. 1e). Under Dox^- conditions, claudin-2 was only downregulated at cell-cell contacts between transfected claudin-8-positive cells, where claudin-8 expression is strong. In contrast, at cell-cell contacts shared by claudin-8-positive and nontransfected claudin-8-negative cells, claudin-8 staining was very weak and claudin-2 appeared to be expressed at normal levels. Thus, in addition to control of gene expression, claudin-8 seems to interfere with claudin-2 trafficking to the tight junctions.

Generation of MDCK I TetOff Cells with Inducible Expression of Claudin-8

Previous investigations showed that claudin-8 increases TER and decreases Na^+ permeability in MDCK II cells (Yu et al., 2003). In order to study the role of claudin-2 replacement and the barrier properties of the host cell line in this process, we stably expressed claudin-8 in high-resistance renal epithelial MDCK I cells, which lack endogenous claudin-2 (Furuse et al., 2001). Expression was controlled by a Tet-responsive system. Screening by high-stringency Northern blot revealed stable expression of claudin-8 in three clones (4, 6 and 10) (*not shown*). Western blot analysis confirmed the positive tested clones and regulation by Dox (Fig. 2a). Levels of claudin-8 protein in transfected MDCK I TetOff claudin-8 cells were comparable to those in MDCK II TetOff claudin-8 cells. Immunofluorescence staining of transfected cells showed that claudin-8 is expressed at the lateral membrane only under Dox^- conditions (Fig. 2b). Furthermore, double-immunofluorescence staining and analysis of vertical sections of confocal images proved that claudin-8 colocalizes with occludin at the tight junctions and is thus correctly trafficked (Fig. 2c).

Effect of Claudin-8 Expression in MDCK I TetOff Cells on Tight Junction Morphology and Composition

Overexpression of tight junction proteins can lead to formation of additional tight junction strands or changes in

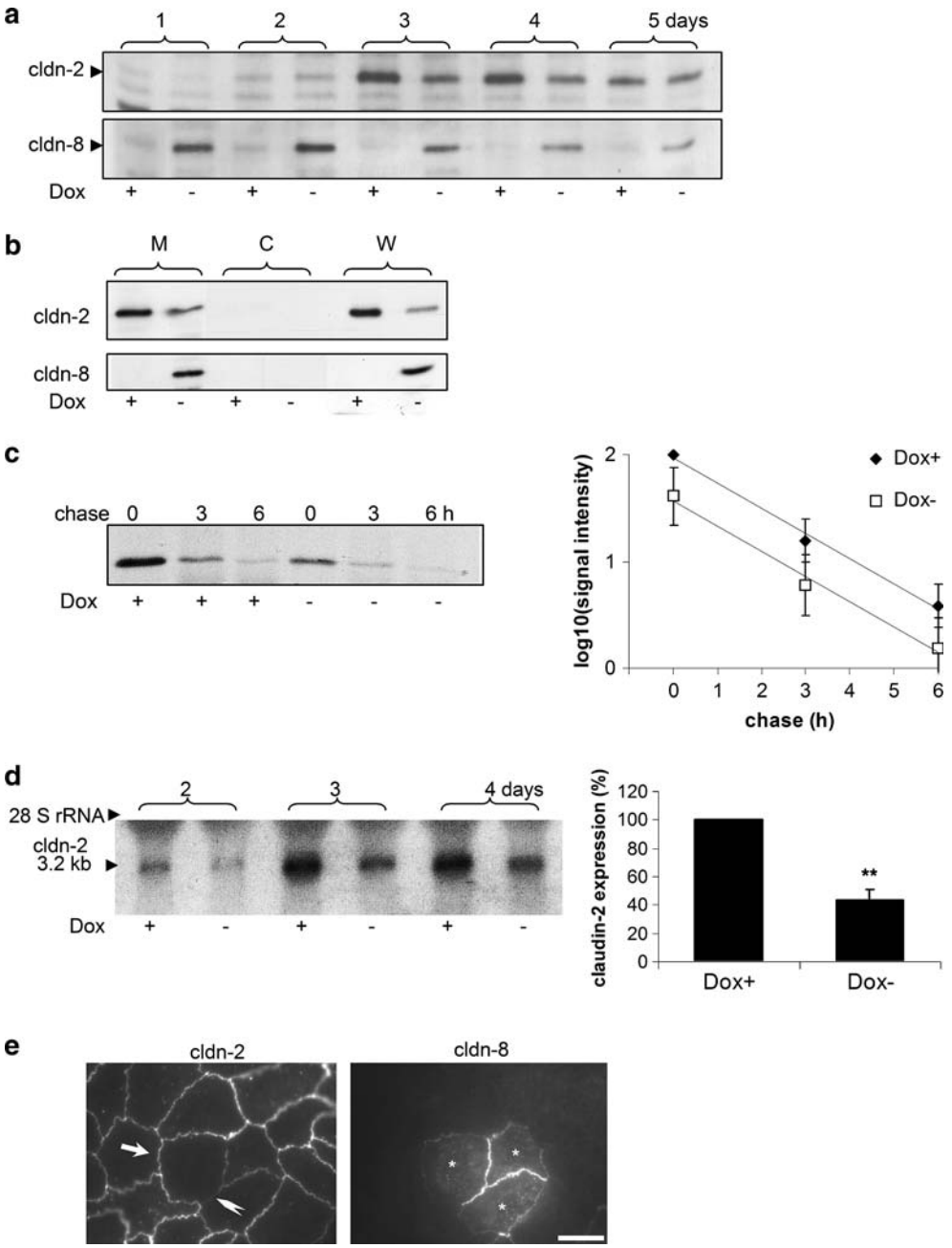

Fig. 1 Analysis of endogenous claudin-2 expression in MDCK II TetOff claudin-8 cells grown under Dox^+/Dox^- conditions. (**a**) Western blot analysis of whole-cell lysates addressing the time course of expression of claudin-2 and claudin-8. (**b**) Western blot analysis of whole-cell lysates (*W*), 100,000 x *g* membrane fractions (*M*) and cytosolic fractions (*C*) isolated from confluent cells after 3 days in culture demonstrate that the overall amount of claudin-2, which is all membrane-bound, is downregulated during claudin-8 expression and that the protein does not redistribute into the cytoplasm. (**c**) Metabolic labeling and pulse chase study of protein degradation of claudin-2 using confluent cells after 3 days in culture. ^{35}S-labeled claudin-2 was chased for the indicated period of time and then immunoprecipitated from the cell lysate. Precipitated protein was analyzed by SDS polyacrylamide gel electrophoresis and quantified by densitometry of the autoradiograph (normalized data shown, Dox^+, 0h = 100%). The plot $\log_{10}$(signal intensity) versus chase time shows that the rate of claudin-2 degradation is similar in Dox^+- and Dox^--treated cells (slope –0.236 and –0.237, respectively) (results of two independent experiments). (**d**) High-stringency Northern blot addressing the time course of claudin-2 expression shows that mRNA levels of claudin-2 are downregulated by induction of claudin-8. The results of quantification of three independent experiments (±SE) for cells cultured for 3 days are presented in the plot. Induction of claudin-8 (Dox^-) causes downregulation of claudin-2 by 57% compared to Dox^+-treated cells (normalized to 100%) (**$p < 0.01$). (**e**) Coculture of claudin-8 transfected and untransfected MDCK II TetOff cells, double-stained for claudin-2 and claudin-8. Claudin-8 expression is strongest at cell-cell contacts between transfected cells (*asterisks*). Endogenous claudin-2 is downregulated at cell-cell contacts between claudin-8-expressing cells (*arrowhead*) but not at the borderline of transected and untransfected cells (*arrow*). Bar = 10 μm

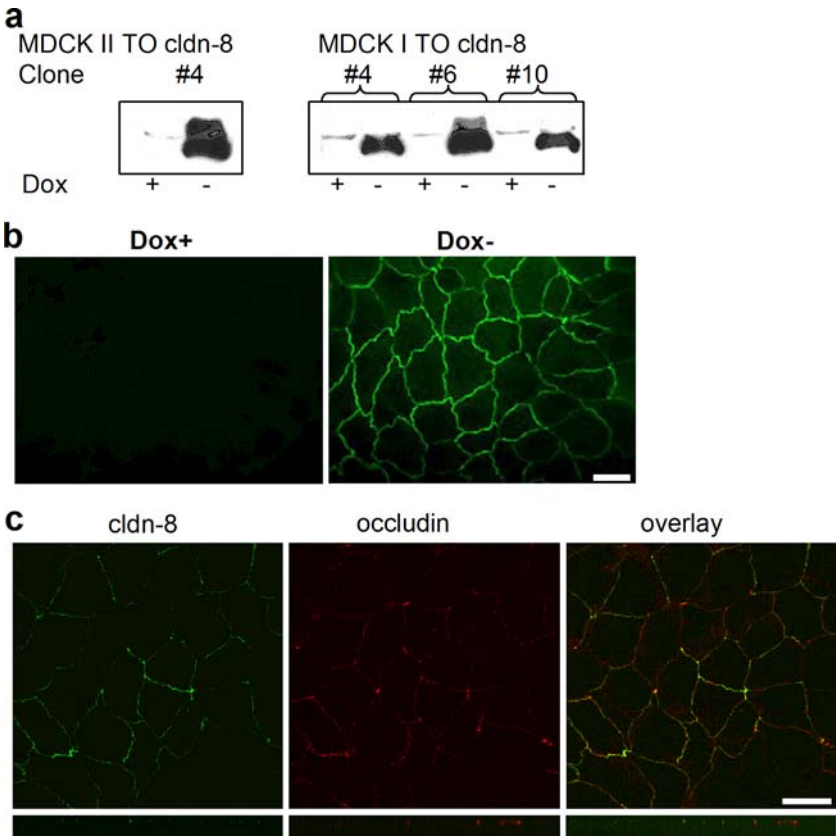

Fig. 2 Generation of novel MDCK I TetOff claudin-8 cell lines. (**a**) Western blot showing induced expression of claudin-8, immunoprecipitated from MDCK II and different clones of MDCK I TetOff claudin-8 cells. (**b**) Immunofluorescence staining of claudin-8 in MDCK I TetOff claudin-8 confirms inducible expression of the protein under Dox^- conditions. (**c**) Double-immunofluorescence staining of claudin-8 and occludin and analysis of vertical sections of confocal images prove functional expression of claudin-8 at the tight junctions of induced (Dox^-) MDCK I TetOff claudin-8 cells. Bar = 10 μm

expression of tight junction proteins endogenous to the host cells that may affect barrier properties. The influence of claudin-8 expression in MDCK I cells on the morphology and number of tight junction strands was investigated by means of electron microscopic analysis of freeze fracture replicas (Fig. 3a). The strand number of MDCK I TetOff claudin-8 cells (clone 4) was found to be slightly increased under Dox^- conditions (median of 3.5 strands in Dox^- cells compared to 3 strands in Dox^+ cells, $p = 0.03$). This clone was used in the expression studies and electrophysiological experiments that are presented below. Another clone (clone 10) showed a higher increase in the strand number (5 compared to 3), but the results of the functional experiments were the same (*not shown*).

Next, we tested whether induction of claudin-8 affects endogenous claudins and occludin in MDCK I TetOff claudin-8 cells. We screened MDCK I for endogenously expressed claudins by Western blot analysis of cell membrane fractions using antibodies targeting claudins 1, 2, 3, 4, 7, 14, 16 and 19. In agreement with an earlier report (Lipschutz et al., 2005), only claudins 1, 3, 4 and 7 could be detected (Fig. 3b). Induction of claudin-8 did not alter the amount of membrane-bound endogenous claudins and occludin. Additionally, immunofluorescence staining showed expression of occludin and claudins 1, 3, 4 and 7 at cell-cell contacts and no differences in the localization and quantity of these proteins between Dox^+- and Dox^--treated cells (Fig. 3c).

TER and Permeability of MDCK I TetOff Claudin-8 Cells

The next set of experiments was performed to test whether claudin-8 affects transepithelial permeability when expressed in MDCK I cells. Monitoring the time course of TER of Dox^+- and Dox^--treated cells, we found an increase in TER over a period of 7 days after seeding before reaching steady state. Claudin-8 expression in MDCK I did not alter the TER during barrier development, when TER is low and comparable to that of a leaky cell line, or under steady-state conditions, when values are in the range 3,300–3,900 $\Omega \cdot cm^2$ (Fig. 4a). Unlike in leaky MDCK II cells, changes in TER in a high-resistance epithelium can potentially reflect not only changes in the resistance of the paracellular route but also alterations in transcellular transport. In contrast to previous observations in MDCK II cells, the data indicate that induction of claudin-8 in MDCK I does not influence either pathway.

In addition, we determined the NaCl dilution potential in order to assess charge selectivity of the epithelium and to exclude the possibility that the apparent lack of change in TER is really due to simultaneous opposing changes in the

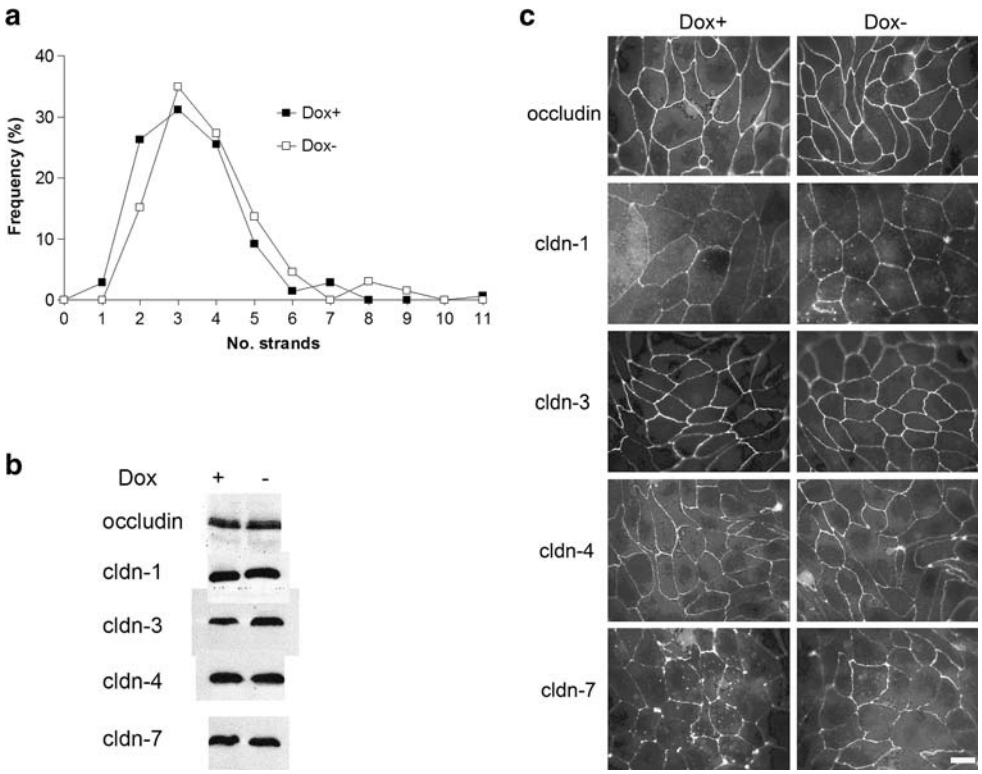

Fig. 3 Effect of claudin-8 induction in MDCK I TetOff claudin-8 cells on tight junction strands and composition. (**a**) Frequency histogram showing tight junction strand counts performed on electron micrographs of freeze-fracture replicas. A small but statistically significant shift to higher strand numbers is observed under Dox^- conditions. (**b**) Western blot of 100,000 x *g* membrane fractions and (**c**) immunofluorescence staining showing similar expression levels of occludin and endogenous claudins in noninduced and induced cells. Bar = 10 μm

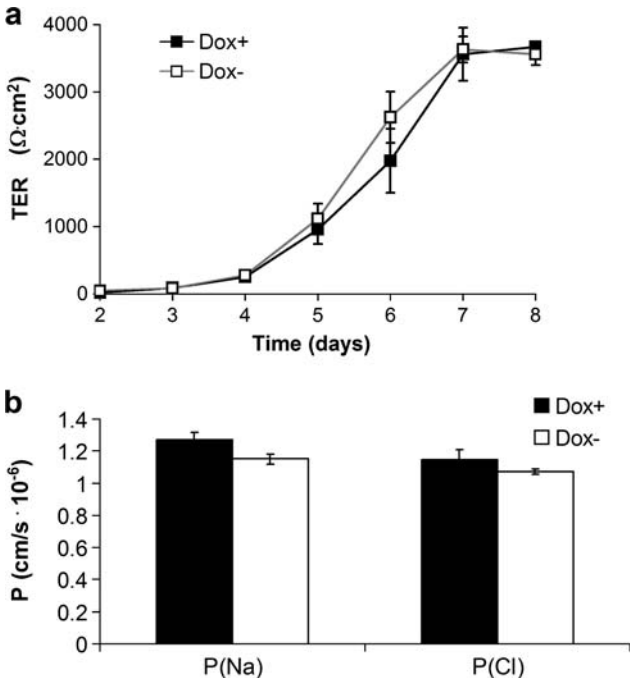

Fig. 4 Permeability properties of MDCK I TetOff claudin-8 cells. (**a**) Time course of the TER in cells which were seeded on filters at confluent density and cultured under Dox^+ and Dox^- conditions. (**b**) Na^+ and Cl^- permeability of confluent monolayers (day 7) determined from transepithelial NaCl dilution potential measurements in Ussing chambers

permeability to Na^+ and Cl^-, the principal transepithelial current carriers. MDCK I TetOff claudin-8 cells, grown on Snapwell filters and mounted in Ussing chambers, were equilibrated in Ringer's solution containing 150 mM NaCl, and transepithelial conductance was determined under current clamp conditions. Subsequently, a transepithelial concentration gradient of NaCl was imposed to determine the dilution potential, from which the permeability ratio P_{Na}/P_{Cl} was calculated. MDCK I TetOff claudin-8 cells were slightly cation-selective (P_{Na}/P_{Cl} = 1.11, Dox^+ cells), and selectivity was not changed under Dox^- conditions. Compared to cation-selective MDCK II cells (P_{Na} in the range 30 to 50 x 10^{-6} cm/s), the absolute value of the P_{Na} of MDCK I TetOff claudin-8 cells was very low. However, neither P_{Na} nor P_{Cl} differed between Dox^+- and Dox^--treated cells (Fig. 4b). Furthermore, permeabilities of other alkali metal cations were determined by bi-ionic potential measurements and were not found to be affected by induction of claudin-8 (*results not shown*).

Use of Epidermal Growth Factor to Downregulate Claudin-2 in MDCK II TetOff Claudin-8 Cells

The striking difference in the effect of claudin-8 on paracellular permeability between MDCK II and MDCK I cells suggests that either the endogenous tight junction proteins

or the baseline TER of the host cell line might have a significant impact on the phenotype observed during the induction of exogenous claudin-8. Comparison of endogenous claudins in MDCK I and II cells showed the presence of claudins 1, 3, 4 and 7 in both cell types whereas claudin-2 was restricted to MDCK II cells. We therefore tested the hypothesis that the lack of an effect of claudin-8 in MDCK I cells is related to the lack of endogenous claudin-2 in these cells. To do this, we had to create a cell model with both low TER and low baseline levels of claudin-2: this was accomplished by treating our MDCK II TetOff claudin-8 cells with epidermal growth factor (EGF).

EGF, which has previously been reported to increase TER in both LLC-PK1 (Saladik et al., 1995) and MDCK II (Singh & Harris, 2004) cells, downregulates cellular claudin-2 expression. Singh & Harris (2004) reported that EGF acts by inhibiting gene expression of claudin-2 in MDCK II cells, presumably by activation of the mitogen-activated protein kinase pathway. We therefore tested whether we could downregulate endogenous claudin-2 in MDCK II TetOff claudin-8 cells by treatment with EGF. MDCK II TetOff claudin-8 cells, grown to confluence in the presence or absence of Dox, were deprived of serum for several hours and exposed to 100 ng/ml EGF for 24 h. Claudins were stained by immunofluorescence (Fig. 5a showing claudin-2, other claudins not shown). In control cells (no EGF), induction of claudin-8 by Dox removal was accompanied by downregulation of claudin-2 at the tight junctions, as described above. In noninduced cells (Dox^+), claudin-2 was downregulated by 87% by EGF, as described by Singh & Harris (2004). When EGF was added to the medium and claudin-8 induced (Dox^-/EGF^+), there was a further, small reduction in claudin-2 expression. The results were confirmed by Western blot analysis of cell membrane fractions (Fig. 5b). Furthermore, no significant alterations in the expression of endogenous claudins other than claudin-2 were observed.

TER and Permeability of EGF-Treated MDCK II TetOff Claudin-8 Cells

If claudin-2 replacement accounts for the changes in permeability observed during claudin-8 induction, these effects should be reduced in cells exhibiting reduced claudin-2 expression. To test this hypothesis, we determined the TER of MDCK II TetOff claudin-8 cells cultured in the presence or absence of Dox and treated with EGF for 24 h as described above (Fig. 6a). In the absence of EGF, removal of Dox significantly increased TER by a factor of 3 compared to baseline conditions. In the presence of EGF, baseline TER was somewhat higher, presumably due to suppression of cation-permeable claudin-2. There was a trend to a relative (1.2-fold) and absolute (29 $\Omega \bullet cm^2$) increase in TER by induction of claudin-8 expression, but it was considerably less with EGF than when EGF was omitted and was not statistically significant.

P_{Na} and P_{Cl} were determined from NaCl dilution potential measurements. In agreement with earlier investigations, induction of claudin-8 in MDCK II TetOff cells reduced P_{Na} by about 70%, while P_{Cl} was not affected (Fig. 6b). Claudin-2 suppression by EGF (Dox^+/EGF^+) strongly decreased P_{Na} but had no effect on P_{Cl}. In EGF-treated cells, induction of claudin-8 (Dox^-/EGF^+) had no additional effect on permeability.

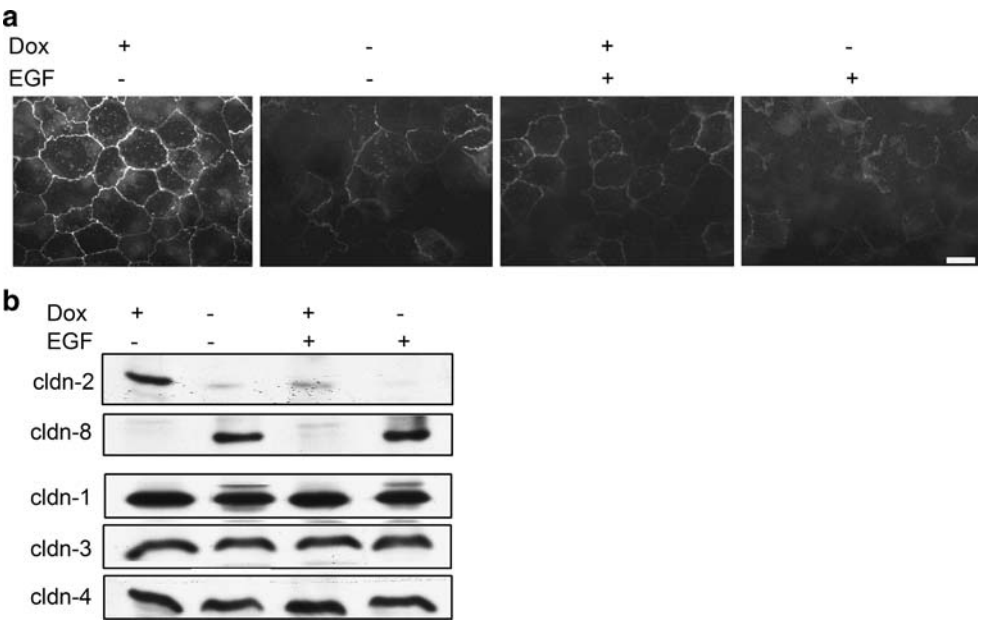

Fig. 5 Effect of EGF on claudins in MDCK II TetOff claudin-8 cells. Monolayers were grown to confluence under Dox^+ and Dox^- conditions (3 days), deprived of serum for 6 h and subsequently treated with 100 ng/ml EGF for 24 h to downregulate claudin-2. The expression of claudins in Dox^+/Dox^--treated cells was analyzed by (**a**) immunofluorescence staining (only shown for claudin-2) and (**b**) Western blots of membrane fractions. Bar = 10 μm

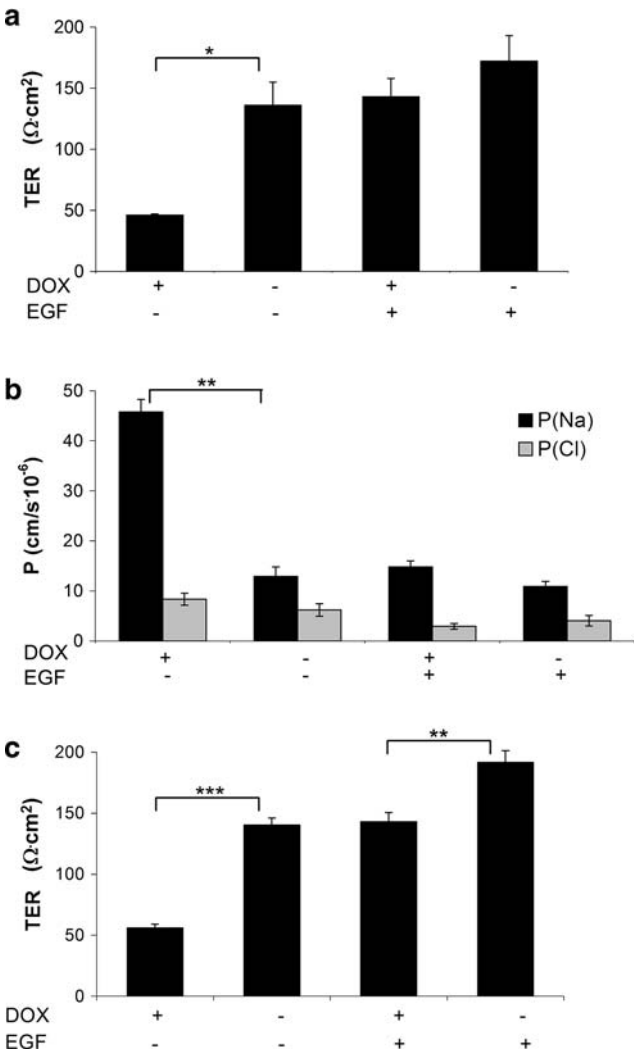

Fig. 6 Permeability properties of EGF-treated MDCK II TetOff claudin-8 cells. Monolayers were grown to confluence on Snapwell filters under Dox^+ and Dox^- conditions (3 days), deprived of serum for 6 h and subsequently treated with 100 ng/ml EGF for 24 h to downregulate claudin-2. TER (**a**) and absolute ion permeabilities (**b**) were then measured. The claudin-8-induced increase in TER as well as the claudin-8-induced decrease in Na^+ permeability were less in cells treated with EGF. (**c**) Identical experiment to (**a**) except that confluent cells were first incubated with EGF (48 h) before Dox was omitted from the medium ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$)

To mimic claudin-8 incorporation into tight junctions that already lack claudin-2, another experimental protocol was devised, in which the sequence of Dox removal and EGF addition was reversed. Confluent cells were first treated with EGF (100 ng/ml, 48 h), and then claudin-8 expression was induced by removal of Dox (Fig. 6c). The level of expression of claudin-8 and endogenous claudins at the tight junctions was similar to the results described above (*not shown*). Furthermore, in the presence of EGF, there was a reduced effect on TER by claudin-8, as described above.

Discussion

Proteins of the claudin family play an important role in the regulation of paracellular transport. The determination of specific characteristics of distinct isoforms is the subject of ongoing investigations, frequently based on heterologous claudin transfection into host cells. In the present study, we used different strains of MDCK cells, stably transfected with claudin-8, as a model to test the influence of a single endogenous claudin on the effect of an exogenously expressed claudin.

Our results provide evidence that exogenously expressed claudins can interfere with endogenous isoforms. Using MDCK II cells, we showed that claudin-8 downregulates endogenous claudin-2 protein and decreases levels of claudin-2 mRNA (Fig. 1a-d). Recent studies have shed light on the transcriptional regulation of claudins in epithelial cells. In MDCK II cells, expression of claudin-2 is suppressed by growth factors like hepatocyte growth factor (Balkovetz et al., 2004; Lipschutz et al., 2005) and EGF (Singh & Harris, 2004). This is accompanied by an increase in TER and prevented by MAPK/ERK kinase (MEK) inhibitors. Analysis of the claudin-2 promoter in epithelial cell lines revealed numerous transcription factors that enhance promoter activity, often acting synergistically, including Cdx1, Cdx2, hepatocyte nuclear factor 1α (HNF1α) GATA-binding protein 4 (GATA-4), and T-cell factor(TCF)/β-catenin (Escaffit, Boudreau & Beaulieu, 2005; Mankertz et al., 2004; Sakaguchi et al., 2002). Claudin-2 in MDCK cells has furthermore been shown to be reduced by Snail (Carrozzino et al., 2005), which regulates claudins and other junctional proteins (Ikenouchi et al., 2003) and triggers barrier breakdown and epithelial-to-mesenchymal transition. The signaling pathways involved in the downregulation of claudin-2 on the mRNA level in MDCK II TetOff claudin-8 cells are still unknown and a target of ongoing investigations.

In addition, coculture experiments showed that the downregulation occurs primarily at sites within the cell where high levels of claudin-8 are expressed, i.e., at tight junctions between claudin-8-transfected cells (Fig. 1e). Claudin-8 could potentially impede insertion of claudin-2 into junctional strands. The other scenario, that claudin-8 destabilizes inserted claudin-2 protein at the junctions, appears to be less likely since no redistribution of claudin-2 into the cytoplasm or increase in the rate of degradation could be observed (Fig. 1b, c). The reasons for the apparent incompatibility of claudin-2 with claudin-8 are unknown. By studying the interaction of heterogeneous claudin species within and between tight junction strands, Furuse, Sasake & Tsukita (1999) found that claudins form heteropolymers which interact in a homotypic and heterotypic manner with claudins of adjacent cell membranes (‘‘head

to head'' interaction). However, some combinations of heterotypic interaction between distinct isoforms seem to be restricted. For example, whereas claudin-2 undergoes head-to-head interaction with claudin-3-based strands, it does not interact with claudin-1. A similar restriction between claudin-8 and claudin-2 could underlie the replacement of claudin-2 in MDCK II cells which express claudin-8. If so, however, it is not clear whether this is caused by claudin-8 of the same tight junction strand or the apposing strand and what sequences determine compatibility of interacting isoforms.

In a model describing the consequences of overexpression of integral tight junction proteins in epithelial cells, we have suggested that the exogenously expressed protein either adds new strands in series with the existing ones or inserts into the existing strands, adding to or replacing endogenous proteins (Yu, 2003). This study is the first to show replacement of an endogenous claudin by an exogenously expressed isoform. Numerous other studies on claudin characterization by heterologous expression have been published, but in most of them no significant changes in the expression and localization of claudins endogenous to the host cell line have been described. It is possible that this is because the phenomenon of replacement is specific for the interaction of claudin-8 with claudin-2. Alternatively, this may be a more general phenomenon that has been missed because of the inability to examine the expression and localization of all claudins endogenous to a particular host cell line. In the present study, we cannot exclude the possibility that additional claudins are endogenously expressed by MDCK I and II cells and affected by exogenously expressed claudin-8 because isoform-specific antibodies are not yet available for all claudins.

By expressing claudin-8 in cells that either lack claudin-2 (MDCK I) or have claudin-2 markedly downregulated (by EGF), we show that the effect of claudin-8 expression on the permeability properties of the epithelium depends directly on replacement of claudin-2 at the tight junctions (Fig. 6). It is important to appreciate that EGF reduced endogenous claudin-2 protein levels in MDCK II cells by ~90% but increased TER only modestly, from a baseline of 50 to approximately 150 $\Omega \bullet cm^2$. Thus, EGF-treated MDCK II cells are still extremely leaky and have a TER that is more than an order of magnitude less than the TER of MDCK I cells (>3,000 $\Omega \bullet cm^2$), our model of a ''tight'' monolayer. Thus, the finding that claudin-8 no longer has a sealing effect in EGF-treated (but still fairly leaky) MDCK II cells constitutes the best evidence we have that the sealing effect is predominantly dependent on claudin-2 and not simply on the tightness of the host cell. We conclude that pores in the paracellular pathway formed by claudin-8 exhibit much lower cation permeability than those formed by claudin-2 or that claudin-8 is totally impermeable to cations. In this model, replacement of claudin-2 by claudin-8 would reduce the magnitude of cation permeability without affecting its activation energy, exactly as we have found previously (Yu et al., 2003). A role for claudin-8 as a paracellular cation barrier protein also makes sense physiologically since it is expressed in tight segments of the distal nephron, where transtubular salt and pH concentration gradients are generated by hormone-regulated transcellular transport (Li et al., 2004).

The observations fit a model in which the permeability characteristics of paracellular pores encoded by different claudin isoforms differ over a wide range, including pores which primarily increase ion permeability and those which are virtually impermeable, thus acting primarily as ''barrier proteins.'' Permeability characteristics of different claudin isoforms are determined by the charges on amino acids of the first extracellular loop, which probably electrostatically interact with ions passing through the pore (Colegio et al., 2002). For example, when claudin-4 is expressed in MDCK II cells, it decreases cation permeability, indicating that claudin-4 forms a paracellular cation barrier. Mutation of lysine-65 in the 1st extracellular loop of claudin-4 to aspartate abolishes its ability to discriminate against cations. Comparing charged amino acids of the first extracellular loops of claudin-8 and claudin-2, a higher ratio of positively charged relative to negatively charged amino acids is found for claudin-8, which agrees with a role for this isoform in enhancing the cation barrier. Moreover, claudin-8 expresses a lysine at position 65, the same amino acid which seems to be important for limiting cation diffusion through claudin-4-based ''pores.'' In contrast, claudin-2 expresses aspartate at position 65, which could be involved in facilitating paracellular cation permeation.

Our data show that the heterologous expression of claudin-8 has different effects on the phenotype of different host cell lines. Changes in Na^+ permeability and TER are only detectable in low-resistance cation-selective MDCK II cells (Yu et al., 2003), whereas no effects on the developing and steady-state TER of less cation-selective MDCK I cells can be observed (Fig. 4). This is consistent with recent findings by other investigators. Claudins with low cation permeablity, like claudin-4, have a much higher impact on permeability properties of cation-selective MDCK II cells compared to anion-selective LLC-PK1 cells. In contrast, claudins with high cation selectivity, like claudin-2, claudin-15 and claudin-16, have stronger effects on anion-selective LLC-PK1 cells (Hou et al., 2005; Van Itallie et al., 2003).

In our study, we were able to manipulate the expression of a single endogenous claudin in our host cells and thereby show that the repertoire of endogenous claudins in the host cell is a key determinant of the phenotype resulting from heterologous claudin transfection. If the endogenous and

exogenous claudins have similar permeability properties (e.g., both are cation-impermeable), then replacement of one by the other would have little apparent effect. By contrast, if the endogenous and exogenous claudins have quite different properties (as with replacement of cation-impermeable claudin-8 by cation-permeable claudin-2), then the functional effect would be quite obvious.

This has important implications for future studies of claudin overexpression in cell lines. If transfection of a claudin isoform into one host cell does not yield any functional phenotype, this might suggest that the permeability properties of this isoform are too similar to those of the claudins endogenous to the host cell. This should prompt studies using other host cell lines with different permeability properties. Furthermore, each claudin overexpression study should be accompanied by analysis of endogenous claudin expression levels (though obviously limited by the availability of antibodies to all isoforms). If changes occur, the resulting phenotype of a host cell must be interpreted as representing the net effect of addition of an exogenous claudin and changes in endogenous claudins. In the specific case of a replacement model, the resultant permeability change is simply the difference between the permeability of the exogenous claudin and that of the replaced endogenous claudin.

In conclusion, the properties of an exogenous claudin cannot be studied independently from the influence of endogenous claudins and properties of the host cells. This observation fits a model in which heterologously expressed claudins add new charge-selective pores to tight junctions or replace existing ones. However, only the combined contribution of pores based on endogenous and exogenous claudins can be directly measured.

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