Study of Claudin Function by RNA Interference

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Claudins are tight junction proteins that play a key selectivity role in the paracellular conductance of ions. Numerous studies of claudin function have been carried out using the overexpression strategy to add new claudin channels to an existing paracellular protein background. Here, we report the systematic knockdown of endogenous claudin gene expression in Madin-Darby canine kidney (MDCK) cells and in LLC-PK1 cells using small interfering RNA against claudins 1–4 and 7. In MDCK cells (showing cation selectivity), claudins 2, 4, 6, and 7 are powerful effectors of paracellular Na⁺ permeation. Removal of claudin-2 depressed the permeation of Na⁺ and resulted in the loss of cation selectivity. Loss of claudin-4 or -7 expression elevated the permeation of Na⁺ and enhanced the proclivity of the tight junction for cations. On the other hand, LLC-PK1 cells express little endogenous claudin-2 and show anion selectivity. In LLC-PK1 cells, claudin-4 and -7 are powerful effectors of paracellular Cl⁻ permeation. Knockdown of claudin-4 or -7 expression depressed the permeation of Cl⁻ and caused the tight junction to lose the anion selectivity. In conclusion, claudin-2 functions as a paracellular channel to Na⁺ to increase the cation selectivity of the tight junction; claudin-4 and -7 function either as paracellular barriers to Na⁺ or as paracellular channels to Cl⁻, depending upon the cellular background, to decrease the cation selectivity of the tight junction.

Tight junctions are cell-cell interactions that provide the primary barrier to the diffusion of solutes through the paracellular pathway, creating an ion-selective boundary between the apical and basolateral extracellular compartments (see reviews in Refs. 1–3). The integral membrane proteins of the tight junction include occludin, a 65-kDa membrane protein bearing four transmembrane domains and two extracellular loops, and claudins, a family with at least 22 homologous proteins of 20–28 kDa that share a common topology with occludin (4–7).

Claudins have been shown to confer ion selectivity to the paracellular pathway. In MDCK cells, claudin-4, -5, -8, -11, and -14 selectively decrease the permeability of cation through tight junction, whereas the permeation of anion is largely unchanged (8–12). MDCK cells express five endogenous claudins, claudin-1–4 and -7. LLC-PK1 cells express four endogenous claudins, claudin-1, -3, -4, and -7. In LLC-PK1 cells, claudin-2, -15, -16 selectively increase the permeability of cation through the tight junction with no significant effects on anions (13–14). When exogenous claudins are added to the tight junction, they constitute new charge-selective channels leading to a physiological phenotype that combines the contributions of both endogenous and exogenous claudins in the cell. A biochemical inventory of claudin-claudin interactions is not yet available, although the principle of specificity has been demonstrated in mouse L-fibroblasts (15). In addition, although efforts have been made to demonstrate the oligomerization properties of claudin-4 in cultured insect cells (16), there are few data documenting the oligomerization states of claudins or whether claudins can co-oligomerize to form additional functional units. FRAP (fluorescence recovery after photobleaching) studies suggest that claudin molecules assembled in tight junctions have limited mobility (17), consistent with their known interactions with proteins in the tight junction scaffold (18–20). However, the assembly of claudins into oligomers is likely to occur before delivery to the cell surface.

To date, all studies of claudin function have been carried out using the overexpression strategy, adding new claudin channels to an existing paracellular protein background. To complement these data, we have studied the function of claudin when cells become deficient in a specific claudin. Small interfering RNA (siRNA) has proven to be a powerful tool to suppress gene expression in mammalian cells through a process known as RNA interference (21–22). In this study, we have systematically knocked down the endogenous expression of claudins 1–4 and 7 in MDCK cells and claudins 4 and 7 in LLC-PK1 cells using siRNA and probed their function. We have also rescued the loss of each claudin function by exogenously expressing its siRNA-resistant counterpart from a different species.

EXPERIMENTAL PROCEDURES

Antibodies and Cell Lines—The following antibodies were used in this study: rabbit polyclonal anti-claudin-1, anti-claudin-2, anti-claudin-3, anti-claudin-7, and mouse monoclonal anti-claudin-4 and anti-occludin antibodies (Zymed Laboratories); fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G and rhodamine-labeled goat anti-mouse immunoglobulin G (Chemicon); and horseradish peroxidase-labeled donkey anti-rabbit and anti-mouse immunoglobulin G (Amersham Biosciences). MDCK II cells were cultured in minimum Eagle’s medium (MEM, Invitrogen) supplemented with

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§ The abbreviations used are: MDCK cells, Madin-Darby canine kidney cells; siRNA, small interfering RNA; EST, expressed sequence tag; FITC, fluorescein isothiocyanate; TER, transepithelial resistance; Ω, ohm (a unit of resistance).
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10% FBS and penicillin/streptomycin; 293T cells and LLC-PK1 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin/streptomycin, and 1 mM sodium pyruvate.

Construction of Canine Gene Sequences of Claudins—The mRNA sequences of claudin-2 and -3 of Canis familiaris are available from the GenBankTM accession numbers AF358907 and AF358908 for claudin-2 and claudin-3, respectively. To obtain the sequences of dog claudins 1, 4, and 7, the coding region of mouse claudin-1 (accession number AF072127), claudin-4 (AF087822), and claudin-7 (AF087825) were used to Blast search the dog genome project (managed by the Human Genome Project at the Sanger Center, Cambridge, UK). The genomic sequence with highest alignment score was retrieved for each claudin. Claudin-1 was located on chromosome 34 (spanning 2 exons from 25389105 to 25391013 and from 25393206 to 25402578). Claudin-4 was located on chromosome 6 (spanning 1 exon from 9222975 to 9224222). Claudin-7 was located on chromosome 5 (spanning 3 exons from 35195628 to 35195793, 35196098 to 35196265, and 35197000 to 35197230). Fragments of canine claudin-1, -4, or -7 mRNA sequence (coding region) were stitched from the extracted exons to form a more complete sequence. This sequence was then realigned to the original mouse claudin sequence (see alignments in supplemental Figs. S1–S5); the overall percentages of nucleotide similarity to mouse claudins were 89% for dog claudin-1, 83% for dog claudin-4, and 89% for dog claudin-7.

Search for Porcine Gene Sequences of Claudins—As the genomic sequence of pig (Sus scrofa) is not yet complete within the public research domain, we have searched the PEDE (Pig EST Data Explorer) data base for the mRNA sequences of porcine claudins. PEDE is a data base of porcine EST (expressed sequence tag) collections derived from full-length cDNA libraries and maintained by the Animal Genome Research Program in Japan. To obtain the sequences of pig claudins-4 and 7, the coding region of mouse claudin-4 (GenBankTM accession number AF087822) and claudin-7 (AF087825) were used to Blast search the PEDE data base. The full-length cDNA sequence with highest alignment score was retrieved for claudin-4 (PEDE designation, 20050322C-005301; length, 1394 bp) and claudin-7 (PEDE designation, 20050322C-001624; length, 1251 bp). The coding region of pig claudin was then realigned to the original mouse claudin sequence (see alignments in supplemental Figs. S6 and S7); the overall percentages of nucleotide similarity to mouse claudins were 89% for pig claudin-4; 86% for pig claudin-7.

Molecular Cloning and Retrovirus Production—The following full-length mammalian claudins were cloned into the retroviral vector pQCXIH (a gift from Dr. Joan Brugge, Harvard Medical School): mouse claudin-2 (GenBankTM accession number AF072128), mouse claudin-4 (AF087822), and mouse claudin-7 (AF087825). For siRNA studies, the siRNA hairpin oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) and annealed and cloned into the Moloney murine leukemia virus retrovirus backbone downstream of the human small nuclear RNA U6 promoter to create the pSIREN-claudin siRNA constructs. Vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped retroviruses were produced in 293T cells and used to infect MDCK cells at a titer of 1 × 10⁶ colony-forming units/ml, as described previously (14).

Protein Electrophoresis and Immunoblotting—Confluent cells were dissolved in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% SDS, and protease inhibitor mixture; Pierce). After shearing with a 23-gauge needle, lysates (containing 15 μg of total protein) were subjected to SDS-PAGE under denaturing conditions and transferred to a nitrocellulose membrane followed by blocking with 3% nonfat milk, incubation with primary antibodies (1:1,000) and the horseradish peroxidase-labeled secondary antibody (1:5000), and exposure to an ECL Hyperfilm (Amersham Biosciences). Molecular mass was determined relative to protein markers (Bio-Rad).

Immunolabeling and Confocal Microscopy—Cells grown on Transwell inserts (Corning) were fixed with cold methanol at −20 °C followed by blocking with phosphate-buffered saline containing 10% fetal bovine serum and incubation with primary antibodies (1:300) and fluorescein isothiocyanate (FITC) or rhodamine-labeled secondary antibodies (1:200). After washing with phosphate-buffered saline, slides were mounted with Mowiol (Calbiochem). Confocal analyses were performed using the Nikon TE2000 confocal microscopy system equipped with Plan-Neofluar ×40 (numeric aperture 1.3 oil) and ×63 (numeric aperture 1.4 oil) objectives and krypton-argon laser (488 and 543 lines). For the dual imaging of FITC and rhodamine, fluorescent images were collected by exciting the fluorophores at 488 nm (FITC) and 543 nm (rhodamine) with argon and HeNe lasers, respectively. Emissions from FITC and rhodamine were detected with the band-pass FITC filter set of 500–550 nm and the long-pass rhodamine filter set of 560 nm, respectively. All images were converted to JPEG format and arranged using Photoshop 6.0 (Adobe).

Electrophysiological Measurements—Electrophysiological studies were performed on cell monolayers grown on porous filters (Transwell), as described previously (14). Voltage and current clamps were performed using the EVC4000 Precision V/I clamp (World Precision Instruments) with Ag/AgCl electrodes and an agarose bridge containing 3 mM KCl. Transepithelial resistance (TER) was measured using the Millicell-ERS and chopstick electrodes (Millipore). The TER of the confluent monolayer of cells was determined in buffer A (145 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES, pH 7.4), and the TER of blank filters was subtracted. Dilution potentials were measured when buffer B (80 mM NaCl, 130 mM mannitol, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose and 10 mM HEPES, pH 7.4) replaced buffer A on the apical side or basal side of filters. Electrical potentials obtained from blank inserts were subtracted from those obtained from inserts with a confluent growth of cells. The ion permeability ratio (PNa/PCl) for the all-effects was determined relative to protein markers (Bio-Rad).
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RESULTS

Knocking Down Claudin Gene Expression in MDCK Cells—The small nuclear RNA promoter (U6) is used for directing expression of siRNAs because it is active in a wide variety of cell types and efficiently directs the synthesis of small noncoding transcripts bearing well defined termini. The siRNA hairpin oligonucleotides were designed and cloned with the strategy described previously (Fig. 1A; Ref. 21). Each oligonucleotide contains a unique complementary 19-nucleotide sequence within the coding region of a canine claudin. Claudin-1, -2, -3, -4, and -7 were each screened with 7 to 10 siRNA sequences spanning their gene coding regions. Each of the siRNA sequences showed different degrees of suppression of claudin expression when assessed by both Western blotting and immunostaining. Only a small number of siRNA sequences suppressed both detectable staining on Western blots (Fig. 1B) and detectable claudin immunostaining (>95% loss, Fig. 1C). Two sequences with these activities were identified for each claudin and used in subsequent studies (sequences shown on supplemental Figs. S1–S5).

Loss of Claudin Function and Paracellular Ion Conductance—To examine the paracellular ion transport in the absence of claudin function, we stably expressed the identified siRNAs in MDCK cells against claudin-1, -2, -3, -4, and -7. As we aimed to have claudin expression suppressed by siRNAs during a prolonged period in MDCK cells, so that they could become fully polarized and form tight junctions, we utilized a retroviral expression system to drive siRNA expression. Each individual siRNA construct was used to infect MDCK cells (with empty vector-infected MDCK cells as control), and the infected cells were seeded onto Transwell inserts to become polarized. On day 9 post-polarization, cell monolayers were subjected to electrophysiological measurements and immunostained in order to visualize the loss of claudin expression. Although all siRNAs were active in protein expression, we observed dramatic changes in paracellular permeability only with claudin-2, -4, and -7. To control for up- or down-regulation of nontargeted claudins, we assayed the protein levels of the remaining four claudins (among the five endogenous claudins studied: claudin-1, -2, -3, -4, and -7) in the absence of claudin-2, -4, or -7 and found no differences compared with empty vector controls (data not shown).

Consistent with previous findings (10, 12), MDCK II cells are leaky, having a TER value of 78.0 ± 185 cm² (measured in 145 mM NaCl and shown in Fig. 2A and Table 1). Loss of claudin-2 expression dramatically increased TER to 248.3 ± 185 cm² (p < 0.001, n = 3); in contrast, loss of claudin-4 or -7 expression significantly decreased TER to 53.7 and 43.0 ± 185 cm², respectively. To determine the ion selectivity (PNa/Pi), we applied an apical-to-basal chemical gradient (145 mM NaCl at the apical side to 80 mM at the basal side) to the MDCK monolayers and recorded the diffusion potential. The base-line value of ion selectivity (PNa/Pi) in MDCK cells was 6.14 ± 0.19, consistent with the cation selectivity reflected by the diffusion potential of 10.83 ± 0.12 mV (with the apical side as zero reference). These results are similar to published values (11–12). Loss of claudin-2 expression significantly lowered the diffusion potential to 4.03 ± 0.03 mV and PNa/Pi to 1.74 ± 0.01 (p < 0.001, n = 3; Fig. 2, B and C, and Table 1). In comparison, a loss of claudin-4 or -7 expression led to a significant increase in the diffusion potential (to 13.10 ± 0.15 or 13.03 ± 0.23

made with the Neuman-Keuls test. Values were expressed as mean ± S.E. unless stated otherwise.
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![Graph A](image1)
![Graph B](image2)

**FIGURE 2. Effects of claudin depletion in MDCK cells on paracellular ion conductance.** TER values (A), dilution potential values (B), the ion permeability ratio of $Na^+$ versus $Cl^-$ (C), and permeability values of $Na^+$ across MDCK cell monolayer depleted of claudins 1–4 and 7, respectively (D), are shown.

**TABLE 1**

| Group                                   | TER (Ω cm$^2$) | Dilution potential (mV) | $P_{Na}/P_{Cl}$ | $P_{Na}$ ($10^{-6}$ cm/s) | $P_{Cl}$ ($10^{-6}$ cm/s) |
|-----------------------------------------|----------------|-------------------------|-----------------|---------------------------|---------------------------|
| MDCK + empty vector                    | 78.0 ± 2.3     | 10.83 ± 0.12            | 6.14 ± 0.19     | 20.23 ± 0.09              | 3.30 ± 0.09               |
| MDCK + claudin1 siRNA                  | 80.0 ± 3.5     | 10.50 ± 0.29            | 5.67 ± 0.41     | 19.48 ± 0.21              | 3.46 ± 0.21               |
| MDCK + claudin2 siRNA                  | 248.3 ± 3.5    | 4.03 ± 0.03             | 1.74 ± 0.01     | 4.70 ± 0.01               | 2.70 ± 0.01               |
| MDCK + claudin2 siRNA + claudin2 mouse | 64.7 ± 0.9     | 9.80 ± 0.12             | 4.76 ± 0.12     | 23.33 ± 0.11              | 4.90 ± 0.11               |
| MDCK + claudin3 siRNA                  | 65.3 ± 1.5     | 11.17 ± 0.12            | 6.74 ± 0.24     | 24.58 ± 0.11              | 3.86 ± 0.11               |
| MDCK + claudin4 siRNA                  | 53.7 ± 2.3     | 13.10 ± 0.15            | 13.89 ± 1.13    | 31.68 ± 0.16              | 2.13 ± 0.16               |
| MDCK + claudin4 siRNA + claudin4 mouse | 65.3 ± 0.3     | 11.27 ± 0.18            | 6.95 ± 0.37     | 24.67 ± 0.16              | 3.57 ± 0.16               |
| MDCK + claudin7 siRNA                  | 43.0 ± 2.6     | 13.03 ± 0.23            | 13.65 ± 1.72    | 39.69 ± 0.31              | 2.99 ± 0.31               |
| MDCK + claudin7 siRNA + claudin7 mouse | 73.0 ± 0.6     | 10.23 ± 0.15            | 5.28 ± 0.19     | 21.13 ± 0.12              | 4.01 ± 0.12               |
| LLC-PK1 + empty vector                 | 61.3 ± 1.5     | -7.97 ± 0.03            | 0.305 ± 0.002   | 7.03 ± 0.03               | 23.06 ± 0.03              |
| LLC-PK1 + claudin4 siRNA               | 118.0 ± 5.3    | -3.00 ± 0.06            | 0.66 ± 0.01     | 6.21 ± 0.03               | 9.35 ± 0.03               |
| LLC-PK1 + claudin4 siRNA + claudin4 mouse | 69.3 ± 3.3    | -7.47 ± 0.03            | 0.334 ± 0.002   | 6.65 ± 0.03               | 19.95 ± 0.03              |
| LLC-PK1 + claudin7 siRNA               | 261.7 ± 1.8    | 1.47 ± 0.03             | 1.22 ± 0.01     | 3.85 ± 0.01               | 3.16 ± 0.01               |
| LLC-PK1 + claudin7 siRNA + claudin7 mouse | 94.0 ± 3.5    | -6.77 ± 0.07            | 0.376 ± 0.004   | 5.34 ± 0.04               | 14.19 ± 0.04              |

mV, respectively) and in $P_{Na}/P_{Cl}$ (to 13.89 ± 1.13 or 13.65 ± 1.72, respectively). Calculation of the permeability value of $Na^+$ ($P_{Na}$) and $Cl^-$ ($P_{Cl}$) indicated that claudin-2, -4, and -7 are powerful modulators of $P_{Na}$. Removal of claudin-2 from MDCK cells depressed the permeation of $Na^+$ (a significant drop of $P_{Na}$ to 4.70 ± 0.01 from the control level of 20.23 ± 0.09 × 10^{-6} cm/s; Fig. 2D and Table 1). On the other hand, removal of claudin-4 or -7 up-regulated $P_{Na}$ (to 31.68 ± 0.16 and 39.69 ± 0.31 × 10^{-6} cm/s, respectively). Interestingly, the permeability of $Cl^-$ was not affected by the knocking down of these claudins. Inhibiting the basolateral Na$^+$/K$^+$-ATPase (1 mm ouabain) had no effects on $P_{Na}$ or $P_{Cl}$ in either control or siRNA-expressing cells, indicating a paracellular pathway for ion flux. The remaining claudins in MDCK cells (claudin-1 and -3) were not paracellular ion effectors (Table 1). This experiment was repeated and confirmed independently with three separate monolayers. A similar effect was also seen for each claudin with the second selected siRNA sequence.

**Rescue of Claudin Function in MDCK Cells**—To selectively rescue the loss of function of claudins in MDCK cells, we stably expressed an siRNA-resistant claudin (mouse claudin-2, -4, and -7) in MDCK cells lacking the relevant endogenous claudin.
The mouse cDNA sequence chosen to rescue dog claudin-2 contained two mismatches compared with the dog claudin-2 siRNA sequence (Fig. 3A), the mouse claudin-4 cDNA sequence contained four mismatches to the dog claudin-4 siRNA sequence, and the mouse claudin-7 cDNA sequence contained one mismatch to the dog claudin-7 siRNA sequence. The protein expression of all three dog claudins was effectively restored with their mouse counterparts (Fig. 3B, Western blot, and 3C, immunostaining), further proving that the siRNA-mediated gene regulation is highly specific and can differentiate a single nucleotide mismatch (1-bp mutation at position 3 of the siRNA sequence of dog claudin-7 against the mouse claudin-7 cDNA sequence). Similarly, Brummelkamp et al. (21) showed that a 1-bp mutation at either position 2 or 9 of the 19-nucleotide siRNA sequence against CDH1 abolished its ability to knock down CDH1 gene expression. Forced expression of the siRNA-resistant mouse claudin-2 in claudin-2-deficient MDCK cells restored the elevated TER (248.3 Ω·cm²) to near the control level (64.7 Ω·cm²), resulting in the return of the dilution potential to its base line (Table 1). The reexpression of claudin-2 accounted for a profound increase in $P_{Na}$ from 4.70 ± 0.01 in the claudin-2-deficient background to 23.33 ± 0.11 (close to the control value of 20.23 ± 0.09 × 10⁻⁶ cm/s), thus providing strong evidence that claudin-2 contributes to a paracellular cation channel. The elevated Na⁺ permeability in cells with depleted endogenous claudin-4 or -7 declined to the baseline level (24.67 ± 0.16 × 10⁻⁶ in claudin-4 rescue; 21.13 ± 0.12 × 10⁻⁶ cm/s in claudin-7 rescue), when the expression of claudin-4 or -7 was restored by the mouse isoform. The function of claudin-4 and -7 contrasted with that of claudin-2 in that both claudin-4 and -7 blocked paracellular cation permeation.

**Loss of Claudin Function in LLC-PK1 Cells**—Our data on claudin-7 function contrast with those of a recent study performed in LLC-PK1 cells using the overexpression strategy, which showed that claudin-7 simultaneously decreases the paracellular Cl⁻ conductance and increases the paracellular Na⁺ conductance (25). The discrepancy could result from the different paracellular protein background, in that MDCK cells showed cation selectivity ($P_{Na}/P_{Cl}$, 2.14 ± 0.19; diffusion potential, +10.83 ± 0.12 mV), whereas LLC-PK1 cells showed anion selectivity ($P_{Na}/P_{Cl}$, 0.305 ± 0.002; diffusion potential, −7.97 ± 0.03 mV). LLC-PK1 cells express endogenous claudins-1, -3, -4, and -7, with very little expression of claudin-2 (maintained at a very low level close to the background (25)). The lack of endogenous claudin-2 expression in LLC-PK1 cells explains the low paracellular Na⁺ permeability (7.03 ± 0.03 × 10⁻⁶ cm/s) compared with that in MDCK cells (20.23 ± 0.09 × 10⁻⁶ cm/s). Our data (in MDCK cells) had ruled out the possibilities of claudin-1

*FIGURE 3. Rescuing expression of siRNA-resistant isoforms of claudins. A, sequence comparison between dog and mouse claudins. Note that the siRNA sequence against dog claudin-2 bears two mismatches to the mouse claudin-2 sequence; the siRNA sequence against dog claudin-4 bears four mismatches to mouse claudin-4; and the siRNA sequence against dog claudin 7 bears one mismatch to mouse claudin 7. B, protein immunoblots of expression of mouse claudin in MDCK cell background depleted of endogenous dog claudin. C, confocal images showing the tight junction staining of claudins is restored when expression of mouse claudins is forced in siRNA-expressing cells.*
and -3 as paracellular ion effectors. Thus we focused upon the function of claudin-4 and -7 in LLC-PK1 cells. The same strategy as described above in MDCK cells was employed to select effective siRNA sequences against porcine claudin-4 and -7 (two effective siRNA sequences for each claudin, labeled in supplemental Figs. S6 and S7). Loss of claudin-4 or -7 expression significantly up-regulated the TER value (Table 1; claudin-4 knockdown, 118.0 Ω·cm²; claudin-7 knockdown, 261.7 Ω·cm²; versus control, 61.3 Ω·cm²; p < 0.001, n = 3) and the value of diffusion potential (claudin-4 knockdown, −3.00 ± 0.06 mV; claudin-7 knockdown, +1.47 ± 0.03 mV; versus control, −7.97 ± 0.03 mV; p < 0.001, n = 3), causing the siRNA-expressing cells to lose their anion selectivity. Calculation of the permeability value of Na⁺ (P_{Na}) and Cl⁻ (P_{Cl}) indicated that claudin-4 and -7 were powerful channels of P_{Cl}. Loss of claudin-4 or -7 function from LLC-PK1 cells resulted in a dramatic drop of the permeation of Cl⁻ (P_{Cl}) to 9.35 ± 0.03 in claudin-4 knockdown cells and to 3.16 ± 0.01 in claudin-7 knockdown cells; from the control level of 23.06 ± 0.03 × 10⁻⁶ cm/s). Exogenous expression of the siRNA-resistant mouse claudin-4 or -7 in LLC-PK1 cells lacking the endogenous claudin selectively rescued the loss-of-function phenotype of claudin-4 or -7, showing a reciprocal trajectory of paracellular ion permeabilities (see data in Table 1 and sequence comparisons in supplemental Figs. S6 and S7).

**DISCUSSION**

The MDCK and LLC-PK1 epithelial cell lines have been particularly useful in the study of tight junction as an *in vitro* cell culture model of renal tubule epithelium (1,26–28). Numerous studies have elucidated the paracellular pathways of the tight junction in these cells and the role of claudins by expression of individual claudins. A major limitation of this approach is that MDCK cells and LLC-PK1 cells already possess a background of multiple claudins. The recorded changes of paracellular conductance cannot reflect the property of each individual claudin but the deviation relative to the background.

Our study aimed to systematically knock down the endogenous claudin expression in MDCK cells and LLC-PK1 cells using siRNA (against claudin-1, -4 and -7, respectively). An epithelium experiencing a loss of claudin expression should show a reciprocal paracellular permeability change to the same epithelium overexpressing the claudin in order to confirm an assignment of function. Using siRNA silencing, we found that in MDCK cells claudin-2, -4, and -7 are powerful effectors of paracellular cation permeation (P_{Na}) with no effects on anion (P_{Cl}). Removal of claudin-2 depressed the permeation of Na⁺ and caused the MDCK cell to lose its cation selectivity (P_{Na}/P_{Cl} drops from >6 to close to 1.7). On the other hand, loss of claudin-4 or -7 expression elevated the permeation of Na⁺ and increased the permeability of the tight junction for cations (P_{Na}/P_{Cl} jumps from 6 to >13). The expression of siRNA-resistant isoforms of claudin-2, -4, or -7 selectively rescued the loss of function of these claudins in MDCK cells, indicating that the changes in paracellular ion permeation caused by removing a specific claudin truly reflect the property of this claudin in the MDCK context. Given that our conclusions of the functions of claudin-2 and -4 are consistent with previous overexpression findings (claudin-2, Refs. 13 and 29–31; claudin-4, Refs. 9–10), our studies provide strong evidence that claudin-2 functions to increase, and claudin-4 to decrease, the cation permeation. However, our data on claudin-7 function contrast with those of a recent study performed in LLC-PK1 cells using the overexpression strategy, which showed that claudin-7 concurrently decreases the paracellular Cl⁻ conductance and increases the paracellular Na⁺ conductance (25). To resolve this discrepancy and complement our studies performed in MDCK cells, we have generated LLC-PK1 epithelial cells with suppressed gene expression of claudin-4 or -7 by RNA interference. Knockdown of claudin-4 or -7 depressed the paracellular permeation of Cl⁻ in LLC-PK1 cells and caused the cells to lose their anion selectivity. The expression of siRNA-resistant isoforms of claudin-4 or -7 selectively rescued the loss of function of these claudins in LLC-PK1 cells, indicating that claudin-4 and -7 function as paracellular channels to Cl⁻ in LLC-PK1 cells. Further studies are needed to reconcile the contrasting conclusions on claudin-7 function drawn by us and by Alexandre et al. (25), although overexpression of foreign claudins in an existing tight junction context could interfere with the structural makeup of the tight junction, especially when claudins are fused to green fluorescent protein to form a large protein moiety. Moreover, the use of constitutively expressing stable cell lines generates a large amount of clonal variation in TER and dilution potentials. So as to obtain convincing results by this method, a sufficiently large number of independent clones may need to be examined to reach a statistical conclusion. Nevertheless, the discrepancy highlights the importance of physiological measurements in both the presence and absence of a particular claudin in a given cell context.

The yin and yang of function of claudin-4 and -7 are particularly interesting. Claudin-4 and -7 function either as paracellular barriers to Na⁺ or as paracellular channels to Cl⁻, depending upon the cellular background, to modulate the ion selectivity of the tight junction. This mode of claudin function is in fact consistent with the established theory that claudins form charge-selective pores and confer the ion selectivity to the tight junction (9,13–14). These charge-selective pores appear to be less discriminative than the conventional ion channels positioned in the cell membrane. The extracellular loops of claudins are enriched with charged amino acids, which could contribute to the ion selectivity of claudins, as highlighted by our previous studies on claudin-16 (parcellin-1) and its mutants bearing point mutations of the charged amino acids in the extracellular loop (14). When claudin-4 or -7 forms ion-selective pores, the positive charges in the extracellular loops facing the pores may hinder the passage of cations (Na⁺) while permitting anions (Cl⁻) to go through. Intriguingly, claudin-4 and -7 function as paracellular barriers to Na⁺ in MDCK cell but as paracellular channels to Cl⁻ in LLC-PK1 cells. It is likely that: 1) the protein composition of the tight junction differs between these two cell types, thus providing alternative partners with which claudin-4 and -7 may interact; or 2) the cellular regulatory pathways differ between the two cell lines, thereby creating alternative protein modifications of claudins (*e.g.* protein phosphorylation, as highlighted by WNK4 phosphorylating claudins 1–4 in MDCK.
cells and selectively up-regulating paracellular Cl\textsuperscript{−} transport; see below).

Our studies have created a platform for the examination of the regulation of claudin function in MDCK cells. The paracellular pathway is highly regulated by a wide range of physiologic inputs, including hormones, cytokines, myosin activity, and many cell signaling pathways (see review in Ref. 32). Because claudins function as effectors of ion transport at the end of regulatory pathways, there must be transducing proteins that modulate the function of claudins and thus link the physiologic inputs to the final effectors. WNK4 is an interesting candidate, first discovered as the gene linked to pseudohypoaldosteronism type II (PHAII), a rare Mendelian form of hypertension (33). Studies have shown the serine-threonine kinase WNK4 phosphorylates claudins 1–4 in MDCK cells and that its gain-of-function mutant selectively increases paracellular anion permeability (P\textsubscript{Cl}) (34, 35). The effect of WNK4 on paracellular ion transport is abolished by missense mutations that impair WNK4 kinase activity (34), suggestive of WNK4-mediated phosphorylation of claudins in the paracellular pathway. It will be informative to study WNK4 and its mutant forms in MDCK cells lacking a specific claudin and to identify the functional substrate of the kinase in the paracellular pathway. This may contribute to our understanding of the roles of claudins in renal diseases and herald novel treatments.

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