Protein Kinase D Promotes Airway Epithelial Barrier Dysfunction and Permeability through Down-regulation of Claudin-1*

Received for publication, August 20, 2013, and in revised form, November 15, 2013. Published, JBC Papers in Press, November 21, 2013, DOI 10.1074/jbc.M113.511527

Huachen Gan1, Guibo Wang1, Qin Hao1, Q. Jane Wang‡, and Hua Tang†1
From the †Department of Cellular and Molecular Biology, The University of Texas Health Science Center at Tyler, Tyler, Texas 75708 and the ‡Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Background: Claudin-1 is a key component of epithelial tight junctions.
Results: PKD, especially PKD3, suppresses claudin-1 expression and promotes airway epithelial barrier dysfunction and permeability.
Conclusion: PKD is a negative regulator of claudin-1 and airway epithelial barrier integrity.
Significant: Our findings offer new insights into the regulation of airway epithelial barrier integrity and a novel therapeutic target for barrier dysfunction-associated airway and lung diseases.

At the interface between host and external environment, the airway epithelium serves as a major protective barrier. In the present study we show that protein kinase D (PKD) plays an important role in the formation and integrity of the airway epithelial barrier. Either inhibition of PKD activity or silencing of PKD increased transepithelial electrical resistance (TEER), resulting in a tighter epithelial barrier. Among the three PKD isoforms, PKD3 knockdown was the most efficient one to increase TEER in polarized airway epithelial monolayers. In contrast, overexpression of PKD3 wild type, but not PKD3 kinase-inactive mutant, disrupted the formation of apical intercellular junctions and their reassembly, impaired the development of TEER, and increased paracellular permeability to sodium fluorescein in airway epithelial monolayers. We further found that overexpression of PKD, in particular PKD3, markedly suppressed the mRNA and protein levels of claudin-1 but had only minor effects on the expression of other tight junctional proteins (claudin-3, claudin-4, claudin-5, occludin, and ZO-1) and adherent junctional proteins (E-cadherin and β-catenin). Immunofluorescence study revealed that claudin-1 level was markedly reduced and almost disappeared from intercellular contacts in PKD3-overexpressed epithelial monolayers and that claudin-4 was also restricted from intercellular contacts and tended to accumulate in the cell cytosolic compartments. Last, we found that claudin-1 knockdown prevented TEER elevation by PKD inhibition or silencing in airway epithelial monolayers. These novel findings indicate that PKD negatively regulates human airway epithelial barrier formation and integrity through down-regulation of claudin-1, which is a key component of tight junctions.

The airway epithelium is the first line of defense against inhaled gases, particulates, pathogens, and other potentially hazardous environmental stimuli (1–3). It has at least two major functions; (i) the epithelial cells form a tight barrier that physically isolates pathogens and particulates and selectively control the passage of ions, water, and other solutes (2); (ii) the epithelial cells produce chemokines and cytokines to recruit immune cells for dealing with immune responses or to limit foreign antigen invasion (2, 3). A little difference in the epithelial barrier could lead to a large impact on disease susceptibility or outcome (3). A growing body of evidence indicates that dysfunction of the airway epithelial barrier is involved in the development and progression of various airway and lung diseases, such as asthma, lung injury and fibrosis, and chronic obstructive pulmonary disease (4–10). The epithelial barrier is composed of two essential elements, an intact epithelial monolayer and the intercellular junctions that connect epithelial cells to their neighboring cells (11). Mature apical junctional complexes (AJCs),2 the main component of intercellular junctions, comprise the most apical tight junctions, underlying adherent junctions and the desmosome (12, 13). Tight junctions seal the paracellular clefts between neighboring cells. Depending on the protein composition, tight junctions define the barrier characteristics and in addition maintain cell polarity (7, 14). Tight junctions are composed of several different components, including transmembrane, peripheral, and cytoskeletal proteins, which act in concert to control paracellular permeability. The membrane proteins that mediate cell to cell contacts include claudins, occludin, tricellulin, and junctional adhesion molecules. These protein extracellular domains interact with homotypic domains of the adjacent cells, and the intracellular domains of these proteins interact with a set of scaffolding proteins, including the PDZ-domain-containing zonula occludens proteins (ZO-1, ZO-2, and ZO-3), thus connecting to cytoskeleton (15). Many lines of evidence have shown that among the

* This work was supported, in whole or in part, by NHLBI, National Institutes of Health Grant R21-HL097216 (to H. T).
† To whom correspondence should be addressed: Dept. of Cellular and Molecular Biology, The University of Texas Health Science Center at Tyler, 11937 U. S. Highway 271, Tyler, TX 75708. Tel.: 903-877-7938; Fax: 903-877-7558; E-mail: hua.tang@uthct.edu.
‡ The abbreviations used are: AJC, apical junctional complex; PKD, protein kinase D; TEER, transepithelial electrical resistance; SAEC, small airway epithelial cells; ZO-1, zonula occludens protein 1; VE, vascular endothelial.
PKD-mediated Down-regulation of Claudin-1 and Barrier Dysfunction

tight junctional components, the integral membrane protein claudins, since their discovery in 1998, are the principle barrier-forming proteins (14, 16–18). The claudin-1 knock-out mice died from severely impaired barrier function (17), whereas occludin null mice were born with no gross barrier defect (19). Claudins (23 kDa) have four transmembrane domains and two extracellular loops and now have a family of 27 isoforms. They are expressed differentially among various tissues, and their expression pattern impacts on epithelial barrier function (20).

It has been reported that claudin-1, claudin-4, claudin-5, claudin-14, and claudin-18 are tightening junctional proteins with sealing function, whereas claudin-2 and claudin-8 are loosening junctional molecules with leaky function (20). Additionally, claudin-3 exhibits sealing function in Madin-Darby canine kidney cells (21) but shows leaky properties in alveolar cells (22).

The serine/threonine protein kinase D (PKD) family kinases include PKD1 (also called protein kinase Cµ-PKCµ), PKD2, and PKD3 (PKCv) (23). PKD contains a tandem repeat of zinc finger-like cysteine-rich motifs at its N terminus that display high affinity for diacylglycerol or phorbol ester, a pleckstrin homology domain, and a C-terminal catalytic domain that shares homology with the calmodulin-dependent kinases (23). PKD has been implicated in cell proliferation, vesicle fission, and trafficking, gene expression, and rearrangement of actin cytoskeleton (23–25). Although PKD family kinases exhibit a homologous catalytic domain, they vary with respect to their subcellular localization, expression, and regulation (23, 26–28). These findings suggest a functional differentiation among PKD isoforms.

We have reported that PKD1 regulates the production of proinflammatory cytokines by vascular endothelial growth factor (VEGF) in endothelial cells (29) and that PKD2 is pivotal for angiogenesis (30). We also found that both PKD2 and PKD3 were novel growth regulators in triple-negative breast cancer cells (31). Moreover, PKD has recently been implicated in the regulation of endothelial cell permeability. Tinsley et al. (32) reported the involvement of PKCδ and PKD in pulmonary microvascular endothelial cell hyperpermeability. Others have reported that PKD mediates endothelial cell permeability by VEGF and uroctin through phosphorylation of guanine nucleotide exchange factor Syx or disruption of VE-cadherin-catenin complex (33, 34). Relatively less is known about the function and molecular basis of PKD in the regulation of airway epithelial barrier function, although it has been shown that the epithelial barrier disruption by polyinosinic-polycytidylic acid (polyI:C) could be attenuated by G6976, an inhibitor of PKD and classical PKC isoforms (35).

In this study we have investigated the role of PKD in epithelial barrier formation and function in 16HBE14o—human bronchial epithelial cell line and primary human small airway epithelial cell monolayers by using multiple approaches. We have identified PKD, especially PKD3, as a critical negative regulator of airway epithelial barrier formation and integrity by suppressing the expression of claudin-1, a key component of tight junctions.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—PKD1 (A-20) and PKD1/2 (C-20) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). PKD2 antibody, G66983, Y27632, and ML-7 were from Millipore (Billerica, MA). PKD3, GFP, phospho-HDAC4(Ser-632)/HDAC5(Ser498)/HDAC7(Ser486), phospho-4E-BP1 (Thr-37/46), and phospho-(Ser/Thr) PKD substrate antibodies and reagents for chemiluminescence detection were from Cell Signaling Technology (Beverly, MA). Actin antibody and blebbistatin were from Sigma. Rat tail collagen (type I) and antibodies against E-cadherin and β-catenin were from BD Biosciences. Occludin, ZO-1, and claudin-1,-2,-3,-4, and -5 antibodies, Alexa fluor 568-labeled anti-mouse and anti-rabbit antibodies, Alexa fluor 647-labeled anti-mouse and anti-rabbit antibodies, Lipofectamine 2000, and G418 were from Invitrogen. PKD inhibitor kb-NB142-70 was from Tocris Bioscience (Minneapolis, MN), and G6976 was from LC Laboratories (Woburn, MA).

**Cell Culture, Transfection, and Generation of Stable Cell Lines**—16HBE14o—human bronchial epithelial cells were kindly provided by Dr. Dieter Gruenert (University of California at San Francisco) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Primary human small airway epithelial cells (SAECs) were obtained from Lonza (Walkersville, MD) and cultured in small airway growth medium and used for experiments within three passages. For immunofluorescence, transcellular electrical resistance measurement, and permeability studies, epithelial cells were seeded at a density of 1.5–3 × 10⁵ cells/cm² on collagen-coated permeable Transwell inserts with a 0.4-μm pore size (Corning), and the medium was changed the following day and subsequently changed every other day for the duration of experiment. All cell cultures were maintained in a humidified 5% CO₂ atmosphere in air at 37 °C. To generate 16HBE14o—cells stably expressing individual PKD isoform, 16HBE14o—cells were transfected with empty pEGFP-C3 vector (BD Bioscience Clontech), pEGFP-C3-PKD2 (36), pEGFP-C3-PKD3 (27) (kindly provided by Dr. Osvaldo Rey, University of California at Los Angeles), pcDNA3 vector (Invitrogen), or pcDNA3-HA-PKD1S738E/S742E (37) encoding a constitutively active PKD1 (Addgene plasmid #10810, kindly provided by Dr. Alex Toker, Harvard Medical School) by using Lipofectamine 2000, and stable cell clones were selected with 1 mg/ml G418. The cell clones were further subjected to FACs sorting of GFP-positive cells via BD FACSAria cell sorter (BD Biosciences), and the stable expression of PKD isoforms was verified by FACs and Western blot analyses. For adenovirus-mediated expression of PKD3, SAECs were infected with recombinant adenovirus expressing control vector, PKD3 wild type (SignaGen Laboratories), or a PKD3 kinase-inactive mutant D720A (38) at a multiplicity of infection of 10–20 plaque-forming units/cell.

**Transepithelial Electrical Resistance (TEER) and Permeability Assay**—TEER was measured with an EVOMX voltommeter (World Precision Instruments). The data, which subtract the basal electrical resistance of cell-free collagen-coated Transwell inserts from each experimental point, were presented either as absolute values (ohm × cm²) or changes relative to the control group. To evaluate the paracellular permeability of cultured 16HBE14o—cells, 0.02% fluorescein sodium salt (Fisher) in DMEM containing 2% FBS was added to the apical side of the inserts, and DMEM containing 2% FBS was added to the lower
PKD-mediated Down-regulation of Claudin-1 and Barrier Dysfunction

well. At a certain time interval after adding sodium fluorescein, 100 μl of fluid was collected from the basolateral compartment of each insert and transferred to Corning 96-well plates. A microplate spectrophotometer (BIO-TEK Instruments) at 490 nm was used to measure the amount of fluorescein sodium that had diffused from the apical to the basal side of the insert. Data are presented as the -fold change from baseline.

**Calcium Switch**—16HBE14o— epithelial cells were seeded at a subconfluent density of 1.5 – 3 x 10⁵ cells/cm² on collagen-coated permeable Transwell inserts, and TEER was monitored every other day. On the appropriate day, cells were incubated with serum-free DMEM overnight, and the next day cells were treated with 4 mM EGTA in serum-free DMEM for 25 min at 37 °C. The cells were allowed to reassemble by replacing the medium with calcium-containing growth medium.

**Immunofluorescence Microscopy**—16HBE14o— cell monolayers grown on Transwell filters were rinsed with pre-cold phosphate-buffered saline (PBS) twice and fixed in pre-cold methanol for 20 min at −20 °C. Filters were washed with PBS three times and blocked in 1% BSA for 1 h at room temperature, then carefully excised from the Transwells and incubated with the indicated primary antibodies against junctional proteins. After 1 h of incubation, filters were washed serially with PBS and incubated for another hour with Alexa Fluor-labeled secondary antibodies at room temperature. After washing with PBS, filters were mounted with mount-G (Southern Biotech), and fluorescence was visualized and captured using a PerkinElmer Life Sciences Ultra VIEW LCI confocal imaging system. Adobe Photoshop-7.0 software was used for image processing.

**Small Interference RNA (siRNA) and siRNA Transfection**—AllStars non-targeting negative control siRNAs (Con-S1, no. 102728; Con-S2, no. 102207) and the validated human PKD1 siRNA (PKD1-S1, no. SI0042378), PKD1 siRNA-2 (PKD1-S2, no. SI0031350), PKD2 siRNA-1 (PKD2-S1, no. SI0379578), PKD2 siRNA-2 (PKD2-S2, no. SI0222678), PKD3 siRNA-1 (PKD3-S1, no. SI04439211), PKD3 siRNA-2 (PKD3-S2, no. SI02223984) were from Qiagen (Valencia, CA). Human claudin-1 siRNAs (siCLDN1, sc-43040) were from Santa Cruz Biotechnology. For siRNA transfection, 16HBE14o— cells were seeded into different plates for 24 h to reach 50 –70% confluence, and then siRNA was transfected into 16HBE14o— cells in a final concentration of 20 nm by using Lipofectamine 2000 and OptimEM I-reduced serum medium according to the manufacturer’s protocols (Invitrogen). After 24 h, the transfected cells were trypsinized and seeded on the Transwell filters, and the medium was changed on the following day and subsequently changed every other day for the duration of experiment. The silencing effects of siRNAs were confirmed by Western blotting analysis.

**Western Blot Analysis**—Western blot analysis were performed as we described previously (39).

**RNA Isolation and Reverse Transcription (RT)-PCR**—Total RNA was isolated using the RNeasy RNA isolation kit according to the manufacturer’s protocol (Qiagen). RT-PCR primers were designed with Oligo6 software (Molecular Biology Insights) as follows: claudin-1 (expected product of 260 bp), 5’-GCC CAG GCC ATG TAC GAG-3’ (forward) and 5’-GCA AGA AGA AAT ATC GCA CCC-3’ (reverse); claudin-4 (323 bp), 5’-ATC TGG GAG GGC CTA TG-3’ (forward) and 5’-GCC ACC AGC GGA TTG TAG AAG-3’ (reverse); internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (608 bp), 5’-CGC TGA GTA CGT CGA G-3’ (forward) and 5’-GAG GAG TGG TGG TGG TAG AAG-3’ (reverse). RT-PCR conditions were 50 °C for 30 min and 95 °C for 15 min followed by 25 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min followed by 72 °C for 10 min. Relative change in mRNA was measured by densitometric analysis and normalized to GAPDH.

**Statistical Analysis**—Data are expressed as the means ± S.E. A Student’s t test was used for statistical analysis. p < 0.05 is considered statistically significant.

**RESULTS**

Inhibition of PKD Activity or Silencing of PKD Increase TEER of Airway Epithelial Cell Monolayers and PKD3 Is the Most Efficient Isoform in the Process—TEER reflects the paracellular and transcellular resistance and is a sensitive measure of barrier integrity. 16HBE14o— human bronchial epithelial cell line retains the differentiated morphology and function of normal human bronchial epithelium (40). When plated on Transwell membrane filters, 16HBE14o— cells formed monolayers of well-differentiated columnar cells and developed TEER upon confluence with a plateau on day 7 (Fig. 1A). Interestingly, we found that TEER was markedly increased by a newly developed selective PKD inhibitor kb-NB142-70 (41) over 7 days of culture on filters in a dose-dependent manner (Fig. 1A). The increase in TEER could be observed as early as 4 h after kb-NB142-70 treatment (Fig. 1B). By using a phospho-Ser/Thr PKD substrate antibody that specifically recognizes the PKD consensus motif LXRXX(S/T) and PKD-mediated protein phosphorylation (30, 42), we found that the PKD-dependent protein phosphorylation in 16HBE14o— cells was markedly suppressed by the selective PKD inhibitor kb-NB142-70 (Fig. 1C, top panel). The phosphorylation of PKD protein substrate class II histone deacetylases HDACA/5/7 (24) on Ser-632/Ser-498/Ser-486 was also greatly inhibited by kb-NB142-70 (Fig. 1C, second panel). The expression of PKD isoforms was not affected by treatment of cells with kb-NB142-70 for 24 h (Fig. 1C) and 7 days (data not shown). We further found that TEER was also significantly increased by another PKD inhibitor G66976 (43) but not by a general PKC inhibitor G66983 (43) (Fig. 1D). G66976 is an inhibitor of PKD and classical PKC isoforms and has been widely used as a PKD inhibitor for intact cell studies (29, 44, 45). These findings indicate that inhibition of PKD activity increases TEER of 16HBE14o— cell monolayers and suggest that PKD may be a negative regulator of epithelial barrier integrity. To confirm the role of PKD in TEER, 16HBE14o— cells were transfected with isoform-specific PKD siRNAs for 24 h in cell culture plates as we described (29–31), the cells were then trypsinized and seeded to Transwell filters at higher density to accelerate the attainment of confluence and processes of epithelial differentiation. We found that all the PKD-silenced 16HBE14o— monolayers exhibited higher TEER than control cells over a 4-day culture period (Fig. 1E) and that among the three PKD isoforms, PKD3 knockdown was the most efficient one to increase TEER (Fig. 1G). Fig. 1F shows that PKD1, PKD2, or PKD3 were...
markedly knocked down by two different isoform-specific siRNAs. Additionally, PKD knockdown did not significantly affect 16HBE14o−/H11002 cell proliferation (data not shown). Taken together, these novel findings indicate that inhibition of PKD activity or silencing of PKD, especially PKD3, markedly increase TEER and barrier integrity of airway epithelial cells.

**Overexpression of PKD Decreases TEER and Increases Epithelial Monolayer Permeability**—We next determined the effect of PKD overexpression on TEER in 16HBE14o−/H11002 and primary human airway epithelial cells. We generated pooled and cloned 16HBE14o−/H11002 cells stably overexpressing PKD isoforms through G418 selection and/or FACS sorting. As shown in Fig. 2A, we found that overexpression of PKD3 dramatically decreased TEER development. Over the 7-day period, the TEER was impaired and could not be developed well in pooled or cloned 16HBE14o−/H11002 cells expressing GFP-PKD3 compared with control cells expressing GFP alone. On day 7, TEERs of GFP-PKD3-pool, -C1, and -C2 cells were only 34, 26, or 10% that of control GFP-pool cells, respectively. The expression of GFP (27 kDa) and GFP-PKD3 (137 kDa) was verified by Western blotting with GFP and PKD3 antibodies (Fig. 2A). We further showed that the transepithelial flux of sodium fluorescein was markedly augmented in pooled and cloned 16HBE14o−/H11002 cells stably expressing PKD3, indicating an increased paracellular permeability by PKD3 overexpression (Fig. 2B). Moreover, PKD2 overexpression also attenuated TEER development in 16HBE14o−/H11002 cells. Thus, overexpression of PKD3 increased TEER development, whereas PKD2 overexpression impaired TEER development.
Similarly, pooled or cloned 16HBE14o-/H11002 cells stably expressing a constitutively active PKD1 (PKD1-S738E/S742E) (37) exhibited an impaired development of TEER over 7 days (Fig. 2D). Although 16HBE14o- cells retain the differentiated morphology and function of normal human bronchial epithelia (40), they are transformed cells and might not fully resemble primary airway epithelial cells in vivo. We utilized primary SAECs to confirm our results. Primary SAECs were grown on Transwell filters and infected with recombinant adenovirus carrying control vector, human PKD3 wild type (PKD3-WT), or a PKD3 kinase-inactive mutant D720A (PKD3-DN), and TEER was measured and normalized to control vector over a 3-day period. PKD3 expression was confirmed by Western blotting. All data are the means ± S.E. (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus control GFP cells or control vector. Results represent four independent experiments.

PKD Impairs AJC Reassembly after Barrier Disruption by Calcium Depletion—We next examined whether PKD regulates AJC reassembly by using the calcium switch technique.
Mature 16HBE14o-H11002 cell monolayers were disrupted by depleting calcium with EGTA and then allowed to reassemble by replacing the medium with calcium-containing growth medium as shown in Fig. 3. We found that barrier reassembly was significantly accelerated in the presence of PKD inhibitor kb-NB142-70 after calcium switch (Fig. 3A). In contrast, overexpression of PKD3 delayed barrier reassembly (Fig. 3B). The PKD3-overexpressed 16HBE14o-H11002 cell monolayers developed only 20% TEER of control cells from 4 to 7 h post-calcium switch, and TEER was recovered only 20–34% in PKD3-overexpressed 16HBE14o-H11002 monolayers and 26–45% in control cells from 4 to 7 h post-calcium switch. Furthermore, the delayed development of TEER was associated with a corresponding increase (3.4-fold) in permeability to sodium fluorescein in PKD3-overexpressed 16HBE14o− cell monolayers compared with control cell monolayers (Fig. 3C). These data indicate that PKD also negatively regulates apical junction reassembly in airway epithelial cells.

**PKD Overexpression Preferentially Down-regulates Claudin-1 but Not Other AJC Proteins**—Because PKD impaired epithelial barrier formation and reassembly, we examined whether PKD suppresses the expression of AJC proteins in airway epithelial cells. As shown in Fig. 4, A–D, forced overexpression of PKD1, PKD2, or PKD3 all resulted in a marked reduction in the protein level of claudin-1 that is a key component of tight junctions in airway epithelial cell monolayers. In particular, the protein level of claudin-1 was profoundly suppressed by overexpression of PKD3 in 16HBE14o− and primary SAEC monolayers (Fig. 4, C and D). In contrast, forced overexpression of PKD isoforms had only minor effects on the protein levels of other tight junctional proteins (claudin-3, claudin-4, claudin-5, occludin, and ZO-1) and adherent junctional proteins (E-cadherin and β-catenin) in the cells. In some cases, we observed that the protein level of claudin-3 was also reduced by PKD3 overexpression in 16HBE14o− cell monolayers (Fig. 4C, third panel). Claudin-2 was not expressed in 16HBE14o− cell monolayers, and claudin-2, claudin-3, and claudin-5 were not detectable in primary SAEC monolayers. Furthermore, we found that the mRNA levels of claudin-1 but not claudin-4 were markedly suppressed (60–70% inhibition) by PKD3 overexpression in 16HBE14o− cell monolayers (Fig. 4E), whereas protein degradation rates of claudin-1 were similar in the control and PKD3-overexpressed cell monolayers (Fig. 4F). Additionally, PKD3 overexpression did not influence the phosphorylation of translation repressor protein 4E-BP1 on threonine-37/46 in primary SAECs (Fig. 4D, eighth panel) and in 16HBE14o− cell monolayers (data not...
It has been shown that phosphorylation of 4E-BP1 results in activation of cap-dependent translation (47). These findings indicate that PKD preferentially down-regulates claudin-1 but not other AJC proteins by modulating claudin-1 mRNA level but not protein stability in airway epithelial cells.

PKD3 Overexpression Abolishes Claudin-1 and Claudin-4 Localization to Apical Junctions and Disrupts Cell-Cell Contacts—Because we found that PKD down-regulated claudin-1 and negatively controlled TEER development and epithelial barrier formation, we used confocal microscopy to visualize the impact of PKD3 overexpression on the structure of epithelial apical junctions. Occludin and ZO-1 are two well known markers of AJC tight junctions, contributing to tight junction stabilization and optimal barrier function (48, 49). As shown in Fig. 5, we found that in polarized monolayers of control GFP-16HBE14o– cells, occludin and ZO-1 predominantly localized to intercellular apical junctions and colocalized with claudin-1 and claudin-4.

PKD-mediated Down-regulation of Claudin-1 and Barrier Dysfunction

![Diagram of PKD-mediated Down-regulation of Claudin-1 and Barrier Dysfunction](https://example.com/diagram.png)

**FIGURE 4. PKD overexpression preferentially down-regulated claudin-1 but not other AJC proteins.** A–C, pooled (Pool) or cloned (C1 or C2) 16HBE14o– cells stably expressing pcDNA3 vector alone (Vec), a constitutively active HA-PKD1S738E/S742E (PKD1CA), GFP alone (GFP), GFP-PKD2 or GFP-PKD3 were grown on Transwell inserts for 7 days and collected. Equal amounts of cell lysates were subjected to Western blotting with specific antibodies against PKD1, PKD2, and AJC proteins as indicated. GFP-PKD3 (C, top panel) was detected with a GFP antibody. n.s., nonspecific band. D, primary SAECs were grown on Transwell inserts to develop TEER to a plateau, then infected with recombinant adenovirus carrying vector alone or human PKD3 for 72 h. Equal amounts of lysates were subjected to Western blotting with specific antibodies against PKD3 and AJC proteins as indicated. E, pooled or cloned 16HBE14o– cells stably expressing GFP alone or GFP-PKD3 were grown on Transwell inserts for 7 days, and the mRNA levels of claudin-1 and claudin-4 were determined and shown in a representative RT-PCR. Relative change in mRNA level was measured by densitometry analysis using ImageJ 1.47 software and normalized to GAPDH (n = 3). **, p < 0.01 versus control cells. F, pooled 16HBE14o– cells stably expressing GFP alone or GFP-PKD3 were grown on Transwell inserts for 7 days and then treated with a protein synthesis inhibitor cycloheximide (20 μM) for 0–16 h. The cells were collected, and equal amounts of lysates were subjected to Western blotting analysis with antibodies against claudin-1 and actin. Relative changes in protein levels were measured by densitometric analysis using ImageJ 1.47 software and normalized to actin and are represented as percentage of claudin-1 at 0 h (n = 3). Results represent three independent experiments.
claudin-4 was also observed in the cytosolic compartment of control 16HBE14o−/H11002 monolayers. It should be noted that PKD3 overexpression caused a significant alteration in the formation of apical junctions. We found that in PKD3-overexpressed 16HBE14o−/H11002 cell monolayers, occludin and ZO-1 were predominantly distributed to sites of disorganized cell-cell contacts that lost tension and had gaps and fragments (Fig. 5, arrows). However, PKD3 overexpression had a minor effect on the signal intensities of occludin and ZO-1, and a small amount of ZO-1 could be observed in the cytosol of PKD3-overexpressed 16HBE14o−/H11002 monolayers. An immunofluorescence study further revealed that claudin-1 level was markedly reduced and almost disappeared from intercellular contacts in PKD3-overexpressed 16HBE14o−/H11002 monolayers. An immunofluorescence study further revealed that claudin-1 level was markedly reduced and almost disappeared from intercellular contacts in PKD3-overexpressed 16HBE14o−/H11002 monolayers. An immunofluorescence study further revealed that claudin-1 level was markedly reduced and almost disappeared from intercellular contacts in PKD3-overexpressed 16HBE14o−/H11002 monolayers. An immunofluorescence study further revealed that claudin-1 level was markedly reduced and almost disappeared from intercellular contacts in PKD3-overexpressed 16HBE14o−/H11002 monolayers.

**Claudin-1 Knockdown Prevents TEER Elevation by PKD Inhibition or Silencing in Epithelial Monolayers**—Claudin-1 is a key barrier-forming protein and plays an essential role in the formation of tight junctions (14, 16–18). Indeed, we found that knockdown of claudin-1 markedly inhibited TEER development in 16HBE14o−/H11002 monolayers during 5 days of culture on Transwell filters (Fig. 6A). Specific knockdown of claudin-1 with a pool of 3 target-specific claudin-1 siRNAs on day 5 was verified in Fig. 6B. Interestingly, we found that the enhanced TEER development in 16HBE14o−/H11002 cell monolayers by PKD inhibitor kb-NB142-70 (41) or PKD3 silencing could be largely prevented or almost completely suppressed by claudin-1 knockdown, respectively (Fig. 6, C and D). In particular, on day 4, PKD3 knockdown increased claudin-1 expression by 55% and TEER by 40% in 16HBE14o−/H11002 cell monolayers, but both effects were abolished by claudin-1 knockdown (Fig. 6, D and E). Moreover, we found that PKD3 overexpression preferentially down-regulated claudin-1 in 16HBE14o−/H11002 cell monolayers and that this effect could be partially reversed by kb-
These findings indicate that the PKD-mediated dysfunction of airway epithelial barrier is largely dependent on claudin-1.

**DISCUSSION**

The airway epithelium is the first line of defense against inhaled gases, particulates, pathogens, and other potentially hazardous environmental stimuli (1–3). A growing body of evidence indicates that dysfunction of airway epithelial barrier is involved in the development and progression of various airway and lung diseases, such as asthma, lung injury and fibrosis, and chronic obstructive pulmonary disease (4–10). In the present study we report that PKD promotes airway epithelial barrier dysfunction and permeability, which is mediated through the down-regulation of a key tight junctional protein claudin-1. Our findings may offer new insights into the regulation of airway epithelial barrier integrity and a novel therapeutic target for epithelial barrier dysfunction-associated airway and lung diseases.

By using multiple approaches, we have obtained substantial evidence demonstrating that PKD, especially PKD3, impairs the formation of apical intercellular junctions and their reassembly, decreases in TEER, and increases in paracellular permeability to sodium fluorescein in 16HBE14o– human bronchial epithelial cell and primary human SAEC monolayers. We further show that PKD preferentially down-regulates claudin-1, but not other tight junctional proteins (claudin-3, claudin-4, claudin-5, occludin, and ZO-1) and adherent junctional proteins (E-cadherin and β-catenin) in the epithelial monolayers. Claudin-1 is a sealing junctional protein and a key compo-

**FIGURE 6. Claudin-1 knockdown prevented TEER elevation by PKD inhibition or silencing in epithelial monolayers.** A–E, 16HBE14o– cells were transfected with 20 nm non-targeting control siRNA (siControl or siCon), a pool of 3 target-specific human claudin-1 siRNAs (siCLDN1 or siC1), or PKD3 siRNA-1 (siPKD3 or siD3) alone or together as indicated and grown for 24 h. A, the transfected cells were trypsinized and seeded to Transwell filters at higher density to grow for 5 days, and then TEER was monitored every day. B, equal amounts of lysates from the cells in panel A on day 5 were subjected to Western blotting analysis, and relative change in claudin-1 protein level was measured by densitometric analysis using ImageJ 1.47 software and normalized to actin. C, the transfected cells were trypsinized, seeded on Transwell filters at higher density, then treated with DMSO (0.02%, v/v) or 1 μM kb-NB142-70 (kb-70) on day 2, and TEER was monitored until day 5. D, the transfected cells were trypsinized and grown on Transwell filters, and TEER was monitored until day 4. E, equal amounts of lysates from cells in panel D on day 4 were subjected to Western blotting with the indicated antibodies, and relative changes in protein levels were measured by densitometric analysis using ImageJ 1.47 software and normalized to actin. F, pooled 16HBE14o– cells stably expressing GFP alone or GFP-PKD3 were grown on Transwell inserts for 7 days then treated with DMSO (0.02%, v/v) or 1 μM kb-NB142-70 (kb-70) for 24 h. The cells were collected, equal amounts of lysates were subjected to Western blotting with specific antibodies as indicated, and relative changes in claudin-1 levels were measured by densitometric analysis and normalized to actin. All data are the means ± S.E. (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus cells treated with control siRNA or PKD3 siRNA. Results represent three independent experiments.
PKD-mediated Down-regulation of Claudin-1 and Barrier Dysfunction

The claudin-1 knock-out mice died from severely impaired barrier function (17). Overexpression of claudin-1 increases TEER 4-fold in Madin-Darby canine kidney cells (50), whereas a reduced expression of claudin-1 and claudin-4 leads to disruption of epithelial barrier (51). Moreover, we found that claudin-1 and claudin-4 were both expressed in 16HBE14o- primary SAEC cell monolayers. Immunofluorescence study revealed that PKD not only reduced claudin-1 localization to apical junctions but also restricted claudin-4 localization to intercellular contacts, although PKD did not affect claudin-4 expression in airway epithelial monolayers. These results suggest that claudin-1 may be required for the appropriate incorporation of claudin-4 into apical junctions. We lastly found that claudin-1 knockdown prevented TEER elevation by PKD inhibition or silencing in airway epithelial monolayers. On the basis of these findings, it is plausible that down-regulation of claudin-1 contributes to the PKD-mediated dysfunction of airway epithelial barrier.

We found that PKD suppressed the mRNA and protein levels of claudin-1 but did not affect claudin-1 protein stability in airway epithelial cells. Additionally, we showed that PKD overexpression did not influence the phosphorylation of translation repressor protein 4E-BP1 on threonine 37/46 in airway epithelial monolayers. It has been shown that phosphorylation of 4E-BP1 results in activation of cap-dependent translation (47). It seems that PKD down-regulates claudin-1 at the mRNA level. Several reports have shown that transcription factors SP1, Cdx1, Cdx2, and GATA-4 positively regulate claudin-1 expression, whereas transcription factors SLUG, SNAIL, and HIFα act as repressors of claudin-1 expression in epithelial cells (52–54). SNAIL also down-regulates other junctional molecules, including E-cadherin and occludin; thus, it is a trigger of epithelial-mesenchymal transition (14, 53). It has been reported that phosphorylation of SNAIL-1 by PKD1 did not alter the nuclear location of SNAIL-1 but removed SNAIL-1 repressor effect on E-cadherin, thus up-regulating E-cadherin in breast epithelial cells (55). If this is the case in airway epithelial cells, overexpression of PKD would up-regulate E-cadherin and claudin-1. However, we observed that PKD3 overexpression abolished claudin-1 expression but did not affect E-cadherin expression. It seems likely that the direct phosphorylation of SNAIL by PKD may not be involved in the PKD-mediated down-regulation of claudin-1 in airway epithelial cells. Studies are ongoing in our laboratory to identify the PKD-targeted transcription factors for claudin-1 down-regulation. Moreover, PKD is able to phosphorylate heat shock proteins (HSP), such as HSP27 and HSP90, and PKD2 is reported to regulate BCL6-associated zinc finger protein mRNA stabilization through phosphorylation of HSP90β (56). The possibility that PKD regulates claudin-1 mRNA stabilization in airway epithelial cells may not be excluded.

In support of our results, PKD has been recently implicated in the regulation of endothelial cell permeability. Tinsley et al. (32) reported the involvement of PKCδ and PKD in pulmonary microvascular endothelial cell hyperpermeability. Ngok et al. (34) has shown that PKD1 phosphorylates the RhoA-specific guanine nucleotide exchange factor Syx and causes its disassociation from endothelial cell junctional anchors and thus mediates the VEGF-induced cell permeability in human umbilical vein endothelial cells. Wan et al. (33) recently reported that urocortin increased lipopolysaccharide-induced endothelial permeability through a PKD-mediated down-regulation of VE-cadherin and subsequent disruption of VE-cadherin-catenin complex in human umbilical vein endothelial cells. The proposed mechanisms for PKD-mediated permeability in human umbilical vein endothelial cell monolayers are different from ours in airway epithelial cells. A recent study has demonstrated that claudin-5 and tight junctions control paracellular permeability in arteriolar and capillary endothelial cell monolayers, whereas human umbilical vein endothelial cell and venular junctions use adherent junctional protein VE-cadherin for regulating barrier function (57). Moreover, in retinal microvascular endothelial cells, VEGF disturbs the barrier of the endothelial cell monolayers by inducing down-regulation of claudin-1 but not claudin-3, claudin-5, ZO-1, and VE-cadherin (58). It is interesting to know whether PKD mediates the down-regulation of claudin-1 by VEGF in microvascular endothelial cells. In airway epithelial cells, it has been shown that the synthetic double-stranded RNA polyI:C induces PKD phosphorylation/activation and epithelial barrier disruption and that the epithelial barrier disruption by polyI:C could be attenuated by Gö6976, a selective inhibitor of PKD and classical PKC isoforms (35). Moreover, Rezaee et al. (59) recently reported that airway epithelial barrier disruption induced by respiratory syncytial virus involved PKD-dependent actin cytoskeletal remodeling, possibly dependent on cortactin activation. These findings support a role of PKD in the regulation of airway epithelial barrier formation.

Tight junctions are composed of transmembrane, peripheral scaffolding, and cytoskeletal proteins, which act in concert to control paracellular permeability. Association with the underlying actin cytoskeleton is known to stabilize the structure of tight junctions and enhance the barrier integrity (15). We found that PKD3 overexpression caused disorganized cell-cell contacts that lost tension and had gaps in airway epithelial cells, which implicates a possibility of losing association with actin cytoskeleton. This effect may result from the down-regulation of claudin-1 as PKD-mediated dysfunction of airway epithelial barrier is largely dependent on claudin-1. Additionally, the down-regulation of claudin-1 by PKD was barely affected by actin cytoskeleton modulators, such as nonmuscle myosin II inhibitor blebbistatin, Rho-dependent kinase inhibitor Y-27632, and the myosin light chain kinase inhibitor ML-7 (data not shown). PKD has been implicated in the rearrangement of actin cytoskeleton through multiple levels of regulation (25). It merits further investigation to examine whether PKD-mediated remodeling of actin filaments influences tight junction association with actin cytoskeleton.

In summary, we have shown that PKD, in particular PKD3, contributes to the dysfunction of airway epithelial barrier through at least in part the down-regulation of a key tight junctional protein claudin-1. Findings from our study have the potential to improve therapeutic possibility with PKD inhibitors for epithelial barrier dysfunction-associated airway and lung diseases.
REFERENCES

1. Hackett, N. R., Shykhaiyev, R., Walters, M. S., Wang, R., Zwick, R. K., Ferris, B., Witover, B., Salti, J., and Crystal, R. G. (2011) The human airway epithelial basal cell transcriptome. PLoS ONE 6, e18378

2. Hirota, J. A., and Knight, D. A. (2012) Human airway epithelial cell innate immunity. Relevance to asthma. Curr. Opin. Immunol. 24, 740–746

3. Proud, D., and Leigh, R. (2011) Epithelial cells and airway diseases. Immuno- nol. Rev. 242, 186–204

4. Koval, M. (2009) Tight junctions, but not too tight. Fine control of lung permeability by claudins. Am. J. Physiol. Lung Cell. Mol. Physiol. 297, L217–L218

5. Ohta, H., Chiba, S., Ebina, M., Furuse, M., and Nukiwa, T. (2012) Altered expression of tight junction molecules in alveolar septa in lung injury and fibrosis. Am. J. Physiol. Lung Cell. Mol. Physiol. 302, L193–L205

6. Lambrecht, B. N., and Hammad, H. (2012) The airway epithelium in asthma. Nat. Med. 18, 684–692

7. Soini, Y. (2011) Claudins in lung diseases. Respir. Res. 12, 70

8. de Boer, W. I., Sharma, H. S., Baelenmans, S. M., Hoogsteden, H. C., Lambrecht, B. N., and Braunstahl, G. J. (2008) Altered expression of epithelial junctional proteins in atopic asthma. Possible role in inflammation. Can. J. Physiol. Pharmacol. 86, 105–112

9. Xiao, C., Puddicombe, S. M., Field, S., Haywood, J., Broughton-Head, V., Puxeddu, I., Haitchi, H. M., Vernon-Wilton, E., Sammut, D., Bedke, N., Cremin, C., Sones, J., Djukanovic, R., Howarth, P. H., Collins, J. E., Holgate, S. T., Monk, P., and Davies, D. E. (2011) Defective epithelial barrier function in asthma. J. Allergy Clin. Immunol. 128, 549–556

10. Shykhaiyev, R., Otaki, F., Bonsu, P., Dang, D. T., Teacher, M., Strulovic- Barel, Y., Salit, J., Harvey, B. G., and Crystal, R. G. (2011) Cigarette smoking reprograms apical junctional complex molecular architecture in the human airway epithelium in vivo. Cell. Mol. Life Sci. 68, 877–892

11. Ogasawara, N., Kojima, T., Go, M., Ohkuni, T., Koizumi, J., Kamekura, R., Milatz, S., Günzel, D., Wolburg, H., Piontek, J., Huber, O., and Blasig, I. E. (2010) Claudin-1 and -2. Novel integral membrane proteins localizing at tight junctional proteins in atopic asthma. A lesson from claudin-deficient mice. J. Cell Biol. 156, 1099–1111

12. Cording, I., Berg, J., Kading, N., Bellmann, C., Tscheik, C., Westphal, J. K., Milatz, S., Günzel, D., Wolburg, H., Piontek, J., Huber, O., and Blasig, I. E. (2013) In tight junctions, claudins regulate the interactions between occludin, tricellulin, and marveld3, which, inversely, modulate claudin oligomerization. J. Cell Sci. 126, 554–564

13. Saitou, M., Furuse, M., Sasaki, H., Schulze, J. D., Fromm, M., Takano, H., Noda, T., and Tsukita, S. (2000) Complex phenotype of mice lacking occludin, a component of tight junction strands. Mol. Biol. Cell 11, 4131–4142

14. Proud, D., and Leigh, R. (2011) Epithelial cells and airway diseases. Immuno- nol. Rev. 242, 186–204

15. González-Mariscal, L., Betanzos, A., Nava, P., and Jaramillo, B. E. (2003) Claudins in lung diseases. Am. J. Physiol. Lung Cell. Mol. Physiol. 301, L40–L49

16. Rozengurt, E., Re, O., and Waldron, R. T. (2005) Protein kinase D signaling. J. Biol. Chem. 280, 13205–13208

17. Put, F., and Rubin, C. S. (2003) Claudins: a novel family of integral membrane proteins localizing at tight junctions. J. Biol. Chem. 278, 23737–23785

18. Olayioye, M. A., Barisic, S., and Haussler, A. (2013) Multi-level control of actin dynamics by protein kinase D. Cell. Signal. 25, 1739–1747

19. Auer, A., von Blume, J., Sturany, S., von Wichert, G., Van Lint, J., Van- denheede, J., Adler, G., and Seufferlein, T. (2005) Role of the regulatory domain of protein kinase D2 in phorbol ester binding, catalytic activity, and nucleocytoplasmic shuttling. Mol. Biol. Cell 16, 4375–4385

20. Reyn, O., Yuan, Y., Xu, S. H., and Rozengurt, E. (2003) Protein kinase C nu/protein kinase D3 nuclear localization, catalytic activation, and intracellular redistribution in response to G protein-coupled receptor agonists. J. Biol. Chem. 278, 23737–23785

21. Haco, Q., Wang, L., and Tang, H. (2009) Vascular endothelial growth factor induces protein kinase D-dependent production of proinflammatory cyto- kinines in endothelial cells. Am. J. Physiol. Cell Physiol. 296, C281–C287

22. Haco, Q., Wang, L., Zhao, Z. J., and Tang, H. (2009) Identification of protein kinase D2 as a pivotal regulator of endothelial cell proliferation, migration, and angiogenesis. J. Biol. Chem. 284, 799–806

23. Zhao, Q., McKenzie, R., Gar, H., and Tang, H. (2013) Protein kinases D2 and D3 are novel growth regulators in HCC1806 triple-negative breast cancer cells. Anticancer Res. 33, 399–399

24. Tinsley, J. H., Teasdale, N. R., and Yuan, S. Y. (2004) Involvement of PKCβ and PKD in pulmonary microvascular endothelial cell hyperpermeability. Am. J. Physiol. Cell Physiol. 286, C1065–C111

25. Wan, R., Guo, R., Chen, C., Jin, L., Zhu, C., Zhang, Q., Xu, Y., and Li, S. (2013) Urocortin increased LPS-induced endothelial permeability by regulating the cadherin-catenin complex via corticotrophin-releasing hor- mone receptor 2. J. Cell Physiol. 228, 1295–1303

26. Ngok, S. P., Geyer, R., Liu, M., Kourtidis, A., Agrawal, S., Wu, C., Seerapur, H. R., Lewis-Tuffin, L. J., Hoodle, K. L., Huveldt, D., Marx, R., Baraban, J. M., Storz, P., Horowitz, A., and Anastasiadis, P. Z. (2012) VEGF and angiopoietin-1 exert opposing effects on cell junctions by regulating the Rho GEF Syx. J. Cell Biol. 199, 1103–1115

27. Rezaee, F., Meednu, N., Emo, J. A., Saatian, B., Chapman, T. J., Naydenov, N. G., De Benedetto, A., Beck, L. A., Ivanov, A. I., and Georas, S. N. (2011) Polyinosinosine-polycytidylic acid induces protein kinase D-dependent disas- sembly of apical junctions and barrier dysfunction in airway epithelial cells. J. Allergy Clin. Immunol. 128, 1216–1224

28. Ray, O., Yuan, Y., and Rozengurt, E. (2003) Intracellular redistribution of protein kinase D2 in response to G-protein-coupled receptor agonists. Biochem. Biophys. Res. Commun. 302, 817–824

29. Storz, P., Döppler, H., and Toker, A. (2004) Protein kinase Cδ selectively regulates protein kinase D-dependent activation of NF-κB in oxidative stress signaling. Mol. Cell. Biol. 24, 2614–2626

30. Chen, J., Lu, G., and Wang, Q. J. (2005) Protein kinase C-independent effects of protein kinase D3 in glucose transport in L6 myotubes. Mol. Pharmacol. 67, 152–162

31. Tang, H., Zhao, Z. J., Landon, E., and Inagami, T. (2000) Regulation of calcium-sensitive tyrosine kinase Pyk2 by angiotensin II in endothelial cells. Roles of Yes tyrosine kinase and tyrosine phosphatase SHP-2. J. Biol. Chem. 275, 8393–8396

32. Cozens, A. L., Yeze, M. J., Kunzelmann, K., Ohru, T., Chin, L., Eng, K., Finkbeiner, W. E., Widdicombe, J. H., and Gruenert, D. C. (1994) CFTR expression and chloride secretion in polarized immortal human bronchial
epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **10**, 38–47

41. Lavalle, C. R., Bravo-Altamirano, K., Giridhar, K. V., Chen, J., Sharlow, E., Lazo, J. S., Wipf, P., and Wang, Q. J. (2010) Novel protein kinase D inhibitors cause potent arrest in prostate cancer cell growth and motility. *BMC Chem. Biol.* **10**, 5

42. Döppler, H., Storz, P., Li, J., Comb, M. J., and Toker, A. (2005) A phosphorylation state-specific antibody recognizes Hsp27, a novel substrate of protein kinase D. *J. Biol. Chem.* **280**, 15013–15019

43. Gschwendt, M., Dieterich, S., Rennecke, J., Kittstein, W., Mueller, H. J., and Johannes, F. J. (1996) Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase c isoenzymes. *FEBS Lett.* **392**, 77–80

44. Ha, C. H., Jhun, B. S., Kao, H. Y., and Jin, Z. G. (2008) VEGF stimulates HDAC7 phosphorylation and cytoplasmic accumulation modulating matrix metalloproteinase expression and angiogenesis. *Arterioscler. Thromb. Vasc. Biol.* **28**, 1782–1788

45. Park, J. E., Kim, Y. I., and Yi, A. K. (2008) Protein kinase D1. A new component in TLR9 signaling. *J. Immunol.* **181**, 2044–2055

46. Tobey, N. A., Argote, C. M., Hosseini, S. S., and Orlando, R. C. (2004) Calcium-switch technique and junctional permeability in native rabbit esophageal epithelium. *Am. J. Physiol. Gastrointest. Liver Physiol.* **286**, G1042–G1049

47. Pause, A., Belsham, G. J., Gingras, A. C., Donzé, O., Lin, T. A., Lawrence, J. C., Jr., and Sonenberg, N. (1994) Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. *Nature* **371**, 762–767

48. Takahashi, S., Iwamoto, N., Sasaki, H., Ohashi, M., Oda, Y., Tsukita, S., and Furuse, M. (2009) The E3 ubiquitin ligase LNX1p80 promotes the removal of claudins from tight junctions in MDCK cells. *J. Cell Sci.* **122**, 985–994

49. Cummins, P. M. (2012) Occludin. One protein, many forms. *Mol. Cell. Biol.* **32**, 242–250

50. Inai, T., Kobayashi, J., and Shibata, Y. (1999) Claudin-1 contributes to the epithelial barrier function in MDCK cells. *Eur. J. Cell Biol.* **78**, 849–855

51. Banan, A., Zhang, L. J., Shaikh, M., Fields, J. Z., Choudhary, S., Forsyth, C. B., Farhadi, A., and Keshavarzian, A. (2005) θ Isoform of protein kinase C alters barrier function in intestinal epithelium through modulation of distinct claudin isotypes. A novel mechanism for regulation of permeability. *J. Pharmacol. Exp. Ther.* **313**, 962–982

52. Bhat, A. A., Sharma, A., Pope, J., Krishnan, M., Washington, M. K., Singh, A. B., and Dhawan, P. (2012) Caudal homeobox protein Cdx-2 cooperates with Wnt pathway to regulate claudin-1 expression in colon cancer cells. *PLoS ONE* **7**, e37174

53. Martínez-Estrada, O. M., Cullerés, A., Soriano, F. X., Peinado, H., Bolós, V., Martínez, F. O., Reina, M., Cano, A., Fabre, M., and Vilaró, S. (2006) The transcription factors Slug and Snail act as repressors of claudin-1 expression in epithelial cells. *Biochem. J.* **394**, 449–457

54. Harten, S. K., Shukla, D., Barod, R., Hergovich, A., Balda, M. S., Matter, K., Esteban, M. A., and Maxwell, P. H. (2009) Regulation of renal epithelial tight junctions by the von Hippel-Lindau tumor suppressor gene involves occludin and claudin 1 and is independent of E-cadherin. *Mol. Biol. Cell* **20**, 1089–1101

55. Bastea, L. I., Döppler, H., Balogun, B., and Storz, P. (2012) Protein kinase D1 maintains the epithelial phenotype by inducing a DNA-bound, inactive SNAI1 transcriptional repressor complex. *PLoS ONE* **7**, e30459

56. Miwa, D., Sakaue, T., Inoue, H., Takemori, N., Kurokawa, M., Fukuda, S., Omi, K., Goishi, K., and Higashiyama, S. (2013) Protein kinase D2 and heat shock protein 90 β are required for BCL6-associated zinc finger protein mRNA stabilization induced by vascular endothelial growth factor-A. *Angiogenesis* **16**, 675–688

57. Kluger, M. S., Clark, P. R., Tellides, G., Gerke, V., and Pober, J. S. (2013) Claudin-5 controls intercellular barriers of human dermal microvascular but not human umbilical vein endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **33**, 489–500

58. Deissler, H. L., Deissler, H., Lang, G. K., and Lang, G. E. (2013) VEGF but not PlGF disturbs the barrier of retinal endothelial cells. *Exp. Eye Res.* **115**, 162–171

59. Rezaee, F., DeSando, S. A., Ivanov, A. I., Chapman, T. J., Knowlden, S. A., Beck, L. A., and Georas, S. N. (2013) Sustained protein kinase D activation mediates respiratory syncytial virus-induced airway barrier disruption. *J. Virol.* **87**, 11088–11095