Claudin-3 and claudin-19 partially restore native phenotype to ARPE-19 cells via effects on tight junctions and gene expression

Shaomin Peng^{1,2,3,*}, Shao-Bin Wang^{2,3,*}, Deepti Singh^{2,3}, Peter Y.C. Zhao^{2,3}, Katherine Davis^{2,3},
Bo Chen³, Ron A. Adelman³, Lawrence J Rizzolo^{2,3,†}

¹Aier School of Ophthalmology, Central South University, Changsha, China

²Dept. of Surgery, Yale University School of Medicine, New Haven, CT, 06520

³Dept. of Ophthalmology, Yale University School of Medicine, New Haven, CT, 06520

*The first two authors contributed equally to this work.

Running title: Claudins restore RPE phenotype

†Corresponding author: Lawrence J Rizzolo
Yale University School of Medicine, Dept of Surgery
PO Box 208062
New Haven, CT 06520-8062
203 785-6277 (o)
203 785-5155 (FAX)
Email: lawrence.rizzolo@yale.edu

Abbreviations

Abstract:

Mutations of claudin-19 cause severe ocular deficits that are not easily reconciled with its role in regulating the outer blood retinal barrier. ARPE-19 is a widely used culture model of the retinal pigment epithelium (RPE). ARPE-19 is unique among epithelial cell lines, because it expresses all tight junction proteins except claudin family members. ARPE-19 also loses aspects of the RPE phenotype with cell passage. This study asks whether exogenous expression of the main RPE claudins, claudin-3 and claudin-19, would restore RPE phenotype, and whether these claudins have distinct roles in RPE. An Ussing chamber was used to measure the transepithelial electrical resistance and transepithelial electrical potential. These measurements were used to estimate the permeability co-efficients of ions. The transepithelial diffusion of polyethylene glycols were used to examine the leak pathway of tight junctions. Wound-healing, quantitative RT-PCR and immunoblotting examined diverse aspects of the RPE phenotype. Over-expression of either claudin decreased the permeability of small ions and polyethylene glycol. Both claudins were slightly cation-specific, but claudin-3 was less permeable to large solutes. Claudin expression widely affected gene expression to partially restore RPE phenotype. Claudins redistributed filamentous actin from stress fibers to circumferential bands associated with tight junctions, and made wound-healing more epithelial-like. Both claudins increased the expression of genes related to RPE core functions and increased steady-state levels of phosphorylated-AKT. In conclusion, claudin-3 and claudin-19 formed general permeability barriers and affected cell morphology, proliferation, migration, AKT signaling, and gene expression. When claudins are exogenously expressed, ARPE-19 more closely model native RPE.

Keywords: Claudin; Tight Junction; AKT; Retinal Pigment Epithelium; Outer blood-retinal barrier; wound-healing

1. Introduction

The ARPE-19 cell line was derived from a 19-year-old man (Dunn et al., 1996; Dunn et al., 1998). A Pubmed search shows it is widely used among vision researchers as a model of RPE, and therefore it is important to understand the strengths and weakness of the model. ARPE-19 expresses many RPE-specific genes and can exhibit RPE-like cell polarity (Cai and Del Priore, 2006; Deora et al., 2004; Dunn et al., 1998; Strunnikova et al., 2010). Low-passage cultures of ARPE-19 exhibited leaky tight junctions that have a transepithelial electrical resistance (TER) of $\sim 50\text{-}100\ \Omega \times \text{cm}^2$ (Dunn et al., 1996). The epithelial phenotype diminishes with cell passage, the TER decreases, and only rudimentary tight junctions form (Luo et al., 2006). ARPE-19 are available from the American Type Culture Collection (Manassas, VA), but the epithelial phenotype can be maintained for only 3 or 4 passages provided the cells are passaged at high density and maintained in low serum medium. High passage ARPE-19 continues to express many tight junction proteins, such as zonula occludens-1 (ZO-1) and occludin, but only a minority of cells express detectable claudin. Claudin-19, the major claudin of human RPE is undetected. Consequently, even under culture conditions that favor differentiation, the tight junctions of high-passage ARPE-19 are only marginally functional. Several labs published culture conditions that enhance differentiation of ARPE-19. However, the effectiveness of the procedures appears to depend on the history of how cultures were passaged and maintained before the cultures were treated. In any event, these procedures do not augment claudin expression (Ahmado et al., 2011; Luo et al., 2006). Mutation of claudin-19 has pleiotropic effects in human eyes that include: macular colobomata, myopia, and horizontal nystagmus (Konrad et al., 2006). Accordingly, ARPE-19 is an intriguing cell line that provides epithelial cell biologists and retinal specialists with a unique opportunity. As the rare cell that expresses

most tight junction proteins except claudins, the barrier properties of individual claudins can be explored in an essentially claudin-null background. For the retinal specialist, ARPE-19 provides an opportunity to explore functions of claudin-19, aside from barrier function.

Claudins are a family of proteins that lend specificity to tight junctions (Günzel and Yu, 2013). The complex of tight junctions encircles the cells of an epithelial monolayer and binds the tight junctional complex of neighboring cells. The result is a semi-permeable, semi-selective seal that retards transmonolayer diffusion of solutes through the spaces between the cells.

Permeability and selectivity vary among epithelia according to the function of the tissue in which they reside. The diversity of functions results from the subset of claudins that are expressed.

Research on claudins has focused on the role of the extracellular domains in determining selectivity. Besides variability in the amino acid sequence of the extracellular domains, there is variability in the sequence of the intracellular, C-terminal domain. The C-terminal domain is a frequent target for raising claudin-specific antibodies. Although the extreme C-terminus of all claudins has a binding motif for the PDZ1 domains of ZO-1, it is unknown whether the cytoplasmic domains might play a role in signal transduction. Recent studies suggest claudins may participate in signaling networks (González-Mariscal et al., 2014; Pope et al., 2014; Shang et al., 2012; Suh et al., 2013).

The retinal pigment epithelium (RPE) lies between a fenestrated capillary bed in the choroid and the photoreceptors of the retina. Because this region is known as the outer retina, the RPE forms the outer blood-retinal barrier (Rizzolo et al., 2011). In human RPE, all cells in the monolayer express claudin-19 and claudin-3, but the monolayer is heterogeneous (Peng et al.,

2012; Peng et al., 2011). A smattering of other claudins are expressed in subsets of cells. RNA knockdown by siRNA demonstrates that claudin-19 is the major contributor to the barrier properties of human RPE. In humans, claudin-19 is found only in the eye and kidney, but in kidney claudin-19 must dimerize with claudin-16 to reach the cell surface (Hou et al., 2009; Hou et al., 2008). Expressed alone in MDCK or LLC-PK1 cells, claudin-19 reaches the cell surface without claudin-16. Conceivably, claudin-19 binds to a different, endogenous claudin, but in RPE it is unclear what that partner might be. A knock-down of claudin-3 failed to affect the localization of claudin-19 and most cells in the RPE monolayer don't express another claudin in large quantity (Peng et al., 2011).

This study used adenoviral vectors to exogenously express claudin-3 and claudin-19. The receptor for adenovirus is found in the apical membrane of RPE rather than the apical junctional complex (Diaz et al., 2009). Consequently, we could use this vector system to transduce RPE without affecting tight junctions. Along with an increase in functionality of the tight junctions, expression of either claudin had pleiotropic effects on ARPE-19 and partially restored an RPE phenotype that had been lost with cell passage.

2. Materials and Methods:

2.1. Cell Culture

ARPE-19 cells were obtained from the American Type Culture Collection (Manassas, VA). To confirm the identity of the cells used in these experiments, cells from older passages were returned to the American Type Culture-Collection for STR analysis. A 100% identity match with ARPE-19 was confirmed. In further verification of the strain used throughout the study, we used quantitative RT-PCR arrays with primers specific for human genes to generate a gene expression profile based on RPE signature genes (Strunnikova et al., 2010). In that study and ours, expression of signature genes in ARPE-19 was compared to that of primary cultures of human fetal RPE (hfRPE). The subset of signature genes that we used is shown in Fig. 1B. Additionally, cultures were immunostained to verify that the human antigen Tra-1-85 was expressed in 100% of the cells. Cultures were used from passages 24-40 and maintained in tissue culture flasks in DMEM (Life Technologies, Grand Island, NY) containing 2% heat-inactivated fetal calf serum (FBS; Atlanta Biologicals, Norcross, GA), 1:100 (v/v) glutamine penicillin-streptomycin, 1:100 (v/v) sodium pyruvate (Life Technologies, Grand Island, NY), 1:100 (v/v) ITS+ Premix Universal culture supplements (BD Biosciences, Bedford, MA). The cells were harvested and initially were reseeded, 1.5×10^5 cells per well, onto Transwell or Snapwell culture inserts (1.12 cm² growth area, 0.4 μ m pores; Corning, Inc., Corning, NY) with 10% FBS. After cultures became confluent (2-4 days), they were returned to maintenance medium and transduced with adenoviral vectors. Adenoviral vectors that expressed either GFP, claudin-3 or claudin-19 were obtained from SignaGen Laboratories (Rockville, MD), and used according to the company's protocol. Briefly, newly confluent cultures of ARPE-19 were transduced using viral

stocks were diluted in media to 1×10^7 infectious units/ml. 0.5 ml was added to the apical medium chamber for a multiplicity of infection ≈ 10 . The culture medium was changed the next day and medium was changed every other day thereafter. Experiments were performed between the first and second week post-transduction.

2.2. Measurement of TER and Ion Selectivity

TER was measured using an EVOM resistance meter with EndOhm electrodes (World Precision Instruments, Sarasota, FL) and Ussing chambers (Physiologic Instruments, San Diego, CA), as described previously (Peng et al., 2012). Briefly, cultures maintained on Snapwell filters were mounted in Ussing chambers and incubated in a modified Ringer's solution to inhibit membrane transport. The solution contained 150 mM NaCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 3 mM BaCl_2 , 10 mM glucose, and 10 mM HEPES, pH 7.4 at 37°C, and continuously bubbled with compressed air. The osmolality was balanced with mannitol. BaCl_2 was added to reduce diffusion through K^+ channels. Silver chloride electrodes with 3 M KCl agar bridges were used for the current producing and voltage sensing electrodes, and were controlled by a model VCC MC6 voltage/current clamp and ACQUIRE & ANALYZE Revision II software (Physiologic Instruments, San Diego, CA). The transepithelial electrical potential (TEP) was referenced to the basolateral chamber. For ion selectivity and permeability, dilution and bi-ionic electrical potentials were examined by replacing the solution in the basolateral chamber. For dilution potentials, the NaCl in the basolateral was reduced to 75 mM NaCl. For bi-ionic potentials, the NaCl was replaced with 150 mM KCl. The relative ionic permeabilities of the monolayers were calculated using the Goldman-Hodgkin-Katz equation. The individual permeation coefficients

for Na^+ (P_{Na}), Cl^- (P_{Cl}) and K^+ (P_{K}) were estimated by the method of Kimizuka and Koketsu (Kimizuka and Koketsu, 1964).

2.3. Paracellular Flux of Methylpolyethylene Glycol (mPEG)

The cultures were pre-incubated with 10 mM NH_4Cl for 30 minutes. The mPEG (average molecular weight, 550 D or 350 D; MP Biomedicals, Solon, OH) was added to the basal medium chamber to a final concentration of 50 $\mu\text{g/mL}$. The cultures were incubated for 1.5 hours at 37°C in a humidified chamber with 5% CO_2 and the medium from the apical medium chamber collected for analysis. The concentration of mPEG was determined using a rabbit monoclonal ELISA kit (PEG ELISA Kit; Epitomics, Burlingame, CA) according to the manufacturer's protocol. The apparent permeation coefficient, P_{app} , was estimated as described previously (Peng et al., 2011; Van Itallie et al., 2008), P_{app} for mPEG550 or mPEG350 will be referenced as P_{550} or P_{350} , respectively.

2.4. Quantitative Reverse Transcriptase–Polymerase Chain Reaction (qRT²-PCR)

Two methods were used. qRT²-PCR was performed as described previously (Peng et al., 2013). Briefly, 2 μg of total RNA was reverse-transcribed to cDNA (Quantitect Reverse Transcription Kit; Qiagen). Real-time RT-PCR was performed (iTaQ Universal SYBR® Green SuperMix and Bio-Rad CFX 96 thermal cycler; Bio-Rad, Hercules, CA) following the manufacturer's suggested protocol. Experiments were performed in triplicate with a minimum of two biological repeats. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control to normalize the data. Relative expression of mRNA was calculated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The primers are listed in Table S1.

A custom made qRT²-PCR array was prepared by (Bio-Rad). The array included controls, house-keeping genes for normalization, and two sets of RPE related genes that are detailed in Fig. 1B.

2.5. Protein Electrophoresis and Immunoblotting

ARPE-19 cultures were solubilized and prepared for immunoblotting, as described (Peng et al., 2011). To normalize samples α -tubulin was used as an internal standard. Primary antibodies are listed in Table S2. Immunoblots were developed using ECL plus chemiluminescence reagent (Amersham Life Science, Arlington Heights, IL) followed by imaging and quantification using a Molecular Imager ChemiDoc XRS System. Images are representative of three or more experiments.

2.6. Immunofluorescence and Confocal Microscopy

The subcellular distribution of the claudins, ZO-1, and occludin was determined by indirect immunofluorescence as described (Peng et al., 2011). Briefly, Cultures were fixed at 4°C in two changes of 100% ethanol (30 minutes total). The primary antibodies listed in Table S2, were used followed by incubation with ML-grade secondary antibodies conjugated with Cy2, Cy3, or Cy5 dyes (Jackson ImmunoResearch Laboratories, West Grove, PA). Alexa Fluor 488 phalloidin (Invitrogen) was used to label F-actin, and DAPI (4,6-diamidino-2-phenylindole) was used to label the nucleus. Fluorescence images were acquired with an LSM 410 spinning-disc confocal microscope and processed using Axiovision or Zen software (Carl Zeiss, Inc, Thornwood, NY). Images are representative of three or more experiments.

2.7. Wound healing assay

TER became stable 1-2 weeks post infection with adenoviral vectors that encoded GFP, claudin-3, or claudin-19. Then, a single linear wound was created with a sterile 10- μ l pipette tip. The wound was 517 ± 37 μ m wide. After rinsing with culture medium to remove cell debris, cells continued to be maintained as above. Culture continued until the control cells repopulated the wound. In some experiments, the following agents were added during the wound-healing phase: Human epidermal growth factor (EGF 10 ng/ml, MP Biomedicals, LLC. Solon, OH); EGF plus an EGFR inhibitor (AG1478, 250-1000 nM, Cell Signaling Technology, Inc. (LY294002, 1000ng/ml, Cayman Chemical Company, Ann Arbor, Michigan); EGF plus an inhibitor of ERK1/2 activation by MEK (UO126, up to 50 μ M Promega Corporation, Madison, WI); or EGF plus an inhibitor of AKT1/2 (A6730, 10.0 μ M, Sigma-Aldrich). Cell proliferation and migration were followed using DAPI and antibodies to the proliferation marker, Ki67 protein (Thermo Fisher Scientific, Waltham, MA). Images were quantified by counting nuclei found within ± 200 μ m of the center of the wound and expressed as nuclei/100,000 μ m². Cells were counted within a field that was 400 $\times$ 300 μ m. Six fields from three experiments were counted and averaged.

2.8. Statistics

P-values were calculated from normally distributed data sets using a two-tailed Student's t-test. The number of independent experiments is indicated in the figure legends. Error bars represent the s.e.m.

3. Results:

The growth medium used in these experiments fostered the differentiation of ARPE-19, as measured by gene expression and TER (Luo et al., 2006). Differentiation required 8-12 weeks post-confluence, but the experiments reported in this study were performed within two weeks after the cultures became confluent. Accordingly, the TER of control cultures was $< 20 \Omega \times \text{cm}^2$ (Fig. 1A). Overexpression of either claudin-3 or claudin-19 increased the TER to $\sim 60 \Omega \times \text{cm}^2$. Control vector that expressed GFP had no effect. This TER was greater than the $\sim 40 \Omega \times \text{cm}^2$ that the control cultures obtained only after 6-12 weeks, but less than the $\sim 200 \Omega \times \text{cm}^2$ observed for native RPE (Quinn and Miller, 1992).

Gene expression in ARPE-19 during this early culture period was compared to human fetal RPE (hfRPE) using a quantitative real time RT-PCR (qRT²-PCR) array that included a subset of signature genes (Strunnikova et al., 2010) and genes related to RPE maturation (Peng et al., 2013). Notably, several mRNAs were barely detectable in ARPE-19 (30 cycles of PCR or more under our protocol): *CDLN-19*, *FRZB*, *SFRP5*, and *TTR* (Fig. 1B). Several other genes were also down-regulated $> 4\times$ relative to hfRPE: *BEST1*, *DCT*, *RBPI*. Several transport genes were up-regulated $> 4\times$.

3.1. Claudin-3 and Claudin-19 exhibited similar, but distinct roles in RPE tight junctions.

Control cultures were transduced with a viral vector that encoded green fluorescent protein (GFP) and test cultures with vectors that encoded claudin-3 or claudin-19. The expression of GFP had no effect on the behavior of ARPE19 in any experiment of this study.

Exogenous gene expression of claudin-3 or claudin-19 had little effect on the expression of other claudins or occludin. Overexpression of claudin-3 increased the expression of its mRNA by nearly 1000× with a corresponding increase in the expression of claudin-3 protein (Fig. 2). Nominally, overexpression of claudin-19 increased the expression of its mRNA by 5 orders of magnitude, but note that the endogenous mRNA was barely detected. A corresponding increase in claudin-19 was also observed on immunoblots.

Both claudin-3 and claudin-19 co-localized with N-cadherin, occludin, and ZO-1. Unlike endogenous claudins that are evident in only a few cells (Luo et al., 2006), the exogenous claudins were expressed in >90% of the cells. Examples are presented in Fig. 2. Exogenous claudin-3 was detected only in the apical junctional complex. Some claudin-19 was evident in the apical junctional complex, but a large fraction was also found in a peri-nuclear compartment. Co-expression of claudin-3 and claudin-19 did not have an additive effect on the TER. Neither did co-expression affect the subcellular localization of either claudin (data not shown).

Two methods were used to explore the properties of the tight junctions formed by claudin-3 or claudin-19. An electrophysiology approach was used to measure the dilution potential for sodium chloride and the bi-ionic potential for Na^+ and K^+ . Under the conditions of the experiment major transcellular transport mechanisms were inhibited (see methods). From these measurements, we estimated the permeation coefficients for Na^+ , K^+ , and Cl^- across the tight junctions (Fig. 3A,B). Claudin-3 and claudin-19 decreased the permeability of the three ions to similar extents. For control cells, the permeation ratios, $P_{\text{Na}}/P_{\text{Cl}}$ and $P_{\text{K}}/P_{\text{Cl}}$ were equivalent to permeation across the filter, 0.77 and 0.95, respectively (Peng et al., 2012). Both

claudin-3 and claudin-19, appeared to be slightly cation selective. There was little if any difference between P_K/P_{Na} and permeation across the filter (1.24).

The second approach revealed a difference between claudin-3 and claudin-19 regarding the selectivity for large, nonionic solutes. The permeation coefficient was estimated for large and small polymers of methylated polyethylene glycol (mPEG). Most of the polymers of mPEG₃₅₀ are small enough to cross tight junctional strands (Peng et al., 2011; Van Itallie et al., 2008). Most of the polymers of mPEG₅₅₀ are so large they require strands to break and reseal as the polymers move across the anastomosing network of strands. Both claudins decrease the permeability of mPEG regardless of size. As would be expected from the size difference of mPEGs and the lack of functional tight junctions in ARPE19, the diffusion coefficient for mPEG₃₅₀ was greater than that of mPEG₅₅₀ (Fig. 3 C,D). For claudin-19 cultures, mPEG₃₅₀/mPEG₅₅₀ was approximately the same as in control cultures, which indicates there is no preference for small or large solutes. For claudin-3 cultures, this ratio increased, which indicates that claudin-3 tight junctions selected against larger solutes.

Claudin expression accelerated the shift in location of filamentous actin from stress fibers to the circumferential bands typical of epithelia. As noted in the introduction, this process typically takes 6-8 weeks in media that foster differentiation (Luo et al., 2006). Like uninfected cultures (not shown), the virus control exhibits ZO-1 at the apical junctional complex, but filamentous actin was mostly in stress fibers. Only a few days post-transduction with *CLDN3* or *CLDN19*, the filamentous actin relocated from stress fibers to circumferential bands that co-

localized with ZO-1 (Fig. 4). Claudin-3 was more effective than claudin-19 in shifting the distribution of actin.

3.2. Claudins affected gene expression and cell migration.

Of commonly studied RPE markers and junction-associated genes, exogenous *CLDN* expression upregulated *BEST1* and downregulated *EGFR* and *cMYC*, as measured by separate qRT²-PCR reactions (Fig. 5A). Gene expression does not always correspond to steady state levels of protein, as illustrated by BEST1 and EGFR. Despite a 5-10× increase in *BEST1* mRNA, immunoblots showed no increase the steady-state level of its translated product, bestrophin (Fig. 5B).

Accompanying the modest decrease in *EGFR* mRNA was a decreased steady-state level of EGFR. The qRT²-PCR array described in Fig. 1B was used to simultaneously examine the effects exogenous *CLDN* expression on a broader spectrum of genes. Several signature genes and one maturation gene were up-regulated more than 10× (Fig. 5C). Where there was overlap, the methodologies for Fig. 5A and Fig. 5C yielded similar results to confirm the validity of the PCR arrays. Six of the 12 genes that were under-expressed in ARPE-19 (Fig. 2) were upregulated when claudin was expressed. The up-regulation of the *WNT* signaling pathway genes, *FRZB* and *SRBP5*, suggest that gene targets of *WNT* signaling might also be regulated.

Expression of exogenous CLDN had little effect on the mRNAs for gap, tight, and adhesion junctions, as determined using a second qRT²-PCR array (Fig. 5D, Table S3).

Tight junctions, WNT signaling, and EGFR modulate cell proliferation and wound healing. A scratch assay was used to study the effects of exogenous *CLDN* expression on wound-healing (Fig. 6A). Two-week post-confluent cultures were scratched with a pipette tip on Day 0.

As revealed by Ki-67, proliferating cells were readily detected in control cultures, but not in cultures expressing either claudin. Within four days, the wound in control cultures was covered with Ki-67 positive cells. In claudin-3 and claudin-19 cultures, Ki-67 cells became evident within the wound, but the wound remained open. Both the density of cells within the wound and the percentage of Ki-67 positive cells was lower in claudin-expressing cultures (Supplemental Fig. 1). Higher magnification images of the wound margin include the fluorescence channel for actin filaments (Fig. 6B). At day four, claudin-expressing cells that lie outside the wound-margins retained circumferential bands of actin and a polygonal shape. As most clearly observed for claudin-3, cells in the wound were of two morphologies: 1) spread cells that expressed Ki-67, but not actin stress fibers; and 2) elongated cells that were Ki-67 negative with actin observed primarily in stress fibers. Claudin-expressing cells within the wound had a more spread morphology than control cells.

The decreased rate of cell proliferation could be due to the reduced amount of the EGFR in claudin-expressing cells or suppression of an EGF-independent signal transduction pathway. The culture medium contains 2% serum, which would include EGF and other growth factors. To eliminate these confounding factors, cultures were incubated in a serum free medium overnight, and wound-healing was assessed after 2.5 days (Fig. 7,8). There was no discernable difference between claudin-expressing cultures and the GFP control. When EGF was added at the beginning of the experiment, wound-healing was accelerated in the GFP cultures, and only 2-3 days were required to close the wound. There was little effect on the claudin-expressing cultures. In GFP cultures, the effect of EGF was substantially reduced by two inhibitors of EGFR kinase activity, AG1478 and PD153035, and an inhibitor of the AKT arm of the EGF signaling pathway.

After 2.5 days in the presence of EGF, claudin-expressing cells invaded the wound, but the cell density was low compared to control cells. Inhibitors of EGFR kinase had a small inhibitory effect on wound-healing by claudin-expressing cultures, but inhibition of AKT phosphorylation blocked wound-healing to the same extent as observed for GFP cultures.

To corroborate this finding, EGF pathways were examined in more detail using immunoblotting. There are three arms of the EGF signal transduction pathway that include either: AKT, ERK1/2, or STAT3. In normal culture media, expression of claudin increased the phosphorylation of AKT without affecting the phosphorylation of EGFR, ERK1/2, or STAT3 (Fig. 9A). When serum-starved cells were treated with EGF, there was a notable effect on control cells. AKT was phosphorylated and this could be reduced to baseline by inhibitors of EGFR. There was no discernable effect on ERK1/2 or STAT3. Surprisingly, AKT was phosphorylated in claudin-expressing cells, even in the absence of EGF. Further, EGF did not increase phosphorylation and EGFR inhibitors did not decrease phosphorylation. Expression of claudin lacked an effect on the phosphorylation of ERK1/2 or STAT3. Consistent with this finding, wound-healing was unaffected by an inhibitor of ERK1/2 phosphorylation by the MEK inhibitor, UO126, except at high concentrations that would also affect AKT (data not shown).

Activated AKT1/2 can be inhibited by a compound developed by Merck (Kenilworth, NJ) and marketed by Sigma-Aldrich (A6730). Unlike inhibitors of EGFR or the ERK or STAT3 arms of the EGF signaling pathway, direct inhibition of AKT1/2 retarded wound healing in control and claudin-expressing cultures (Figs. 7,8). These data suggest that claudins affect ARPE-19 via an EGF-independent, AKT pathway.

4. Discussion

The transcriptome of ARPE-19 has been reported by several labs (Cai and Del Priore, 2006; Sharma et al., 2005; Strunnikova et al., 2010). Comparisons between laboratories is complicated by the use of different passages of ARPE-19 and different culture conditions (Luo et al., 2006). Nonetheless, the uniform conclusion was that the transcriptome of ARPE-19 differed substantially from primary cultures of RPE. The expression of *CLDN19* in ARPE-19 is not reported in any of these studies. Using a subset of the RPE signature genes (Strunnikova et al., 2010), and maturation genes (Peng et al., 2013), the current study confirmed that many RPE genes are present, but under-expressed, in ARPE-19. One study used culture conditions that were similar to ours in terms of passage number, low-serum medium, and newly confluent cells (Ablonczy et al., 2011). Those authors used assays of function to conclude that ARPE-19 resembled aged or pathologic RPE.

4.1. Claudin permeability and selectivity.

Three-weeks post confluence, the low TER of ARPE-19 indicates a lack of functional tight junctions. Exogenous expression of claudins established functional tight junctions within days. Claudin-3 and Claudin-19 are considered to be barrier rather than pore proteins, which means that their addition to existing tight junctions raises TER rather than decrease it (Günzel and Yu, 2013). The ARPE-19 model allowed us to confirm this impression by expressing these claudins in a claudin-null background that included most other tight junction proteins. The decreased ion permeability and modest cation selectivity indicates a barrier was formed, but selectivity was too low for claudin-3 and claudin-19 to be considered cation-specific claudins (Yu et al., 2009).

Selectivity based on size involves a second mechanism. Early studies revealed independent regulation of TER and the permeability of non-ionic tracers (Balda et al., 1996; Ban and Rizzolo, 2000; Peng et al., 2011; Van Itallie et al., 2008; Watson et al., 2001). These findings suggested a “pore” and “leak” model of tight junction permeability (Liang and Weber, 2014; Rizzolo, 2007; Van Itallie and Anderson, 2006). The pores are thought to have a size limit of Stoke’s radius $\sim 4\text{\AA}$. Because they are found all along tight junctional strands, they have a high capacity and discriminate according to size and charge. The polydisperse mixtures of mPEG used in this report have a Stoke’s radius that is predominantly below (mPEG₃₅₀), or above (mPEG₅₅₀), this cutoff. The “leak” pathway has a low capacity and does not discriminate among solutes. It is thought to involve transient breaks in the tight junctional strands, or channels formed where three cells meet. Whereas the pore path is thought to be regulated by claudins, the leak pathway is less well understood, but involves occludin, ZO-1, and tricellin. The current study indicates that claudin-3 also affects the leak pathway. Perhaps claudin-3 exerts its effect by affecting how occludin and ZO-1 are organized in the junctional complex to reduce strand-breaks. Mechanism aside, this finding offers an explanation for why RPE would express two different barrier proteins. Claudin-19 forms the major barrier. When it is knocked down by siRNA the TER of RPE is very low, whereas knockdown of claudin-3 had no effect (Peng et al., 2011). The presence of claudin-3 may modulate the capacity of the “leak” pathway in native RPE.

A limitation of this study was that only a fraction of claudin-19 was expressed in tight junctions with the remainder residing in a peri-nuclear compartment. The reason for this mislocalization is suggested by studies of claudin-19 in the kidney. In kidney, claudin-19 binds

claudin-16 and does not reach the cell surface if the expression of claudin-16 is abrogated (Hou et al., 2009; Hou et al., 2008), Claudin-16 is not expressed in native RPE (Peng et al., 2011) or ARPE-19 cells. Surprisingly, in cultures of cell lines derived from kidney that lack claudin-16, MDCK and LLC- PK1, exogenous claudin-19 is transported to tight junctions (Hou et al., 2006), It is unclear how many claudins are expressed in these lines, or what a possible binding partner might be. In our ARPE-19 cultures, there is no evidence for any claudin that is uniformly expressed or in sufficient quantity to explain our data. Co-expression of claudin-3 and claudin-19 demonstrated that claudin-3 could not fulfill this role. Claudin-19 expressing ARPE-19 cells would provide means to test putative binding partners for their ability to localize claudin-19 to the tight junction.

4.2. Restoration of the RPE phenotype.

Claudin-3 and claudin-19 partially restored a native RPE phenotype to ARPE-19. Tight junctions are part of the apical junctional complex, which regulates gene expression, cell morphology, and cell proliferation (Caplan et al., 2008; Cereijido et al., 2008; Collinet and Lecuit, 2013; Farkas et al., 2012; Mellman and Nelson, 2008; Steed et al., 2010), Recent studies suggest claudins contribute to this regulation (González-Mariscal et al., 2014; Pope et al., 2014; Shang et al., 2012; Suh et al., 2013). It is unclear whether claudin-3 or claudin-19 regulated signaling pathways for gene expression and protein turnover directly, or indirectly by reorganizing the apical junctional complex. Better than occludin, claudins form the extensive anastomosing network of strands that is the skeleton of tight junctions. By reorganizing pre-existing tight junctional proteins into a functional barrier and signaling complex, claudins might indirectly affect gene expression and AKT signaling.

Exogenous claudins fundamentally changed wound-healing by ARPE-19. Cells engaged in the cell cycle were readily apparent in confluent cultures of control ARPE-19, but rarely detected when exogenous claudin was expressed. Cell proliferation was rapidly activated in control ARPE-19, and was principally responsible for closing the wound. When claudin was expressed, wound healing more closely followed the slow process exhibited by other epithelia (Matsubayashi et al., 2011; Rørth, 2009). Cells attempted to close the wound by cell spreading. Actin redistributed into long cables of stress fibers that were oriented perpendicular to the edge of the wound, and in the direction of cell migration. Cell proliferation was evident, but was not the sole mechanism for closing the wound.

EGF stimulates cell proliferation and migration in various tissues, and EGF accelerated wound healing in ARPE-19 by activating the AKT arm of the EGF signaling pathway (Paul et al., 2013). A similar finding was reported earlier for ARPE-19 (Zhang et al., 2013). An alternate mechanism for wound-healing became evident when claudin-3 or claudin-19 were expressed. AKT was constitutively activated, even though the vast majority of the cells were not near the wound edge and were quiescent. The role of phosphorylated-AKT in quiescent cells remains to be determined, as does the mechanism that regulates whether or not activated AKT induces the cell-cycle. These data imply that claudins activated additional signaling pathways that modulated the effects of activated AKT proximal and distal to the wound edge to restore epithelial-like wound-healing properties to ARPE-19. This is an area of active investigation in our laboratory.

This study demonstrates how two claudins with similar effects on the paracellular diffusion of ions can have different effects on the paracellular diffusion of large solutes. Together, they fine-tune the barrier function of RPE. Other claudins expressed by native RPE appear in only subsets of cells (Peng et al., 2011). It would be valuable to learn whether these “minor” claudins are expressed in distinct regions of the RPE monolayer and how they might fine tune barrier properties. Directly or indirectly, claudin-3 and claudin-19 activate AKT, which is involved in many cellular processes. The pleiotropic effects of claudin-19 may help explain the extensive developmental eye defects suffered by patients with mutations in claudin-19 (Konrad et al., 2006). Ongoing experiments are exploring which effects of claudin can be attributed to AKT or not. AKT might prove to be a therapeutic target for preventing the dedifferentiation of RPE that occurs in proliferative vitreoretinopathy (Fisher et al., 2005). Exogenous expression of claudin provides a means to improve the ARPE-19 culture model to more closely resemble native tissue. Further, claudins reduce the time required for cultures to mature from months to days post-confluence.

Acknowledgements

The authors thank Maryam Ghassi-Nejad and Laurel Tainsh for critically reviewing the manuscript. This work was supported by grants from the Connecticut Regenerative Medicine Research Fund, 14-SCB-YALE-18 (LJR); Department of Defense W81XWH-15-1-0029 (LJR); Alonzo Family Fund (LJR); National Natural Science Foundation of China, 81570867 (SP); Science Research Foundation of Aier Eye Hospital Group. AF151D06 (SP); Newman's Own Foundation (RAA); and Leir Foundation (RAA); Research to Prevent Blindness (Yale University).

References

Ablonczy, Z., Dahrouj, M., Tang, P.H., Liu, Y., Sambamurti, K., Marmorstein, A.D., Crosson, C.E., 2011. Human Retinal Pigment Epithelium Cells as Functional Models for the RPE In Vivo. *Invest. Ophthalmol. Vis. Sci.* 52, 8614-8620.

Ahmado, A., Carr, A.-J., Vugler, A.A., Semo, M.a., Gias, C., Lawrence, J.M., Chen, L.L., Chen, F.K., Turowski, P., da Cruz, L., Coffey, P.J., 2011. Induction of Differentiation by Pyruvate and DMEM in the Human Retinal Pigment Epithelium Cell Line ARPE-19. *Invest. Ophthalmol. Vis. Sci.* 52, 7148-7159.

Balda, M.S., Whitney, J.A., Flores, C., González, S., Cereijido, M., Matter, K., 1996. Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. *J. Cell Biol.* 134, 1031-1049.

Ban, Y., Rizzolo, L.J., 2000. Differential regulation of tight junction permeability during development of the retinal pigment epithelium. *Am. J. Physiol.* 279, C744-C750.

Cai, H., Del Priore, L.V., 2006. Gene expression profile of cultured adult compared to immortalized human RPE. *Molecular vision* 12, 1-14.

Caplan, M.J., Seo-Mayer, P., Zhang, L., 2008. Epithelial junctions and polarity: complexes and kinases. *Current Opinion in Nephrology and Hypertension* 17, 506-512.

Cereijido, M., Contreras, R.G., Shoshani, L., Flores-Benitez, D., Larre, I., 2008. Tight junction and polarity interaction in the transporting epithelial phenotype. *Biochim. Biophys. Acta* 1778, 770-793.

Collinet, C., Lecuit, T., 2013. Chapter Two - Stability and Dynamics of Cell–Cell Junctions, in: Frans van, R. (Ed.), *Progress in Molecular Biology and Translational Science*. Academic Press, pp. 25-47.

Deora, A.A., Gravotta, D., Kreitzer, G., Hu, J., Bok, D., Rodriguez-Boulan, E., 2004. The basolateral targeting signal of CD147 (EMMPRIN) consists of a single leucine and is not recognized by retinal pigment epithelium. *Mol. Biol. Cell* 15, 4148-4165.

Diaz, F., Gravotta, D., Deora, A., Schreiner, R., Schoggins, J., Falck-Pedersen, E., Rodriguez-Boulan, E., 2009. Clathrin adaptor AP1B controls adenovirus infectivity of epithelial cells. *Proc. Natl. Acad. Sci. U S A* 106, 11143-11148.

Dunn, K.C., Aotaki-Keen, A.E., Putkey, F.R., Hjelmeland, L.M., 1996. ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. *Exp Eye Res* 62, 155-169.

Dunn, K.C., Marmorstein, A.D., Bonilha, V.L., Rodriguez-Boulan, E., Giordano, F., Hjelmeland, L.M., 1998. Use of the ARPE-19 cell line as a model of RPE polarity: basolateral secretion of FGF5. *Investigative ophthalmology & visual science* 39, 2744-2749.

Farkas, A.E., Capaldo, C.T., Nusrat, A., 2012. Regulation of epithelial proliferation by tight junction proteins. *Ann N Y Acad Sci* 1258, 115-124.

Fisher, S.K., Lewis, G.P., Linberg, K.A., Verardo, M.R., 2005. Cellular remodeling in mammalian retina: results from studies of experimental retinal detachment. *Prog. Retin. Eye Res.* 24, 395-431.

González-Mariscal, L., Domínguez-Calderón, A., Raya-Sandino, A., Ortega-Olvera, J.M., Vargas-Sierra, O., Martínez-Revollar, G., 2014. Tight junctions and the regulation of gene expression. *Seminars in Cell & Developmental Biology* 36, 213-223.

Günzel, D., Yu, A.S.L., 2013. Claudins and the Modulation of Tight Junction Permeability. *Physiol. Rev.* 93, 525-569.

Hou, J., Gomes, A.S., Paul, D.L., Goodenough, D.A., 2006. Study of claudin function by RNA interference. *J. Biol. Chem.* 281, 36117-36123.

Hou, J., Renigunta, A., Gomes, A.S., Hou, M., Paul, D.L., Waldegger, S., Goodenough, D.A., 2009. Claudin-16 and claudin-19 interaction is required for their assembly into tight junctions

and for renal reabsorption of magnesium. *Proceedings of the National Academy of Sciences* 106, 15350-15355.

Hou, J., Renigunta, A., Konrad, M., Gomes, A.S., Schneeberger, E.E., Paul, D.L., Waldegger, S., Goodenough, D.A., 2008. Claudin-16 and claudin-19 interact and form a cation-selective tight junction complex. *J. Clin. Invest.* 118, 619-628.

Kimizuka, H., Koketsu, K., 1964. Ion transport through cell membrane. *J. Theor. Biol.* 6, 290-305.

Konrad, M., Schaller, A., Seelow, D., Pandey, A.V., Waldegger, S., Lesslauer, A., Vitzthum, H., Suzuki, Y., Luk, J.M., Becker, C., Schlingmann, K.P., Schmid, M., Rodriguez-Soriano, J., Ariceta, G., Cano, F., Enriquez, R., Juppner, H., Bakkaloglu, S.A., Hediger, M.A., Gallati, S., Neuhauss, S.C., Nurnberg, P., Weber, S., 2006. Mutations in the tight-junction gene claudin 19 (CLDN19) are associated with renal magnesium wasting, renal failure, and severe ocular involvement. *Am. J. Hum. Genet.* 79, 949-957.

Liang, G.H., Weber, C.R., 2014. Molecular aspects of tight junction barrier function. *Current Opinion in Pharmacology* 19, 84-89.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402-408.

Luo, Y., Zhuo, Y., Fukuhara, M., Rizzolo, L.J., 2006. Effects of culture conditions on heterogeneity and the apical junctional complex of the ARPE-19 cell line. *Invest. Ophthalmol. Vis. Sci.* 47, 3644-3655.

Matsubayashi, Y., Razzell, W., Martin, P., 2011. 'White wave' analysis of epithelial scratch wound healing reveals how cells mobilise back from the leading edge in a myosin-II-dependent fashion. *Journal of cell science* 124, 1017-1021.

Mellman, I., Nelson, W.J., 2008. Coordinated protein sorting, targeting and distribution in polarized cells. *Nat. Rev. Mol. Cell Biol.* 9, 833-845.

Paul, I., Bhattacharya, S., Chatterjee, A., Ghosh, M.K., 2013. Current Understanding on EGFR and Wnt/ β -Catenin Signaling in Glioma and Their Possible Crosstalk. *Genes & Cancer* 4, 427-446.

Peng, S., Gan, G., Qiu, C., Zhong, M., An, H., Adelman, R.A., Rizzolo, L.J., 2013. Engineering a blood-retinal barrier with human embryonic stem cell-derived retinal pigment epithelium: transcriptome and functional analysis. *Stem cells translational medicine* 2, 534-544.

Peng, S., Gan, G., Rao, V.S., Adelman, R.A., Rizzolo, L.J., 2012. Effects of proinflammatory cytokines on the claudin-19 rich tight junctions of human retinal pigment epithelium. *Invest. Ophthalmol. Vis. Sci.* 53, 5016-5028.

Peng, S., Rao, V.S., Adelman, R.A., Rizzolo, L.J., 2011. Claudin-19 and the barrier properties of the human retinal pigment epithelium. *Invest. Ophthalmol. Vis. Sci.* 52, 1392-1403.

Pope, J.L., Bhat, A.A., Sharma, A., Ahmad, R., Krishnan, M., Washington, M.K., Beauchamp, R.D., Singh, A.B., Dhawan, P., 2014. Claudin-1 regulates intestinal epithelial homeostasis through the modulation of Notch-signalling. *Gut* 63, 622-634.

Quinn, R.H., Miller, S.S., 1992. Ion transport mechanisms in native human retinal pigment epithelium. *Invest. Ophthalmol. Vis. Sci.* 33, 3513-3527.

Rizzolo, L.J., 2007. Development and role of tight junctions in the retinal pigment epithelium. *Int. Rev. Cytol.* 258, 195-234.

Rizzolo, L.J., Peng, S., Luo, Y., Xiao, W., 2011. Integration of tight junctions and claudins with the barrier functions of the retinal pigment epithelium. *Prog. Retin. Eye Res.* 30, 296-323.

Rørth, P., 2009. Collective Cell Migration. *Annual Review of Cell and Developmental Biology* 25, 407-429.

Shang, X., Lin, X., Alvarez, E., Manorek, G., Howell, S.B., 2012. Tight Junction Proteins Claudin-3 and Claudin-4 Control Tumor Growth and Metastases. *Neoplasia* 14, 974-IN922.

Sharma, R.K., Orr, W.E., Schmitt, A.D., Johnson, D.A., 2005. A functional profile of gene expression in ARPE-19 cells. *BMC Ophthalmol* 5, 25.

Steed, E., Balda, M.S., Matter, K., 2010. Dynamics and functions of tight junctions. *Trends in Cell Biology* 20, 142-149.

Strunnikova, N.V., Maminishkis, A., Barb, J.J., Wang, F., Zhi, C., Sergeev, Y., Chen, W., Edwards, A.O., Stambolian, D., Abecasis, G., Swaroop, A., Munson, P.J., Miller, S.S., 2010. Transcriptome analysis and molecular signature of human retinal pigment epithelium. *Hum. Mol. Genet.* 19, 2468-2486.

Suh, Y., Yoon, C.H., Kim, R.K., Lim, E.J., Oh, Y.S., Hwang, S.G., An, S., Yoon, G., Gye, M.C., Yi, J.M., Kim, M.J., Lee, S.J., 2013. Claudin-1 induces epithelial-mesenchymal transition through activation of the c-Abl-ERK signaling pathway in human liver cells. *Oncogene* 32, 4873-4882.

Van Itallie, C.M., Anderson, J.M., 2006. Claudins and Epithelial Paracellular Transport. *Annu. Rev. Physiol.* 68, 403-429.

Van Itallie, C.M., Holmes, J., Bridges, A., Gookin, J.L., Coccaro, M.R., Proctor, W., Colegio, O.R., Anderson, J.M., 2008. The density of small tight junction pores varies among cell types and is increased by expression of claudin-2. *J. Cell Sci.* 121, 298-305.

Watson, C.J., Rowland, M., Warhurst, G., 2001. Functional modeling of tight junctions in intestinal cell monolayers using polyethylene glycol oligomers. *Am J Physiol Cell Physiol* 281, C388-397.

Yu, A.S., Cheng, M.H., Angelow, S., Gunzel, D., Kanzawa, S.A., Schneeberger, E.E., Fromm, M., Coalson, R.D., 2009. Molecular basis for cation selectivity in claudin-2-based paracellular pores: identification of an electrostatic interaction site. *J Gen Physiol* 133, 111-127.

Zhang, L., Wang, F., Jiang, Y., Xu, S., Lu, F., Wang, W., Sun, X., Sun, X., 2013. Migration of retinal pigment epithelial cells is EGFR/PI3K/AKT dependent. *Front. Biosci. (Schol Ed)* 5, 661-671.

Figures and Legends

Figure 1. ARPE-19 exhibits a TER near baseline and has low expression of key RPE mRNAs. A) Control cultures exhibited little evidence of functional tight junctions, but exogenous expression of either claudin-3 or claudin-19 rapidly increased TER. B) ARPE-19 exhibited substantial differences from hfRPE in the expression mRNA of key RPE mRNAs. ARPE-19 and hfRPE

were assayed using qRT²-PCR arrays. Dashed lines indicate a 4× difference from hfRPE. Note the inverted x-axis. (A&B) Quantification: mean±s.e.m., n=5.

Figure 2. Exogenous claudins localized to the apical junctional complex, but did not affect the expression of other tight junctional genes that are expressed by native RPE. A) Claudin-3: qRT²-PCR compared the expression of each mRNA relative to the expression of that mRNA by control cultures of ARPE19. Only the expression of claudin-3 mRNA increased post-transduction, but other claudin genes and occludin were unaffected. B) Claudin-19: qRT²-PCR compared the

expression of each mRNA relative to the expression of that mRNA by control cultures. Only the expression of claudin-19 mRNA increased post-transduction, but other claudin genes and occludin were unaffected. Note that claudin-19 mRNA was barely detected in control cells. C) Immunoblots show a large increase in the expression of claudin-3, post-transduction. D) Immunoblots show a large increase in the expression of claudin-19, post-transduction, but other claudin genes and occludin were unaffected E) Immunofluorescence revealed that claudin-3 (red) was detected in few cells transduced with empty vector (control). Exogenous claudin-3 localized in apical junctional complexes (arrowheads in merged images) revealed by N-Cadherin (green). F) Indirect immunofluorescence revealed that claudin-19 (red) was undetected in cells transduced with empty vector (control). Exogenous claudin-19 localized primarily in internal perinuclear structures (arrows. It was detected in apical junctional complexes (arrowheads in merged images) revealed by ZO-1 and occludin (green). Nuclei were revealed in the merged image (blue). The apical (ap) and basal (ba) orientation of the xz images are indicated by double arrowheads. Quantification: mean $\pm$ s.e.m., n=3. Bar, 10 μ m

Figure 3. Claudin 3 and claudin 19 had similar, but distinct, effects on permeability. (A and B) Permeation coefficients were estimated from bi-ionic and dilution potentials under conditions that inhibited membrane transport. Claudin-3 and claudin 19 decreased the permeability of small ions and made tight junctions slightly cation-specific. Differences between claudin-3 and claudin-19 were slight. (C and D) The apparent permeability coefficients were estimated for polydisperse mixtures of mPEG that averaged 350 or 550 Daltons. Claudin-3 and claudin-19 decreased the permeability of non-ionic solutes, but claudin-3 discriminated against larger solutes. Quantification: mean $\pm$ s.e.m., n=6. *p<0.001; **p<0.05

Figure 4. Exogenous claudin reorganizes the actin cytoskeleton. Freshly confluent monolayers of ARPE19 were infected with empty viral vector or adenovirus harboring claudin-3 or claudin-19. The cultures were imaged 3 days later after staining with phalloidin (green) to reveal filamentous actin and anti-occludin or anti-ZO-1 (red). In controls actin co-localized with tight junction proteins to form orange dots (arrowheads) apical to lateral membranes that were labeled by phalloidin alone. Additionally, actin filaments were found throughout the cytoplasm (arrows). When claudin-3 was expressed, filamentous actin was detected only in tight junctions and lateral membranes. Claudin-19 also rearranged actin, but it was less effective than claudin-3. Bar, 10 μm

Figure 5. Exogenous expression of claudin-3 or claudin-19 affected gene expression. A) Common RPE marker and junction associated genes by standard qRT²-PCR techniques. Significant increases were observed for Best 1 mRNA, an RPE-marker gene. Significant decreases were observed for proliferation-related mRNAs EGFR and c-MYC. B) Immunoblots revealed a decrease in the steady-state level of the EGF receptor, but the steady-state level of bestrophin and TRPC4 was unaffected. Similar results were obtained for cMYC (not shown). C and D) Custom-made qRT²-PCR arrays were used to examine genes important for RPE

differentiation and maturation (C) or junctional complexes (D). Actin and GAPDH were used to normalize the data. C) Exogenous CLDN expression upregulated the expression of 6 of the 12 genes that were under-expressed by ARPE-19 (see Fig. 1B). D) Exogenous CLDN expression had minimal effect on the expression of genes for tight, adherens, gap, and integrin-based junctions. See the supplemental Table S3 for the estimated expression of each gene relative to housekeeping genes. Note that expression $< 10^{-5}$ was at or below the limits of detection.

Quantification: mean $\pm$ s.e.m., n=3.

Figure 6. Exogenous CLDN expression reduced cell proliferation, migration and wound healing. Newly confluent cultures were infected with the viral vector indicated at the top of each column. When the TER of all the cultures were stable (1-2 weeks) the cultures were scratched with a pipette tip (Day 0). The cultures were stained to reveal nuclei of proliferating (purple) and non-proliferating cells (blue). Phalloidin was used to reveal filamentous actin (green). (A) Low magnification (Actin not shown): On Day 0, proliferating cells were evident only in the virus control cultures. The cells were maintained in medium containing 2% FBS. On Day 4, proliferating cells were numerous and the wound was nearly closed. In the claudin-expressing cells, there were fewer proliferating cells and the wounds were still open. High magnification (Actin channel included): In the GFP-control cultures, filamentous actin commonly appeared in

stress fibers even at Day 0. Stress fibers were absent for CLDN3 cultures and greatly reduced in CLDN19 cultures on Day 0, but became evident in cells close to the wound edge on Day 4. On Day 4, claudin-expressing cells were more spread than in control cells indicating that cell spreading contributed to the partial healing of the wound when claudin was expressed. Arrowheads, circumferential bands of filamentous actin at cell-cell junctions. Arrows, filamentous actin in stress fibers. Bar (A), 100 μm ; Bar (B), 25 μm

Figure 7. EGF-stimulated wound healing was reduced in claudin-expressing cells. Cells were cultured as described in the legend to Fig. 6, except cells were switched to a serum-free medium the night before the start of the experiment. Control cultures (-EGF) receive no additions. Test cultures received EGF alone or with an inhibitor. EGF-accelerated wound healing in cells transduced with an irrelevant gene, but not when transduced by CLDN3 or CLDN19. The effect of EGF was reduced by two classes of EGFR inhibitor. The slow wound-healing in the claudin expressing cells was slightly affected by EGFR inhibitors. In contrast, direct inhibition of AKT1/2 phosphorylation (iAKT) inhibited wound-healing in control and claudin expressing cultures. Inhibitors of MEK, or STAT3 had little or no effect (not shown). Bar, 100 μ m

Figure 8. Wound-healing in the presence of CLDN3 and CLDN19 is independent of EGF stimulation and involves an AKT signaling pathway. Cells were cultured in serum-free medium as described in the legend to Fig. 7 and quantified as described in Materials and Methods. EGF failed to simulate wound-healing in claudin expressing cells. Nonetheless inhibitors of EGFR kinase (AG1478 and PD153053) slightly reduced wound-healing those cultures. An inhibitor of AKT phosphorylation (iAKT) reduced wound-healing to similar levels in all cultures. Bars indicate significant differences with the GFP cultures ($p < 0.02$). Quantification, mean $\pm$ s.e.m., $n=6$ fields from 3 experiments.

Figure 9. Exogenous expression of CLDN3 and CLDN19 increase the phosphorylation of AKT independent of EGF. A) In unstimulated cells, exogenous expression of claudin resulted in the phosphorylation of AKT, but not EGFR, ERK, or STAT3. B) Cultures were placed in serum-free medium overnight and incubated with EGF in the presence or absence of the EGFR inhibitors, AG1378 and PD153053. EGF induced the phosphorylation of AKT in control ARPE-19 that expressed GFP, and phosphorylation could be prevented by inhibitors of EGFR. ERK 1/2 and STAT3 were unaffected. In claudin-expressing cells, EGF failed to increase the phosphorylation of AKT and inhibitors of EGFR failed to decrease phosphorylation.