A connected tale of claudins from the renal duct to the sensory system

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Claudins are tight junction membrane proteins that regulate paracellular permeability to ions and solutes in many physiological systems. The electric property of claudin is the most interesting and pertains to two important organ functions: the renal and sensorineural functions. The kidney comprises of three major segments of epithelial tubules with different paracellular permeabilities: the proximal tubule (PT), the thick ascending limb of Henle's loop (TALH) and the collecting duct (CD). Claudins act as ion channels allowing selective permeation of Na⁺ in the PT, Ca²⁺ and Mg²⁺ in the TALH and Cl⁻ in the CD. The inner ear, on the other hand, expresses claudins as a barrier to block K⁺ permeation between endolymph and perilymph. The permeability properties of claudins in different organs can be attributed to claudin interaction within the cell membrane and between neighboring cells. The first extracellular loop of claudins contains determinants of paracellular ionic permeability. While analogous to transmembrane ion channels in many ways, the biophysical and biochemical properties of claudin based paracellular channels remain to be fully characterized.

Introduction

A fundamental question in cell biology is how cells transport ions in highly complex tissue structures such as the kidney tubule, the gastrointestinal tract, the lung alveolus and the organ of Corti of the inner ear. The cells lining these ductal structures coordinate with each other to acquire the apicobasal polarity through the formation of a key cellular organelle—the tight junction (TJ) that separates the extracellular space into different compartments. The tight junction is responsible for the barrier to movement of ions and molecules between these compartments. The TJ in thin section electron microscopy is composed of a series of direct membrane contacts. Freeze-fracture electron microscopy reveals the membrane protein interactions at the tight junction as a branching and anastomosing reticulum of “fibrils” or “strands” on the P fracture face. These fibrils have been demonstrated to be partly composed of integral membrane proteins directly involved in cell-cell interaction. Deep-etch microscopy and subcellular fractionation expose a matrix of densely packed globular proteins on the cytoplasmic surfaces of the junctional membranes that are detergent resistant and sandwiched between the membrane surface and the actin cytoskeleton.

The known integral membrane proteins of the tight junction include occludin (a 65 kDa membrane protein bearing four transmembrane domains and two uncharged extracellular loops), the Junctional Adhesion Molecules (JAMs), a four-member group of glycosylated proteins and the claudins. A remarkably complex submembrane scaffold protein complex such as ZO-1 interacts with cytoplasmic portions of the integral proteins.

Claudins are 21–28-kDa proteins consisting of a large family of at least 26 members in humans that were first identified by Furuse et al. in 1998. Topological analyses revealed a common structure in claudins made of four transmembrane domains, two extracellular loops, amino- and C-terminal cytoplasmic domains and a short cytoplasmic turn. The first extracellular loop (ECL1) of claudin consists of -50 amino acids with charged residues that contribute to paracellular ion selectivity. The second extracellular loop (ECL2) consists of -25 amino acids with a predicted helix-turn-helix motif that mediate trans claudin interactions and claudin interactions with the Clostridium perfringens enterotoxin (CPE). The C-terminal domain of claudin contains a PDZ (postsynaptic density 95/discs large/zonula occludens-1)-binding motif (YV) that is critical for interaction with the submembrane scaffold protein ZO-1 and intracellular trafficking.

Claudin mutations have serious consequences, consistent with its primary role in ion homeostasis. Claudin-1 deficient mice die within one day of birth and show a loss of the water barrier of skin. Claudin-2 knockout mice lose salt through the kidney, accompanied by hypercalciuria and polyuria. Targeted deletion of claudin-5, which is predominantly expressed in vascular endothelia, results in a selective increase in the blood-brain barrier to small molecules. Knockout of claudin-11 results in male infertility and severe demyelination in the central nervous system, consistent with its function to maintain proper ion balance in Sertoli tight junctions and at the Nodes of Ranvier. Mutations in claudin-14 cause nonsyndromic recessive deafness DFNB29, ostensibly due to a failure in ion balance in the organ of Corti. Mutations in claudin-16 have been associated with human FHHNC syndrome (familial hypomagnesemia with
Numerous recordings have led to an important conclusion: the permeability of an ion across the tight junction is significantly different from its free-water mobility. The paracellular transport is not a simple diffusion but requires interaction and facilitation from proteins in the tight junction. Claudin is the primary factor underlying the conductance process. The best example is claudin-2. Amasheh et al. showed that ectopic expression of claudin-2 in high-resistance MDCK I cells increased paracellular conductance by over 20-fold. The tight junction also demonstrates selectivity allowing permeation of only a small number of ions. The paracellular ion selectivity clearly depends on claudins. For example, overexpression of claudin-16 in anion selective LLC-PK1 cells reversed the tight junction selectivity to cation. The structural basis for paracellular ion selectivity is encoded in the ECL1 of claudins. Through a series of chimera studies, Colegio et al. showed that claudin-4 adopted the ion selectivity of claudin-2 when the ECL1 domains of claudin-2 and -4 were swapped. Yu et al. proposed a single-pore model to explain the observed ion selectivity of claudin-2, in which the pore is a cylinder with conical vestibules and charged side chains from ECL1 positioned facing into the lumen and electrostatically interacting with permeating ions. While Yu’s model well explained the cation over anion selectivity of claudin-2, emphasizing a role of the effective charge in its extracellular domain, the paracellular conductance appeared not solely dependent upon the extracellular charge. In addition to ion permeability and selectivity, claudins demonstrate pH dependence, thermodynamics and anomalous mole fraction effects similar to conventional ion channels, which led to a new concept of claudins forming the paracellular channel, a novel class of channels oriented perpendicular to the membrane plane and joining two extracellular compartments.

The existence of paracellular channels in the tight junction has long been debated, largely due to the lack of recording technology that allows definitive separation paracellular from transcellular conductance. Conductance scanning, first introduced by the access resistance to result in a measured change in potential on the pipette that depends on the conductance of the pore in the membrane. PE: pipette electrode, RE: reference electrode and WE: working electrode.

**Electric Properties of Claudin**

The electric properties of claudin are defined by its ability to alter the ion permeability and selectivity of the tight junction. Measurement of paracellular permeability using cell membrane impermeable tracers indicates that there are 7–8 Å diameter size-selective pores in the tight junction that allow passage of small charged or uncharged solutes. Most inorganic ions are permeable through the tight junction, including major extracellular ions: Na⁺, K⁺, Cl⁻, Ca²⁺ and Mg²⁺. In a non-selective epithelium, the paracellular conductance represents the overall permeability of tight junction to all ions present in the extracellular space. The conductance of tight junction (G_TJ) is the reciprocal of its resistance (R_TJ) that can be determined using a direct current (DC) circuit according to Ohm’s law (Fig. 1A). A more accurate measurement takes cell membrane conductance into account by using an alternating current (AC) circuit (Fig. 1B). An alternating current (I) with an angular frequency (ω) generates an oscillating potential (E) across the tight junction with the same frequency but different phase. The impedance (Z_TJ), deriving from E/I and its reciprocal (1/Z_TJ) reflect tight junction conductance when ω approaches zero (Fig. 1C). The structural basis for paracellular ion selectivity is encoded in the ECL1 of claudins. Through a series of chimera studies, Colegio et al. showed that claudin-4 adopted the ion selectivity of claudin-2 when the ECL1 domains of claudin-2 and -4 were swapped. Yu et al. proposed a single-pore model to explain the observed ion selectivity of claudin-2, in which the pore is a cylinder with conical vestibules and charged side chains from ECL1 positioned facing into the lumen and electrostatically interacting with permeating ions. While Yu’s model well explained the cation over anion selectivity of claudin-2, emphasizing a role of the effective charge in its extracellular domain, the paracellular conductance appeared not solely dependent upon the extracellular charge. In addition to ion permeability and selectivity, claudins demonstrate pH dependence, thermodynamics and anomalous mole fraction effects similar to conventional ion channels, which led to a new concept of claudins forming the paracellular channel, a novel class of channels oriented perpendicular to the membrane plane and joining two extracellular compartments.
by Frömter in 1972, is based on the analysis of local differences in current density above the surface of an epithelial monolayer, allowing quantitative determination of the size and distribution of local conductivity.\(^{27}\) The limiting factor is spatial resolution. Earlier efforts had to rely upon optic lens to position the recording probe that neither provided sufficient lateral resolution nor allowed precise control of probe-surface distance.\(^{28,29}\) A breakthrough was made by Chen et al. in 2013 by using scanning ion conductance microscopy (SICM) to record the paracellular conductance in MDCK cells.\(^{30}\) SICM makes use of a "virtual seal" to monitor the access resistance of the gap between the probe and surface (\(R_{\text{acc}}\)) (Fig. 1D).\(^{31}\) \(R_{\text{acc}}\) in the PE-RE circuit can be recorded to draw a topographic image of the surface and position the probe in a fixed distance above surface. When the pipette resistance (\(R_{\text{pipette}}\)), access resistance (\(R_{\text{acc}}\)) and resistance of a conducting pore (\(R_{\text{pore}}\)) in a membrane are of comparable scale (order of magnitude), the conductance of the pore (\(G_{\text{pore}} = 1/R_{\text{pore}}\)) can be separated through a circuit PE-WE. In MDCK cell monolayers, Chen et al. recorded local conductance over an area within nominal radii of 265 nm at discrete locations: cell junction (for paracellular pathway) and cell center (for transcellular pathway).\(^{30}\) The recorded paracellular conductance differed significantly from the transcellular conductance in size and distribution. Deletion of claudin-2 gene abolished the paracellular conductance without affecting the transcellular conductance. Further improvements to increase the spatial resolution to below 10 nm will allow capture of single paracellular channel conductance, open probability, ion selectivity and most importantly the structure-function relationship.

**Claudin Interaction Plays More than an Adhesion Role**

Claudins associate by *cis* interactions within the plasma membrane of the same cell into dimers or higher oligomeric states\(^{32,33}\) and by *trans* interactions between claudins in adjacent cells. Additional *cis* interactions likely assemble claudin oligomers into tight junction strands. The *cis* interaction can involve a single type of claudin (homomeric interaction) or different types of claudins (heteromeric interaction); the *trans* interaction can occur in homotypic or heterotypic mode (Fig. 2).\(^{34}\) Earlier anatomical studies revealed the membrane protein interactions at the tight junction as a branching and anastomosing reticulum of "fibrils" or "strands" on the freeze-fracture face.\(^{3}\) These fibrils had been hypothesized to be partly composed of integral membrane proteins directly involved in cell-cell adhesion. Using the fibroblast cells that normally have no tight junction, Furuse et al. have demonstrated that ectopically introduced claudins were able to form well-elaborated strands at cell-cell junctions that resemble the tight junction strands.\(^{34}\) It was clear at that time that claudin *trans* interaction played a crucial adhesion role. Co-culture experiments revealed that *trans* interactions of claudin-1 and...
patients of the FHHNC syndrome, which caused claudin-16 mis-trafficking to the lysosome due to a selective loss of its binding affinity with ZO-1. In a more general study, Umeda et al. have knocked out both ZO-1 and ZO-2 in mammary epithelia and demonstrated their functional requirement for de novo TJ formation and claudin localization. The model of ZO-1 facilitated claudin localization was soon contested by Hou et al. using double claudin knockout animals. Despite normal distribution of ZO-1 and occludin in claudin-16 or -19 KO animal, loss of either claudin gene rendered mis-localization of both claudins on the protein level of the tight junction, suggesting a ZO-1 independent mechanism for claudin trafficking.

The Channel Theory of Claudin in the Kidney

The Kidney functions by initially excreting many salts and small molecules found in the blood, then selectively taking back those that need to be conserved while allowing others to be excreted in the urine.

The traditional view of the renal reabsorption process is that of a tandem array of ion channels and transporters located in the plasma membrane of tubular epithelial cell conducting ion transport in a coordinated manner at the expense of energy. While many mutations have been found in these transmembrane ion channels or transporters causing human diseases of electrolyte imbalances, there are growing numbers of cases caused by mutations in the claudin genes, pointing to a new mechanism by which the kidney utilizes the paracellular channel to conduct ion transport. It should also be emphasized that in addition to an independent transport route, the paracellular pathway provides a feedback mechanism to coordinate with the transcellular pathway and form a complete electric circuit.

There are segment-specific claudin expression profiles along the length of the nephron. Several studies using probes specific for claudin-1–19 mRNAs and proteins reveal that claudin-6,-9 and -13 are not detectable, and claudin-5 and -15 only in endothelial cells; the rest are specifically expressed in different segments of the nephron. While claudin-3, -10, -11, -14, -16 and -19 were observed in the thick ascending limb of Henle’s loop (TALH) and claudin-3, -4, -7 and -8 in the distal tubules and the collecting duct, claudin-2, -10 and -17 are highly expressed in the “leaky” proximal tubule consistent with its high cation permeability when expressed in MDCK cells. Claudin-4 and -8 are expressed primarily along the entire “tight” distal nephron and in inner medullary segments of the thin descending limbs of juxtamedullary nephrons. This pattern of expression is consistent with their role as paracellular cation barrier.

Immunofluorescence analysis has shown that claudin-7 is expressed in the TALH and the collecting ducts of porcine and rat kidneys, although another study described claudin-7 in the distal nephron as located primarily on the basolateral membrane. Claudin-14, -16 and -19 are expressed exclusively in the TALH where the tight junction has low resistance but high cation selectivity. In summary, while there are still some conflicting published data, claudin-2, -10 and -17 are expressed in proximal tubules, while claudin-3, -4, -7, -8, -10, -14, -16 and -19 are found in the distal nephron including
the TALH, the distal tubule and the collecting duct. It is also clear that the patterns of claudin expression along the nephron changes with development, with claudin-7 and -8 upregulated postnatally.49

A single-pore model in the proximal tubule

The proximal tubule of the kidney is a leaky epithelium that reabsorbs up to 60% of the filtered Na\(^+\) and Cl\(^-\) load as well as 60–65% of filtered Ca\(^{2+}\) and 25–30% of filtered Mg\(^{2+}\). Almost half of the NaCl is reabsorbed through the paracellular pathway.50 The driving force for paracellular reabsorption is not clear. Unlike the transcellular pathway that utilizes ion transporters in the membrane at the expense of energy, the paracellular pathway is passive and only depends upon the electrochemical gradient across the epithelium. A prevailing hypothesis is that paracellular reabsorption of NaCl is driven by the Cl\(^-\) electrochemical gradient that builds up owing to preferential reabsorption of HCO\(_3^-\) over Cl\(^-\).51 The early proximal tubule reabsors Na\(^+\) via the Na\(^+\)/H\(^+\) exchanger (NHE3) and HCO\(_3^-\) via the Na\(^+\)/HCO\(_3^-\) co-transporter (NBC). This makes the luminal fluid delivered to the mid to late proximal tubule low in HCO\(_3^-\) concentration but higher in Cl\(^-\) concentration compared with the levels in the plasma. Here, the lumen to basolateral Cl\(^-\) concentration gradient drives paracellular Cl\(^-\) reabsorption and in turn generates a lumen-positive potential.52 This electrical gradient then drives paracellular reabsorption of Na\(^+\) (Fig. 3A).

Claudin-2, which acts as a paracellular cation pore, is highly expressed in the proximal tubule. Knockout of claudin-2 decreases the paracellular cation selectivity of the proximal tubule, causing urinary loss of both Na\(^+\) and Cl\(^-\) when fed with high-salt diet.15 The molecular insight of claudin-2 channel permeability is provided by Yu et al.26 Using Brownian dynamics, Yu’s single-pore model suggests that the channel pore of claudin-2 is made of two protruding carboxyl groups on the residue aspartate-65 of ECL1 from two apposing claudin molecules in adjacent cells (Fig. 3B). The electrostatic field generated by the apposing carboxyl groups is predicted to be cylindrical and symmetrical. Ablating the charge on aspartate-65 by mutation to serine indeed abolished the cation selectivity of claudin-2 and reduced its permeability to Na\(^+\) (P\(_{Na}^\text{iso}\)). Yu’s model also predicts a single-pore conductance of 70 pS at physiological Na\(^+\) concentrations. Ca\(^{2+}\) permeability, on the other hand, is predicted to be low owing to its high activation energy barrier for dehydration. Paradoxically in claudin-2 knockout animals, urinary loss of Ca\(^{2+}\) is much more pronounced than Na\(^+\), suggesting claudin-2 channel is prone to divalent cation.15 In fact, extracellular Ca\(^{2+}\) competes with Na\(^+\) and inhibits Na\(^+\) permeability in transfected claudin-2 channels.53 It appears that aspartate 65 has higher than predicted charge density, thus favoring binding of Ca\(^{2+}\) over that of Na\(^+\). This hypothesis was soon corroborated by van Itallie et al. in a biochemical study demonstrating that claudin-2 forms a homodimer through cis-interaction of the second transmembrane domain.53

The molecule underlying paracellular Cl\(^-\) transport remains elusive. In addition to claudin-2, the proximal tubule also expresses claudins 10a,16 12,54 and 17.44 Claudin-10a and -17 can function as anion-selective pores in vitro and may be responsible for paracellular Cl\(^-\) reabsorption in the proximal tubule.44,54 In intestinal epithelium, claudin-12 interacts with claudin-2 and functions as a Ca\(^{2+}\)-selective pore.56 This claudin interaction may play a similar role in paracellular Ca\(^{2+}\) reabsorption in the proximal tubule.

An anti-porter model in the loop of Henle

The thick ascending limb of the loop of Henle (TALH) reabsorbs 25–40% of filtered Na\(^+\), 50–60% of filtered Mg\(^{2+}\) and 30–35% of filtered Ca\(^{2+}\). The TALH actively transports Na\(^+\) and Cl\(^-\) via the transcellular route and provides a paracellular pathway for reabsorption of Mg\(^{2+}\) and Ca\(^{2+}\).57,58 Na\(^+\), K\(^+\) and Cl\(^-\) enter the cell through the Na\(^+\)/K\(^+\)-2Cl\(^-\) cotransporter (NKCC2) in the luminal membrane. Na\(^+\) exits the cell through the Na\(^+\)/
K’-ATPase in exchange for K’ entry. K’ is secreted into the lumen through the renal outer medullary potassium channel (ROMK). Cl’ leaves the cell through a basolateral Cl’ channel (CIC-kb and barttin). Mg’’ and Ca’’ are passively reabsorbed through the paracellular channel, driven by a lumen-positive transepithelial voltage ($V_{tc}$) (Fig. 4A). The generation of this lumen-positive $V_{tc}$ can be attributed to two mechanisms: (a) the active-transport $V_{tc}$ due to apical K’ secretion and basolateral Cl’ exit and (b) the diffusion $V_{tc}$ generated because of the transepithelial NaCl concentration gradient and the cation selectivity of the paracellular channel in the TALH.99 Because the TALH expresses no aquaporin, continuous NaCl reabsorption generates a diluted luminal fluid that facilitates the generation of a NaCl concentration gradient from basolateral down to lumen. The backflux of Na’ through a cation selective paracellular channel generates a lumen-positive diffusion potential that drives Mg’’ and Ca’’ reabsorption. (B) An anti-porter model of claudin-16 channel that is made of two apposing hemi-channels each with five negatively charged residues in ECL1 protruding into the junctional space.

Figure 4. Ca’’ and Mg’’ transport mechanism in the thick ascending limb of Henle’s loop (TALH). (A) NaCl is reabsorbed through a concerted action of the Na’-K’-2Cl’ cotransporter (NKCC2), the renal outer medullary potassium channel (ROMK) and basolateral Cl’ channel (CIC-kb and barttin). Because water is not reabsorbed in the TALH, continuous NaCl reabsorption generates a diluted luminal fluid that facilitates the generation of a NaCl concentration gradient from basolateral down to lumen. The backflux of Na’ through a cation selective paracellular channel generates a lumen-positive diffusion potential that drives Mg’’ and Ca’’ reabsorption.

K’-ATPase in exchange for K’ entry. K’ is secreted into the lumen through the renal outer medullary potassium channel (ROMK). Cl’ leaves the cell through a basolateral Cl’ channel (CIC-kb and barttin). Mg’’ and Ca’’ are passively reabsorbed through the paracellular channel, driven by a lumen-positive transepithelial voltage ($V_{tc}$) (Fig. 4A). The generation of this lumen-positive $V_{tc}$ can be attributed to two mechanisms: (a) the active-transport $V_{tc}$ due to apical K’ secretion and basolateral Cl’ exit and (b) the diffusion $V_{tc}$ generated because of the transepithelial NaCl concentration gradient and the cation selectivity of the paracellular channel in the TALH.99 Because the TALH expresses no aquaporin, continuous NaCl reabsorption generates a diluted luminal fluid that facilitates the generation of a NaCl concentration gradient from basolateral down to lumen. The backflux of NaCl through a cation selective paracellular pathway generates a lumen-positive diffusion potential according to the Goldman-Hodgkin-Katz equation. This diffusion $V_{tc}$ is the major driving force of Mg’’ and Ca’’ reabsorption.

The molecular basis for TALH divalent cation reabsorption remained unknown until the discovery of mutations in claudin-16 and -19 as the cause of the rare autosomal recessive renal disorder FHHNC without ocular involvement (OMIM#248250)19 and with ocular involvement OMIM#248190,60 respectively. FHHNC is characterized by uncontrolled loss of serum Mg’’ and Ca’’ through the kidney. Renal immunohistological studies reveal that claudin-16 and claudin-19 are exclusively localized in the TALH.6 It was initially hypothesized that claudin-16 formed a selective paracellular Mg’’ and Ca’’ channel. Several studies tested this hypothesis in vitro but only came to ambiguous conclusion. The mild effect and peculiar unidirectional rectification of transfected claudin-16 in MDCK cells precluded definitive conclusion.61,62 Hou et al. transfected a model cell line, LLC-PK1, that has low endogenous paracellular cation permeability and found that claudin-16 profoundly increased Na’ permeability ($P_{Na}$) accompanied by only moderately enhanced Mg’’ permeability ($P_{Mg}$).24 $P_{Na}$ was greatly reduced or completely disappeared in all FHHNC disease mutants of claudin-16, suggesting that changes in $P_{Na}$ rather than changes in $P_{Ca}$ or $P_{Mg}$ are pathogenic for this disease.24 Claudin-16 deficient KD mice showed significantly reduced plasma Mg’’ levels, increased urinary excretion of Mg’’ and Ca’’ and nephrocalcinosis.63 This phenotype recapitulated that of FHHNC. Consistent with the hypothesis that claudin-16 acts primarily as a $P_{Na}$ pathway, when TALH segments were isolated and perfused ex vivo, the paracellular ion selectivity ($P_{Na}/P_{Cl}$) of the TALH was significantly reduced in claudin-16 deficient mice.65 The structural insight of claudin-16 function was provided by Hou et al.24 Unlike claudin-2 that is dependent upon one charged amino acid in ECL1 to permeate Na’, claudin-16 appears to require a string of negatively charged residues interspersed along ECL1 (D104S, D105S, E119T, D126S and E140T). Mutation of each of the five functionally important residues had a modest effect (11–33% reduction in $P_{Na}$), and combining the mutations generated additive effects.24 Because in the TALH reabsorption of Mg’’ and Ca’’ depends upon backflux of Na’ in order to generate the diffusion potential, claudin-16 must be able to permeate Mg’’ or Ca’’ with Na’ at the same time but in opposite direction (Fig. 4B). This anti-porter mode requires that Mg’’ or Ca’’ does not inhibit Na’ permeation by blocking the channel pore as in the case of claudin-2. Because of the size selectivity of claudin channel, it is less likely that the claudin-16 channel pore allows simultaneous occupation of two cations. Because Mg’’ and Ca’’ concentrations are far less than Na’ on both sides of the epithelia, an alternation mechanism allowing absorption of divalent cations will not affect the overall level of Na’ backflux or diffusion potential.

The other major cause of FHHNC is mutations in claudin-19. Hou et al. found that claudin-19 profoundly decreased Cl’ permeability ($P_{Cl}$) and functioned as a Cl’ barrier when expressed in LLC-PK1 cells.57 The FHHNC mutations from human patients either partially or completely abolished the claudin-19
effects on $P_{Cl^-}$. Coexpression of claudin-16 and claudin-19 in LLC-PK1 cells resulted in the simultaneous upregulation of $P_{Na^+}$ and downregulation of $P_{Cl^-}$ and hence in a large increase in $P_{Na^+}/P_{Cl^-}$, generating a highly cation-selective paracellular pathway. Mutations in claudin-19 allows backflux of Cl$^-$, which neutralizes the positive potential of the tubular lumen and eliminates the driving force for Mg$^{2+}$ and Ca$^{2+}$ reabsorption. In addition to demonstrating cooperativity between two different claudins, these data also suggest that, whereas some claudins form pores that allow preferential passage of specific ions, other claudins may contribute to barriers that prevent passage of specific ions. Claudin-19 KD animals phenocopy claudin-16 KD animals and develop the FHHNC manifestations of reduced plasma Mg$^{2+}$ levels and excessive renal wasting of Mg$^{2+}$ and Ca$^{2+}$. The phenotypic similarities of claudin-19 KD with claudin-16 KD can be explained by the cis heteromeric interaction between the two claudins. Several FHHNC mutations in claudin-16 and claudin-19 disrupted this interaction and abolished their cation selectivity when coexpressed in LLC-PK1 cells, suggesting a role for claudin interaction in the development of FHHNC.

A recent genome-wide association study (GWAS) identified claudin-14 as a major risk gene for hypercalciuric nephrolithiasis and bone mineral loss. Using several biochemical and genetic criteria, Gong et al. revealed predominant gene expression of claudin-14 in the TALH of the kidney. The aldosterone-sensitive distal nephron (ASDN) encompasses the distal convoluted tubule (CNT) and the collecting duct (CD). It is collectively responsible for the reabsorption of approximately 5% of filtered NaCl and plays a vital role in the regulation of extracellular fluid volume (ECFV) and blood pressure. The Na$^+$ reabsorption in the CNT and the CD is driven by apical entry through the epithelial Na$^+$ channel (ENaC) and by basolateral efflux through the Na’/K’-ATPase and in the principal cell. The unidirectional reabsorption of Na$^+$ generates a lumen-negative transepithelial potential ($V_\text{p}$) of $-25$ mV. The Cl$^-$ transport in the CNT and the CD depends on anion channels or transporters located in both principal cells and intercalated cells, dictated by two independent cellular mechanisms. The transcellular mechanism utilizes the Na’-driven Cl$^-$ bicarbonate exchanger (NDCBE; encoded by the Slc4a8 gene) in the apical membrane of β-intercalated cells to reabsorbs Na$^+$ and HCO$_3^-$ in exchange of Cl$^-$ secretion and the pendrin exchanger (encoded by the Slc26a4 gene) in the apical membrane of β-intercalated cells to take back Cl$^-$ in exchange of HCO$_3^-$ secretion. The net result is an equal quantity of Cl$^-$ and Na$^+$ reabsorbed with no effect on $V_\text{p}$. The paracellular mechanism depends upon the lumen-negative $V_\text{p}$ and a Cl$^-$ conductance in the tight junction (“chloride shunt”) (Fig. 5A).

In principle, the level of Cl$^-$ reabsorption must match that of Na$^+$ reabsorption so as to maintain luminal fluid electroneutrality, because the NDCBE–pendrin-mediated transcellular route carries no net charge. Conversely, defects in Cl$^-$ transport will increase the magnitude of $V_\text{p}$, depolarize the luminal membrane and subsequently inhibit ENaC.

The molecular mechanism of chloride shunt has been revealed by a systematic study of claudin function in the collecting duct. Several claudins, including claudin-3, -4, -7 and -8, are expressed in the collecting duct. Using RNA interference, Hou et al. found that loss of claudin-4 significantly decreased paracellular anion permeabilities, including P$_{Cl^-}$ in cultured collecting duct cells (M-1 and mIMCD3). Mutagenesis has identified a key amino acid—lysine-65 in ECL1 of claudin-4 required for Cl$^-$ permeability (Fig. 5B). Lysine-65 in claudin-4 is homologous to aspartate-65 in ECL1 of claudin-4 required for Cl$^-$ permeability (Fig. 5B). Lysine-65 in claudin-4 is homologous to aspartate-65 in claudin-2. This positively charged residue confers anion selectivity to claudin channel pore in a similar Brownian model. A direct support for claudin-4’s role in chloride shunt comes from its KO study. Constitutive knockout of claudin-4 resulted in higher urinary excretion of Cl$^-$ as well as Ca$^{2+}$. Increased urine volume, accompanied by urothelial hyperplasia, caused urinary tract obstruction and hydronephrosis as animals aged. Claudin-4 was found in the TJs of the ureter in addition to the DCT, the CNT and the CD. Despite the electrolyte imbalances, the morphology of TJ in urothelia and renal epithelium appeared to be normal. Claudin-8 also plays a potentially important role in collecting duct $P_{Cl^-}$. In both M-1 and mIMCD3 cells, claudin-8 KD decreased $P_{Cl^-}$ to a level similar to that in claudin-4 KD. Claudin-8 does not appear to make an individual channel to permeate Cl$^-$. Instead, it interacts with claudin-4 and recruits claudin-4 to the tight junction. Loss of claudin-4 in KO animals reduced the TJ localization level of claudin-8, providing in vivo support for their interaction.

To determine the role of claudin-3 and -7, Hou et al. knocked down their expression in collecting duct M-1 and mIMCD3

A chloride shunt in aldosterone sensitive distal nephron

The aldosterone-sensitive distal nephron (ASDN) encompasses the distal convoluted tubule (DCT), the connecting
is present in the tight junction of the collecting duct.\textsuperscript{73} The PHAII-related mutations in WNK4 increase paracellular Cl\textsuperscript{−} conductance in transfected MDCK cells, compatible with the original hypothesis that a gain-of-function in chloride shunt conductance causes PHAII.\textsuperscript{74} Nevertheless, transgenic animals expressing a gain-of-function mutation in WNK4 failed to show any increase in transepithelial Cl\textsuperscript{−} permeation in the collecting duct.\textsuperscript{75} In ex vivo perfused cortical collecting ducts, the tight junction was only slightly anion-selective (P\textsubscript{Cl}/P\textsubscript{Na}: 1.64 in presence of amiloride),\textsuperscript{75} not substantially different from the ratio of their free-water mobilities (1.54). WNK4 stimulation increased amiloride sensitive Na\textsuperscript{+} current but not the paracellular anion permselectivity,\textsuperscript{75} contrasting with the in vitro data.

**The Barrier Theory of Claudin in Sensory Systems**

In contrast with the kidney, the functions of sensory organs rely upon highly specialized signal transduction mechanisms employed by neurons and receptor cells, which require an independent environment well insulated from general circulation. Tight junctions fulfill such a role. The claudins made by the sensory system therefore must play a very different role from those in the kidney. Interestingly, some claudins play a conserved role from renal to sensory systems while others are unique to each organ. On the basis of whether their expression increases or decreases TER in epithelial recordings, claudins are classified as channel or barrier claudins. This loose categorization helps to separate the renal from neural claudins.

**A K\textsuperscript{+} barrier in the organ of Corti**

The cochlea of the inner ear has two compartments with different ionic composition (Fig. 6A). The perilymph of the scala vestibule and scala tympani is low in K\textsuperscript{+} concentration but high in Na\textsuperscript{+} concentration, similar to the cerebrospinal fluid.\textsuperscript{76} The endolymph of the scala media is unusually rich in K\textsuperscript{+} (~150 mM) and is held at a positive potential of +80 to +100 mV relative to the perilymph.\textsuperscript{77} The endocochlear K\textsuperscript{+} potential is critical for the depolarization of sensory hair cells in the organ of Corti. Hearing depends upon two types of mechanosensitive hair cells: inner hair cell (IHC) and outer hair cell (OHC). Both types of hair cells respond to movements of their apical hair bundles by modulation of cation influx through mechanosensitive channels. The depolarizing current across the apical membrane of hair cells is carried by K\textsuperscript{+}, instead of Na\textsuperscript{+}.\textsuperscript{77,78} K\textsuperscript{+} is then taken up by the apposing supporting cells, secreted into the perilymph of scala tympani and recycled back to the scala media across the stria vascularis. The enrichment of endocochlear K\textsuperscript{+} and the development of endocochlear potential are...
Claudin-11 is only found in the tight junction between basal cells of the stria vascularis. Two independent lines of claudin-11 KO animals revealed the same phenotypes. The tight junction completely disappeared from the basal cells; the endocochlear potential was less than 30 mV; and the hearing thresholds were elevated 50 dB across the entire frequency spectrum, despite a normal K⁺ concentration in the endolymph. There was no morphological abnormality in sensory hair cells or degeneration of hair cells. Normal K⁺ accumulation in endolymph suggests that (1) tight junctions in the basal cells are not required for barrier to K⁺ backflux; or (2) the uptake of K⁺ by the marginal cell is very efficient and not affected in absence of basal cell tight junction. The major source of endocochlear potential is the K⁺ diffusion potential across the apical membrane of intermediate cell through Kir4.1 (Fig. 6B). Loss of claudin-11 presumably creates a shunt between basal cells that allows current passage and

Figure 6. Structure of the cochlea and its tight junction. (A) Schematic presentation of the cochlea and extracellular fluid compartments. (B) Cellular model of the stria vascularis that generates the endocochlear potential (EP) through K⁺ secretion from perilymph to endolymph. The stria vascularis is a multilayered epithelium in which tight junctions between the marginal cells, which face directly the endolymph, as well as tight junctions between the basal cells isolate an intrastrial space that has a low K⁺ concentration. (C) Tight junctions are also found in the organ of Corti between hair cells and supporting cells and between supporting cells.
rapid dissipation of the apical potential. It is not known which ion carries the current. Recording of claudin-11 permeability has not been performed either in vitro in epithelial cell cultures or in vivo in the stria vasculare.

The first clinical evidence of claudin mutations in deafness comes from a linkage study of claudin-14 in nonsyndromic recessive deafness DFNB29. In two Pakistani consanguineous cohorts, two causal mutations (398delT and T254A) were found in the claudin-14 gene. The 398delT mutation causes a frameshift within the codon of Met133, which substitutes 23 incorrect amino acids and prematurely terminates translation 69 nucleotides later. The T254A mutation substitutes aspartic acid for valine (V85D). Valine 85 is a conserved site within the second transmembrane domain (TM2) of claudin-14 protein. Aspartic acid at position 85 is predicted to affect hydrophobicity and disrupt the secondary structure of TM2. Therefore, both 398delT and T254A are loss-of-function mutations. Claudin-14 gene expression in the inner ear was developmentally regulated. At P4, claudin-14 was found primarily in the IHC and OHC and to a lesser extent, in the supporting cells. By P8, claudin-14 expression was observed in IHC, OHC and supporting cells throughout the organ of Corti. In contrast, no expression of claudin-14 was found in the stria vascularis or the Reissner’s membrane. Claudin-14 KO mice have a normal endocochlear potential but are deaf due to degeneration of OHC. The tight junction between supporting cells and between supporting cells and hair cells are normal in absence of claudin-14, suggesting these TJs contain other redundant claudins. The K+ level in endolymph was not recorded for claudin-14 KO. In vitro, claudin-14 functions as a cation barrier, reducing $P_K$ and $P_{Na^+}$. One hypothesis of OHC degeneration is the diffusion of K+ via OHC tight junctions from the endolymph to the space of Nuel, which is normally filled with low K+ cortilymph. Long-term exposure of the basolateral membrane of OHC to high K+ levels is toxic and causes OHC depolarization and degeneration.

A recent large-scale mutagenesis has identified a novel claudin – claudin-9, mutation of which causes deafness (nmf329) in mice. Positional cloning revealed a missense mutation (F35L) in claudin-9 from the nmf329 strain. In vitro, heterologous expression of wildtype claudin-9 reduced $P_K$ and $P_{Na^+}$. One hypothesis of OHC degeneration is the diffusion of K+ via OHC tight junctions from the endolymph to the space of Nuel, which is normally filled with low K+ cortilymph. Long-term exposure of the basolateral membrane of OHC to high K+ levels is toxic and causes OHC depolarization and degeneration.

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Claudins and Human Diseases

Claudins can play direct causative roles in various human diseases. The best example is the claudin trio – claudin-14, -16 and -19. The loss-of-function mutations in claudin-14 are found in children with inherited deafness, owing to a K+ barrier defect in the organ of Corti. Its genetic locus is also linked to a common disease – kidney stone by a recent GWAS study. Using biochemical and biophysical analyses, Gong et al. have elegantly shown that claudin-14 interacts with and inhibits the claudin-16 and claudin-19 channel permeability, both genes found in a fatal inherited disease due to uncontrolled loss of serum Ca2+ and Mg2+. Claudins may be involved in the signaling pathways influenced by many disease conditions. For example, CaSR, the Ca2+ sensing receptor, has been recognized with pivotal importance in extracellular Ca2+ metabolism and causing familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism (NSHPT), two inherited conditions characterized by altered calcium homeostasis. The loss of CaSR in the kidney significantly reduces the gene expression level of claudin-14, hence upregulating claudin-16 and -19 permeability for urinary Ca2+ reabsorption. WNK4, the causative gene for pseudo-hypoaldosteronism type II, has been found to hyper-phosphorylate claudin-4 and concomitantly increase paracellular Cl- permeability in the collecting duct of the kidney.

Future Directions

(1) The single-channel conductance of a paracellular channel has never been recorded. With recent advances in SICM technology, paracellular conductance can be revealed at sub-micron scales. Further refinement in spatial resolution will allow capture of “discrete” paracellular channels along the tight junction strand.

(2) The oligomeric nature of paracellular channel is a major mystery. It is now well known that claudin function is dictated by its cellular background and combination with other claudin molecules. What is the basic functional unit of claudin along the tight junction strand? How is the tight junction strand organized from these claudin units? Recent advances in super-resolution microscopy may provide the much-needed resolution in claudin molecule localization and interaction.

(3) How does claudin switch between channel and barrier function? Because all claudins possess a similar extracellular loop (ECL1) that presumably dictates channel or barrier function, how is the ECL1 structurally arranged to achieve such a diverse function? The single-pore theory lacks the complexity to be extended to organ systems beyond the renal proximal tubule.

(4) How is claudin function regulated? Current knowledge of claudin regulation is limited to its intracellular C-terminus where numerous phosphorylation and interaction sites have been characterized. These modifications seem only to affect claudin protein trafficking. A bigger challenge would be to identify regulatory mechanisms that alter claudin function extracellularly or through structural changes.

(5) The clinical evidence for claudin dysfunction in human diseases is still very limited. Despite recent progresses in human genomic studies, causative mutation or SNP has yet been found in claudin genes from common diseases such as kidney stone or hypertension. Physiological analyses of transgenic animals for claudin dysfunction will provide pathogenic candidate genes that
can be examined under higher genomic resolution and within more focused population.

(6) No drugs that modulate claudin function have been developed. Despite several attempts to target the ECL2 domain of claudin with *Clostridium perfringens* enterotoxin in cancer therapy, our knowledge of drugs that can target the larger ECL1 domain is limited. Screening for paracellular channel inhibitors will be challenging but rewarding. Such inhibitors will not only provide new treatments to related diseases but also allow structural analyses of paracellular channel permeation.

(7) Finally and most importantly, no structural information is available for claudin. Since the discovery of the first claudin in 1998, no lab has ever been able to crystalize a claudin molecule. The technical difficulties will eventually be overcome with growing knowledge of claudin protein biochemistry both in Petri dish and under native environments.

Disclosure of Potential Conflicts of Interest
No potential conflict of interest was disclosed.

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