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Review

Transmembrane proteins of tight junctions

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Abstract

Tight junctions contribute to the paracellular barrier, the fence dividing plasma membranes, and signal transduction, acting as a multifunctional complex in vertebrate epithelial and endothelial cells. The identification and characterization of the transmembrane proteins of tight junctions, claudins, junctional adhesion molecules (JAMs), occludin and tricellulin, have led to insights into the molecular nature of tight junctions. We provide an overview of recent progress in studies on these proteins and highlight their roles and regulation, as well as their functional significance in human diseases.

Keywords: Tight junction; Cell junction; Cell polarity; Claudin; Occludin; Junctional adhesion molecule

Contents

Tight junctions are the apicalmost constituent of the apical junctional complex in vertebrate epithelial cell sheets (Fig. 1A). They are also observed in vascular endothelial cells and mesothelial cells, as well as several other types of cells (i.e., Schwann cells, oligodendrocytes and Sertoli cells). By transmission electron microscopy, tight junctions appear as a series of very close membrane appositions (so-called 'kissing points') of adjacent cells [1]. On freeze–fracture electron microscopy, these contact sites principally correspond to continuous networks of intramembranous particle fibrils (tight junction strands) (Fig. 1B) and complementary grooves in the protoplasmic and extracellular faces, respectively [2].

Tight junctions act as a semipermeable barrier (or gate) to the paracellular transport of ions, solutes and water, as well as cells, and are considered to function as a fence that divides apical and

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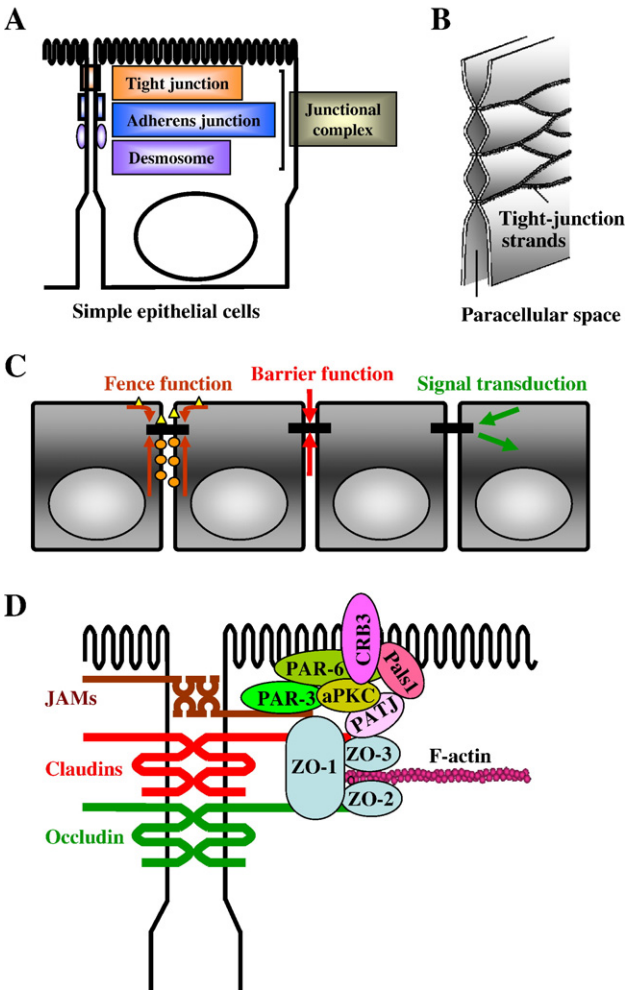

Fig. 1. Apical junctional complex and tight junctions. (A) Schematic drawing of the junctional complex. (B) Schematic structure of tight junction strands. (C) Functions of tight junctions. (D) Molecular components of tight junctions. Three families of tight junction transmembrane proteins, occludin, claudins and JAMs, as well as some scaffold proteins and polarity proteins are presented.

basolateral domains of plasma membranes (Fig. 1C) (for reviews, see [3–5]). In addition, like other cell–cell and cell–extracellular matrix junctions (for review, see [6]), tight junctions coordinate a variety of signaling and trafficking molecules regulating cell differentiation, proliferation and polarity, thereby serving as multifunctional complex (for reviews, see [4,7–11]). These functions of tight junctions are critical for epithelial and endothelial cell sheets to establish distinct tissue compartments within the body and maintain homeostasis.

Moreover, disturbance of the functions of tight junctions is considered to cause or contribute to a variety of pathological conditions, such as inflammatory bowel disease (IBD), infections and cancers, as well as vasogenic edema and blood-borne metastasis (for reviews, see [12–18]). In turn, the barrier function of tight junctions also restricts drug delivery to underlying tissues and therefore how to overcome the paracellular barrier (i.e., blood–brain barrier [BBB]) is critical for treatment of human diseases.

Numerous studies have disclosed various molecular components of tight junctions, including the integral membrane proteins, occludin [19], claudins [20], junctional adhesion molecules (JAMs) [21] and tricellulin [22], as well as increasing numbers of their scaffold proteins (Fig. 1D) (for reviews, see [4,5,11,12,23–25]). In this section, we will provide overview of relatively new findings on transmembrane proteins of tight junctions with focus on their physiological roles and regulation, as well as on their contributions to human diseases. We will primarily review mammalian tight junctions.

1. Occludin

Occludin, the first identified component of tight junction strands, is an approximately 60-kDa tetraspan membrane protein with two extracellular loops, a short intracellular turn, and N- and C-terminal cytoplasmic domains [19]. Among these domains, the first extracellular loop is exceptionally rich in tyrosine and glycine residues (about 60%) and contains very few charged amino acids. On the other hand, the long C-terminal domain is rich in serine, threonine and tyrosine residues, which are frequently phosphorylated by various protein kinases (for reviews, see [7,26]). In addition, the C-terminal region directly binds to ZO-1 [27], which in turn associates with the actin cytoskeleton. Although two isoforms of occludin are produced by alternative splicing [28], no clear difference in their tissue distribution and functions has been reported.

Overexpression experiments using full-length and mutated occludin in Madin–Darby canine kidney (MDCK) cells or *Xenopus* cells [29,30], as well as a study using a synthetic peptide corresponding to the second extracellular loop of occludin [31], initially suggested a role of occludin in the barrier and fence functions of tight junctions. Surprisingly, by contrast, occludin-deficient embryonic stem cells differentiated into polarized epithelial cells bearing well-developed tight junctions that exhibited no barrier dysfunction [32]. Furthermore, occludin-null mice were born with no apparent abnormalities in the structure or barrier function of tight junctions in intestinal epithelia [33]. However, occludin$^{-/-}$ mice showed postnatal growth retardation and complex phenotypes in various organs, including inflammation and hyperplasia of the gastric mucosa, absence of cytoplasmic granules in striated duct cells of the salivary gland, and thinning of the compact bone, as well as brain calcification and testicular atrophy, suggesting an unexpected role of occludin.

Several studies have indicated that occludin may be involved in the transduction of signals. First, forced expression of occludin suppresses the v-Raf-induced transformation and tumorigenesis of the salivary gland epithelial cell line Pa4 [34,35]. Second, it has been reported that occludin interacts with transforming growth factor (TGF)-β type I receptor and regulates its localization for TGF-β-dependent dissolution of tight junctions during epithelial-to-mesenchymal transition (EMT) [36] Third, by using an RNAi approach to knockdown the expression of occludin in MDCK cells, it has been demonstrated that occludin controls the actin cytoskeleton via activation of the small GTPase RhoA to extrude apoptotic cells without altering the degree of apoptosis [37]. Fourth, experiments using hepatocytes from occludin$^{-/-}$ mice have revealed that occludin inhibits apoptosis through activation of mitogen-

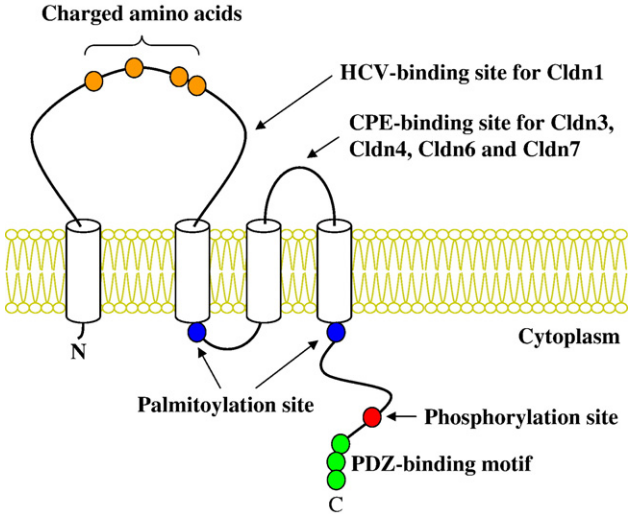

Fig. 2. A model of claudin structure.

activated protein kinase (MAPK) and Akt signaling pathways [38]. Fifth, overexpression of occludin in several cancer cells results in enhanced sensitivity to apoptotic factors via modulation of the expression of apoptosis-related genes, leading to inhibition of their tumorigenicity [39]. Thus, occludin seems to function as a signal transmitter from and to tight junctions, though there are discrepancies concerning the role of occludin in apoptosis among the latter three studies. In this sense, it is noteworthy that occludin can also associate with a variety of signaling molecules such as the nonreceptor tyrosine kinase c-Yes, atypical protein kinase C (aPKC) and PI3-kinase, as well as protein phosphatases 2A and 1 [40,41] (for review, see [10]).

2. Claudins

2.1. Structure and distribution of claudins

Claudins are 18- to 27-kDa tetraspan proteins with a short cytoplasmic N-terminus, two extracellular loops and a C-terminal cytoplasmic domain, and do not show any sequence similarity to occludin (Fig. 2) [4,20]. Claudins are capable of forming tight junction strands [42] and thus the backbone of the tight junction. The claudin family consists of 24 members in mice and humans and exhibits distinct expression patterns in tissue- and cell-type-specific manners (for reviews, see [4,43–45]). The C-terminal cytoplasmic tail of claudins is required for their stability and targeting [46–48]. In addition, through a PDZ-binding motif at their C-terminus, claudins directly bind to PDZ (PSD-95/Dlg/ZO-1) domain-containing peripheral membrane proteins such as ZO-1, ZO-2, ZO-3, PATJ and MUPP1 (Fig. 2) [49–51].

More than two claudin species are generally expressed in most types of epithelial cells (for reviews, see [43–45]). Because claudins comprise a large gene family as described above, and some anti-claudin antibodies react not only with the corresponding claudin but also with other claudin species [52], it is of particular importance to verify the specificity of the antibodies used. Along this line, we and others have demonstrated, by immunoblot and immunohistochemical analyses using antibodies that selectively recognize the corresponding claudins (Fig. 3) [53–55], as well as by gene expression analysis, that claudin-2 (Cldn2), Cldn3, Cldn4, Cldn7, Cldn8, Cldn12 and Cldn15 are abundantly expressed in the duodenum, jejunum, ileum and/or colon with remarkable variations in the expression levels throughout the segments of the intestinal tract (Fig. 4A) [55–57]. In addition, these claudin species show distinct subcellular localization in the intestinal epithelium (Fig. 4B) [55–57]. Moreover, Cldn2, Cldn4 and Cldn15 are expressed in gradients along the crypt-to-villous axis of the intestine (Fig. 4B). Cldn2 is only detected in the deep crypt of the entire intestine [56–58], and Cldn15 is restricted to the crypt of the colon with no gradients along the crypt-to-villous axis of the duodenum, jejunum or ileum [55,57,59]. By contrast, Cldn4 is predominantly expressed in the upper villi/surface of the whole intestine [57]. Concerning gradients within tissues, we also have found that Cldn7 is expressed on cell membranes in Brunner's glands of the duodenum much less than in the crypt epithelium [55]. The segment- and/or axis-specific expression of different claudins has also been reported in several other epithelial tissues, including the liver lobule [56], nephron [60] and inner ear [61].

Another issue that should be mentioned is basolateral localization of claudins (Fig. 4B). For example, Cldn7 is expressed more strongly on basolateral surfaces of the intestinal epithelium than on the apical tips [55,57,62]. Similar or identical patterns of expression are observed for Cldn7 in simple epithelial cells of the lung and kidney [63,64], as well as for Cldn1, Cldn3 and Cldn4 in the intestinal epithelium [56–58]. Although the significance of basolateral claudins remains obscure, extrajunctional claudins may serve as pools for junctional claudins and alternatively play a role in cell–cell and cell–matrix adhesion and/or signaling [64,65]. In fact, the cell–cell adhesion molecule EpCAM directly associates with Cldn7 on basolateral membranes [62], implying a novel role of basolateral claudins.

2.2. Physiological functions of claudins

Claudins, which also show Ca^{2+}-independent cell adhesion activity [66], are the major determinant of the barrier function of tight junctions, including the paracellular charge and size selectivity. This concept has been drawn from studies of claudin genes using knockout mice (for reviews, see [44]), transgenic mice [67,68] and in inherited human diseases [69–72], as well as overexpression experiments using wild-type and mutated claudins in cultured cell lines as described below.

Importantly, the first extracellular loop, in which there is a wide variation in the position and number of charged amino acids depending on each claudin (Fig. 2, unlike occludin, creates paracellular pores (channels) for selective ions between neighboring cells (for review, see [44,45]). For example, Cldn15 has two negatively charged residues (positions 53 and 64) in the latter half of this loop, and substitution of those by positive ones converts Cldn15 from cation- to anion-permissive [73,74]. In both mice and humans, Cldn2 has three negatively charged amino acids (positions 53, 65 and 75) within the first extracellular loop [74], and Cldn12 contains four negatively charged residues

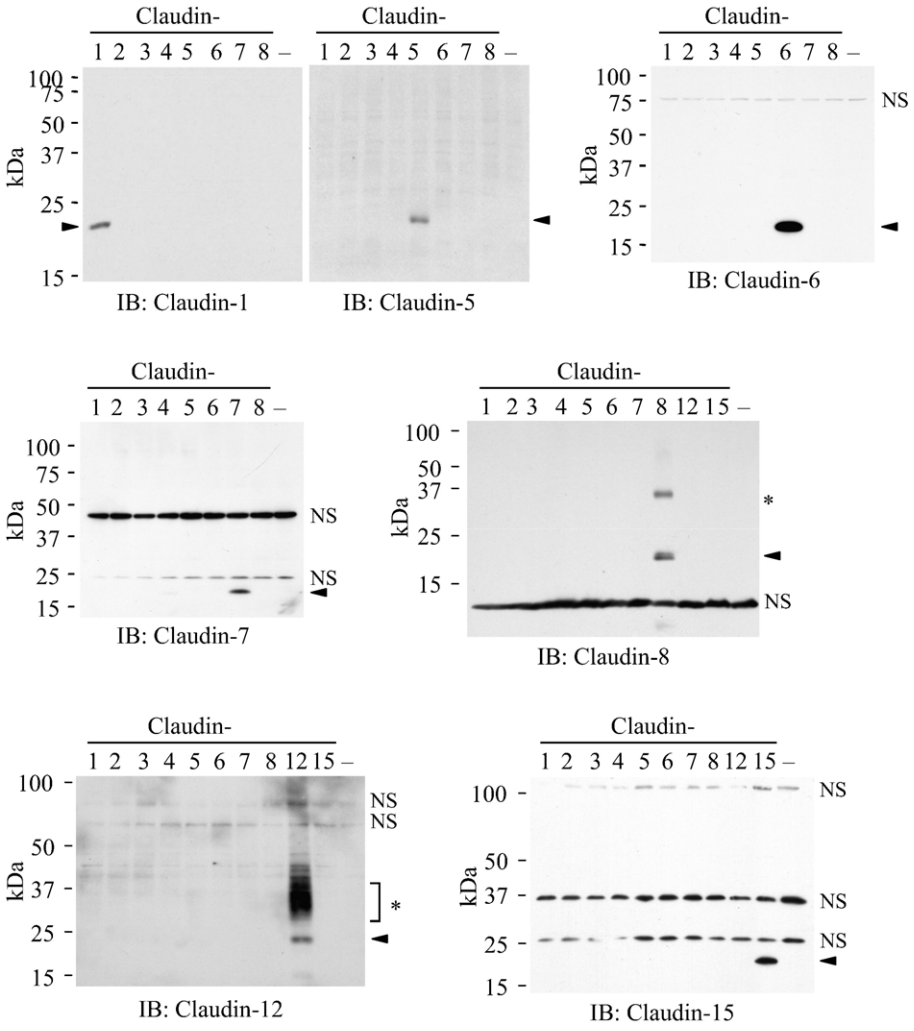

Fig. 3. Specificity of anti-Cldn1, anti-Cldn5, anti-Cldn6, anti-Cldn7, anti-Cldn8, anti-Cldn12 and anti-Cldn15 antibodies. COS-7 cells were transfected with either empty or claudin expression vectors. Five μg of total cell lysate was separated by SDS-PAGE and immunoblotted (IB) with the indicated antibodies, followed by chemiluminescence detection. The transfected mouse claudin cDNAs are indicated at the top of each lane. The mobility of molecular mass markers (kDa) is indicated on the left. Arrowheads and asterisks indicate specific signals. NS, nonspecific signal.

(positions 62, 66, 71 and 74) in the same domain [55], suggesting that these claudins may also form paracellular pores for cations. Similarly, Cldn7 likely acts as a paracellular pore to cations [75], and it contains three negatively charged amino acids within the first extracellular loop. By contrast, Cldn4, Cldn8 and Cldn14 function primarily in cation barriers [73,76–78], and Cldn13 might create an anion-selective pore as it contains four positively charged amino acids in the second half of the first extracellular loop [55]. It is assumed that the combination and proportion of different claudin species contribute to the barrier properties of tight junctions in a given cell (tissue) type.

Several lines of evidence have suggested that Cldn16/paracellin-1 (PCLN-1) plays a key role in the reabsorption of Mg^{2+} and Ca^{2+}. First, positional cloning has identified that familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC), an autosomal recessive disease showing severe Mg^{2+} and Ca^{2+} wasting, is caused by mutations in the *Cldn16/PCLN-1* gene [69]. It is also known that Cldn16 is highly expressed at tight junctions of epithelial cells in the thick ascending loop of Henle, where the reabsorption of divalent cations occurs [69]. Second, disease-associated mutations in patients with FHHNC indeed affect the intracellular traffic and paracellular Mg^{2+} transport functions of Cldn16 [79]. Third, forced expression of Cldn16 enhances Mg^{2+} and Ca^{2+} transport in MDCK cells [80,81]. Fourth, using the pig renal epithelial cell line LLC-PK1 and transgenic mice, Cldn16, which also seems to cause profound effects on permeability of monovalent cations, is shown to drive the reabsorption of Mg^{2+} and Ca^{2+} [82,83].

On the other hand, Cldn2 is known to act as paracellular channel to Na^+ in renal epithelial cells without altering Cl^- conductance. For instance, overexpression of Cldn2 in MDCK strain I cells lacking endogenous Cldn2 increases paracellular Na^+ permeability and decreases transendothelial electrical resistance (TER) [84,85]. Similar effects are observed when Cldn2 is overexpressed in LLC-PK1 cells expressing little endogenous Cldn2 [74]. Conversely, knockdown of endogenous Cldn2 expression in MDCK strain II cells reduces paracellular Na^+ permeation and elevates TER [82].

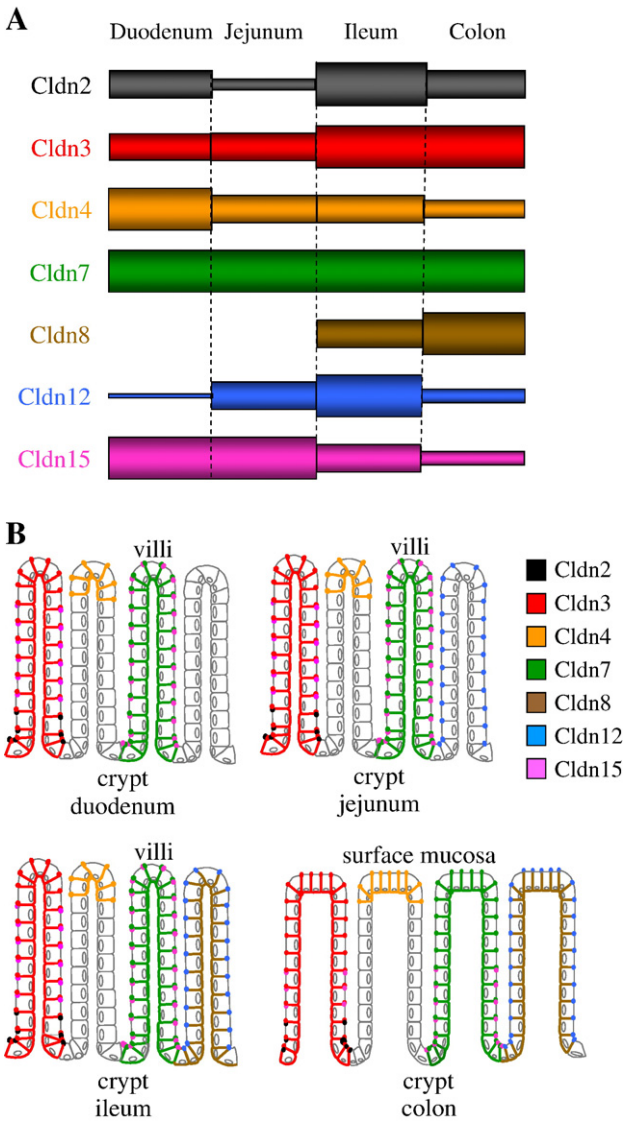

Fig. 4. Differential expression and subcellular localization of claudins along the mouse intestine. Relative expression levels (A) and schematic distribution (B) of Cldn2, Cldn3, Cldn4, Cldn7, Cldn8, Cldn12 and Cldn15 in the duodenum, jejunum, ileum and colon are summarized [47,49]. (A) Bar height represents the expression level. (B) The colors represent the same claudins as in panel A. Colored dots and lines correspond to localization at tight junctions and basolateral membranes, respectively.

Interestingly, we have recently found, by using RNAi and overexpression strategies, that Cldn2 and Cldn12 facilitate paracellular Ca^{2+} absorption in intestinal epithelial cells (our unpublished results). We have also reported that forced expression of Cldn2, but not Cldn12, in the intestinal epithelial cell line Caco-2 results in enhancement of Na^{+} permeability. Furthermore, we have demonstrated that expression of Cldn2 and Cldn12 is upregulated in enterocytes in vitro and in vivo by an active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$]. These findings strongly suggest that Cldn2- and/or Cldn12-based tight junctions form paracellular Ca^{2+} channels in intestinal epithelia, and highlight a novel mechanism behind vitamin D-dependent calcium homeostasis.

Compared with the significance of claudins in charge selectivity, our knowledge on the contribution of claudins to size selectivity is still fragmentary. In Cldn5-deficient mice, the BBB is severely affected with regard to small molecules (less than about 800 Da), but not larger molecules [86]. In contrast, induced expression of Cldn5 reconstitutes the paracellular barrier against inulin (5 kDa), but not mannitol (182 Da), in the leaky rat lung endothelial (RLE) cell line [87]. The discrepancy concerning the role of Cldn5 in paracellular size selectivity might be explained by a difference in expression of endogenous claudins between these endothelial cell types. In other words, at least Cldn5 and Cldn12 are expressed in BBB endothelial cells [86], whereas no endogenous claudins are detected in RLE cells as far as we could determine [87–89].

We should reconsider the role of claudin-based tight junctions in the fence function. Umeda et al. [90] have recently demonstrated that loss of ZO-1 and ZO-2 in the mouse mammary epithelial cell line Eph4 lacking endogenous ZO-3 completely disrupts the polymerization of claudins and formation of tight junctions, but surprisingly does not perturb overall apicobasal cell polarity (i.e., formation of microvilli and cell junctions, as well as the asymmetrical distribution of membrane proteins) even if polarity proteins PAR-3, aPKC and Pals1 are localized in lateral membranes. These findings strongly suggest that claudin-based tight junctions are dispensable for the fence function, at least in monolayer culture of mature epithelial cells [91].

2.3. Physiological regulation of claudins

Concerning posttranslational modification of claudins, we have reported that cyclic AMP (cAMP)-dependent protein kinase (PKA) phosphorylates Cldn5 at Thr^{207}, which is accompanied by enhanced detergent insolubility and junctional immunoreactivity of Cldn5, as well as a change in the barrier function of tight junctions (Fig. 5) [53,87]. Since detergent insolubility of proteins is considered to indicate their integration into macromolecular complexes such as intercellular junctions, phosphorylation of claudins might be involved in its incorporation into tight junctions, leading to promotion of tight junction functions. Note that gene expression of Cldn5 in brain microvascular endothelial

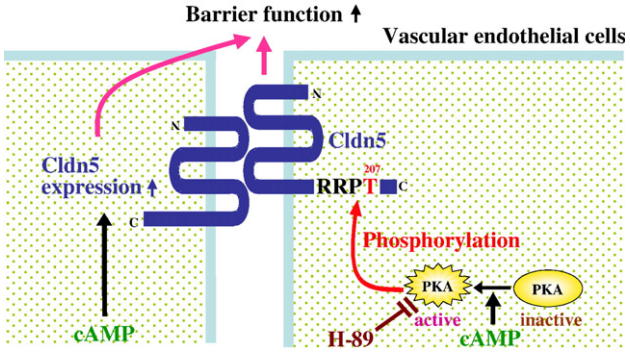

Fig. 5. Models depicting the enhancement of vascular endothelial barrier by cAMP and PKA. cAMP induces phosphorylation of Cldn5 protein at Thr^{207} via a PKA-dependent pathway and activates expression of the *Cldn5* gene in a PKA-independent manner.

cells is activated by cAMP in a PKA-independent manner, indicating that cAMP regulates Cldn5 and elevates the barrier function via PKA-dependent and -independent pathways [53]. It is also reported that PKA phosphorylates Cldn16, resulting in enhancement of its detergent insolubility and junctional localization [81]. By contrast, PKA-dependent phosphorylation of Cldn3 leads to its cytoplasmic localization and barrier dysfunction in ovarian cancer cells [92].

Several other protein kinases also phosphorylate claudins at their C-terminal tail and influence the barrier function of tight junctions. For example, we demonstrated that MAPK promoted phosphorylation of Cldn1 at Thr^{203} and increased its insolubility to detergent and the barrier function [88]. PKC is also known to phosphorylate Cldn1 [93,94] and Cldn4 [92]. In addition, it has been shown that WNK4 phosphorylates Cldn1, Cldn2, Cldn3 and Cldn4, and that the disease (pseudohypoaldosteronism type II)-causing mutant increases their phosphorylation levels [95]. Moreover, the receptor tyrosine kinase EphA2 associates with Cldn4 via their extracellular domains and phosphorylates Cldn4 in the C-terminal tail at Tyr^{208}, resulting in inhibition of its interaction with ZO-1 and reduction of its integration into cell junctions, as well as barrier dysfunction of tight junctions [96]. As another type of posttranslational modification, it has been reported that Cldn2, Cldn4 and Cldn14 are palmitoylated [97]. Palmitoylation of Cldn14 at two sets of membrane-proximal cysteins following the second and forth transmembrane domains, which are well conserved among claudin species, is required for efficient tight junction localization and function [97].

Among transcription factors, the nuclear receptor superfamily is one of the most important for regulating expression of claudins. First, we found that two types of nuclear receptors, retinoid receptors and hepatocyte nuclear receptor 4α (HNF4α), triggered expression of Cldn6 and Cldn7 in the embryonal carcinoma cell line F9 [54,98,99]. Subsequently, Cldn1 was shown to be absent in hepatocytes in mice with conditional knockout of the *HNF4α* gene [100]. Note that HNF4α acts as a morphogen to induce the formation of cell junctions [54,99–101] and microvilli [102], establishing at least two aspects of apicobasal cell polarity. It is also reported that retinoid receptors and the kinase IKK1 cooperatively regulate the expression of retinoid target genes, including *Cldn23*, in keratinocytes [103]. In addition, comparison of gene expression profiling between wild-type and Sertoli cell-specific androgen receptor (AR)-knockout mice revealed that AR mediated activation of the Cldn3 expression in Sertoli cells [104]. Furthermore, we have recently provided evidence showing that expression of Cldn2 and Cldn12 is upregulated in enterocytes in vitro and in vivo by $1\alpha,25(OH)_2D_3$ through its receptor VDR (our unpublished results). Taken collectively, expression of different claudin species seems to be upregulated by distinct members of the nuclear receptor superfamily in a cell-specific manner.

Regarding regulation of Cldn2 expression, the caudal-related homeobox proteins Cdx1 and Cdx2 activate its gene expression, and HNF1α augments the Cdx2-induced transcriptional activation of the human *Cldn2* promoter [105]. In addition to Cdx2 and HNF1α, GATA4 cooperatively upregulates expression of the *Cldn2* gene [106]. Cldn2 expression is also upregulated through activation of RhoA and c-Jun N-terminal kinase (JNK) [107]. On the other hand, activation of MAPK is known to downregulate Cldn2 expression in several epithelial cell types [38,108,109], although MAPK activation by the inflammatory cytokine IL-17 upregulates Cldn1 and Cldn2 expression in the human intestinal cell line T84 [110].

For other regulators of claudin expression, there is a review summarizing data on the topics [45]. Among them, we should mention that the transcriptional repressor SNAI1 (Snail homologue 1) downregulates the expression of Cldn3, Cldn4 and Cldn7 [111] in Eph4 epithelial cells and Cldn1 in MDCK cells [112] during EMT. It is of particular interest that SNAI1 binds the promoter of *VDR* and *HNF4α* genes and represses their expression [113,114] (for review, see [115]). Hence, SNAI1 directly and indirectly inhibits the expression of several claudin species.

2.4. Claudins and human disease

To date, mutations in four *claudin* genes have been reported to cause human hereditary diseases. First, various mutations in the *Cldn16* gene were identified in patients with FHHNC as mentioned above [69,79,116]. Second, mutations in the *Cldn14* gene, the expression of which is abundantly observed in outer hair cells of the cochlea, cause autosomal recessive deafness [70,117]. Third, mutations in the *Cldn1* gene are reported in neonatal sclerosing cholangitis with ichthyosis [71]. Fourth, mutations in the *Cldn19* gene, whose expression is highly detected in renal tubules and the retina, have recently been identified in families affected with severe hypomagnesemia due to renal wasting, nephrocalcinosis, progressive renal failure and severe ocular abnormalities [72].

Unlike other junction molecules, expression of claudins is often increased in various cancers. For example, Cldn3, Cldn4 and Cldn7 are frequently upregulated in a variety of cancers such as those originating from the ovary, fallopian tubes, breast, uterus, prostate, thyroid, pancreas, stomach, colon and bladder [18,118–120]. Since not only Cldn3/RVP1 (rat ventral prostate-1) [121] and Cldn4/CPE-R (receptor for *Clostridium perfringens* enterotoxin [CPE]) [122], but also Cldn7, bind to CPE with high affinity (Fig. 2) [123,124], these claudins are expected to be promising targets of CPE-mediated therapy for cancers. Although the exact roles of overexpressed claudins in cancer are unclear, expression of some claudins in epithelial cells promotes cell invasion, motility and/or survival [125,126] (for review, see [109]), suggesting potential functions of claudins in tumorigenesis and metastasis. On the other hand, it is noteworthy that reduced expression of Cldn4 and Cldn7 at the invasive front of colorectal and esophageal cancer, respectively, significantly correlates with tumor invasion and metastasis [127,128].

The expression and/or distribution of claudins are also altered in numerous other pathological conditions, and these changes are proposed to play important roles in the pathophysiology of such diseases. For instance, the interferon-γ-triggered internalization of claudin-1 and -4, as well as that of occludin and JAM-A, was suggested to contribute to the pathogenesis and barrier dysfunction in IBD using the human intestinal

epithelial cell line T84 [129] (for review, see [15]). Moreover, hypoxia, which disrupts the barrier properties of neural vascular endothelial cells and accelerates the progression of disorders such as cerebral ischemic diseases and diabetic retinopathy, is reported to provoke a reduction in expression levels of Cldn5 protein and its disappearance from the plasma membrane [130]. Interestingly, we have found that retinoids increase and decrease the expression of glial cell line-derived neurotrophic factor (GDNF) and vascular endothelial growth factor (VEGF) in glial cells, respectively, attenuating the barrier dysfunction of vascular endothelial cells in diabetic retinopathy [131].

Recently, Cldn1 was identified as a coreceptor for hepatitis C virus (HCV) and shown to be required for HCV entry into host cells (Fig. 2) [132]. It has also been reported that ectopic expression of Cldn1 in non-hepatic cells confers susceptibility to HCV infection. In addition, the C-terminal portion within the first extracellular loop of Cldn1 is critical for HCV susceptibility (Fig. 2).

3. Tricellulin

Tricellulin has been identified as a four-pass transmembrane protein that that is concentrated at the vertically oriented tight junction strands of tricellular contacts of epithelial cell sheets [133]. Its C-terminal sequence (~130 amino acids) is 32% identical to that of occludin, whereas the N-terminal cytoplasmic domain is longer than that of occludin. Tricellulin is phosphorylated, and its expression is repressed by SNAI1. Using an RNAi approach, it has been shown that tricellulin participates in the epithelial barrier and organization of not only tricellular tight junctions but also bicellular tight junctions. More recently, it has been found that recessive mutations in the *tricellulin* gene cause nonsyndromic deafness (DFNB49), and that tricellulin in fact is assembled at tricellular tight junctions in cochlear and vestibular epithelial cells [134]. Interestingly, in spite of its ubiquitous distribution in epithelial cells, the only phenotype of the *tricellulin* mutations appears to be deafness. It is also reported that multiple isoforms of tricellulin are generated by alternative splicing.

4. Junctional adhesion molecules

JAMs are glycosylated transmembrane proteins that belong to the immunoglobulin (Ig) superfamily and consist of two extracellular Ig-like domains, a single transmembrane region and a C-terminal cytoplasmic domain. The JAM family is divided into two subgroups based on their sequence similarities (for reviews, see [24,25]). The first subgroup, JAM-A (also designated JAM/JAM-1/106 antigen/F11R), JAM-B (also known as VE-JAM/mJAM-3/hJAM-2) and JAM-C (also named mJAM-2/hJAM-3) has a class II PDZ domain-binding motif at their C-terminal ends, and directly interacts with ZO-1 and PAR-3 [135–137]. By contrast, members of the second subgroup, coxsackie and adenovirus receptor (CAR), endothelial cell-selective adhesion molecule (ESAM) and JAM4 contain a class I PDZ domain-binding motif at their C-terminus. CAR and JAM4 associate with Ligand-of-Numb protein X1 (LNX1) [138,139], and JAM4 and ESAM bind to the membrane-associated guanylate kinase protein (MAGI-1) [140,141].

In addition to association with intracellular binding partners, JAMs show homophilic and heterophilic interactions through their extracellular domains (for reviews, see [24,25,142]). Moreover, JAMs exhibit heterophilic interactions via the extracellular domains with β1 and β2 integrins. So far, it has been reported that JAM-A, JAM-B and JAM-C interact with integrins αLβ2 (LFA-1), α4β1 (VLA-4) and αMβ2 (Mac-1), respectively, which are found on circulating blood cells (for reviews, see [24,25,142]). Since JAMs are expressed on endothelial cells, epithelial cells and/or blood cells, they function as cell–cell adhesion molecules not only between the same types of cells but also among distinct types of cells (i.e., leukocytes, platelets and/or endothelial cells) through homophilic and heterophilic interactions.

4.1. JAM-A, JAM-B and JAM-C

JAM-A is involved in the barrier function of tight junctions in both endothelial and epithelial cells [21,143,144], and development of apicobasal cell polarity in epithelial cells [145]. It is also reported that JAM-B and JAM-C are found on Sertoli cells and spermatids, respectively, and that JAM-C is essential for the polarization of round spermatids [146]. JAM-A represents not only a component of tight junctions but also a receptor for reoviruses [147].

JAM-A also participates in monocyte transmigration [21]. In addition, by analyzing JAM-A$^{-/-}$ mice, endothelial JAM-A$^{-/-}$ mice and/or those treated with an anti-JAM-A antibody, JAM-A was revealed to control transmigration of neutrophils, but not lymphocytes, across endothelial cells [148,149]. In addition to JAM-A, JAM-C is involved in transmigration of neutrophils and monocytes across endothelial and epithelial cells [150–153] (for reviews, see [44,45,142]). On the other hand, JAM-A also regulates endothelial cell migration [154–156]. Furthermore, it is demonstrated that dendritic cells (DCs), in which occludin and Cldn1 are found [157], also express JAM-A, and JAM-A-deficient mice show enhanced motility of DCs and their migration to lymph nodes [158].

Another issue that should be mentioned is the role of JAM-C in the malignant potential and angiogenesis of tumors. First, homophilic interaction of JAM-C occurs between tumor cells and endothelial cells [159]. Second, JAM-C is expressed in most cancer cells showing metastatic properties and promotes metastasis of the human fibrosarcoma cell line HT1080 by enhancing its adhesion to extracellular matrices and the subsequent invasion [160]. Third, JAM-C is implicated in angiogenesis of tumor grafts and hypoxia-induced neovascularization, suggesting that JAM-C may be a putative target for antitumor therapies [161].

4.2. CAR, ESAM, JAM4

CAR contributes to the barrier function of tight junctions in epithelial cells [162,163]. It is predominantly expressed in a variety of epithelial cells lining the body cavities, but not

endothelial cells [164]. Epithelial CAR shows heterophilic interaction with neutrophil JAML (JAM-like molecule, [165]), thereby modulating neutrophil migration [166]. CAR is also concentrated in intercalated discs between cardiomyocytes [164,167] and is essential for early embryonic cardiomyocyte development [168,169]. In addition, CAR-like membrane protein (CLMP) has also been identified [170].

Coyne and Bergelson [171] have demonstrated the mechanism by which group B coxsackieviruses (CVBs) approach from the apical surface of polarized epithelial cells and access CAR in tight junctions. In brief, the attachment of CVBs to the GPI-anchored protein decay-accelerating factor (DAF) on the apical membrane provokes two independent signaling pathways required for viral entry: activation of tyrosine kinase Abl initiates Rac-dependent actin rearrangements that permit virus movement to tight junctions, where association with CAR leads to conformational changes in the virus capsid that are essential for viral entry and release of viral RNA; in parallel, activation of Src tyrosine kinase Fyn phosphorylates and activates caveolin-1, which in turn promotes virus internalization into vesicles [171,172].

ESAM is restricted to endothelial cells and platelets, but not epithelial cells or leukocytes [173,174]. ESAM$^{-/-}$ mice show no obvious abnormalities in physiological angiogenesis but exhibit impairment in pathological angiogenesis such as in tumor growth [175]. Extravasation of neutrophils, but not recruitment of lymphocytes, is also significantly reduced in ESAM-deficient mice at an early step of the inflammatory process [176]. In addition, loss of ESAM enhances VEGF-initiated permeability [176]. JAM4$^{-/-}$ mice are reported to possess no apparent abnormalities as far as examined [177].

5. Conclusions

The discoveries of occludin, claudins, JAMs and tricellulin were undoubtedly breakthroughs in the field and have greatly contributed to our understanding of the molecular nature of tight junctions. Recently, Bves (blood vessel/epicardial substance)/Pop1a, which belongs to the Popeye family Popdc (Popeye domain containing), has been suggested to be another transmembrane protein of tight junctions [178,179]. Bves is predicted to be a three-pass integral membrane protein with an extracellular N-terminus and an intracellular C-terminal domain, and its sequence is well conserved across a variety of vertebrate species. The majority of Bves is ultrastructurally found at tight junctions of polarized epithelial cells, and it is involved in the epithelial barrier. Thus, our knowledge on integral membrane proteins of tight junctions has been accumulated, several unanswered questions remain.

First, the structural basis for claudin-based paracellular pores (channels) should be characterized by electron crystallography. This will help us to understand the mechanisms by which claudins determine the paracellular charge and size selectivity. Second, the undefined roles of tight junction transmembrane proteins must be determined. For instance, overexpression of Cldn1 on the right side but not on the left side of the chick embryo results in abnormal heart looping [94]. The exact functions of extrajunctional claudins also remain to be elucidated. Third, the functional relevance of posttranslational modifications of claudins must be determined. Fourth, the significance of tight junction membrane proteins in human diseases should be further clarified. Do several pathogens access their cognate receptors in tight junctions via mechanisms similar to those by which CVBs target CAR? How do tight junction proteins contribute to carcinogenesis and metastasis? Finally, are there other proteins that are concentrated at tricellular tight junctions? In addition, it should also be examined how cells and substances pass through tricellular contact sites.

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