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Review

Knockout animals and natural mutations as experimental and diagnostic tool for studying tight junction functions *in vivo*

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ABSTRACT

Two sides of functions of tight junctions; the barrier and the channel in the paracellular pathway are believed to be essential for the development and physiological functions of organs. Recent identification of molecular components of tight junctions has enabled us to analyze their functions by generating knockout mice of the corresponding genes. In addition, positional cloning has identified mutations in the genes of several components of tight junctions in hereditary diseases. These studies have highlighted *in vivo* functions of tight junctions.

Contents

1. Introduction

Tight junctions (TJs) contribute to epithelial and endothelial barrier functions by restricting the diffusion of solutes through the paracellular pathway in vertebrate species [1]. This function of TJs enables isolation from the external environment and maintenance of distinct fluid compartments within the body, which are fundamental aspects for the functions of most organ systems [1]. TJs are not complete barriers, but rather contain pores through which ions and small molecules pass passively with charge and size selectivities. Importantly, the barrier properties of TJs vary among different types of epithelia depending on their physiological functions [2–6]. Transepithelial electric resistance (TER), which is the easiest and most

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sensitive measure of barrier strength toward ions, varies from 5 to 31, 000 $\Omega \times cm^2$ [52]. The selectivities between cations and anions in the paracellular pathway also vary among different types of epithelia *in vivo* and *in vitro* [2–6]. Not only for leaky epithelia but also for tight epithelia, TJs are important routes of epithelial transport, which is the sum of transcellular and paracellular transport.

Although these functions of TJs are believed to be essential for the development and physiological functions of organs, there have been no experimental demonstrations of these aspects due to the lack of appropriate methods for specifically modulating the functions of TJs *in vivo*. However, the recent identification of molecular components of TJs has enabled us to analyze their functions by generating knockout mice of the corresponding genes (Table 1). In addition, positional cloning has identified mutations in the genes of several TJ components in hereditary human and cattle diseases, further demonstrating critical roles for TJs in various organs (Table 2). In this review, the pathologies of knockout or knockdown mice and natural mutations of the genes of TJ-associated structural proteins are summarized, and the roles of TJs *in vivo* are discussed.

2. Knockouts and natural mutations of claudin family genes

Accumulating evidence has revealed that claudins are the major barrier-forming proteins of TJs [2–6]. When claudins are overexpressed in cultured fibroblasts, exogenous claudins are concentrated from both sides of adjacent cells into cell–cell contact planes, where well-developed TJ strands, the core structures of TJ barriers, are formed *de novo* [7]. Claudins comprise a multigene family containing 24 members in the mouse and human genomes [3,4]. In most cell types, multiple claudin types are coexpressed in individual cells and the combinations and proportions of different claudins vary among cell types [9]. This manner of claudin expression is thought to provide functional diversity for the barrier properties of TJs, such as conductance and charge selectivity of ions, depending on the environment of the extracellular domains of the claudins [10]. Indeed, a number of studies have revealed that overexpression of a certain claudin in cultured epithelial cells changes the barrier or channel properties of TJs, as evaluated by measuring the TER and diffusion potential, which indicate the charge selectivity of TJs [10–18]. The effects of overexpression of each claudin depend not only on the type of claudin overexpressed but also on the cell lines used, since each cell line has its own barrier properties of TJs based on its unique background expression pattern of claudins [19]. The aspects that can be measured in such experiments are the barrier properties of TJs generated from particular combinations of claudins, namely the endogenous claudins and the overexpressed claudin. It is possible, however, that the effects reflect the barrier-forming or channel-forming properties of the added claudin types, with positive and negative charge selectivities. Thus, the overall barrier properties of TJs in each epithelial type are determined by the combination of claudin types expressed in each cell type.

Table 1
Tight junction gene knockout (KO) and knockdown (KD) mice

Gene	Phenotype	Ref.
Cldn-1 KO	Skin barrier defect to the water loss	[23]
Cldn-5 KO	Blood-brain barrier defect	[30]
Cldn-11 KO	CNS myelin defect, Blood-testis barrier defect	[34]
	Deafness	[36,37]
Cldn-14 KO	Phenocopy of human non-syndromic deafness	[41]
Cldn-15 KO	Megaintestine	[44]
Cldn-16 KD	Phenocopy of familial hypomagnesemia with hypercalciuria and nephrocalcinosis	[49]
Cldn-19 KO	Schwann cell barrier defect	[57]
Occludin	Viable with complex phenotype	[63]
ZO-1	Embryonic lethal at E10.5	[77]
ZO-2	Embryonic lethal	[85]
ZO-3	No phenotype	[72,85]

Table 2
Tight junction-associated hereditary diseases

Gene	Disease	Ref.
Cldn-1	Neonatal ichthyosis and sclerosing cholangitis	[24]
Cldn-14	Non-syndromic deafness (*DFNB29*)	[8]
Cldn-16	Familial hypomagnesemia with hypercalciuria and nephrocalcinosis (human)	[48]
	Chronic interstitial nephritis and renal tubular dysplasia (bovine)	[53,54]
Cldn-19	hypomagnesemia with hypercalciuria and nephrocalcinosis with visual impairment	[59]
Tricellulin	Non-syndromic deafness (*DFNB49*)	[67]
ZO-2	Familial hypercholamenia	[78]

To date, many claudin gene knockout mice and mutations in claudin genes related to hereditary human and cattle diseases have been reported. Various pathologies have therefore come to be interpreted from the viewpoint of TJ barrier/channel deficits. In addition, mutation and morpholino suppressions of claudin genes in zebrafish have been reported.

2.1. Claudin-1-deficient mice and claudin-1 gene mutations

The mammalian epidermis consists of a stratified epithelium with four types of layers, namely the stratum basale, stratum spinosum, stratum granulosum and stratum corneum. It is well known that cornified cell envelopes and the lipid lamella between them create a strong barrier in the skin against physical stress, infection and water dispersion [20]. On the other hand, the roles of TJs in the epidermis have been ignored for a long time due to the difficulties associated with identifying TJs in the skin by electron microscopy [21]. However, the recent identification of TJ-associated proteins has enabled us to re-examine the existence of TJs in the mammalian epidermis. Immunofluorescence microscopy analyses have identified continuous TJs surrounding keratinocytes in the stratum granulosum that include, at least, occludin, claudin-1 and claudin-4 [22,23].

Claudin-1-deficient ($Cldn1^{-/-}$) mice are born alive, but die within 1 day of birth due to excessive dehydration from the skin [23]. The skin of $Cldn1^{-/-}$ mice looks macroscopically normal at birth, but gradually begins to show a wrinkled appearance. The epidermis in $Cldn1^{-/-}$ mice does not exhibit any overt abnormalities in the organization of the keratinocyte layers, except that the stratum corneum appears to be more compact than those of wild-type and heterozygous mice under conventional fixation/embedding conditions during sample preparation for microscopy [23]. Morphological analyses by immunofluorescence and electron microscopy suggested the presence of continuous TJs containing occludin and claudin-4 in the stratum granulosum in $Cldn1^{-/-}$ mice [23]. However, these TJs are permeable to a water-soluble tracer of ~600 Da injected subcutaneously, whereas this tracer cannot pass through TJs in wild-type mice. The lipid lamella formed between corneocytes appears normal in $Cldn1^{-/-}$ mice [23]. Although claudin-1 was demonstrated to be essential for the TJ barrier in granular cells[23], the barrier function of the stratum corneum to the water loss in these mice may also be affected, which should be examined in the future study. The reason why claudin-1-deficient TJs, which still include at least claudin-4, become leaky is also an open question.

Mutations in the claudin-1 gene have been identified in neonatal ichthyosis and sclerosing cholangitis (NISCH) syndrome [24]. Originally, this syndrome was reported as autosomal recessive ichthyosis with scalp hypotrichosis, scaling alopecia, sclerosing cholangitis, oligodontia, enamel dysplasia and leukocyte vacuolization in four patients in two inbred kindreds of Moroccan origin [25]. The disease gene was mapped to chromosome 3q27–28, where a deletion of two nucleotides led to a frameshift mutation that resulted in a premature stop codon at amino acid 67 of claudin-1 [24]. Subsequently, a different frameshift mutation leading to a premature stop codon at

amino acid 120 was also reported in NISCH syndrome in a Swiss family [26]. In the liver of these patients, no claudin-1 protein was detected by immunoblotting analyses using anti-claudin-1 antibodies generated against the C-terminal cytoplasmic domain of claudin-1 [24]. Although the detailed mechanism of this syndrome is not yet understood, the major symptoms of the syndrome, namely sclerosing cholangitis and ichthyosis, appear to be related to the lack of expression of claudin-1 in the liver and skin [23,27]. In the liver, claudin-1 is a component of TJs in the bile canaliculi, through which the bile acid generated in hepatocytes flows into the bile duct [27]. In the skin, claudin-1 is a component of TJs in the stratum granulosum in the epidermis [22,23]. The mechanism behind the skin defects in NISCH syndrome may be further clarified by analyzing the phenotypes of claudin-1-deficient mice, especially those of skin grafts to nude mice.

2.2. Claudin-5-deficient mice

The TJs in brain endothelial cells are well-developed compared with those in endothelial cells in other organs and establish the blood-brain barrier (BBB) [28,29]. The BBB is thought to protect the brain against harmful materials circulating in the blood and maintain a stable solute environment in the cerebrospinal fluid. Claudin-5 is the major known component of TJs in brain endothelial cells [30].

Cldn-5$^{-/-}$ mice are born alive in the expected Mendelian ratio, but die within 1 day of birth [60]. The development of blood vessels in Cldn-5$^{-/-}$ mice appears normal and no bleeding or edema is observed. In ultrathin section electron microscopy, the brain endothelial cells of newborn Cldn-5$^{-/-}$ mice have TJs with normal appearances at cell–cell contact sites [60]. However, tracer experiments revealed that the BBB against small water-soluble molecules, but not larger molecules, is selectively affected in these mice. When a tracer of 443 Da (a primary amine-reactive biotinylation reagent) was perfused via the heart of wild-type 18.5-d embryos for 5 minutes, the tracer was retained in the blood vessels in the brain and excluded from the central nervous system (CNS). In contrast, the tracer was distributed throughout the brain parenchyma in Cldn-5$^{-/-}$ mice, indicating that the BBB is severely affected in these mice [60]. When microperoxidase (1.9 kDa) was used as a tracer, however, its leakage from blood vessels was not observed [60]. The mechanism behind this size discrimination remains elusive. The reason why Cldn-5$^{-/-}$ mice die just after birth is also unclear.

2.3. Claudin-11-deficient mice

Claudin-11 was originally identified as oligodendrocyte-specific protein since it represents one of the major components of oligodendrocytes [31]. Claudin-11 is expressed in specific organs, including oligodendrocytes, in the brain [31,32,34], Sertoli cells in the testis [32,34] and the basal cell layer of the stria vascularis (SV) in the cochlea [33,34,36]. Since claudin-11 seems to be the sole claudin in these cell types, claudin-11-deficient mice exhibit various pathological conditions without compensation by other claudin types.

CNS myelin, which is generated by oligodendrocytes, is not an epithelium, but contains TJs formed from claudin-11 [32,34]. These TJs run parallel to the axon axis and radially through the myelin sheath, comprising the so-called radial component. Cldn-11$^{-/-}$ mice do not exhibit demyelination, but lack TJ strands in the CNS myelin, as observed by freeze-fracture replica electron microscopy [34]. Cldn-11$^{-/-}$ mice show hind limb weakness and slowed conduction velocities in the CNS, suggesting that TJs in oligodendrocytes are involved in the tight electric sealing of myelin in the CNS [34].

The blood-testis barrier (BTB) separates the environment of the testis, where sperm is generated, from the blood. The proposed function of the BTB is to provide a specialized compartment to facilitate the development of spermatozoa [79,80]. Furthermore, BTB is thought to protect developing spermatozoa, which mature after immunotolerance is established, against autoimmune responses [35]. In Sertoli cells, which are specialized epithelial cells for generating seminiferous tubules, TJs are the major component of the BTB [35]. Cldn-11$^{-/-}$ male mice lack TJ strands in their Sertoli cells and show male sterility accompanied by small seminiferous tubules without spermatozoa, but not autoimmune responses [34]. These observations suggest that TJs in the BTB function in the generation of the solute environment critical for spermatogenesis rather than in the avoidance of autoimmune responses.

Cldn-11$^{-/-}$ mice also suffer from deafness [36,37]. The cochlea includes a peculiar compartment filled with endolymph [38]. This compartment is characterized by a very high K^+ concentration (~150 mM) and a positive endocochlear potential (EP; ~90 mV), which are both indispensable for cochlear hair cells to transduce acoustic stimuli into electrical signals [39,40]. These conditions are thought to be generated by the SV, which faces toward the endolymph [39,40]. The SV has two epithelial barriers, termed the marginal and basal cell layers. The TJs of the basal cell layer are primarily composed of claudin-11 [33,34]. In Cldn-11$^{-/-}$ mice, the basal cells lack TJs and the hearing ability is markedly reduced [36,37]. In these mice, the EP is suppressed to ~30 mV, although the K^+ concentration of the endolymph is maintained around the normal level, indicating that the TJs in the basal cells play a role in establishing the EP [36,37].

Due to the peculiar tissue expression pattern of claudin-11, Cldn-11$^{-/-}$ mice exhibit rather diverse symptoms. To date, no human hereditary syndromes with a combination of slowed conduction velocities in the CNS, male sterility and deafness have been reported.

2.4. Claudin-14 gene mutations and claudin-14-deficient mice

Mutations in the claudin-14 gene were identified as the cause of non-syndromic recessive deafness *DFNB29*, which was mapped on chromosome 21q22.1, in two Pakistani families [8]. In the affected individuals in one family, a homozygous single nucleotide deletion was found within the codon for Met133, which is located in the third transmembrane domain. The resulting frameshift mutation causes 23 incorrect amino acids and premature termination, with the loss of the C-terminal half of the full-length protein. Another mutation identified in the affected individuals is a homozygous missense mutation that substitutes aspartic acid for valine 85 in the second transmembrane domain. *In situ* hybridization and immunofluorescence staining revealed claudin-14 expression in the hair cells of the organ of Corti in the mouse cochlea [8], which includes a peculiar compartment filled with endolymph that is characterized by a very high K^+ concentration and a positive EP [38–40].

As a mouse model for *DFNB29*, claudin-14-deficient mice were generated. Cldn-14$^{-/-}$ mice exhibit profound hearing loss with a normal EP [41]. In Cldn-14$^{-/-}$ mice, inner hair cells and outer hair cells (OHCs) develop normally by P7, but the OHCs subsequently disappear or become disorganized. By P18, most of the OHCs are lost [41]. Since the OHC degeneration coincides with elevation of K^+ in the endolymph during development, it has been proposed that leakage of the high K^+ concentration to the basolateral domain of OHCs due to the reduced TJ barrier under the loss of claudin-14 may cause prolonged OHC depolarization, thereby leading to death of the OHCs [41]. Claudin-14 is expressed in the liver and kidney, but abnormalities in these organs have not been observed.

2.5. Claudin-15-deficient mice

Intestinal epithelial cells express various claudin types, including at least claudin-2, -3, -4, -7, -12 and -15, as evaluated by immunofluorescence staining [42,43]. Among these, claudin-15 is concentrated in the TJs of all intestinal epithelial cells throughout the mouse intestinal tube [43]. Claudin-15-deficient mice are born alive and grow

normally [44]. Until 3–4 weeks after weaning, there are no remarkable differences in the overall morphologies of the small intestine between wild-type and Cldn-15$^{-/-}$ mice. However, adult Cldn-15$^{-/-}$ mice show an abnormally expanded intestine, referred to as a mega-intestine phenotype [44]. The upper small intestine, duodenum and jejunum in Cldn-15$^{-/-}$ mice are increased by approximately 2-fold compared to those in wild-type mice. Histologic examination revealed that the sizes of the intestinal villi are significantly increased in the upper intestine without a pathological state [44]. A BrdU incorporation study revealed that the proliferation zone of the small intestine is expanded by approximately 2-fold in length along the crypt in Cldn-15$^{-/-}$ mice, while apoptosis is unaffected, indicating that cell proliferation is enhanced in Cldn-15$^{-/-}$ mice [44]. Polyp/cancer formation was not observed in 2-year-old Cldn-15$^{-/-}$ mice [44]. Electrophysiological analyses demonstrated that the ion conductance in the distal portion of the jejunum in Cldn-15$^{-/-}$ mice is decreased, although neither wild-type nor Cldn-15$^{-/-}$ mice show remarkable permeability to water-soluble tracers of more than ~400 Da [44]. The molecular mechanisms for how claudin-15-deficiency induces these interesting phenotypes remain unclear.

2.6. Claudin-16 mutations and claudin-16-deficient mice

In the kidney, nutrients and electrolytes are reabsorbed while primary urine flows in nephron segments, characterizing the intense epithelial transport in this organ. The epithelial cells in each nephron segment express specific sets of membrane transporters and claudin types to achieve their unique properties of epithelial transport, which is composed of transcellular and paracellular pathways [45]. In the kidney, Mg^{2+} is predominantly reabsorbed in the thick ascending limb (TAL) of Henle via a paracellular pathway [46]. A luminal positive transepithelial potential is established in the TAL, and this potential and the chemical gradient are thought to provide the driving force for Mg^{2+} reabsorption [47].

Claudin-16 was initially reported as paracellin-1, which is the causative gene for a rare human autosomal recessive hereditary disease termed familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC) with severe renal Mg^{2+} wasting [48]. Expression of claudin-16 is restricted to the TAL, where Mg^{2+} ions are reabsorbed. The claudin-16 gene was mapped on chromosome 3q27 [49], and several nonsense mutations and many point mutations in the extracellular, transmembrane and cytoplasmic domains have been identified in FHHNC [48–50,81,82]. Since the symptoms of FHHNC are excessive urinary excretions of Mg^{2+} and Ca^{2+} ions, it was once hypothesized that claudin-16 forms a selective channel for divalent cations, including Mg^{2+} and Ca^{2+}, for their reabsorption [48]. However, recent intensive studies of claudin-16-deficient (knockdown) mice as well as claudin-16-overexpressing epithelial cells support the idea that claudin-16 creates a non-selective cation channel.

Claudin-16-deficient mice were recently generated using RNA interference (RNAi) technology [51]. These claudin-16 knockdown (Cldn-16 KD) mice show significantly reduced plasma Mg^{2+} levels and excessive urinary excretions of Mg^{2+} and Ca^{2+}. Furthermore, calcium deposits are observed in the basement membranes of the medullary tubules and the interstitium in the kidney of adult Cldn-16 KD mice. These phenotypes of Cldn-16 KD mice are very similar to the symptoms in human FHHNC patients.

Electrophysiological analyses of isolated TALs revealed that the paracellular pathway of the TAL is cation-selective without discrimination among monovalent (Na^+) and divalent (Mg^{2+}/Ca^{2+}) cations in wild-type mice and that this cation selectivity is significantly reduced in Cldn-16 KD mice, suggesting that claudin-16 works as a non-selective cation channel in the TAL [51]. These conclusions for the TAL are consistent with those obtained from *in vitro* analyses of claudin-16-overexpressing LLC-PK1 epithelial cells by the same research group [15]. Based on these studies, a model explaining claudin-16-deficiency has been proposed [51]. According to this model, the luminal positive transepithelial potential in the TAL, which is the driving force for Mg^{2+} reabsorption, is primarily generated by the diffusion potential. In the TAL, NaCl is electrogenically absorbed, resulting in NaCl concentration in the basolateral extracellular space and a diluted fluid in the lumen. A diffusion potential occurs between these spaces since the TJs formed by claudin-16 in the TAL are cation-selective, thereby generating the luminal positive potential of the TAL. Claudin-16-deficiency results in the loss of cation selectivity of TJs in the TAL, finally leading to dissipation of the luminal positive potential and loss of Mg^{2+} reabsorption.

In addition to the hereditary human disease FHHNC, a null mutation of claudin-16 has been reported in cattle, which results in chronic interstitial nephritis and renal tubular dysplasia [53,54]. The clinical features of the bovine disease differ from those of the human disease, but the mechanisms behind these differences are not yet understood.

2.7. Claudin-19-deficient mice and claudin-19 gene mutations

In the myelinated axons of the peripheral nervous system (PNS), the existence of TJ strand-like structures has been reported within Schwann cells [55,56]. These structures are densely and spirally concentrated at the Schmidt–Lanterman incisures and paranodes, and occur as two parallel lines along the outer and inner mesaxons. Claudin-19 is expressed in large amounts in the PNS, but not the CNS, and constitutes these TJ-like structures in Schwann cells [57]. Claudin-19-deficient mice are born alive and grow normally, but show behavioral abnormalities that can be attributed to PNS deficits [57]. Consistently, ultrathin election electron microscopy has revealed that membrane contacts of the TJ-like structures in the outer and inner mesaxons observed in wild-type mice are never observed in Cldn-19$^{-/-}$ mice [57]. The overall organization of the myelin remains unchanged in Cldn-19$^{-/-}$ mice. One possibility is that claudin-19-based TJs in Schwann cells function for electric sealing in myelin, although this has not yet been demonstrated.

Claudin-19 is also expressed in the TAL, the major site of paracellular Mg^{2+} reabsorption in the kidney [58]. Mutations in the claudin-19 gene are associated with recessive magnesium wasting, whose locus was mapped on chromosome 1p34.2 [59]. The renal phenotype in these patients is very similar to that of FHHNC patients with claudin-16 mutations, although the patients also have visual impairment. Consistent with the latter phenotype, claudin-19 is expressed in retinal pigmental epithelial cells, at least in zebrafish [59]. *In vitro* analyses demonstrated that claudin-19 functions as a Cl^- blocker at TJs in LLC-PK1 epithelial cells, while claudin-16 functions as a Na^+ channel [17]. Very recently, it has been shown that claudin-16 and claudin-19 form a physical complex within cells and generate cation selectivity of TJs in a synergistic manner [17].

2.8. Claudin-deficient zebrafish

Zebrafish *Danio relio* is another useful vertebrate model for genetic analyses of gene function *in vivo*. An insertional mutation in the claudinj gene at the 3′ untranslated region results not only in smaller otoliths in the ear but also in hearing and vestibular dysfunction [83]. *In situ* hybridization revealed that claudinj is expressed in ear and brain. Morpholino inhibition of cldnj expression showed similar defects in otoliths formation [83]. Although the mechanism how otoliths formation is affected remains unclear, one possibility is that the assembly of otolith material is inhibited due to altered ionic environment in the ear caused by claudinj mutation [83].

Zebrafish claudin-15 is expressed in the gut under the control of Tcf2 transcription factor [84]. Morpholino suppression of claudin-15 results in the multi-lumen formation in the gut [84]. The expression of claudin-15 creates cation pores and promotes single lumen formation in 3D-cultured MDCK cells [84]. Therefore, claudin-15 seems

to be involved in the lumen expansion in the gut development via fluid accumulation, which is controlled by the paracellular transport of ions through claudin-15-containing TJs [84].

3. Occludin-deficient mice

Occludin was identified as the first transmembrane component of TJs [61]. Occludin has a molecular mass of ~65 kDa and is composed of four transmembrane domains, two extracellular loops, a long C-terminal cytoplasmic domain, an N-terminal cytoplasmic domain and one intracellular turn. The TJs in most epithelial cells contain occludin, which is thought to be incorporated into claudin-based TJ strands [62]. However, its physiological function is not yet understood. Occludin-deficient ($OCLN^{-/-}$) mice possess morphologically normal TJs [63]. Intensive electrophysiological analyses in Ussing chambers have revealed no obvious differences in the epithelial transport and barrier functions between normal and $OCLN^{-/-}$ mice [63,64]. However, $OCLN^{-/-}$ mice exhibit various complex phenotypes, including chronic inflammation and hyperplasia of the gastric epithelium, calcification in the brain, testicular atrophy, loss of cytoplasmic granules in striated duct cells of the salivary gland and thinning of the compact bone [63]. These phenotypes cannot be simply explained in terms of barrier dysfunction of TJs. The gastric phenotype of $OCLN^{-/-}$ mice indicates that occludin may be involved in epithelial differentiation.

4. Tricellulin mutations

TJs circumscribe cells and function as barriers/channels in the paracellular pathway. To be more exact, TJs should be divided into two types, namely bicellular TJs (bTJs), which are formed by claudins between two adjacent cells, and tricellular TJs (tTJs), which occur at regions where three cells come into contact [65]. Since claudins act to form TJs between two cells, tTJs cannot simply be sealed by claudin-based TJ strands like bTJs. Freeze-fracture electron microscopy analyses have revealed that the TJ strands of bTJs approach from both sides to the very limit of the edge of the tricellular contact region, and then extend basolaterally [65]. As a result, a very narrow tube is formed at the extracellular region of a tricellular contact in the apicobasal direction. Although tTJs have been ignored for a long time, it is obvious that bTJs and tTJs should be considered separately in the context of the barrier functions of TJs.

Tricellulin is a recently discovered membrane protein that is concentrated in tTJs in various epithelial cells [66]. Tricellulin has four transmembrane domains and exhibits sequence similarity to occludin. In particular, its C-terminal cytoplasmic domain shows high sequence similarity to the same region of occludin, to which ZO-1 binds [66]. *In vitro* analyses revealed that this domain of tricellulin also binds to ZO-1 [67]. When the expression of tricellulin was suppressed by RNAi in mouse cultured epithelial cells, the barrier function of the epithelial cellular sheet became compromised and not only tTJs but also bTJs were disorganized, indicating crucial roles for tricellulin in the formation tTJs as well as bTJs [66].

Recessive mutations of the tricellulin gene cause human non-syndromic deafness *DFNB49* [67,68]. In mouse inner ears, tricellulin is found at tricellular contacts in most epithelial cells of the cochlea. In particular, the sensory epithelium of the organ of Corti shows remarkable localization of tricellulin along the entire depth of the tricellular region of the lateral membrane, reflecting well-developed tTJs between the OHCs and supporting cells [67]. All four mutations reported to date result in the loss of all or most of the conserved region in the C-terminal cytoplasmic domain, namely the occludin-ELL domain, which is required for ZO-1 binding [67]. These findings suggest that tricellulin mutants lacking the C-terminal cytoplasmic domain are sufficient for epithelial barrier functions in many tissues, but cannot maintain a very strong barrier in the inner ear due to its unusually steep concentration gradient of electrolytes.

5. Knockout mice and mutations of ZO-1, ZO-2 and ZO-3 genes

Many peripheral membrane proteins underlying the cytoplasmic surface of TJs have been identified. Among them, three closely related proteins, ZO-1, -2 and -3, are thought to be directly involved in TJ formation [69]. These proteins belong to the membrane-associated guanylate kinase (MAGUK) family, containing three PDZ domains (PDZ1, -2 and -3), one src homology 3 (SH3) domain and one guanylate kinase-like (GUK) domain from their N-termini. The PDZ1 domains of all these proteins bind to the cytoplasmic tails of claudins *in vitro* and normal claudins overexpressed in mouse L fibroblasts recruit these MAGUKs to reconstituted TJ strands, whereas C-terminal-deleted claudins do not [70]. The establishment of mouse epithelial cells lacking expression of ZO-1, -2 and -3 has revealed that ZO-1 or ZO-2 is required for TJ formation by claudin polymerization in epithelial cells, suggesting redundant functions of ZO-1 and ZO-2, at least for TJ formation [71]. In contrast, ZO-3 is dispensable for TJ formation in these cells, although it is specifically expressed in epithelial cells [72]. Overexpression of various ZO-1-deleted constructs in mouse epithelial cells lacking expression of ZO-1, -2 and -3 suggested that dimerization of ZO-1 beneath the plasma membrane triggers TJ formation by claudins [71]. Interestingly, in addition to the role of ZO-1 in TJ formation, detailed analyses of these cells further showed that ZO-1 is involved in adherens junction (AJ) formation through activation of Rac [73]. This observation seems to be consistent with the previously reported relationship between ZO-1 and AJs. ZO-1 is initially recruited to cadherin-based AJs during cell contact formation and then becomes distributed to TJs according to cell polarity formation [74]. In addition, ZO-1 and ZO-2 are components of AJs in, for example, cardiac muscle cells, which do not have TJs [75,76].

ZO-1-deficient mice have been reported very recently [77]. *ZO-1*$^{-/-}$ mice die at embryonic stage E10.5. *Tjp1*$^{-/-}$ embryos appear normal up to E8.5, but exhibit severe growth defects at E9.5, a significant reduction in size and the absence of turning with apoptosis in the notochord, neural tube area and allantois [77]. In the yolk sack, *Tjp1*$^{-/-}$ mice show defects in vascular development with abnormal angiogenesis, possibly due to defects in cell–cell adhesion-related tissue remodeling [77]. These phenotypes indicate critical roles for ZO-1 in development, and should be further analyzed from the viewpoint of AJ formation as well as TJ formation.

A nucleotide substitution in the ZO-2 gene was identified in familial hypercholanemia (FHC), which was mapped to chromosome 9q12–q13 [78]. The affected patients have a point mutation (135C), which is predicted to cause a valine-to-alanine substitution (V48A) in the ZO-2 protein [78]. The mutated valine lies within the PDZ1 domain of ZO-2 and is highly conserved in many PDZ domains as a branched hydrophobic residue. *In vitro* analyses demonstrated that the V48A mutation affects binding to the C-terminal region of claudins. This mutation is not the sole cause of this type of FHC, which is very rare, since the frequency of this mutation is high (7%), indicating oligogenic inheritance [78]. Although the V48A substitution has been proposed to cause increased paracellular permeability to bile acids from the bile to the plasma, its detailed mechanism remains totally unknown.

ZO-2-deficient mice have been reported very recently [85]. *ZO-2*$^{-/-}$ embryos die shortly after implantation. Egg cylinders of *ZO-2*$^{-/-}$ embryos do not show mesoderm development with reduced cell proliferation followed by apoptosis [85]. Ectoderm of E6.5 and E7.5 embryos show reduced electron dense plaque of the cytoplasmic region of TJs and barrier defect to lanthanum tracer although their epithelial polarity appears normal [85]. Although *ZO-2*$^{-/-}$ embryoid bodies do not show obvious abnormality in the structure and barrier function of TJs probably due to redundant function of ZO-1 [85], subtle change in TJ function by ZO-2-deficinecy may be enough to affect the development of mouse embryo.

ZO-3-deficient mice exhibit no phenotypes despite the epithelia-specific expression of ZO-3 [72,85]. On the other hand, morpholino inhibition of ZO-3 in zebrafish embryogenesis results in various abnormalities such as edema, loss of blood circulation, and tail fin malformations [86]. These phenotypes accompany with partial disruption of TJ structure and permeability barrier [86], suggesting the difference in the contribution of ZO-1, ZO-2, and ZO-3 to TJs among species.

6. Conclusions

Many pathologies of knockout mice and mutations of the claudin and tricellulin genes in hereditary human diseases have been interpreted as defects in TJ functions. As observed for claudin-16 mutations, however, the pathologies are also caused by the impaired channel properties of TJs for selective paracellular transport, i.e. abnormal strengthening of the TJ barrier function. We should note that these two sides of TJ-associated diseases are brought about by variations in the types of claudins, which display unique barrier or channel properties. Due to the complex expression patterns of claudins, the pathologies of the mice and natural disorders vary. As seen in NISCH syndrome with a claudin-1 mutation and Cldn-11$^{-/-}$ mice, syndromic symptoms appear to be derived from the peculiar tissue expression patterns of these claudins.

Furthermore, it is of interest that mutations in tricellulin, which is expressed in most epithelial cells and seems to be involved in the barrier functions of TJs in general, were identified in one of the familial non-syndromic deafness (DFNB) diseases. Since the inner ear has to be equipped with very strong TJ barriers to maintain its unusually steep concentration gradient of electrolytes for hearing, it is a very sensitive system for detecting defects in TJ barrier functions from the pathology of deafness. Further positional cloning of causative genes for DFNB diseases may lead to the identification of novel molecular components required for the barrier functions of TJs.

On the other hand, knockout mice for several claudins and occludin exhibit their phenotypes through cell death or cell growth, and these effects still cannot be directly interpreted as defects in the barrier functions of TJs. Indeed, the signaling function of TJs has been so far proposed [87,88]. Further analyses of these phenotypes will be useful toward clarifying the complex roles of TJs *in vivo*.

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