Author correction

Turksen, K. and Troy, T.-C. (2004). Barriers built on claudins. *J. Cell Sci.* **117**, 2435-2447.

We wish to correct some errors that we inadvertently introduced in revising and editing our recent Commentary. In the text, we incorrectly cited (Wolburg et al., 2003) in reference to the downregulation of claudin-23 in gastric cancer; the reference should be (Katoh and Katoh, 2003) as correctly cited in Table 3. (Peacock et al., 1997) was mistakenly inserted in relation to the discussion of the number of claudin genes. We also wrote the number of human claudin genes as 24, the number currently ascribed to the mouse claudin genes (GenBank). The correct number ascribed to human claudin genes by (Katoh and Katoh, 2003) is 23, up from the originally predicted 20 (Venter et al., 2001). In this regard, it is worth noting that, since our Commentary was published, (Loh et al., 2004) have annotated the claudins in the teleost *Fugu rubripes* genome and reported 56 claudin genes, of which only 35 can be assigned orthology to 17 mammalian claudin genes, with the remaining 21 being specific to the fish lineage and most of the 56 expressed in a more or less tissue-specific fashion, or at particular developmental stages. This, along with other issues we raised, suggests that additional annotation of multiple other genomes and other functional genomics approaches will be useful to advance our understanding of claudin biology and physiology. Finally, based on a Clustal analysis of full-length claudins, we reported that there is a highly conserved WWCC motif of unknown function within the first loop of the claudins analysed; a motif also reported in alignments of claudins carried out by (Katoh and Katoh, 2003).

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Barriers built on claudins

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This review is dedicated to Rosa Beddington who unintentionally encouraged us to work on claudins.

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Summary

The fundamental functions of epithelia and endothelia in multicellular organisms are to separate compositionally distinct compartments and regulate the exchange of small solutes and other substances between them. Tight junctions (TJs) between adjacent cells constitute the barrier to the passage of ions and molecules through the paracellular pathway and function as a 'fence' within the plasma membrane to create and maintain apical and basolateral membrane domains. How TJs achieve this is only beginning to be understood. Recently identified components of TJs include the claudins, a family of four-transmembrane-span proteins that are prime candidates for molecules that function in TJ permeability. Their identification and characterization have provided new insight into the diversity of different TJs and heterogeneity of barrier functions in different epithelia and endothelia.

Key words: Claudins, Tight junctions (TJs), Paracellular permeability, Permeability barrier, Cell polarity

Introduction

In multicellular organisms, certain tissues must be separated from each other and protected against the external environment. Epithelial and endothelial sheets achieve this by providing cellular borders that cover external and internal surfaces throughout the body. Complexes between adjacent cells in these sheets include gap junctions, desmosomes, adherence junctions and tight junctions (TJs) – also known as the zonula occludens. Early ultrastructural and morphological data revealed TJs as continuous circumferential intercellular contacts between epithelial cells (Farquhar and Palade, 1963) that create a barrier to the paracellular movement of water, solutes and immune cells (Madara, 1998; Nusrat et al., 2000). Later work showed that this epithelial barrier is heterogeneous in tightness and dynamics depending on the tissue. In addition it is physiologically regulated, and its disruption contributes to human disease. Numerous in-depth reviews on TJs exist (Begley and Brightman, 2003; Fanning et al., 1999; Gonzalez-Mariscal et al., 2003; Heiskala et al., 2001; Madara, 1989; Mitic and Anderson, 1998; Stevenson, 1999; Tsukita and Furuse, 1999; Tsukita and Furuse, 2000a; Tsukita and Furuse, 2000b; Tsukita et al., 1999; Tsukita et al., 2001; Zahraoui et al., 2000). Here we therefore only briefly summarize their essential features and then focus on the recent identification of claudins and our evolving understanding of their contribution to TJ structure and function.

Tight junctions – morphology and molecules

The morphological basis of TJs became apparent during the 1960s with the emergence of the electron microscope. Early studies described TJs as a series of apparent fusions between the outer leaflets of plasma membranes of adjacent cells to form so-called 'kissing points' where the intercellular space disappears (Gonzalez-Mariscal et al., 2003; Tsukita et al., 2001). These ultrastructural studies also indicated that TJs might provide a barrier to the diffusion of solutes through the paracellular pathway in addition to functioning as a diffusion barrier within the plasma membrane to create and maintain apical and basolateral membrane domains (for reviews see Cereijido et al., 1998; Heiskala et al., 2001; Schneeberger and Lynch, 1992; Tepass, 2003; Tsukita and Furuse, 1999; Tsukita and Furuse, 2002). More details on the morphology of TJs came from freeze-fracture transmission electron microscopy, which showed TJs as a continuous network of intramembranous protein-containing particles (Staehelin, 1974; Tsukita and Furuse, 1999). Notably, the TJ strands observed in one cell are associated laterally with TJ strands in opposing membranes of adjacent cells to form paired TJ strands (Sasaki et al., 2003).

During the late 1980s, biochemical and immunolocalization studies identified the 225 kDa protein zonula occludens-1 (Z0-1) as the first polypeptide exclusively associated with the TJ (Stevenson et al., 1986). ZO-2 and ZO-3, which are highly related to ZO-1, were identified later (Gumbiner et al., 1991; Haskins et al., 1998; Jesaitis and Goodenough, 1994). Immunolocalization by both light- and electron microscopy has revealed that all three known ZOs (ZO-1, ZO-2 and ZO-3) are located exclusively at the cytoplasmic surface of TJs in the immediate vicinity of the plasma membrane. ZOs are modular molecules comprising three PDZ domains (PDZ1, PDZ2, PDZ3), one SH3 domain, and one guanylate kinase (GUK) domain that belongs to the membrane-associated guanylate kinase (MAGUK) protein family (Gonzalez-Mariscal et al., 2000; Haskins et al., 1998; Itoh et al., 1993; Jesaitis and Goodenough, 1994; Willott et al., 1993). Occludin was the first integral membrane protein found in TJ strands of many different cell types (Ando-Akatsuka et al., 1996; Furuse et al., 1993). The N- and C-termini of occludin reside in the cytoplasm and two extracellular loops project into the paracellular space. The loops within the paracellular space are thought to interact with loops originating from occludin in

neighboring cells to promote ‘sealing’ of the paracellular space. Systematic structure-function analysis has indicated that the C-terminal cytoplasmic domain of occludin is important for the interaction with ZO-1 (Furuse et al., 1994).

Knockout studies demonstrated that loss of occludin does not stop the formation of TJs in embryonic stem (ES) cells. Considering that differentiating ES cells normally aggregate to form simple and then cystic embryoid bodies (EBs) that express TJs (Troy and Turksen, 2002; Turksen and Troy, 2001), the observation that occludin-deficient ES cells form simple and then cystic EBs with the same time course as wild-type ES cells was surprising. Furthermore, immunofluorescence microscopy and ultrathin-section electron microscopy revealed that the cells form EBs that have polarized epithelial (visceral endoderm-like) cells and TJs (Saitou et al., 1998). Similarly, freeze-fracture analyses indicated no significant differences in the number or morphology of TJ strands between wild-type and the knockout cells, which is consistent with the observation that the knockout mice survive to adulthood. Although these animals exhibit a variety of abnormalities in tissues requiring TJs, it is not clear which are directly attributable to occludin deficiency and, surprisingly, at least some TJs (e.g. those of intestinal epithelium) have apparently normal structures and functions (Saitou et al., 2000). Other occludin-like molecules might therefore exist, but attempts to identify them have so far failed [although a shorter form of occludin, occludin 1B, is known (Muresan et al., 2000)]. An alternative scenario is that other molecules can form strand structures and form effective barriers without occludin.

The claudins

Biochemical efforts to identify other TJ molecules initiated by Tsukita and coworkers (Furuse et al., 1998a) yielded potential candidates in TJ fractions from chicken liver. In the TJ fraction was a 22 kDa protein whose purification and peptide sequencing allowed cloning of two full-length cDNAs encoding related proteins of 211 and 230 residues, which they named claudins from the Latin word ‘close’. The identification of claudin-1 and claudin-2 led to the recognition that several genes that had already been cloned [e.g. TMVCF (Sirotkin et al., 1997); RVP.1 (Briehl and Miesfeld, 1991); SEMP1 (Swisshelm et al., 1999); Skulin (Turksen and Troy, 2001) and BCMP1 (Christophe-Hobertus et al., 2001)] also belong to the claudin family (Morita et al., 1999a). To date, 24 distinct claudin family genes have been identified in human (Peacock et al., 1997). Many orthologues have also been reported in mouse (Morita et al., 1999a), rat (Briehl and Miesfeld, 1991), frog (Behr et al., 2003; Fujita et al., 2002), fly (Behr et al., 2003), worm (Asano et al., 2003; Simske et al., 2003) and zebrafish (Kollmar et al., 2001). Sequence and expression analysis indicate that they have evolutionarily conserved functions (Kollmar et al., 2001), and recent studies discussed below suggest that the claudins might explain the previous mysterious finding that TJs can form in the absence of occludin.

Claudin structure

Claudins are in the 22 kDa to 27 kDa range. They are integral membrane proteins that have four hydrophobic transmembrane domains and two extracellular loops (the first loop is larger than the second), which appear to be involved in the homophilic and/or heterophilic interactions implicated in TJ formation (Fig. 1, Fig. 2 and Fig. 3). The WWCC motif, W-X(17-22)-W-X(2)-C-X(8-10)-C, within the first loop is conserved among members of the claudin family (Fig. 1 and Fig. 4, sequence highlighted in yellow). The cytoplasmic tail, although relatively constant in length has a sequence that diverges among the claudins. Several other sequences provide clues to the potential role of the cytoplasmic tail, including several potential phosphorylation sites and a PDZ-binding sequence (Fig. 1 and Fig. 4, YV highlighted in pink).

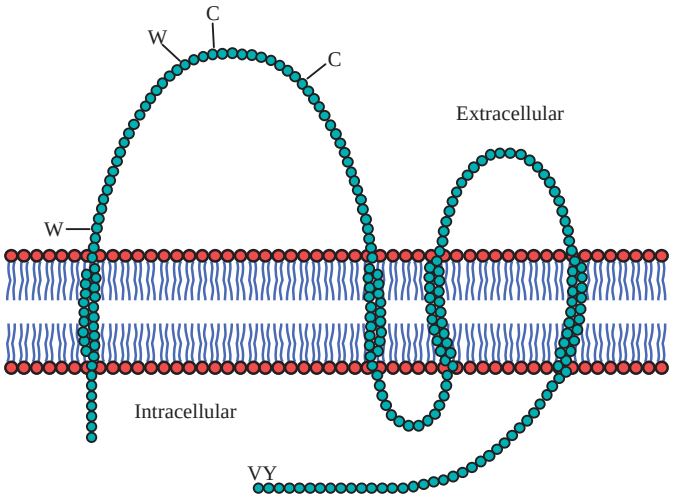

Fig. 1. Diagrammatic depiction of claudin.

Claudins as structural and functional components of TJs

Tagged claudins introduced into cultured Madin-Darby canine kidney cells (which have TJs) or mouse L fibroblasts (which do not have TJs) co-distribute at cell contact sites with other TJ molecules, such as ZO-1 and occludin (Furuse et al., 1998b). Ultrastructural analysis of cells transfected with claudin-1 and claudin-2 indicated that these proteins are also associated with the fibrils that form at points of contact between cells. Interestingly, however, claudin-1-containing strands are largely associated with the protoplasmic face of the cell and are mostly continuous structures. Claudin-2-containing strands, by contrast, are discontinuous at the protoplasmic face.

Furuse et al. co-transfected claudin-1, claudin-2 and claudin-3 into L cells in pairs and detected them at cell-cell borders in elaborate networks (Furuse et al., 1999). Immunoreplica electron microscopy confirmed that different claudins are indeed co-incorporated into individual TJ strands. Interestingly, when two L cell single transfectants expressing claudin-1, claudin-2 or claudin-3 are co-cultured, claudin-3-containing strands associate with claudin-1- and claudin-2-containing strands on adjacent cells to form paired strands, whereas claudin-1-containing strands do not interact with claudin-2-containing strands (Furuse et al., 1999). This indicates that there are specific claudin interactions within and between TJ strands. Kinetic analysis of GFP-claudin-1-

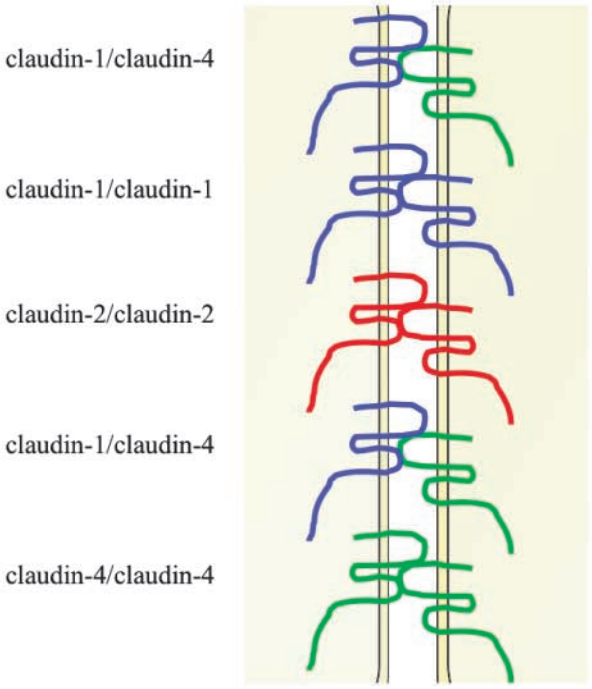

Fig. 2. Proposed paired TJ strands consisting of different claudin combinations.

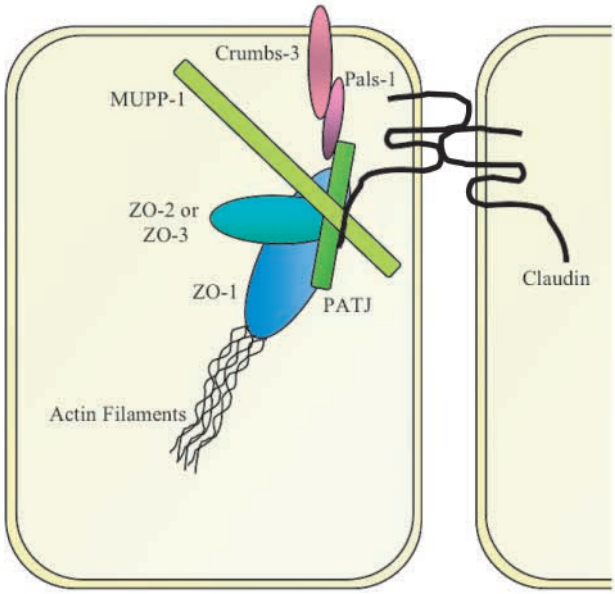

Fig. 3. Schematic representation of claudin and interacting molecules at the tight junction.

containing strands in vitro has also indicated that, although claudins are not mobile within paired strands, claudin-1-containing strands are dynamic: strands occasionally break and anneal, dynamically associating with each other in both an end-to-side and side-to-side manner. Similar experiments with other claudin family members remain to be done; nevertheless, different modes of assembly of claudins clearly could increase the diversity of the structure and functions of TJ strands and provide a molecular basis for the heterogeneity of barrier function seen in different tissue types.

When claudin-2 is introduced into MDCK I cells (which normally express claudin-1 and claudin-4), the transepithelial resistance (TER) values fall (>20-fold decrease) to characteristic levels of MDCK II cells (which normally express claudin-2) (Furuse et al., 2001). By contrast, when claudin-3 is introduced into MDCK I cells, no change in their TER is detected. Claudin-2 thus markedly decreases the 'tightness' of individual claudin-1/claudin-4-based TJ strands, providing support for the hypothesis that different combinations of claudins determine the barrier properties of individual TJ strands. In vivo support for this hypothesis came from studies of transgenic mice over-expressing claudin-6 in the epidermis. In these mice, the endogenous levels of multiple claudins are perturbed, and the epidermal permeability barrier is disrupted in the transgenic epidermis with deadly consequences (Turksen and Troy, 2002). Collectively, these studies support the notion that specific claudins contribute to fibril formation and barrier function in specific tissues, but the precise relationship between fibril formation, barrier formation and barrier tightness remains to be elucidated.

The extracellular loops, whose sequences are distinct in different claudins, contribute to the formation not only of TJ strands but also of ion-selective channels (Schneeberger, 2003). For example, overexpression of wild-type claudins and chimeras containing swapped extracellular domains increases both the number and depth of fibrils; but switching extracellular domains does not alter the characteristic fibril morphologies generated by claudin-4 or claudin-2. Claudin-2-based chimeras containing the first or both extracellular domains of claudin-4 increase TER severalfold and profoundly decreased the permeability to Na^+ relative to Cl^- (Colegio et al., 2003). By contrast, claudin-4-based chimeras containing the first or both extracellular domains of claudin-2 increase TER by only ~60% and ~40%, respectively, and only modestly alter charge selectivity. These results support a model (Fig. 2) in which the combination and mixing ratios of claudins create paracellular channels (Furuse et al., 2001) and the first extracellular domain is sufficient to determine both paracellular charge selectivity and transepithelial resistance (Colegio et al., 2003). The precise sequences responsible have not been established.

Tsukita and co-workers have proposed that TJ strands are linear co-polymers of occludin and various claudins (Furuse et al., 1999; Tsukita and Furuse, 1999). These co-polymers probably present large linear clusters of YV sequences toward the cytoplasm, which might attract cytoplasmic proteins containing PDZ domains that have high affinity for the C-terminal sequences of claudins (Harris and Lim, 2001; Pawson and Nash, 2003; Sheng and Sala, 2001). ZO-1, ZO-2 and ZO-3, for example, bind in vitro to the C-terminal YV sequences of several claudins through their PDZ domains (Itoh et al., 1999). During the past few years, several membrane proteins that participate in TJ-scaffolding complexes have been identified; some of these might directly interact with claudins (Hamazaki et al., 2002). MUPP1 (multi-PDZ domain protein 1) contains 13 PDZ domains that are binding partners for the C-terminus of claudin-1 (Poliak et al., 2002; Tiwari-Woodruff et al., 2001). OSP/claudin-11-associated protein (OAP) 1 (a novel member of the tetraspanin family, identified in a yeast-

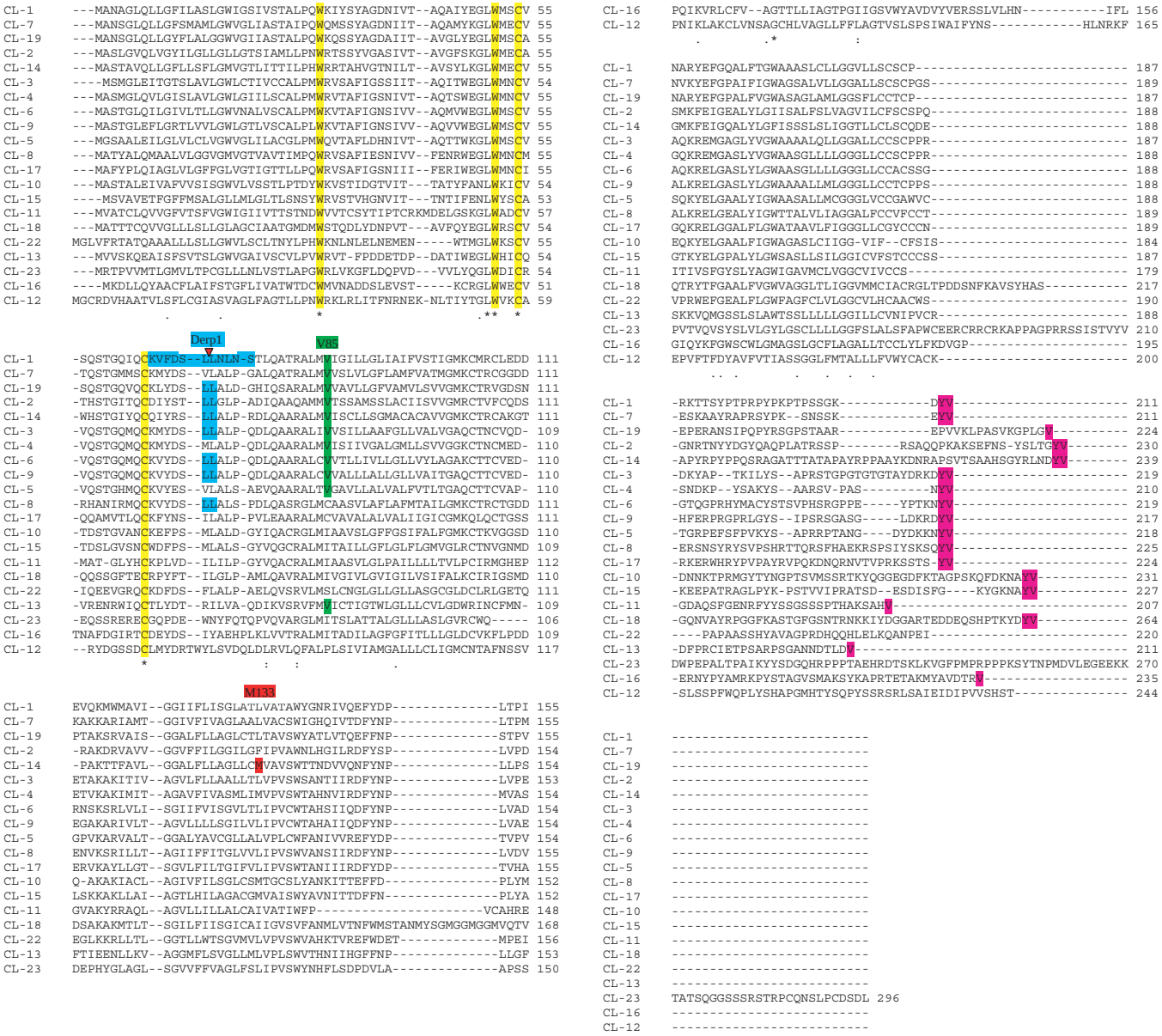

Fig. 4. ClustalW analysis of claudins.

two-hybrid screen using the tail of claudin-11 as bait), claudin-11 and β-1 integrin form a complex, which might have a role in adhesion and integrin signaling (Tiwari-Woodruff et al., 2001). PATJ (PALS1-associated tight junction protein) interacts with Pals1 (protein associated with Lin-seven; a member of the MAGUK family) and CRB1 (Crumbs 1; an apical transmembrane protein) to form a tripartite TJ complex involved in epithelial cell polarity (Hurd et al., 2003; Lemmers et al., 2002; Poliak et al., 2002). The sixth and eighth PDZ domains of PATJ can interact with the C-termini of ZO-3 and claudin-1, respectively. Notice, that not all TJ components directly interact with claudins. JEAP (junction-enriched and associated protein) (Nishimura et al., 2002) and PILT (protein incorporated later into TJs) (Kawabe et al., 2001), for example, are two newly identified peripheral TJ proteins that do not interact with claudins. Notice also, that the molecular linkages between proteins in the complex might be dynamically regulated (for discussion see (Itoh et al., 1999). For example, phosphorylation of some or all of the potential phosphorylation sites in claudins could play an important role in binding dynamics; however, this has not yet been investigated.

Barrier diversity

Early studies on claudin distribution suggested that the diverse barrier functions of different epithelial and endothelial tissues reflect the use of different claudins, and more recent work indicates claudin expression patterns could indeed be responsible for the known variations in permeability. For example, claudin-1, claudin-2, claudin-3, claudin-4, claudin-8, claudin-10, claudin-11 and claudin-16 exhibit a very

complicated, segment-specific expression pattern in the developing nephron (Kiuchi-Saishin et al., 2002). An equally complex distribution of claudins is also apparent in other tissues that have regionally diverse barrier function, such as the intestine (Rahner et al., 2001). In general, however, the data so far suggest that claudin-1 is rather ubiquitous (Furuse et al., 1998b) whereas claudin-6 is developmentally restricted (Sousa-Nunes et al., 2003; Turksen and Troy, 2001) and not expressed in adult tissues (Turksen and Troy, unpublished observations). Claudin-5 is more or less specific to endothelial cells (Morita et al., 1999b) (see Table 1).

Emerging evidence supports the view that claudins are both dynamically regulated under normal conditions to respond to the selective permeability needs of the tissues and that they are developmentally regulated. Studies of claudin expression patterns in zebrafish and *Xenopus* support the concept that their role in development is evolutionarily conserved (Brizuela et al., 2001; Kollmar et al., 2001). Analysis of trophectodermal epithelial intercellular junction formation during different cleavage stages of human embryos indicated that claudin-1 is expressed in blastocysts and throughout human preimplantation development, whereas claudin-4 is upregulated during implantation (Riesewijk et al., 2003). Claudin-1 appears to play no part in the formation of endothelial tight junctions in the first-trimester placenta but is required later (Orchard and Murphy, 2002). Claudin-11 is present at TJs in testes; its expression depends upon male gonad development and systemic and local signaling molecules. It appears early in fetal development in Sertoli cells, immediately after the peak of SRY (sex-determining region, Y gene) expression but just before that of the anti-Mullerian hormone. It is also regulated by tumor necrosis factor-alpha (TNFα) and follicle-stimulating hormone (FSH), which indicates a critical role for these molecules in the (re)modeling process of the hematotesticular barrier during spermatogenesis (Bronstein et al., 2000).

Mouse ES cell cultures have also been used to reveal the expression dynamics of claudin-6 during aggregation-induced commitment to epithelial differentiation in vitro. Whole-mount in situ hybridization studies indicate that claudin-6 is one of the earliest molecules to be expressed in ES cells committed to the epithelial fate in the ectoderm, and the onset of its expression coincides with the expression of the early epithelial marker keratin 8 (K8). The initiation of expression of claudin-6 in EBs in vitro depends on the bone morphogenic protein (BMP) signaling pathway, which suggests that BMPs are involved in claudin-6 expression and epithelialization (Turksen and Troy, 2001).

Claudin genes and claudin gene expression

The chromosomal localizations of the currently known claudin family genes within the human and mouse genomes are outlined in Table 2, and some interesting clues are emerging from sequence analysis. For example, the human claudin-6 and claudin-9 genes are linked in an inverted convergent (tail-to-tail) configuration, with a 1.4 kb intergenic region (Turksen and Troy, unpublished observations). Interestingly, other claudin genes have a similar tail-to-tail organization (e.g. claudin-3 and claudin-4). There are interesting parallels between this and the known *Dlx* genes [*Dlx* genes encode homeobox-containing transcription factors related to Drosophila Distal-less (Dll)], which are convergently transcribed and linked as tail-to-tail pairs in a combinatorial manner in several species (Ghanem et al., 2003; Zerucha and Ekker, 2000). Whether similar combinatorial regulation applies to the claudin gene pairs remains to be seen, however.

Analysis of individual claudin promoters has now begun to provide an understanding of their tissue-specific regulation (Sakaguchi et al., 2002). For example, the 5′-flanking region of the claudin-2 gene, which is expressed in the kidney, liver and intestine, contains binding sites for the intestine-specific Cdx homeodomain proteins and hepatocyte nuclear factor 1 (HNF-1), factors conserved in human and mouse. HNF-1α augments the Cdx2-induced, but not Cdx1-induced, transcriptional activation of the promoter. In mice, HNF-1α is required for claudin-2 expression in the villus epithelium of the ileum and within the liver, but not in the kidneys, which indicates an organ-specific function of HNF-1α in the regulation of claudin-2 gene expression. Deletion analysis of the claudin-18 promoter provided information that *T/EBP/NKX2.1*, a member of the NKX family of homeodomain-containing transcription factors, regulates the expression of claudin-18 in lung and thyroid (Niimi et al., 2001). Interestingly, the claudin-18 gene has two promoters, each of which uses a unique exon one that is spliced to common exons two through five. Alternative usage of these promoters produces lung- and stomach-specific transcripts. The downstream lung-specific promoter contains two T/EBP/NKX2.1-binding sites that drive expression in lung cells. Analysis of *T/EBP/NKX2.1*-null embryo lungs indicated that only claudin-18 is downregulated among 11 claudin transcripts examined. The fact that barrier function of the lungs in these animals is aberrant supports the hypothesis that claudins have tissue-specific roles in barrier formation.

The promoters of the genes encoding claudin-3, claudin-4 and claudin-7 contain multiple E-boxes [E-box motifs with the sequence CAC(G/A)TG bind to transcription factors of the basic helix-loop-helix/leucine zipper (bHLH/zip) family, including the Myc, upstream stimulatory factor (USF) and transcription factor E (TFE) subfamilies]. Transfection as well as electrophoretic mobility shift assays revealed that the transcription factor Snail binds directly to these and completely represses promoter activity. For example, when Snail is overexpressed in cultured mouse epithelial cells, the transition occurs with the concomitant repression of claudin expression (Ikenouchi et al., 2003). This is consistent with studies showing that Snail is a transcriptional repressor that plays a central role in the epithelial-mesenchymal transition, during which epithelial cells lose their polarity during development. This also points to a role for claudins in both normal epithelial cytoarchitecture and the epithelial-mesenchymal transition during metastasis.

Anomalies in claudin expression are responsible for aberrant barrier function

Aberrant TJ barrier function and increased tissue permeability are common characteristics of several diseases, such as pulmonary edema, jaundice, inflammatory bowel disease, diarrhea, several kidney diseases, diabetic retinopathy inflammation, blood-borne metastasis and numerous

Table 1. Tissue and cell line distribution of claudins

	Known claudin expression (+ve or –ve)	Reference
Tissues		
Skin		
Periderm	6^+	Morita et al., 2002
Epidermis	$1^+ 2^- 3^+ 4^+ 6^+ 8^+ 12^+ 17^+$	Brandner et al., 2002; Furuse et al., 2002; Tebbe et al., 2002; Turksen and Troy, 2002
Hair follicle	1^+	Tebbe et al., 2002
Dermis		
Endothelium	5^+	Morita et al., 2003
Cornea		
Epithelium	$1^+ 2^+ 3^+ 7^+ 9^+ 14^+$	Ban et al., 2003
Lung		
Endothelium	5^+	Favre et al., 2003
Testis	1^+	Gye, 2003b; Gye and Ohsako, 2003
Sertoli cells	$1^+ 11^+$	Gye, 2003a
Endothelium	5^+	Kamimura et al., 2002
Retina		
Vascular endothelium	5^+	Barber and Antonetti, 2003
Breast	$4^+ 7^+$	Kominsky et al., 2003; Nichols et al., 2004
Arteries	10^+	Qin et al., 2003
Prostate	4^+	Nichols et al., 2004
Bladder	4^+	Nichols et al., 2004
Pancreas	4^+	Nichols et al., 2004
Duct epithelia	$2^+ 3^+ 4^+$	Rahner et al., 2001
Acinar cells	$3^+ 4^+ 5^+$	Rahner et al., 2001
Ovary	$1^+ 3^+ 4^+$	Rangel et al., 2003a; Zhu et al., 2004
Brain	5^+	Nitta et al., 2003
Cerebral endothelia cells	1^+	Kis et al., 2003
Myelinating Schwann cells		
Paranodal loop	1^+	Poliak et al., 2002
Incisures	5^+	Poliak et al., 2002
Kidney		
Nephron	$7^+ 8^+$	Li et al., 2004; Muller et al., 2003
Distal tubule	$1^+ 3^+ 8^+$	Kiuchi-Saishin et al., 2002; Reyes et al., 2002
Collecting duct	$1^+ 3^+ 4^+ 8^+$	Kiuchi-Saishin et al., 2002; Reyes et al., 2002
Proximal tubule	$2^+ 10^+ 11^+$	Kiuchi-Saishin et al., 2002; Reyes et al., 2002
Thin descending limb of the loop of Henle	2^+	Kiuchi-Saishin et al., 2002
Thin ascending limb of the loop of Henle	$3^+ 4^+ 8^+$	Kiuchi-Saishin et al., 2002
Thick ascending limb of the loop of Henle	$3^+ 10^+ 11^+ 16^+$	Kiuchi-Saishin et al., 2002; Yu et al., 2003
Bowman's capsule	$1^+ 2^+$	Kiuchi-Saishin et al., 2002
Vasculacure	$5^+ 15^+$	Kiuchi-Saishin et al., 2002; Reyes et al., 2002
Inner ear		
Cochlea	$1^+ 2^+ 3^+ 9^+ 10^+ 12^+ 14^+ 18^+$	Kitajiri et al., 2004
Inner hair cells	14^+	Ben-Yosef et al., 2003
Outer hair cells	14^+	Ben-Yosef et al., 2003
Supporting cells	14^+	Ben-Yosef et al., 2003
Organ of Corti	$1^+ 2^+ 3^+ 9^+ 10^+ 12^+ 14^+ 18^+$	Kitajiri et al., 2004
Stria vascularis		
Marginal cells	$1^+ 3^+ 8^+ 9^+ 10^+ 12^+ 14^+ 18^+$	Florian et al., 2003; Kitajiri et al., 2004
Basal cells	11^+	Kitajiri et al., 2004
Reissner's membrane	$1^+ 2^+ 3^+ 8^+ 9^+ 10^+ 12^+ 14^+ 18^+$	Kitajiri et al., 2004
Spiral limbus	$1^+ 2^+ 3^+ 8^+ 9^+ 10^+ 12^+ 14^+ 18^+$	Kitajiri et al., 2004
Vestibule		
Sensory epithelium	$1^+ 3^+ 9^+ 12^+ 14^+ 18^+$	Kitajiri et al., 2004
Dark cells	$1^+ 3^+ 9^+ 12^+ 14^+ 18^+$	Kitajiri et al., 2004
Lung	$1^+ 3^+ 4^+ 5^+ 18^+$	Coyne et al., 2003a; Niimi et al., 2001; Wang et al., 2003
Type II alveolar epithelium	$3^+ 4^+ 5^+$	Wang et al., 2003
Alveolus	5^+	Wang et al., 2003
Liver	$1^+ 2^+$	Kojima et al., 2003; Mazzon and Cuzzocrea, 2003; Rahner et al., 2001
Hepatocytes	$2^+ 3^+ 4^-$	Rahner et al., 2001
Endothelium	5^+	Rahner et al., 2001
Gut		
Gastrointestinal mucosa	4^+	Nichols et al., 2004
Peyer's patches		
Follicle associated epithelium	$2^+ 3^+ 4^+$	Tamagawa et al., 2003
Placenta	23^+	Katoh, 2003
Stomach	$23^+ 18^+$	Katoh, 2003; Niimi et al., 2001; K.T. and T.T.C., unpublished observations
Uterus		
Epithelium	1^+	Orchard and Murphy, 2002

Table 1. *Continued*

	Known claudin expression (+ve or –ve)	Reference
Cells in vitro		
ES cells		
Endothelial cells	5^+	Watabe et al., 2003
Epithelial cells	6^+	Turksen and Troy, 2001
EC cells		
Endodermal epithelium	$6^+ 7^+$	Chiba et al., 2003; Kubota et al., 2001
HaCat	$1^+ 3^+$	Tebbe et al., 2002
MDCK	1^+	Ohkubo and Ozawa, 2004
Eph4	3^+	Matsuda et al., 2004
OSE	1^+	Zhu et al., 2004
Caco-2	1^+	Dorkoosh et al., 2004
MDA-MB361	1^+	Hoevel et al., 2004
T84	$1^+ 4^+$	Bruewer et al., 2003
Endothelium-brain	$3^+ 5^+$	Hamm et al., 2004; Ishizaki et al., 2003; Stamatovic et al., 2003
Brain microvascular endothelium	$1^+ 3^+ 5^+$	Andras et al., 2003; Song and Pachter, 2003
b.End3	$1^+ 5^+$	Omidi et al., 2003
MDCK-II	$1^+ 3^+ 4^+$	Singh and Harris, 2004
PANC-1	4^+	Michl et al., 2003
HMEC	1^+	Swisshelm et al., 1999
Thymocyte	1^+	Tedelind et al., 2003
Retinal pigment epithelium	$1^+ 3^+$	Abe et al., 2003; Rajasekaran et al., 2003
COMMA-1D	1^+	Stelwagen and Callaghan, 2003
HECV-endothelium	$1^+ 5^+$	Ye et al., 2003
HPMC	1^+	Ito et al., 2003
T84	1^+	Yoo et al., 2003
Human airway epithelium	$1^+ 4^+$	Coyne et al., 2003b
Lung alveolar epithelial cells	$3^+ 4^+ 5^+$	Wang et al., 2003
Osteoblast	$1^+ 2^+ 3^+$	Prele et al., 2003

pathological conditions (Mazzon et al., 2002; Wolburg et al., 2003). In experimental autoimmune encephalomyelitis, brain and spinal cord sections reveal the selective loss of claudin-3 from the TJs of venules surrounded by inflammatory cuffs (Wolburg et al., 2001). Increased inflammation and proinflammatory cytokine expression are associated with various diseases of barrier function, such as the inflammatory bowel diseases ulcerative colitis and Crohn's (Kucharzik et al., 2001). Analyses of colonic mucosa from patients with ulcerative colitis and inflamed mucosa from Crohn's disease patients reveal a dramatic downregulation in the expression of claudin-1 in the epithelial cells immediately adjacent to the transmigrating neutrophils (Kucharzik et al., 2001). Similarly, collagenous colitis (an inflammatory disease of unknown etiology in which diarrhea is the leading symptom) is associated with a decrease in claudin-4 expression, which is associated with the barrier dysfunction that contributes to diarrhea by causing a leak in the flux mechanism (Burgel et al., 2002).

Inflammation-related changes in barrier function are also observed in the lung (Coyne et al., 2002); for example, TJs provide an important route for electrolyte transport across airway epithelium and provide a barrier to the migration of toxic materials from the lumen to the interstitium (Nishiyama et al., 2001). Interestingly, the airway epithelium of cystic fibrosis patients has abnormal TJ strands, which suggests that claudin expression might be modulated.

Various viruses and bacteria are able to disturb barrier function (Balkovetz and Katz, 2003; Gruenheid and Finlay, 2003; Hofman, 2003). For example, disruption of the blood-brain barrier is widely believed to be the main route by which HIV enters the CNS. The Tat protein, which is released from HIV-infected cells, alters vascular inflammatory responses as well as blood-brain barrier structure and function both in vitro and in vivo (Andras et al., 2003; Toborek et al., 2003). Exposure of primary cultures of brain microvascular endothelial cells (BMEC) to Tat(1-72) for 24 hours results in a decrease of claudin-1, claudin-5 and ZO-2 expression, whereas total levels of occludin and ZO-1 remained unchanged. In addition, even a short (3 hours) exposure of BMEC to Tat(1-72) induces cellular redistribution of claudin-5 immunoreactivity, which has been confirmed in vivo (Andras

Table 2. Chromosomal location of claudins

Claudin	Human	Mouse	Rat
1	3q28/3q21	16	11
2	X	X	X
3	7q11	5	7
4	7q11, 23	5	12
5	22q11.2	16	
6	16p13.3	17	10
7	17p12	11	
8	21q22.11	16	11
9	16p13.3	17	10
10	13q21.2	14	15
11	3q26.2	3	2
12	7	7	
13		5	
14	21q21	16	11
15	7q21.3	5	12
16	3q28	16	11
17	21q22.11	16	11
18	3	9	
19	1	4	5
21	4q35.1		
22	4q35.1		
23	8q23.1		
24	11q23		

et al., 2003). Considering that TJ proteins are crucial for the barrier function of the blood-brain barrier, such alterations could lead to disturbances of the blood-brain barrier integrity and contribute to HIV trafficking into the brain.

Allergens are important factors in the increasing prevalence of asthma (Shen et al., 2001; Wan et al., 1999). Under normal circumstances, the epithelial barrier prevents allergen invasion. The house dust mite allergen (Derp1 – a cysteine proteinase), however, disrupts intercellular TJs. In fact, a putative Derp1-cleavage site is present in the first extracellular loop domain of claudin-1 (Wan et al., 1999) and several other claudins (see Fig. 4, Derp1 site highlighted in blue). TJ breakdown increases epithelial permeability, allowing Derp1 to cross the epithelial barrier. Furthermore, the transepithelial movement of Derp1 to dendritic antigen-presenting cells via the paracellular pathway might be promoted by its proteolytic activity. The opening of TJs by environmental proteinases might thus be the initial step in the development of asthmatic responses to a variety of allergens. It is conceivable that other organisms use similar strategies to disrupt barrier function in order to gain access.

Mutations in claudins underlie at least two human diseases and are good candidates in others. Claudin-16 (initially termed paracellin-1) (Simon et al., 1999) was identified following positional cloning of a mutated gene responsible for familial hypomagnesemia with hypercalciuria and nephrocalcinosis, an autosomal recessive disease that is characterized by severe renal magnesium and calcium loss (Weber et al., 2001b). Weber et al. found that claudin-16 is expressed exclusively in the kidney, where it is primarily restricted to distal tubular segments, including the thick ascending limb of the loop of Henle, the distal tubule and the collecting duct (Weber et al., 2001a). Mutations in human claudin-16 have implicated TJs in the paracellular resorption of Mg^{2+} and Ca^{2+} but not monovalent cations. Of the mutant alleles, 67% exhibit a missense mutation affecting highly conserved amino acids (L145P, R149L, L151T, L151F) in the extracellular loop of claudin-16. Forty-eight percent of the mutant alleles exhibit a L152F mutation. The mutations are such that they must be influencing the tertiary structure of the loop, thereby resulting in changes in the cation sensitivity of the paracellular pathway in the thick ascending limb of the loop of Henle. More recently, Muller et al. (Muller et al., 2003) reported a claudin-16 mutation that results in inactivation of the PDZ-domain-binding motif and leads to loss of association of ZO-1 with claudin-16 and accumulation of claudin-16. In the inner ear, claudin-14 is restricted to the organ of Corti and is not seen in other regions of the membranous labryrinth.

Mutations in claudin-14 have been found to cause nonsyndromic recessive deafness DFNB29 in two large consanguineous Pakistani families (Wilcox et al., 2001). Affected individuals in one family have a homozygous single nucleotide deletion, 398delT within codon M133, which is located in transmembrane domain 3 (Fig. 4, M133 highlighted in red). This frame shift results in the substitution of 23 incorrect amino acids. A missense mutation (T254A) substituting aspartic acid for valine (V85D) was identified in another family. ClustalW comparison of the deduced amino acid sequence of transmembrane domain 2 (TM2) of 21 mouse claudins indicates that valine 85 is conserved among 11 of the 21 claudins, while isoleucine is present in six claudins, and the remaining three claudins have either a cysteine or proline residue at this position of the consensus molecule (Fig. 4, V85 highlighted in green). Aspartic acid is predicted to disrupt the hydrophobicity of the region, as well as the predicted α and β regions in transmembrane domain 2 (Kyte and Doolittle, 1982). The prevalence of these mutations in different populations is not universal; for example, no disease-associated mutations in claudin-14 have been found in Turkish patients with deafness (Uyguner et al., 2003). Nevertheless, recently, Ben-Yosef et al. reported that claudin-14-null mice have a normal endocochlear potential but are deaf owing to rapid degeneration of cochlear outer hair cells, followed by slower degeneration of the inner hair cells (Ben-Yosef et al., 2003), which supports a role of claudin-14 in hearing.

Claudin-11 expression is restricted in the adult to the myelin sheaths of oligodendrocytes in the CNS, Sertoli cells in the testis, the organ of Corti, the choroid plexus, and the collecting ducts in the kidney (Gow et al., 1999). Claudin-11-knockout mice show a complete loss of TJ fibrils in Sertoli and CNS myelin cells, which leads to male sterility and neurological defects consistent with slowing of neuronal conduction times in the CNS (Gow et al., 1999). Presumably, these defects result from loss of the TJ barrier in the testis and CNS oligodendrocytes, respectively.

The role of claudins in the formation and integrity of the epidermal permeability barrier (EPB) was demonstrated by studies indicating that overexpression and deletion of claudins results in skin defects that have clinical relevance. A defective EPB in premature birth remains a leading cause of neonatal death as a result of its associated complications, which include poor temperature stability, infection by micro-organisms through the skin, and the outflow of water. When claudin-6 is overexpressed under the control of the involucrin promoter, the mice die within 2 days of birth, apparently owing to the lack of an intact EPB – as evidenced by increased water-loss and the penetration of X-gal through the skin (Turksen and Troy, 2002). These results provide new insights into the role of claudin-6 in epithelial differentiation and EPB formation. In addition, the epidermal phenotype of these transgenic mice, which is very reminiscent of that in pre-term infant skin, suggests that they will be an important and novel model for studies of human premature EPB-related morbidity (Turksen and Troy, 2002). Similarly, claudin-1-deficient mice die within 1 day of birth, exhibiting wrinkled skin (Furuse et al., 2002). Dehydration assays and trans-epidermal water-loss measurements revealed that in these mice the EPB is severely affected. To date, however, no mutations in claudins have been identified in human skin diseases.

As already mentioned, claudin-5 is considered to be endothethial cell specific. In claudin-5-deficient mice (Nitta et al., 2003), development and morphology of blood vessels does not appear to be altered, and no bleeding or edema is observed in the brain. Tracer experiments and magnetic resonance imaging, however, reveal that the blood-brain barrier against small molecules (<800 Da), but not larger molecules, is disrupted. The relevance of these observations for human disease remains to be seen.

One of the profound effects of tumorigenesis in epithelial tissues is the loss of cell polarity with concomitant disruption of the EPB (Mullin, 2004) (see Table 3). Hence, it is not surprising that several studies have suggested that claudins are modulated in tumorigenesis, implicating them in tumor

Table 3. Claudin distribution in cancerous tissues

Cancer	Known claudin expression (high or dull)	Reference
Pancreas cancer		
Primary infiltrating	4^{high}	Nichols et al., 2004
Metastatic	4^{high}	Nichols et al., 2004
Intraductal papillary mucinous	4^{high}	Sato et al., 2004
Synovial sarcoma	1^{high}	Billings et al., 2004
Ovarian cancer	3^{high} 4 ^{high} 16^{high}	Hough et al., 2000; Rangel et al., 2003a; Rangel et al., 2003b
Gastric-intestinal cancer	23^{dull}	Katoh, 2003
Glioblastoma multiforme	3^{dull}	Wolburg et al., 2003
Breast cancer		
Ductal carcinoma in situ	7^{dull}	Kominsky et al., 2003
Invasive ductal carcinoma	7^{dull}	Kominsky et al., 2003
Head/neck squamous cell carcinoma	7^{dull}	Al Moustafa et al., 2002
Colorectal cancer	1^{high}	Miwa et al., 2000

suppression (Hough et al., 2000; Li and Mrsny, 2000). Expression of claudin-3 and claudin-4 is modulated in various ovarian cell lines and tissues, including primary cancers, ovarian surface epithelia cells and cystadenoma cells. Claudin-23 (Wolburg et al., 2003) is downregulated in gastric, breast and pancreatic cancer cells, whereas claudin-3 expression is modulated in head and neck as well as ovarian cancer (Al Moustafa et al., 2002). A cDNA microarray analysis provided evidence that claudin-7 is downregulated in head and neck squamous cell carcinoma cells compared with normal cells. Furthermore, both claudin-1 and claudin-7 (Kominsky et al., 2003) have been implicated in breast cancer. In both invasive ductal carcinomas and ductal carcinoma in situ is a loss of claudin-7 expression. Claudin-1 is expressed in normal mammary-gland-derived epithelial cells but is absent in most human breast cancer cell lines (MDA-MB-435 and MDA-MB-361) (Kramer et al., 2000). However, it is worth noting that when the claudin-1 gene was analysed in sporadic tumors and hereditary breast cancer patients, no evidence was found to support the involvement of aberrant claudin-1 expression in breast tumorigenesis, which suggests that other regulatory or epigenetic factors are involved in the downregulation of this gene during breast cancer development. Further data on expression profiles of the claudins in various tumors and hyperproliferative diseases are beginning to accumulate. Together with studies that uncover the contributions of individual claudins in the tightness of tissue-specific barriers, this will contribute to our current understanding of tumorigenesis and might contribute to the development of novel claudin-based therapeutic tools (e.g. Michl et al., 2001).

Future prospects

The identification and characterization of the claudin family is providing a great opportunity for us to explain not only how TJs are formed but also how they participate in generating the heterogeneity observed in the tightness of the various junctions of diverse tissues. Dissecting the molecular and regulatory basis of this diversity is clearly one of the challenges in the field. Generation of specific antibodies and other reagents that will reveal the distribution of each claudin during development, differentiation and injury as well as in the disease state is required. It is also essential that we understand how claudins are regulated in a tissue-specific manner, an area about which there is currently little information. Some of these challenges will probably be the topics of many a grant proposal within the next decade.

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