Cell. Mol. Life Sci. 64 (2007) 17–28
1420-682X/07/010017-12
DOI 10.1007/s00018-006-6314-1

Cellular and Molecular Life Sciences

Review

Claudins: multifunctional players in epithelial tight junctions and their role in cancer

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Received 5 July 2006; received after revision 29 August 2006; accepted 29 September 2006
Online First 20 November 2006

Abstract. The molecular architecture of tight junctions has been a subject of extensive studies that have shown tight junctions to be composed of many peripheral and integral membrane proteins. Claudins have been considered the main tight junction-forming proteins; however, the role they play in a series of pathophysiological events, including human carcinoma development, is only now beginning to be understood. Increasing evidence from *in vitro* and *in vivo* studies have identified the influence of claudins on tight junction structure and function, although claudins also participate in cellular contexts other than tight junctions. The aim of this review is to summarize and discuss the conceptual framework concerning claudins, focusing on the involvement of these proteins in epithelial cell polarity establishment, paracellular transport control, signal transduction and tumorigenesis.

Keywords. Claudin, tight junction, cell signaling, permeability barrier, cell polarity, tumor suppression.

Introduction

Distinct fluid compartmentalization in the body and isolation from the external environment are crucial for the function of most organ systems in multicellular organisms and these features are mediated by epithelium. The defining characteristics of epithelium include its ability to create selective barriers between tissue spaces and to generate polarity of cellular structure and function. The tight junction (TJ) is part of the apical junctional complex and is intimately involved in both paracellular permeability and cell polarity [1, 2]. The TJ acts as a barrier, allowing tissues to regulate paracellular movements of solutes down their electroosmotic gradients. The TJ also promotes the 'fence' function that maintains the differential composition of the basolateral and apical domains by preventing the free diffusion of lipids and proteins between these compartments. This function allows the membrane surface to recognize signals directionally and to transport material across the epithelium.

Earlier studies using transmission electron microscopy showed that the TJ appears as a series of very close membrane appositions, involving the outer leaflets of the plasma membrane of adjacent cells, forming the so-called 'kissing points' [3]. In freeze-fracture replicas, the TJ corresponds to circumferential networks of anastomosing strands of varying complexity in the plane of the plasma membrane [4]. Several molecular constituents of the TJ have been identified, the most prominent being claudin, occludin and junctional adhesion molecule (JAM). A detailed review of the molecular composition of the TJ was provided by Gonzalez-Mariscal et al. [5] in 2003. As revealed by freeze-fracture immunoreplica electron microscopy, occludin is part of TJ fibrils [6], and is also capable of promoting intercellular adhesion [7]. Although occludin seems to play a minor role in TJ structure and function, since occludin knockout mice present structurally and functionally normal TJs [8, 9], several

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studies have shown that occludin contributes to the barrier function in cell culture models [10–12]. JAM is also located at the TJ, where it is capable of promoting cell-cell adhesion and affecting paracellular permeability to some extent [13], but its role in the TJ has been related to the establishment of cell polarity [14].

Since their discovery in 1998 [15], many lines of evidence have shown that claudins are the main TJ protein constituents. Claudins are members of a multigene family, with ~24 members in humans/mice [16], presenting a unique tissue expression pattern [17]. When claudin-1 or -2 is expressed in fibroblasts, which lack the endogenous claudins, they are able to concentrate at cell-cell adhesion planes through homophilic interactions and to reconstitute a well-developed network of strands similar to TJ strand networks [18]. Furthermore, claudin-11 ablation eliminates TJ strands from central nervous system myelin and from Sertoli cells in the testis [19]. Alterations of claudin expression strongly affect epithelial paracellular permeability [for a review see ref. 20]. Indeed, several studies in cultured epithelial cells have reported changes in TJ fibril network characteristics following expression of individual claudins through transfection. Analysis by freeze-fracture electron microscopy revealed an increase in number, depth and complexity of TJ fibrils when claudins were overexpressed in epithelial cells [21–23]. Due to their ability to dramatically affect tight junction structure and function, they are considered the backbone of the TJ. Here we summarize and discuss the conceptual framework concerning claudins, focusing on recent discoveries of the involvement of claudins in essential physiological events that contribute to TJ structure and function.

Claudin structure, protein domains and binding partners

Claudins are integral transmembrane proteins with four membrane-spanning regions, two extracellular loops and one intracellular loop, and N- and C-terminal cytoplasmic domains. Their molecular weight ranges from 20 to 27 kDa, and they are recognized by a set of highly conserved amino acids in the first extracellular loop, which contains the residues GLWxxC(8-10aa)C. This motif is also found in non-claudin molecules such as PMP22, EMP, MP20 and the γ5 subunit of voltage-gated calcium channels [24]. The first loop influences paracellular charge selectivity [23], the second loop is the receptor for a bacterial toxin [25] and the C terminus binds TJ cytoplasmic proteins, such as zonula occludens (ZO)-1, -2 and -3, multi-PDZ domain protein (MUPP)-1 and PALS-1-associated TJ protein (PATJ), through a PDZ motif [26–29]. The claudin C terminus seems to influence protein stability, altering protein turnover and consequently the paracellular permeability [30]. In the membrane, claudins form oligomers with a very dynamic and unstable configuration, although the kind and stability of this interaction may vary within/between cell and claudin types [31–33]. Heterogeneous claudin species were shown to copolymerize to form individual TJ strands as heteropolymers, and between adjacent cells, claudin molecules adhere with each other in both homotypic and heterotypic ways, except in some combinations [34]. Even when claudins lack the PDZ-binding sites they still localize to cell-cell contacts and form freeze-fracture strands [35], suggesting an inherent ability to polymerize, independent of PDZ interactions. Claudins have not yet been crystallized successfully, and the mechanism by which claudins polymerize and how they exert their own charge and/or size selectivity remain elusive.

Claudins and paracellular permeability

Claudins have been reported as being the main constituents of the TJ and important regulators of paracellular permeability. Claudin-14 mutation is associated with hereditary deafness due to an increase in TJ permeability in the organ of Corti in both mice and humans [36, 37]. Claudin-1 ablation in mouse epidermis results in dramatic transepidermal water loss, the protein being indispensable for creating and maintaining the epidermal barrier [38]. *In vitro* studies using epithelial cell monolayers have shown that expression of claudin-1 [21, 39], -4 [22], -7 [40], -8 [27, 41], -14 [36] or -15 [23] dramatically increases transepithelial electrical resistance (TER). In contrast, expression of claudin-2 in high-resistance MDCK type I monolayers decreased the TER [42]. Claudin-6, when overexpressed in mice, can also induce a barrier dysfunction in the epidermis [43]. The claudins that are able to induce a high TER in cell culture models are often found *in vivo* in high-resistance epithelial tissues such as the distal nephron segments [44]. Claudin-2, which induces lower transepithelial resistance [42], is found *in vivo* on leaky epithelium, such as the proximal renal tubule [45] and intestinal crypts [17]. These studies can explain the different barrier properties of the TJ observed among different epithelial tissues as a consequence of the heterogeneity of claudin composition at the plasma membrane. Claudin regulation of TER is based on selective ion permeability. For example, two different mutations in claudin-16 are associated with renal magnesium wasting or childhood hypercalciuria, suggesting that claudin-16 might form an intercellular pore permitting paracellular passage of divalent cation through the TJ in the kidney [46, 47]. In the case of claudin -4 [22], -8 [41] and -14 [36], the reduced conductance observed after the expression of these proteins results from selective discrimination against cations.

An emerging model to explain the ion selectivity mediated by claudins suggests that the fixed charges on the extracellular loops of claudins line aqueous pores and electrostatically influence the passage of ions. Replacing some negative residues with positive ones in the first extracellular loop of claudin-15 and -4 converts them from a cation-selective to an anion-selective pore [23]. This finding is particularly important because it demonstrates for the first time that claudins do not simply regulate paracellular permeability, but must have a more direct role in the permeation pathway, probably acting as paracellular pores.

Charged and non-charged solutes appear to have different barrier-regulating permeabilities. In fact, there is frequently a dissociation of epithelial permeability between ions and non-charged molecules [21, 48–50]. It is improbable that an aqueous pore with ion selectivity could also accommodate large non-charged molecules. Thus, it has been proposed that non-charged molecules pass though transient breaks in TJ fibril networks. Dynamic claudin oligomers may promote this proposed strand breakage and reformation, thereby enabling the uncoupling of TER and flux. The molecular basis for size selectivity of TJs is obscure; however, recent *in vivo* studies have provided the first suggestion that claudins also influence this property. This hypothesis has been supported by the observation that a mouse knockout for claudin-5 presents increased permeability for small but not large non-charged solutes in the blood-brain barrier [51]. An *in vitro* approach has shown that selective removal of claudins-3 and -4 but not -1 from epithelial intestinal cells by the mycotoxin ochratoxin increased the paracellular permeability to small but not large non-charged markers [52]. Although these studies reveal a connection between claudins and size discrimination, the molecular mechanism that governs this process is unclear and whether different claudins form pores with different dimensions remains to be determined. Taken together, these studies point to claudins as the main constituents of the TJ where each claudin can exert a unique barrier property in terms of charge selectivity. Furthermore, the combination and mixing ratios of claudins in a given cell determine the selectivity and strength of the TJ, regulating structural and functional features.

Claudin regulation

In biological systems, post-translational modifications of a protein can determine its activity state, localization, turnover and interaction with other proteins. Phosphorylation, nitrosylation, acylation and glycosylation are some examples. Claudin post-translational modifications by phosphorylation can induce alterations to the paracellular permeability to both ions and non-charged molecules, which are modulated by different signaling mechanisms. In ovarian cells, claudin-3 and -4 have been shown to be substrates of protein kinase A (PKA) and protein kinase C (PKC), respectively [53]. In this study, threonine-phosphorylated claudins decreased detergent solubility and were no longer localized in the cell membrane, moving to the cytosol. This caused an increase in paracellular permeability, probably due to the lack of claudin targeting to the membrane [53]. In epithelial cells, claudin-4 phosphorylation, after aldosterone exposition, also leads to an increase in paracellular permeability to non-charged solutes and ions, although the abundance and cellular localization of the claudin were unaltered [54]. In endothelial cells, claudin-5 is a substrate for PKA and, as in epithelium, its phosphorylation leads to increased permeability for small molecules and ions, but not for large molecules, even though staining of claudin-5 in the cell membrane is maintained [55]. In contrast, other studies have associated claudin phosphorylation with an enhanced barrier function. Claudin-1 phosphorylation in epithelial cells was required for proper barrier function, whereas dephosphorylations mediated by protein phosphatase 2A (PP2A) activity negatively regulate the TJ, enhancing claudin solubility in detergent and increasing paracellular permeability [56]. In endothelial cells, where claudin-5 is a PKA substrate, its phosphorylation enhanced cell membrane association and barrier function [57]. In endothelial cells, claudin-1 phosphorylation by mitogen-activated protein kinase (MAPK) is also required to enhance barrier function, which probably occurs via claudin recruitment into TJs [58]. Furthermore, considering that phosphorylated claudin-7 seems to be restricted to glycolipid-enriched membrane microdomains, phosphorylation seems also to participate in claudin cellular location [59].

Palmitoylation is another protein modification present in claudins. It promotes an increased association of claudin with membranes and caveolae-rich domains [60]. As TJs are considered membrane microdomains [61], palmitoylation would enhance claudin association to the TJ. Indeed, non-palmitoylated claudins are not able to increase transepithelial resistance in the way that palmitoylated ones can [60].

Several lines of evidence have indicated that the apical junctional complex is not static but rather an extremely dynamic structure, undergoing endocytosis even under physiological conditions during epithelial morphogenesis, cell migration and cell shedding [62]. Claudins seem to have special endocytic mechanisms. During epithelial cell migration, claudins present a different behavior in comparison to the other TJ molecules. Whereas JAM, occludin and ZO-1 remained at the junction boundaries during migration, claudin-3 became internalized during cell movement [63]. The tightly opposed membranes of the TJ are endocytosed together into one of the adjoining

cells, and during internalization, claudins segregate away from occludin, JAM and ZO-1 to generate claudin-enriched vesicles [63]. The mechanisms that regulate these phenomena are not well understood.

Claudin can also be transcriptionally regulated. Snail, a transcriptional repressor implicated in regulation of the epithelial-mesenchymal transformation, directly represses the transcription of claudin-3, -4, -7 and occludin in addition to its previously described inhibition of cadherin expression [64, 65]. Snail also reduces claudin-1 expression, however, whether this happens at a transcriptional [66] or a post-transcriptional [67] level remains to be clarified.

Claudin expression can be regulated by several growth factors and cytokines, giving a different response depending on the combination of stimulus and the claudin type analyzed. Claudin-3 and -4 expression increases after epidermal growth factor (EGF) treatment of the renal epithelial cell monolayer, whereas claudin-2 decreases; all these changes are mediated by MAPK activation [68]. The MAPK signaling pathway negatively controls claudin-2 expression in renal epithelial cells and provides evidence for regulation of TJ paracellular transport by alterations in claudin composition within TJ complexes [69]. Transforming growth factor (TGF)-beta is also capable of inducing an increase in claudin-1 expression and reducing paracellular permeability in epithelial cells [70]. However, when thyrocytes are incubated simultaneously with EGF and TGF-beta, paracellular permeability decreases and the amounts of claudin-1 are reduced [71]. Ras-transformed epithelial cells (which present constitutively active MAPK) lose claudin-1 staining at cell-cell contacts but present the protein in the cytoplasm. After MAPK inhibition, claudin-1 staining returns to the normal pattern [72]. Extensive evidence supports the likelihood that the TJ barrier is downregulated at transcriptional and post-transcriptional levels by cytokines associated with inflammation [73]. Patients with inflammatory bowel disease present a strong expression of claudin-2 along the intestinal crypts, while normal colons have low levels of claudin-2. In contrast, claudin-3 and -4 were present throughout normal colonic epithelium and were reduced or redistributed in the inflamed epithelium [74]. The same pattern can be observed in patients with ulcerative colitis: a dramatic increase in claudin-2, accompanied by a decrease in claudin-1 and -4 expression levels in the membrane fraction [75]. As mentioned previously, increased expression of claudin-2 can cause a TJ network to become leaky for small cations [42]. Indeed, inflammatory cytokines such as tumor necrosis factor-(TNF)-alpha, interferon-gamma and interleukin (IL)-13 downregulate claudins and induce a marked increase in paracellular permeability in epithelial cells in culture [74, 76].

Claudin expression in human tumors

TJ structure and function are often found altered in human carcinomas, where TJ loss can account for cancer progress, especially through the loss of cell-cell adhesion and cell differentiation. Some studies have shown that claudins are downregulated in some cancer types. For example, claudin-1 has been found reduced in breast carcinoma, where it has been suggested as a potential tumor suppressor [77, 78] as well as in colon cancer, where it was associated with recurrence and decreased survival [79]. Claudin-7 was reported downregulated in invasive breast carcinoma, correlating with histological grade and metastasis [80, 81], and likewise in head and neck carcinoma [82], corroborating the idea that expression of adhesion-related proteins is reduced in cancer. The decreased expression of the TJ-associated proteins, especially claudins, in tumorigenesis is in agreement with the hypothesis that tumorigenesis is accompanied by TJ disruption and loss of cell-cell adhesion, a process that might play a role in the loss of differentiation, uncontrolled proliferation, loss of cohesion and invasiveness. On the other hand, many studies have shown that certain claudins are upregulated in cancer. Claudin-4 upregulation in pancreatic cells caused reduced invasiveness, tumorigenicity and metastatic potential [83]. Claudin-1 has been found upregulated in colorectal cancer [84, 85] and in pancreatic cancer [86]. Claudin-1 upregulation was also found in thyroid carcinomas, except in aggressive ones [87]. Claudin-3 and -4 have also been reported as upregulated in colorectal, ovarian, gastric, breast, prostate and pancreatic cancers [84, 88–93]. Claudin-7 expression was reported as nearly undetectable in normal cells but highly induced in both primary and metastatic breast tumors [94], and its expression was also elevated in hepatocellular carcinogenesis, as induced by EGF overexpression [95]. In cervical neoplasia, claudin-1 and -7 are expressed at undetectable levels in normal cervical epithelium, but expression of both proteins, mainly staining in the membrane, gradually increased along tumor progression from low-grade lesions to invasive neoplasia [96]. Claudin overexpression seems to be an early event in carcinogenesis, at least for some cancer types, as it can be found in precursor lesions. For example, esophageal adenocarcinoma precursor lesions presented increased protein expression of claudin-3, -4 and -7 [97], and the same happened for claudin-4 in gastric adenocarcinoma precursor lesions [98]. The reasons for the discrepancy observed in claudin expression among the different tissues are unclear but may be related to tissue-specific differences in claudin function or even in the tissue microenvironmental features. Other claudins are differentially expressed in a number of human carcinomas and these data are summarized in Table 1.

Table 1. Claudins expression in human tumors.

Cancer type	Claudin-1	Claudin-2	Claudin-3	Claudin-4	Claudin-7
Colorectal	up [84, 83] down [79]	up [103]	up [84]	up [84]	–
Stomach	up [89]	–	up [89]	up [89, 98]	up [101]
Esophagus	maintained [100]	–	up [97]	up [97]	down [100] up [97]
Ovarian	–	–	up [88]	up [88]	–
Breast	down [77, 78]	down [102]	up [90] maintained [77]	up [90] maintained [77]	down (80, 81] up [94]
Prostate	–	down [102]	up [102]	up [102]	–
Head and Neck	–	–	–	–	down [82]
Thyroid	up [87]	–	–	–	–
Pancreas	up [86]	–	–	up [92, 93, 99]	–
Cervix	up [96]	–	–	–	up [96]

Up, down, maintained: protein expression compared with the normal tissue.

Recently, we suggested that claudin upregulation in colorectal cancer can be associated with a leaky epithelium, as shown by an increased permeability, mainly as a consequence of an abnormal distribution of TJ strands [84] (Fig. 1). However, it remains to be determined if beyond permeability, claudin upregulation could also be associated with polarity loss. The increased permeability found in colorectal cancer, despite claudin overexpression, could be explained by the decreased expression of other TJ proteins such as ZO-1 and occludin, which were reported to be downregulated in colorectal cancer [104, 105]. Whether changes in claudin expression are a cause or consequence of carcinogenesis is not clear, and how they might contribute to tumor progression should be an interesting theme of future studies.

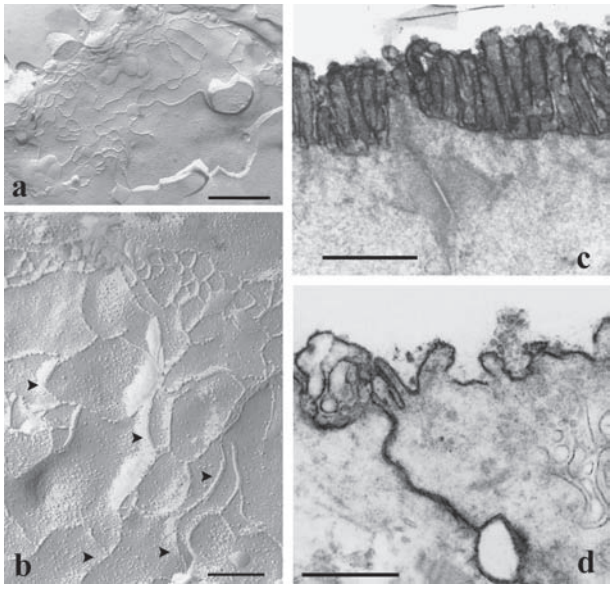

Figure 1. Increased permeability is associated with an abnormal distribution of tight junction strands in human colorectal cancer. (*a*, *b*) Freeze-fracture replicas of colorectal normal mucosa (*a*) and carcinomas (*b*) show the tight junction as an anastomosing fibril network. Observe the expansion through the basolateral side of the tight junction fibrils in the cancerous tissue (arrowheads). Bar, 0.3 µm. (*c*, *d*) Evaluation of paracellular permeability using the electron-dense dye ruthenium red. Normal epithelium (*c*) blocks the dye passage across the tight junction, whereas cancer epithelia (*d*) let the dye pass through the tight junction. Bar, 0.6 µm.

Role of claudins during tumorigenesis

The loss of contact inhibition, which reflects disorder in the signal transduction pathways that connect cell-cell interactions, is typical of both early (loss of cell polarity and growth control) and late (invasion and metastasis) stages of tumor progression. The TJ is central in intercellular adhesion signaling and is involved in the regulation of cell proliferation, differentiation and polarization [106]. Furthermore, it is the site for vesicular targeting and fusion to establish epithelial polarization. In this context, it is interesting to note that the TJ recruits tumor suppressor proteins, like phosphatase protein homologue to tensin (PTEN) [107], oncogenes such as phosphatidylinositol-3 phosphate kinase (PI3K) [108], cell polarity-related proteins such as partitioning defective (PAR)-3, PAR-6, CDC42 and PATJ [109, 110] and vesicular transport-related proteins such as Rab3b, Rab13 and Sec6/8 [111–113].

Unfortunately, few studies have investigated the functional significance of claudin alterations in tumorigenesis. Claudins interact with important proteins related to

cell polarity. It is known that the agouti signaling protein (ASIP) PAR3/PAR6/atypical PKC and the PATJ/protein associated with Lin seven 1 (Pals-1)/Crumbs-3 protein complexes localize to the TJ, and alteration of their expression results in a dramatic loss of cell polarity [114], mislocation of junction proteins [115–117] and disruption of paracellular permeability [118, 119]. The molecular mechanisms involved in these processes are poorly understood, but presumably involve direct interactions between members of these complexes and TJ proteins. It was shown, for example, that claudin-1 and ZO-3 bind to PATJ [29] and the ZO-3-binding site on PATJ is required for localization of PATJ at the TJ in epithelial cells [115]. Claudin also interacts with JAM, a protein that is directly associated with ASIP/PAR-3 and seems to contribute to the early steps of polarity establishment, tethering the ASIP/PAR-3 atypical PKC complex to the TJ [14]. Claudin and JAM associate in their cytoplasmatic domain with ZO-1 and MUPP1, binding in different PDZ domains of these proteins. Thus ZO-1 and MUPP1 are able to bind claudin and JAM at the same time, cross-linking JAM oligomers to claudin-based TJ strands [28]. Thus, components of the paracellular barrier and cell polarity complexes may be reciprocally regulated and interdependent. In this regard, claudin interaction with polarity proteins may cooperate to establish cell polarity and, consequently, alterations in claudin localization and expression would disturb cell polarity, an event frequently observed in cancer. The role of claudin in cell polarity is elusive, although its interaction with partners and cellular functions suggest that it might exist. More studies on polarity and claudin could improve our understanding of the role of claudin in epithelial pathophysiology events.

Claudins may also account for the invasive behavior of cancer cells, since they are able to interact with membrane-type matrix metalloproteinases (MT-MMPs) and promote MMP activity. The colocalization of these proteins is not limited to TJ areas but is also found in cell-cell borders and cytoplasm [120]. Colon cancer cells expressing high levels of claudin-1 were able to increase cell migration and MMP-2 and -9 activity. Tumor xenografts from claudin-overexpressing cells produced much more metastasis than cells expressing low levels of claudin-1 [121]. In oral squamous cell carcinoma, claudin-1 expression promoted cell migration accompanied by MMP-2 activity, laminin-5 $\gamma 2$ cleavage and EGF receptor activation [122]. Overexpression of claudin-3 and -4 in ovarian epithelial cells also resulted in increased activity of MMP-2, motility and invasion [123]. In general, increased claudin expression seems to increase the invasive and metastatic capacity of these carcinomas. A recent clinic-experimental study corroborated this idea by showing a strong association between higher claudin-4 staining and poor survival in gastric adenocarcinomas [89]. Claudin-4 expression is also associated with a more invasive phenotype in human pancreatic cancer [99]. In some breast carcinomas, claudins seem to have an opposing role in tumorigenesis, since downregulation of these proteins observed in the tumors is related to increased invasion and poorer patient prognosis [77, 80].

Claudin expression has also been linked to increased cell survival, which is an important feature that favors cancer cells in initial and late steps of tumorigenesis. Claudin-1 overexpression in colorectal cancer cells was able to improve cell survival, reducing the apoptotic rate. These cells were able to form larger tumors in mouse xenograft in comparison to cells expressing low levels of claudin-1 [121]. Claudin-3 and -4 can also enhance cell survival, without affecting cell proliferation in ovarian cells [123]. A recent proteomic study using cisplatin-resistant ovarian cancer cells revealed a sevenfold increase in claudin-4 expression when compared to the sensitive cells, indicating that this protein is related to cell survival following chemotherapy [124]. Claudins are likely involved in an anti-apoptotic/pro-survival activity, but the molecular mechanisms involved in this event remain unclear. Thus, claudin expression can generate signals relevant to both initial and late steps of carcinogenesis, promoting cell survival, motility and invasion.

Recent studies have reported that claudin can be associated with cell cycle control pathways. Claudin overexpression was shown to lead to TCF-LEF/beta-catenin activation [121]. This complex is known to act as a transcriptional factor, inducing expression of important oncogenes, related to cell proliferation, survival and invasion (myc, cyclin D1, MMP-7), however, the mechanism by which claudin overexpression can cause activation of this signaling is unknown. In fact, mice overexpressing claudin-6 display increased proliferation of epidermal cells and also dysregulated epidermal and hair follicle differentiation, demonstrating the influence of claudins on cell cycle control and cell differentiation [125, 126].

As previously mentioned, claudins bind at their C terminus to ZO-1 and ZO-2 proteins, which are considered potential tumor suppressors. ZO-1 binds to ZO-1-associated nucleic acid-binding protein (ZONAB)/DbpA (ZO-1-associated nucleic acid-binding protein) sequestering it to the TJ. ZONAB regulates the transcription of proliferation-associated proteins, such as cyclin D1 and erbb2 [127]. Thus, claudin could indirectly mediate the gene cell cycle regulatory mechanism acting through ZONAB. Another partner of claudin, the protein MUPP1, is associated with the c-kit proto-oncogene, which is also involved in proliferative pathways. Wild-type c-kit can bind MUPP1, while oncogenic c-kit is not able to [128]. Finally, MUPP1 is a paralogue of PATJ, a key component of the evolutionarily conserved CRB3-PALS1-PATJ polarity complex required for TJ establishment and proper apicobasal polarity in epithelial cells [129]. Like PATJ, MUPP1 also binds Pals-1, suggesting that PATJ and MUPP1 may

share common functions [114]. PDZ proteins are localized at the membrane-cytoskeleton interfaces of cell-cell contacts and form multiprotein signaling complexes at the inner surface of the membrane to modulate cell growth, cell polarity and cell adhesion in response to cell contact [114]. Structural and signaling functions of PDZ proteins are critical for their antitumor activities. Together, these findings suggest that inactivation of certain PDZ proteins such as ZO-1, ZO-2 and MUPP1 in cells may provoke the development of cancer. Therefore, alterations in claudin proteins may alter their binding PDZ-containing proteins and in turn provoke changes in their functions, leading to proliferative signals.

Claudin could also contribute to carcinogenesis by inducing the leakiness of epithelium. As discussed above, phosphorylation can enhance epithelial paracellular permeability. Thus, phosphorylated claudins could enhance the access of growth factors and nutrients to tumor cells. PKA and PKC, for example, which are able to phosphorylate claudins, are often activated in human cancers.

Claudins as potential therapeutic targets

The findings mentioned above have led to the suggestion that claudin proteins, either alone or in combination with other proteins, may represent useful biomarkers for the detection, diagnosis and prognosis of certain cancers, such as gastrointestinal carcinoma, mainly through their pro-metastatic activity enhancing cell survival and migration.

Although well-developed barriers help tissues to maintain homeostasis, they in turn prevent efficient delivery of therapeutic drugs, especially their absorption by the gastrointestinal tract, and their entry into the central nervous systems across the blood-brain barrier. Thus, research into the development of approaches to modulate barrier function for efficient drug delivery has increased in the last few years. Considering that claudins are the main regulators of the barrier function of endothelium and epithelium, studies aiming to modulate the claudin expression pattern in a tissue might also be a useful tool for barrier function modulation. An interesting possibility is the use of *Clostridium perfringens* enterotoxin (CPE), which uses claudin-3 and -4 as its receptors [130, 131]. CPE is a single polypeptide with a molecular mass of 35 kDa that causes food poisoning. CPE triggers lysis of epithelial cells through interaction with claudin-3 and -4, resulting in the initiation of massive permeability changes, osmotic cell ballooning and lysis. CPE is made up of two functionally distinct domains: an approximately 22-kDa N-terminal domain that mediates cytotoxicity and an approximately 13-kDa C-terminal domain that mediates binding [132]. Modulation of claudin by CPE could be used to enhance drug absorption. Interestingly, the C terminal fragment of the toxin, which binds directly to claudin-4 and lacks the cytotoxic domain, enhances intestinal tracer flux without causing cell damage. In fact, the modified toxin produced a better effect capric acid, an enhancer of absorption used in the clinic [133, 134].

Certain human tumors, notably those of the ovary, intestine and pancreas, dramatically upregulate claudin-3 and -4, suggesting that these proteins might be potential therapeutic targets using CPE as the chemotherapeutic agent [93]. CPE toxin elicits cytolysis of breast cancer cell lines in culture and also of isolated primary breast carcinoma cells. Furthermore, when xenografts of breast cancer cells are placed into immunodeficient mice, treatment with CPE resulted in a significant reduction in tumor volume, caused mainly by cell lysis [90]. A similar effect was observed in pancreatic cancer cell xenografts, where intratumoral injections of CPE led to necrosis of large areas of tumor and a significant reduction of tumor mass [93]. In fact, CPE can eliminate human ovarian cancer cells *in vitro* in isolated primary cultures as well as *in vivo* when grown in the peritoneal cavity of mice. In this case, even chemotherapy-resistant primary cancer cells were susceptible to CPE-mediated cytolysis, and no adverse events were observed throughout the CPE treatment protocol in this study. [135]. A claudin-targeting molecule made of the C-terminal of CPE fused with a peptide was reported to inhibit protein synthesis, presenting cytotoxicity only in cells that expressed endogenous claudins [133]. This kind of therapeutic strategy would allow targeting not only of antitumor agents but also of liposomes to claudin-overexpressing tumor cells. Such therapy might provoke some collateral effects since normal tissues, like normal colon epithelium and several glands, show expression of many claudin types. However, these effects could be overcome by the use of a dose that would affect only tumor cells that express much higher amounts of claudin than normal tissues [91, 136]. Another important point favoring the target of tumor cells is the lower CPE cytotoxicity in polarized cells, which generally shows loss of differentiation and polarity during tumorigenesis. It is possible that claudins in healthy epithelium are better shielded from access by the toxin because of their engagement in the TJ. Otherwise, when TJ functionality and structure are altered, as in cancer, a considerable pool of free claudin would be present and CPE access would be facilitated. Other factors might disfavor CPE administration, such as the induction of neutralizing antibodies in patients, which may prevent or reduce the efficacy of repeated CPE administrations. Furthermore, passive CPE diffusion into solid tumors may also reduce the efficacy of local CPE therapy in patients harboring large solid-tumor masses. On the other hand, claudin-expressing carcinomas would also be suitable for anti-claudin antibody-mediated therapy to target antitumor agents of claudin-positive tumors [137]. Taken together, these studies highlight the possibility for a target-specific

and efficient systematic therapy using claudins as therapeutic targets in human cancers that express high levels of these proteins, especially claudin-4.

Concluding remarks

Since the discovery of claudins in 1998 by the Tsukita group [18], characterization and identification of the claudin family have provided not only understanding at the molecular level of the TJ but also the role of claudins in different tissues. Much experimental evidence has revealed that claudins define directly the barrier properties and that the combination and mixture ratios of different claudin species determine the TJ barrier characteristics in a tissue-specific manner. Claudin misregulation found in many types of human carcinoma could be acting to favor initial and late steps of carcinogenesis by evasion of programmed cell death (apoptosis), limitless replicative potential, tissue invasion and metastasis. Enhanced cell migration, disturbed cell polarity, increased cell survival and increased paracellular permeability are some of the events through which claudins could account for tumorigenesis. The mechanisms that govern these molecular events are as yet poorly understood, and future studies are needed to determine the physiological and pathological relevance of claudins, particularly in epithelial cancer. Finally, more detailed studies would be interesting to exploit the claudins as potential therapeutic targets either for drug delivery or as epithelial paracellular barrier modulators.

Acknowledgements. We thank F. Costa e Silva for critical reading of the manuscript and all laboratory members for helpful discussions. Work in our laboratory is supported by Fundação Ary Frauzino para Pesquisa e Controle do Câncer (FAF), Ministerio da Saúde – Brasil, Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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