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Targeting tight junction proteins-significance for drug development

Masuo Kondoh, Takeshi Yoshida, Hideki Kakutani and Kiyohito Yagi

Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan

The choice of drug target and the ability to deliver drug to those targets are pivotal in drug development. Most druggable targets are membrane proteins, such as G-protein-coupled receptors, channels and transporters. However, little attention has been paid to potential druggable targets in the membrane proteins of tight junctions (TJs), through which adjacent cell membranes contact one another. Recent progress in the cell biology of TJs provides new insights into the barrier and fence functions of TJs, suggesting that TJ components are promising candidates for drug discovery. In this review, we summarize the cell biology of TJs and discuss the TJ-based drug discovery.

Introduction

Of the ~30,000 genes in the human genome a subset encodes for proteins that bind, and respond to, bind drug-like molecules [1]. A consensus number of current drug targets for all classes of approved therapeutic drugs are estimated to be 324 drug targets. At present, the total number of proteins targeted by approved therapeutics numbers 324; among these targets, 266 are human genome-derived proteins, and the remainders are proteins of bacteria, viruses, funguses, or other pathogenic organisms. Notably, 60% of drug targets are located at the cell surface and include G-protein-coupled receptors, ion channels, transporters and integrins [2].

The production of compounds with high target potency alone will not lead to a candidate drug; optimization of the pharmacokinetic properties of compounds is essential for successful development. Drug transporters are expressed on the membranes in the intestine, kidney and liver; some drug transporters are responsible for drug transport into tissue and often determine the pharmacokinetic characteristics of drugs, including intestinal absorption, tissue distribution and elimination [3]. The passive targeting of drugs by using a tissue-specific ligand on the membrane is also a potent method to enhance the amount of drug at the disease sites, such as tumors [4]. Thus, the targets for drug delivery are almost always membranous proteins.

The junctional complex of epithelial cells is located at the apical compartment of the lateral membrane and consists of three components: TJs, adherens junctions and desmosomes [5]. Adjacent cell membranes are 15–20 nm apart at adherens junctions and desmosomes; however, the adjacent membranes contact each other at TJs. The intercellular spaces, which are completely obliterated at TJs, are called kissing points. In simple epithelial cell sheets, adherens junctions and desmosomes play a role in mechanical linkage between adjacent cells, and TJs are critical for intercellular sealing [6].

TJs have two important roles, barrier and fence functions. The barrier function is critical for compartmentalization of tissues, indicating that its modulation may be useful for drug delivery to a target tissue [7]. Cells have polarity (basal, apical and lateral sides), and the localization of membranous molecules, such as transporters and receptors, is controlled by TJs, which prevent free movement on the membrane [8]. The cellular polarity is often disrupted by dysfunction of the TJs in disease conditions, such as cancers [9], indicating that TJ proteins may be a druggable target molecule.

Components of TJ strands

Freeze-fracture replica electron microscopy analysis revealed that TJs appear as a series of continuous, anastomotic and intramembranous particle strands or fibrils (Figure 1). The structure and composition of TJs had been a long-standing controversy. In 1993, Furuse *et al*. [10] found the first component of TJ strands, occludin; their research indicated that TJ strands consist of integral membrane proteins polymerizing linearly within a lipid layer of the cell

Corresponding author: Kondoh, M. (masuo@phs.osaka-u.ac.jp)

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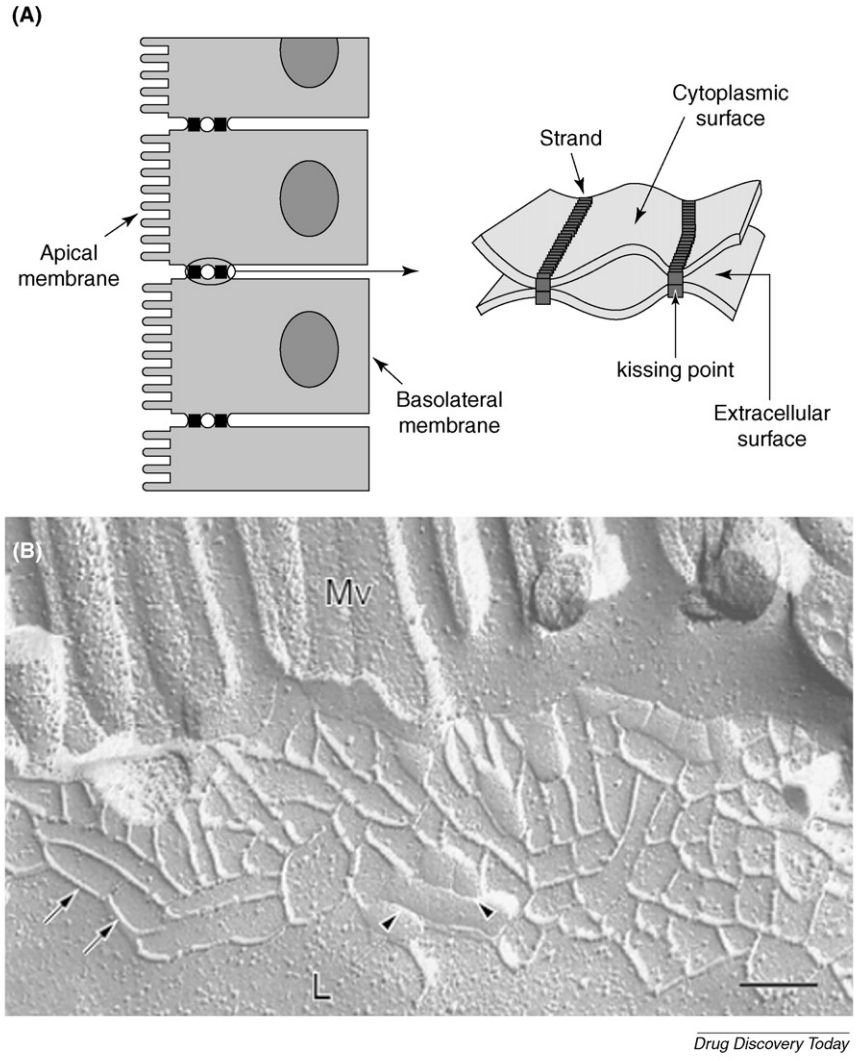

FIGURE 1

Structure of tight junctions. **(A)** Schematic drawing of tight junctions. Tight junctions (TJs; circled) are located at the apical part of lateral membranes. TJs comprise an elaborate network of paired strands, which form the so-called 'kissing points' that eliminate the extracellular space. TJ strands also function as a fence between apical and basolateral membrane domains. **(B)** Freeze-fracture replica image of glutaraldehyde-fixed mouse intestinal epithelial cells. Between apical microvilli (Mv) and the lateral membrane (L), TJs appear as a set of continuous, anastomosing strands in the P-face (arrows), with complementary grooves in the E-face (arrowheads). Bar, 0.2 μm. Tsukita and Furuse [17]. Copyright (1999), with permission from Elsevier.

membrane. Occludin is a transmembrane protein with four transmembrane domains and a molecular mass of ~60 kDa, and it incorporates into TJ strands. However, the formation of TJ strands without occludin was observed in endothelial cells in non-neuronal tissue and in Sertoli cells [11,12]. Moreover, genetic disruption of occludin led to the formation of TJ strands [13].

In 1998, Furuse *et al.* [14] identified novel components of TJ strands, claudin-1 and -2. These proteins, which have a molecular mass of ~23 kDa and contain four transmembrane domains, were localized in TJ strands [15]. The exogenous expression of claudin led to the formation of TJ strands in fibroblast cells that lacked claudin [16]. The claudin protein family contains more than 24 members, whose expression profiles and barrier function differs among tissues [7]. For example, claudin-1 and claudin-5 are widely expressed in a variety of tissues, and the expression of claudin-6 is observed before birth [15,17]. Claudin-1 and Claudin-5 deficient mice lose the barrier function of the epidermis and the blood–brain barrier, respectively [18,19]. The blood–testis barrier was eliminated in claudin-11 knockout mice [20,21]. Most cell types express more than two members of the claudin family, and each TJ strand contains several members of the claudin family. The claudin strands between adjacent cell membranes pair in heterotypic and homotypic manners [22,23]. It has been proposed that the complexity of TJ strands is controlled by the combination and ratio of the 24 members of the claudin family [24]. Indeed, exogenous expression of claudin-11 or -15 increased the barrier function of TJs in MDCK II cells, but not in LLC-PK1 cells [25,26].

Thus, occludin and claudins are key components for regulating the movement of substances across the bicellular junctions in endothelial and epithelial cells (Figure 2).

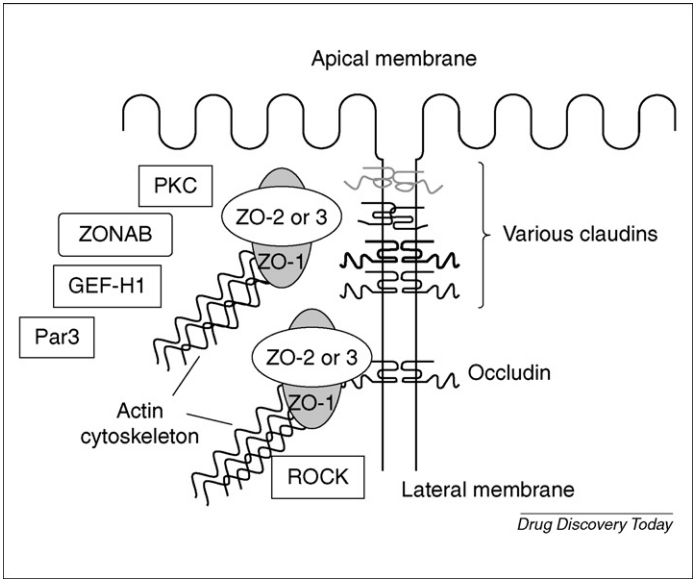

FIGURE 2

Composition of tight junction. Occludin and a family of claudins form cell–cell bridging portions. ZO-1, ZO-2 and ZO-3 play a role as linkers to the cytoskeleton, and protein kinase C (PKC) family, partitioning-defective protein3 (Par3), a guanine nucleotide exchange factor (GEF-H1) and Rho-associated kinase (ROCK) modify the TJ integrity. Activation of a Y-box transcription factor (ZONAB) is regulated by TJ.

Barrier-function of TJs and drug development

TJ barrier function and drug delivery: first generation of TJ modulators

The uptake of hydrophobic molecules occurs by passive transport into the epithelial cells by simple diffusion through the apical cell membrane. By contrast, hydrophilic molecules are impermeable to the epithelial cell sheets. Modulating TJ barriers offers promise for the delivery of drugs with low permeability. Indeed, TJ modulators have been investigated as permeation enhancers since the 1960s. Initially, surfactants and chelators were used to enhance the permeation of drugs [27]; later, bile salts and fatty acids were also found to enhance drug permeation [28]. Research into the mechanisms behind these permeation enhancers reveals that some of them enhance the permeation of drugs through the paracellular route. Depletion of extracellular calcium by EDTA activates protein kinase C and modulates junctional integrity, resulting in expansion of the paracellular route [29,30]. Sodium caprate is currently used clinically as an absorption enhancer and is thought to work through phospholipase C activation–induced contraction of calmodulin-dependent actin-myosin filaments, opening TJs [28]. Other enhancers that modulate TJs are nitric oxide donors, steroidal detergents acyl carnitine and mucoadhesive polymers [28,31]. Although these agents enhance drug permeation, there are problems associated with their use: the target molecules have very low tissue-specificity, there is an influx of toxic substances with the drug, and cell membrane damage may occur.

Although absorption enhancers date back to the 1960s, occludin and claudin were not identified until the 1990s, after which there was gradual progress in the research that lead to the understanding of the paracellular route/TJ mechanism (Table 1). Thus, when these drug delivery systems were developed, the components of TJs, had not been identified. We propose that drug delivery systems targeting TJs should be classified into two categories: first-generation TJ modulators (that do not target TJ-specific components) and second-generation TJ modulators, (that target TJ-specific components) (Table 2).

TABLE 1

Progress in cell biology of TJ

Years	**Events**
1993	Identification of occludin
1998	Identification of claudin
1999 onwards	Clarification of TJ-barrier function of claudin
2005	Identification of tricellulin

TJ barrier function and drug delivery: the second-generation of TJ modulators

Occludin modulators

The putative approach for modulating TJs by using occludin is the prevention of interactions between adjacent occludin molecules. Indeed, Wong and Gumbiner [32] showed that a synthetic peptide, corresponding to the putative extracellular domain of occludin, modulated TJ permeability. A correlation between the perturbation of TJ permeability and a decrease in occludin levels was observed, while other components of TJs, such as ZO-1, ZO-2 and cingulin, were not affected when cells were treated with the occludin peptide [32]. Lancaz-Vieira *et al.* [33] found that peptides consisting of 9 or 10 amino acids, homologous to segments of the extracellular loop of occludin, impaired junction resealing. Another short peptide (14 amino acids) corresponding to the extracellular loop region of occludin could be a prototype for a new class of TJ modulators in intestinal and airway epithelial cells [34,35]. Moreover, a testis-specific, ligand-fused occludin peptide modulated the blood–testis barrier in a testis-specific and reversible manner [36]. Although the molecular mechanisms of these occludin peptides have never been fully clarified, the turnover of occludin, but not its synthesis, was affected by treating cells with the occludin peptides [32,34]. Thus, the peptides may act as antagonists of the occludin extracellular loop and perturb interactions between adjacent occludin molecules in the lateral membrane; the resulting increase in occludin turnover could result in the modulation of TJs. Fusion of a targeting molecule to an occludin modulator could represent a new strategy for drug delivery.

TABLE 2

TJ-modulators

Categories	**TJ-modulators**	**Target molecules**
1st generation TJ modulators	EDTA	Ca^{2+}
	Oleic acid	Cell membrane
	NO	Unknown
	Sodium caprate	Phospholipase C
2nd generation TJ modulators	Occludin peptide	Occludin
	C-CPE	Claudin-4
	FSH-fused occludin peptide	Occludin in BTB

EDTA: ethylenediaminetetraacetic acid; NO: nitric oxide; FSH: follicle-stimulating hormone; BTB: blood–testis barrier.

Claudin modulation

Claudin is a key molecule for TJ properties such as barrier function and selective ion transport [7]. Like occludin, claudin is also a four-transmembrane domain protein with two extracellular domains; however, there have been no reports that TJs can be modulated by using synthetic peptides that correspond to the extracellular region of claudin, although a study that used an enterotoxin peptide has been reported. *Clostridium perfringens* enterotoxin (CPE) has two functional domains: an N-terminal cytotoxic domain and a C-terminal receptor-binding domain [37]. The receptor for CPE was identified by Katahira *et al*. in 1997 [38]. In 1999, Morita *et al*. [15] found that claudin-4 is identical to the receptor for CPE. Fujita *et al*. [39] found that CPE interacts with claudin via the second claudin extracellular loop domain. Treatment of cells with the C-terminal region of CPE (C-CPE) resulted in decreased TJ integrity, accompanied by a decrease in claudin-4 levels [40]. We also found that the ability of C-CPE to enhance jejunal absorption was 400-fold greater than that of sodium caprate, a clinically used absorption enhancer [41]. Moreover, data on claudin-1- and claudin-5-deficient mice suggest that claudin-1 and claudin-5 modulation is effective for drug delivery in the epidermis and across the blood–brain barrier, respectively [18,19]. C-CPE may be a useful prototype for the development of claudin modulators, and RNA interference is a promising method for modulating claudin function. Indeed, the knockdown of claudin by RNA interference resulted in the dysfunction of claudin in cells [43].

Fence-function of TJs and drug development

Downregulation of claudin and tumors

Epithelium-mesenchyme transition (EMT), by which epithelial cells lose their polarity, is speculated to cause acquisition of an invasive and/or malignant phenotype of epithelial tumors [44]. Snail is a transcription repressor that plays a central role in the EMT. The expression of snail reduced claudin/occludin protein levels, followed by EMT [45]. Thus, a decrease in claudin levels may be associated with dysregulation of polarity and malignant tumors. Indeed, claudin-1 is reduced in breast cancer and colon cancer [46–48]. Claudin-7 is downregulated in invasive breast cancer and in head and neck cancer [49,50]. These reports of decreased TJ protein expression in cancer are consistent with the generally accepted idea that tumorigenesis is accompanied by a disruption of TJs, a process that may play an important role in the loss of cohesion, invasiveness, and lack of differentiation observed in cancer cells. Indeed, overexpression of claudin-4 was associated with a significant reduction in the invasive potential of pancreatic cells *in vitro* and *in vivo* [51]. Transfection of oncogenic Raf-1 into a salivary gland epithelial cell line resulted in downregulation of claudin-1 and the loss of TJ function [52]. These findings indicate that an activator of the claudin barrier that maintains cellular polarity may be a novel anti-tumor agent that prevents the progression of the malignancy. Very recently, Litkouhi *et al*. [53] showed that hypomethylated claudin-4 allele is consistent with overexpression of claudin-4 in ovarian cancer cell lines, and moreover treatment of the cells with a demethylating agent resulted in claudin-4-expression in claudin-4-negative ovarian cancer cells. In addition to claudin, CpG island of the occludin promoter is densely methylated in human hepatocellular carcinoma and mouse melanoma. Epigenetic silencing of occludin may be also, at least in part, involved in the tumor development [54,55]. Taken together, epigenetic control of claudin and occludin expression may be also a novel strategy for tumor therapy.

Upregulation of claudin and tumors

As discussed above, the loss of claudins has been interpreted as a mechanism for the loss of cell adhesion and an important step in the progression of cancer to metastasis. However, many claudins, such as claudin-3 and claudin-4, are typically upregulated in many cancers, such as ovarian, prostate and breast cancers, suggesting that claudins have a positive effect on tumorigenesis [56]. Claudin-1 and claudin-4 are expressed in squamous cell carcinoma [57]. The expression of claudin-3 and claudin-4 was associated with an increase in invasion, motility and cell survival in ovarian cancer cells [58]. Considering that claudins play roles in TJ barriers and the maintenance of cellular polarity, upregulation of claudins seems to be inconsistent with tumorigenesis. There are two explanations for this discrepancy. First, the overexpression of claudins may result in the dysfunction of TJ barriers. Claudin forms heterotypic and homotypic strands in the lateral membrane, and the adjacent strands associate with each other, resulting in the formation of the TJ barrier [22,23]. The combination of the 24 claudin family members in the strands is a critical determinant of the properties of TJ barriers. An altered ratio of claudin family members can cause the formation of leaky TJ barriers; indeed, the overexpression of claudin-2 reduced the TJ integrity in MDCK I cells, indicating that in some physiological conditions claudin plays a dominant negative role in TJ barriers [23]. Thus, upregulated claudin may be a dominant negative factor for TJ integrity. Secondly, claudin may play a direct role in the initial stage of metastasis. Overexpression of claudin promoted the activation of matrix metalloproteinases (MMPs), a pro-metastasis factor, mediated by membrane-type matrix metalloproteinases (MT-MMPs) [59]. Claudin-1 and claudin-3/claudin-4 enhanced the invasive activity by processing MMPs in oral squamous cell carcinoma cells and ovarian epithelial cells, respectively [58,60]. Claudin and MT-MMPs were not restricted to TJ strands and co-localized diffusely in cell membranes; so, claudin may be responsible for anchoring MMPs and MT-MMPs, resulting in the formation of invasion-machinery complex. Taken together, these results indicate that some claudins can target drugs to tumors. Indeed, CPE, a toxin sensitive to claudin-4-expressing cells, exhibited anti-tumor activity in claudin-expressing tumors, such as breast, ovarian and pancreatic cancer cells [61–63]. Severe side effects were not observed after the administration of CPE. Cellular polarity is maintained in normal tissues, and claudins are localized in TJs and incorporated into TJ strands. Thus, it may be difficult that claudin extracellular region-targeted agents access the claudin in TJs of normal tissues.

In summary, agents that target abnormalities in cellular polarity are promising candidates for drug discovery. Molecules that target abnormal cellular polarity cannot be detected by using standard genomic drug discovery because the process focuses on whole cells or tissues with no consideration of the different localization of a molecule. In this respect, a claudin-based strategy can lead to the development of a novel type of pharmaceutical agents.

Perspective of TJs-based drug discovery

TJs play pivotal roles in the barrier function and fence function. The identification of functional TJ components has revealed new

insights into TJ-component-based drug discovery. Briefly, they are classified into novel strategies for enhancement of drug permeation through the paracellular route, for targeting to claudin-expressing cells, and for gene therapy for diseases caused by dysfunction of claudins.

Differences exist between the drug delivery systems designed for transcellular and paracellular routes. Hydrophobic compounds easily cross epithelial and endothelial cell sheets via simple diffusion, in contrast to hydrophilic compounds. To deliver hydrophilic drugs to a target tissue, drugs are often modified to be recognized by a transporter or receptor in the target tissue. Claudin is the only molecule known to form a variety of epithelial TJs-barriers with different properties; the combination and mixing ratios of over 24 members of the claudin family determine the barrier properties of the epithelial TJs-barriers, suggesting that modulation of claudin in a member-specific manner might allow us to deliver drugs to a target tissue through the paracellular route [22,23]. The three-dimensional structure of claudin remains to be determined, and theoretical preparation of claudin modulator from structure of claudin is impossible. For now, C-CPE is the only claudin modulator, a claudin-4 modulator [40]. We are attempting to prepare claudin modulators with narrow and broad ranges of specificity to claudin members by preparation of C-CPE library with changes of functional residues in a claudin-4 modulator, C-CPE [64–66]. Occludin is not always critical for TJs-barrier, and occludin does not form a variety of TJs-barrier since occludin is not a family protein like claudin. However, fusion of a targeting ligand with occludin peptide, an occludin modulator may be a potent method to modulate TJ-barrier. Very recently, Wong *et al*. [42] showed that a testis-targeting, ligand-fused occludin peptide modulated the blood–testis barrier. The combination of claudin modulators with occludin modulators will be useful for the modulation of the TJ barrier. Nasal and epidermal route for administration provides promising alternative for systemic delivery of drugs with potentially improved patient comfort, compliance and convenience. Development of claudin modulator for nasal mucosal barrier is a near future problem of pharmaceutical therapy [67].

It is true that strategies for TJ modulation are extremely difficult to apply for pharmaceutical therapy, because opening of TJs leads to the influx of substances other than the drug of interest, potentially resulting in global adverse drug reactions (ADRs), but lessons from claudin-deficient mice have provided strategies to overcome such side effects. Deletion of claudin-5 resulted in dysfunction of blood–brain-barrier, but TJs consisting of at least claudin-12 and morphologically normal vessels were observed in the blood–brain-barrier [19]. Importantly, claudin-5-deficient mice showed no bleeding and edema in the brain. If these findings are applied to other claudin members, a transient modulation of claudin might be a drug delivery system free from serious ADRs.

TJs also play a vital role in the maintenance of cellular polarity by their fence functions [6]. Polarized cells have an asymmetrical distribution of cell-surface proteins, such as ion channels, transporters and receptors [8,68]. Sixty percent of available druggable targets are membrane proteins. Dysregulation of the fence function of TJs causes change in polar localization of membrane proteins, indicating that claudin may be indirectly involved in the druggable targets. The abnormal localization of claudin might result in tumorigenesis and the increased malignancy of tumors. Differences of claudin protein and mRNA levels between normal tissue and tumor tissue does not reflect the abnormal claudin localization. Unlike cell proliferation, disruption of cell polarity is never observed under normal physiological conditions in adult tissue and is unique to diseases states such as cancer and inflammation [69]. Together, a concept of claudin in cellular polarity will produce a novel strategy for tumor targeting.

The transmembrane proteins in TJs form the paracellular barrier, and the cytoplasmic regions play a role in scaffolds of signaling molecules that participate in the regulation of cell proliferation, gene expression and differentiation [70]. Claudin and occludin contain PDZ (PSD95/DlgA/ZO-1 homology) binding domains in the cytoplasmic region, which bind and recruit adaptor proteins containing PDZ motifs, such as ZO-1, ZO-2, ZO-3 and MUPP1. Signaling molecules, such as small GTP binding proteins, Rho family GTPases, phosphatases, and kinases are recruited to the cytoplasmic region of TJs [71]. Thus, various signaling proteins and transduction pathways are associated with TJs. A transcriptional factor ZO-1-associated nucleic acid-binding protein (ZONAB) regulates expression of erbB-2 and several cell cycle regulators thorough its localization to nucleus and TJs determined by interaction between ZO-1 and ZONAB [72]. A receptor tyrosine kinase erbB2 is a dominant oncogene in breast cancer, and amplifying erbB2 signaling is common in aggressive breast tumors. ErbB2 is also the target of the drug Herceptin [73]. ErbB2 enhances activation of the transcription factors STAT3 and c-Jun, which contributes to disruption of epithelial adhesion and hyperproliferation of the breast tumors, respectively [74]. Together, activation of ZONAB induced by dysfunction of TJs may cause tumorigenesis. An inhibitor of ZONAB might be a candidate for anti-tumor agents.

In summary, recent progress in the cell biology of TJs has pioneered novel TJ-based strategies for drug delivery and drug development. We strongly believe that progress in the biology of TJs will provide further breakthroughs and that TJ-based drug discovery will be useful for pharmaceutical therapy in the near future.

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