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Current progress in a second-generation claudin binder, anti-claudin antibody, for clinical applications

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Claudins (CLDNs) are a 27-member family of tetra-transmembrane proteins that have pivotal roles in maintaining cellular polarity and sealing the spaces between adjacent cells. Deregulation of their functions is often associated with pathological conditions, including carcinogenesis and inflammation. Some CLDNs are co-receptors for hepatitis C virus. Because CLDN-driven regulation of intercellular seals might be manipulated to enhance drug absorption, CLDNs are attractive targets for drug development. Monoclonal antibodies recognizing the extracellular domain of CLDNs are the first choice for therapeutics, but their development has been delayed. Here, we overview recent advances in the creation of anti-CLDN antibodies and discuss CLDNs as drug development targets.

Introduction

During their evolution, multicellular organisms have developed a series of machineries to separate the body and the outside environment and to compartmentalize tissues such as the epithelium and endothelium. These machineries function as gatekeepers, selecting and regulating solute movement across the epithelium and endothelium in a tissue-specific manner [1]. The spaces between adjacent cells are sealed by tight junctions (TJs), which in the epithelium and endothelium have barrier and fence functions [1–3]. For instance, TJs prevent free movement of solutes across the epithelium and endothelium through the paracellular spaces. Representative barriers involving TJs are the mucosal barrier, epidermal barrier, blood–brain barrier and blood–testis barrier [1,4]. Regulation of these barriers has been one strategy for drug delivery [4,5]. TJs also maintain cellular polarity by preventing free movement of membrane proteins and lipids between the apical membrane and the basolateral membrane [6]. Disruption of the cellular polarity of the epithelium is an early event in malignant transformation [7]. Moreover, most pathological microorganisms invade the body via the mucosal epithelium [8]. TJs are therefore promising targets for developing drug delivery and cancer therapy and for treating inflammatory diseases and infection.

TJs have complex biochemical structures. They contain transmembrane proteins [occludin, claudins (CLDNs), tricellulin, junctional adhesion molecules] and intracellular proteins (zonula occludens) [9]. CLDNs are the major structural components of TJs [1]. To date, 27 CLDN family members have been identified; each CLDN has different tissue-expression patterns and functions and has a pivotal role in therapeutic areas involving TJs [1,4,10,11] (Table 1). CLDNs were initially described as ~23 kDa tetra-transmembrane proteins with intracellular N and C termini and two extracellular domains [2] (Fig. 1). The first extracellular domain is thought to comprise ~50 amino acids and the second ~22 (Table 2). Recent crystal structure analysis of murine CLDN-15 and -19 revealed that CLDNs have a five-stranded extracellular antiparallel β-sheet domain (four strands of which are in the first extracellular domain and one of which is in the second extracellular domain), a typical left-handed four-helix bundle-type transmembrane domain and a short extracellular helix at the end of the first extracellular domain [12,13] (Fig. 1).

Because CLDNs are transmembrane proteins, binders that interact with their extracellular domains are the first choice as pharmaceutical agents. The first CLDN binder examined was *Clostridium perfringens* enterotoxin (CPE) [14]. CPE and its receptor-binding domain, C-CPE, have been widely used to prove that CLDNs are potent targets for enhancing mucosal absorption of drugs, mucosal vaccination, cancer therapy and stem cell therapy

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TABLE 1

Therapeutic potentials of claudin (CLDN) binders.

Potential therapeutic area	CLDN involved	Background	Refs
HCV entry inhibitor	1	This CLDN is involved in HCV entry into hepatocytes	[38,39]
Drug delivery	1 and 5	CLDN-1 knockout mice showed impairment of the epidermal barrier, and CLDN-5 knockout mice showed partial impairment of the blood–brain barrier.	[3,52]
IBD therapy	2	CLDN-2 is overexpressed in IBD	[59]
Cancer therapy	3, 4, 6 and 18.2	These CLDNs are frequently overexpressed in many tumors	[7,10,50]
Antigen delivery for mucosal vaccination	4	Mucosa-associated lymphoid tissues are covered by highly CLDN-4-expressing epithelium	[60]
Regenerative medicine	6	CLDN-6 is a marker of undifferentiated stem cells	[18]

Abbreviations: HCV, hepatitis C virus; IBD, inflammatory bowel disease.

[5,15–18] (Table 1). However, because of immunogenicity of CPE, the clinical applications of CPE and C-CPE are naturally limited [16]. To overcome the problems associated with the use of CPE and C-CPE, attention has been paid to technologies for developing monoclonal antibodies for CLDNs, and several research groups have had success in this area. In this review, we describe the technologies used to create monoclonal antibodies for the extracellular domains of CLDNs and their application to pharmaceutical therapy (Fig. 2).

Approaches to creating antibodies against CLDN family members

Selection of antigens and screening systems is crucial in the creation of anti-CLDN antibodies. Various methods have been used for this purpose. The methods are classified into non-immunization methods (naive phage display technology) and immunization methods (Table 3).

Peptide immunization

The first anti-CDLN antibodies were successfully created by immunizing chickens with a synthetic peptide corresponding to the extracellular domain of CLDNs [19]. The antigens were peptides corresponding to the following regions of the predicted extracellular domains of human CLDN-1, -3 and -4: one-third of the first domain, two-thirds of the first domain and the second domain. In general, the animals immunized have been the mouse and rat. However, the chicken was chosen as an immunization host because the homology between human and chicken CLDNs is less than 80%–CLDNs have a high degree of homology among species, and the homology between human and rodent CLDNs is more than 90% [19] (Table 2). The resulting polyclonal antibodies successfully reacted with CLDN-3- or CLDN-4-expressing cells.

The peptides derived from the extracellular domains of CLDNs rarely reflect the native CLDN conformations. For example, the peptide derived from the extracellular domain of CLDN-4 cannot interact with a C-CPE fragment even though the extracellular domain of CLDN-4 is the binding domain of C-CPE [20]. Generally, most antibodies raised from peptide immunization bind only to the immunogen and do not bind to the native protein on the cell surface, probably because of conformational differences between the peptide and the native protein on the cell membrane [21]. Indeed, the study mentioned above is the only success in the creation of anti-CLDN antibodies by immunization with a peptide corresponding to the extracellular domain [19].

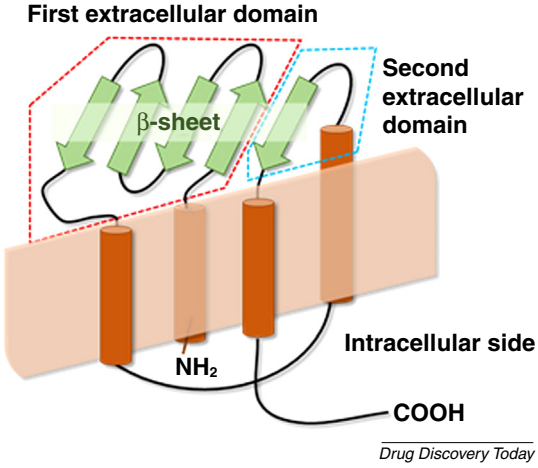

FIGURE 1

Schematic illustration of claudin structure. Cylinders and arrows indicate α-helices and β-strands, respectively.

Antibody phage display

The second successful creation of anti-CLDN antibodies was achieved by using phage display technology [22,23]. Phage display technology is commonly used to obtain binders [24]. A single-chain variable fragment (scFv) library (library size: $<10^9$) was subjected to screening for CLDN-3 binding by using a 31-amino-acid peptide corresponding to the second extracellular domain of CLDN-3 [22]. The resulting human anti-CLDN-3 scFv bound to the CLDN-3 peptide and to cell-expressed CLDN-3. However, the affinity of the scFv for CLDN-3 on the cells was more than 20-fold lower than that for the CLDN-3 peptide. In another study, a synthetic fragment antigen-binding (Fab) phage display library (library size: $>10^9$) was subjected to screening for CLDN-1 binding by using CLDN-1 protein purified from CLDN-1-displaying baculovirus, resulting in the creation of CLDN-1-binding Fab [23].

Immunization with DNA and CLDN-expressing cells

To develop an antibody that binds to the native extracellular domain of membrane proteins, immunization with

antigen-encoding DNA vector and endogenous or exogenous antigen-expressing cells in rodents has been used [21,25]. Immunization with DNA encoding CLDN-1, -6 and -9 has successfully led to the generation of anti-CDLN-1, -6 and -9 antibodies [26,27]. Immunization with CLDN-3- and -4-expressing cells has successfully created anti-CLDN-3 and -4 antibodies in rodents [28,29]. Immunization with plasmid DNA encoding the first extracellular domain of CLDN-18 splicing variant 2 (CLDN-18.2) with secretory signal peptide has led to creation of anti-CLDN-18.2 antibodies [30].

Nanoparticle immunization

Nanoparticles, including liposomes, baculoviruses, virus-like particles (VLPs) and exosomes, have antigen present at high density on their surfaces [31]. Generally, the presence of epitopes at high density in antigens on the surfaces of nanoparticles can induce a robust antibody response without the action of T cells and can sometimes break B cell tolerance [32]. The CLDN family can be categorized as poorly immunogenic proteins as a result of their high homology (Table 2). For instance, a previous report showed no success on generation of anti-CLDN-1 antibody [33]. Therefore,

TABLE 2

Primary amino acid sequences of putative extracellular domains of claudin (CLDN)-1, -2, -3, -4, -5, -6, -18.1 and -18.2.

CLDN (accession number)	First extracellular domain	Second extracellular domain
Human CLDN-1 (NP_066924)	PQWRIYSYAGDNIVTAQAMYEGLWMSCVS QSTGQIQCKVFDSLLNLSST	NRIVQEFYDPMTPVNARYEFGQ
Rat CLDN-1 (NP_113887)	PQWKIYSYAGDNIVTAQAIYEGLWMSCVS QSTGQIQCKVFDSLLNLNST	NRIVQEFYDPMTPINARYEFGQ
Mouse CLDN-1 (NP_057883)	PQWKIYSYAGDNIVTAQAIYEGLWMSCVS QSTGQIQCKVFDSLLNLNST	NRIVQEFYDPLTPINARYEFGQ
Human CLDN-2 (NP_001164566)	PSWKTSSYVGASIVTAVGFSKGLWMECAT HSTGITQCDIYSTLLGLPAD	HGILRDFYSPLVPDSMKFEIGE
Rat CLDN-2 (NP_001100316)	PNWRTSSYVGASIVTAVGFSKGLWMECAT HSTGITQCDIYSTLLGLPAD	HGILRDFYSPLVPDSMKFEIGE
Mouse CLDN-2 (NP_057884)	PNWRTSSYVGASIVTAVGFSKGLWMECAT HSTGITQCDIYSTLLGLPAD	HGILRDFYSPLVPDSMKFEIGE
Human CLDN-3 (NP_001297)	PMWRVSAFIGSNIITSQNIWEGLWMNCVV QSTGQMQCKVYDSLLALPQD	NTIIRDFYNPVVPEAQKREMGA
Rat CLDN-3 (NP_113888)	PMWRVSAFIGSSIITAQITWEGLWMNCVVQ STGQMQCKMYDSLLALPQD	NTIIRDFYNPLVPEAQKREMGT
Mouse CLDN-3 (NP_034032)	PMWRVSAFIGSSIITAQITWEGLWMNCVVQ STGQMQCKMYDSLLALPQD	NTIIRDFYNPLVPEAQKREMGA
Human CLDN-4 (NP_001296)	PMWRVTAFIGSNIVTSQTIWEGLWMNCVV QSTGQMQCKVYDSLLALPQD	HNIIQDFYNPLVASGQKREMGA
Rat CLDN-4 (NP_001012022)	PMWRVTAFIGSNIVTAQTSWEGLWMNCV VQSTGQMQCKMYDSMLALPQD	HNVIRDFYNPLVASGQKREMGA
Mouse CLDN-4 (NP_034033)	PMWRVTAFIGSNIVTAQTSWEGLWMNCV VQSTGQMQCKMYDSMLALPQD	HNVIRDFYNPMVASGQKREMGA
Human CLDN-5 (NP_003268)	PMWQVTAFLDHNIVTAQTTWKGLWMSCV VQSTGHMQCKVYDSVLALSTE	NIVVREFYDPSVPVSQKYELGA
Rat CLDN-5	PMWQVTAFLDHNIVTAQTTWKGLWMSCV	NIVVREFYDPTVPVSQKYELGA

TABLE 2 (*Continued*)

(NP_113889)	VQSTGHMQCKVYESVLALSAE	
Mouse CLDN-5 (NP_038833)	PMWQVTAFLDHNIVTAQTTWKGLWMSCV VQSTGHMQCKVYESVLALSAE	NIVVREFYDPTVPVSQKYELGA
Human CLDN-6 (NP_067018)	PMWKVTAFIGNSIVVAQVVWEGLWMSCV VQSTGQMQCKVYDSLLALPQD	HAIIRDFYNPLVAEAQKRELGA
Rat CLDN-6 (NP_001095834)	PMWKVTAFIGNSIVVAQMVWEGLWMSCV VQSTGQMQCKVYDSLLALPQD	HAIIQDFYNPLVADAQKRELGA
Mouse CLDN-6 (NP_061247)	PMWKVTAFIGNSIVVAQMVWEGLWMSCV VQSTGQMQCKVYDSLLALPQD	HSIIQDFYNPLVADAQKRELGA
Human CLDN-18.1 (NP_057453)	DMWSTQDLYDNPVTSVFQYEGLWRSCVR QSSGFTECRPYFTILGLPAMLQA	NMLVTNFWMSTANMYTGMGGM VQTVQTRYT
Rat CLDN-18.1 (NP_001014118)	DMWSTQDLYDNPVTSVFQYEGLWRSCVQ QSSGFTECRPYFTILGLPAMLQA	NMLVTNFWMSTANMYSGMGGM VQTVQTRYT
Mouse CLDN-18.1 (NP_062789)	DMWSTQDLYDNPVTAVFQYEGLWRSCVQ QSSGFTECRPYFTILGLPAMLQA	NMLVTNFWMSTANMYSGMGGM GGMVQTVQT
Human CLDN-18.2 (NP_001002026)	DQWSTQDLYNNPVTAVFNYQGLWRSCVR ESSGFTECRGYFTLLGLPAMLQA	NMLVTNFWMSTANMYTGMGGM VQTVQTRYT
Rat CLDN-18.2 (XP_006243691)	DQWSTQDLYNNPVTAVFNYQGLWRSCVR ESSGFTECRGYFTLLGLPAMLQA	NMLVTNFWMSTANMYSGMGGM VQTVQTRYT
Mouse CLDN-18.2 (NP_001181850)	DQWSTQDLYNNPVTAVFNYQGLWRSCVR ESSGFTECRGYFTLLGLPAMLQA	NMLVTNFWMSTANMYSGMGGM GGMVQTVQT

The accession numbers of these CLDNs in NCBI database are shown. The TMHMN program (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict the extracellular domains. Red letters indicate amino acids mismatched among human, rat and mouse CLDNs.

TABLE 3

Methods of generating anti-claudin (anti-CLDN) antibodies.

Method		Target	First screening	Product	Refs
Non-immunization methods					
Phage display library		CLDN-3	Peptide-based ELISA	scFv	[22]
Phage display library		CLDN-1	Purified soluble CLDN conjugated beads	Fab, mAb	[23]
Immunization methods					
Antigen	**Immunization host**				
Peptide	Chicken	CLDN-3 and -4	Peptide-based ELISA	pAb	[19]
Cell	Immunocompetent rodents	CLDN-3 and -4	Cell-based assay	mAb	[28]
Cell	Lupus-prone mice	CLDN-3 and -4	Cell-based assay	mAb	[29,46]
DNA	Immunocompetent rodents	CLDN-1, -4, -6, -9 and -18.2	Cell-based assay	mAb	[26,27,30,45]
DNA	Lupus-prone mice	CLDN-1	Cell-based assay	mAb	[40]
VLP	Immunocompetent rodents	CLDN-18.2	Cell-based assay	pAb	[34]

Abbreviations: Fab, fragment antigen-binding; mAb, monoclonal antibody; pAb, polyclonal antibody; scFv, single-chain variable fragment; VLP, virus-like particle.

an active immune enhancer needs to be used to raise an antibody response.

One pioneer study succeeded in inducing the production of anti-CLDN-18.2 antibody by using VLPs based on chimeric hepatitis B core antigen (HBcAg) [34]. Antigen can be inserted into a surface-exposed site on HBcAg. The authors inserted a peptide corresponding to part of CLDN-18.2 by using a G_4SG_4 linker at the N- and C-terminal ends of the peptide. The HBcAg-based VLPs that they designed induced the production of human–mouse cross-reactive anti-CLDN-18.2 antibodies upon immunization of mice.

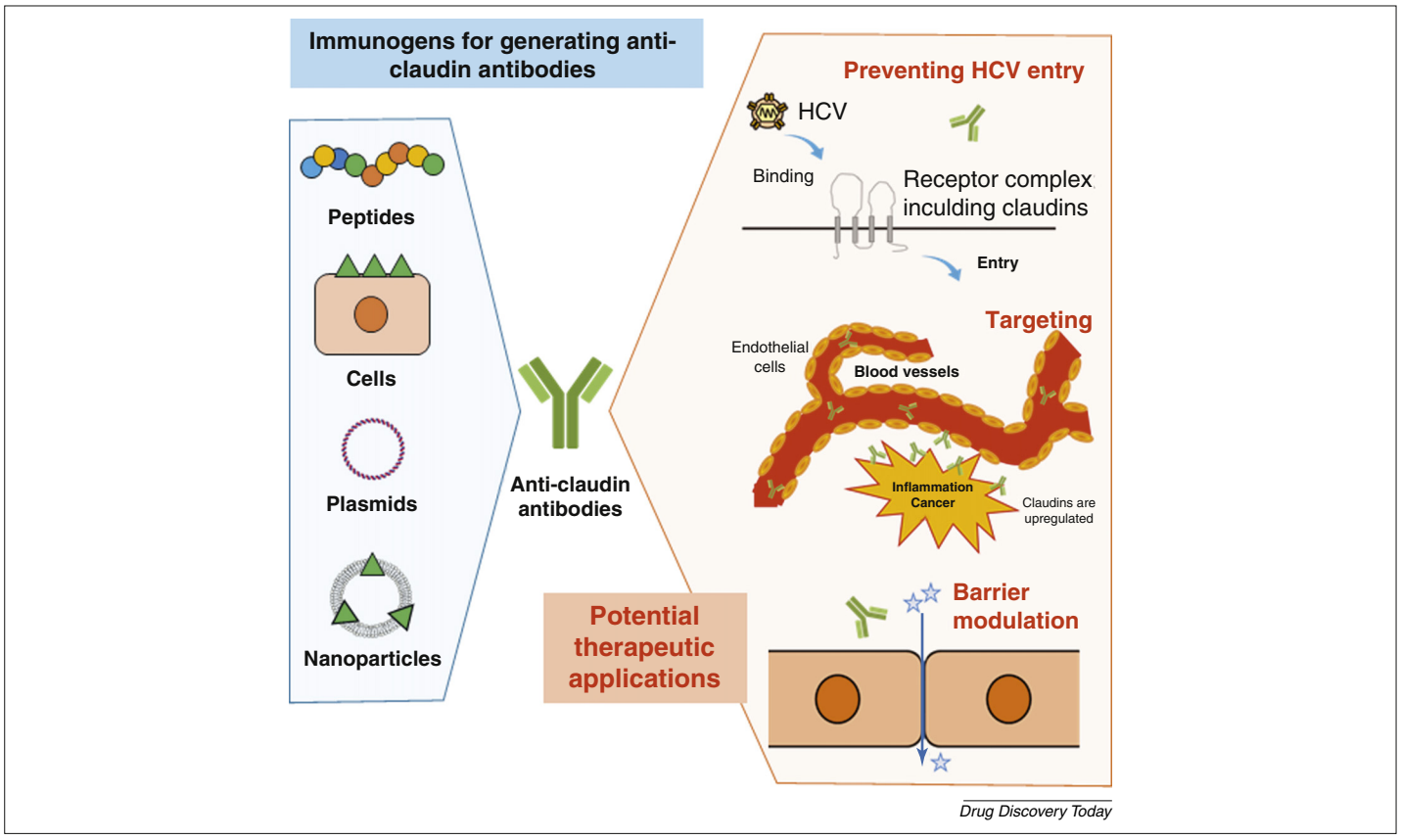

FIGURE 2

A representative cartoon showing the different approaches for creating antibodies against claudin (CLDN) family members and their therapeutic applications.

TABLE 4

Key features of anti-claudin (anti-CLDN) antibodies.

Antibody name	Target	Epitope	Key feature	Therapeutic indication	Refs
OM-7D3-B3	CLDN-1	First ECD	Cross-reacted with mouse CLDN-1	HCV entry inhibitor	[41]
3A2	CLDN-1	Second ECD	Commercially available	HCV entry inhibitor	[40]
7A5	CLDN-1	Second ECD	Commercially available	Epidermal barrier modulator	[53]
KM3900	CLDN-4	Second ECD	Human CLDN-4 specific	Anticancer reagent	[46]
KM3907	CLDN-3 and -4	First ECD	Cross-reacted with mouse CLDN-4 but not mouse CLDN-3	Anticancer reagent	[28]
5A5	CLDN-3 and -4	First ECD	Human specific	Anticancer reagent	[45]
IMAB362	CLDN-18.2	First ECD	Ongoing Phase IIb study	Anticancer reagent	[30]
IMAB027	CLDN-6	Unknown	Ongoing Phase I/II study	Anticancer reagent	[50]
342927	CLDN-6	Unknown	Commercially available	Eliminator of residual undifferentiated cells	[18]

Abbreviations: ECD, extracellular domain; HCV, hepatitis C virus.

The authors of the study indicated that the flanking linker was required for efficient production of antibodies that recognized the native form of CLDN-18.2 on the cell surface.

Therapeutic applications of anti-CLDN antibodies

Finally, we summarize the possible therapeutic applications of anti-CLDN antibodies. The key features of the anti-CLDN antibodies described in this section are summarized in Table 4.

Anti-CLDN-1 antibody as an inhibitor of hepatitis C virus infection

It has recently been estimated that between 64 and 103 million individuals have chronic hepatitis C virus (HCV) infection [35]. Chronic HCV infection can lead to liver failure and hepatocellular carcinoma, resulting in approximately 0.5 million deaths per year [36]. Liver failure and hepatocellular carcinoma caused by chronic HCV infection are the most common indications for liver

transplantation. However, following liver transplantation, HCV re-infection of fresh liver is inevitable, and allograft loss occurs in 30% of patients [37].

HCV entry into hepatocytes is a promising target for a therapeutic strategy; host receptors, in particular, are considered to be HCV genetic barrier-free targets and useful targets of inhibitors of HCV re-infection following liver transplantation [38]. HCV enters hepatocytes through multiple processes involving heparan sulfate, low-density lipoprotein receptor, CD81, the scavenger receptor B1, epidermal growth factor receptor, CLDN-1 and occludin [39]. CLDN-1 is probably required for a HCV internalization step following a binding step involving CD81, because direct interaction with CLDN-1 and virus proteins has not been observed, but interaction between CLDN-1 and CD81 has been observed. The CLDN-1 binding Fab prevented infection of HCV with human hepatocytes [23]. Therefore, anti-CLDN-1 antibody could be a novel host-targeting antiviral agent. Indeed, several groups using human-liver chimeric mice have shown that anti-CLDN-1 antibody inhibits the entry of HCV pseudoparticles into, and their infection of, hepatocytes without obvious toxicity [40,41]. Anti-CLDN-1 antibody inhibited the cell entry of HCV pseudoparticles harboring virus envelope genomes sequenced from patients with chronic genotype 1b HCV infection (and thus mimicking highly variable HCV quasispecies) [26]. Anti-CLDN-1 antibody disrupts the interaction between CD81 and CLDN-1, and disruption of this interaction can inhibit HCV entry [42]. The potential of anti-CLDN-1 as a HCV inhibitor has been studied by other groups using pseudoparticles comprising all HCV genotypes and *in vivo* mouse studies, and anti-CLDN-1 antibody is now considered a potent host-targeting antiviral agent.

Anti-CLDN antibodies as anticancer drugs

The majority of human cancers (~90%) are derived from epithelial cells. In normal epithelial cells, the integrity and polarity of epithelial cells are maintained by the TJ; however, the TJ is often disrupted during carcinogenesis, resulting in uncontrolled tumor growth and detachment [7]. The expression levels of members of the CLDN family change dramatically in tumor tissues compared with those in parental normal tissues [10]. Although the reason why CLDNs are upregulated or downregulated during carcinogenesis is still not fully understood, the changes in the expression profiles of CLDNs have been used to diagnose carcinoma stage. From this perspective, in addition to being used as diagnostic markers, CLDNs are considered to be therapeutic targets.

CLDN-3 and -4 were originally identified as CPE receptors and are expressed in various types of cancers [14]. In particular, CLDN-3 and -4 are expressed in nearly 80% and 70%, respectively, of ovarian cancer tissues, and relationships between CLDN-3 or -4 expression levels (or both) and cancer progression have also been observed in many cancers [7,43,44]. Antibody against CLDN-3 or -4, or both, has also been shown to have antitumor effects in preclinical rodent models [28,29,45,46]. However, CLDN-3 and -4 are also expressed in normal tissues of the breast, ovaries, prostate, bladder and gastrointestinal mucosa, indicating that the safety profiles of anti-CLDN-3 and -4 need to be studied thoroughly. C-CPE and anti-CLDN-4 antibody accumulate in tumors rather than in healthy tissues [47,48]. Consistent with these data, Romani *et al.* [22] speculated that TJ-incorporated CLDNs in well-structured normal tissues might be less accessible to large-molecule CLDN-binders, whereas the non-TJ-incorporated CLDNs, which are frequently observed on the surfaces of tumor cells, could be more accessible to such binders. If this hypothesis is true, anti-CLDN-3 or -4 antibodies should prove to be safe and efficient antitumor reagents.

CLDN-18 has two splicing variants with only an eight-amino-acid difference between their first extracellular domains (Table 2). These variants show different tissue-expression patterns: CLDN-18.1 is expressed in alveolar epithelial cells in the lung whereas CLDN-18.2 is expressed in short-lived differentiated mucosal epithelium of the stomach in a specific promoter-dependent manner [30]. CLDN-18.2, but not CLDN-18.1, is frequently overexpressed in several cancers, including gastric, esophageal, pancreatic, lung and ovarian cancers [30,49]. In particular, it is overexpressed in up to 80% of gastrointestinal adenocarcinomas (primary and metastasized) and in 60% of pancreatic cancers. Therefore, CLDN-18.2 is a suitable target for antibody-based cancer therapy. However, cross-reactivity of the antibody between CLDN-18.2 and CLDN-18.1 could easily lead to unintended side-effects. A CLDN-18.2-specific antibody has been developed [30]; the chimeric antibody IMAB362 is now in a Phase IIb clinical trial for gastroesophageal cancer [50].

CLDN-6 is considered an immature-cell marker because it is not expressed in healthy adult tissues and is expressed only in immature cells such as stem cells, and in undifferentiated cancer cells [51]. These characteristics mean that, if anti-CLDN-6 antibody is administered to humans, it can bind only to CLDN-6-expressing undifferentiated cells and could have an antitumor effect without toxicity. Recently, the anti-CLDN-6 antibody IMAB027 has entered a Phase I/II clinical study for ovarian cancer [50].

Anti-CLDN antibodies for barrier modulation

Several CLDN knockout mice have shown loss of specific-site barriers without the loss of TJ strands, and some have died shortly after birth [3,52]. TJs are complicated barriers; their barrier functions, such as ion permeability and size selectivity, vary markedly in terms of tightness, depending on the composition and mixing ratio of the CLDN molecules in each tissue [4,10]. These barriers sometimes hamper drug delivery to target tissues, indicating that the strategy of opening an 'indispensable' CLDN gate by modulating CLDN in target tissues could be a novel drug delivery system.

The anti-CLDN-1-antibody clone 7A5 significantly inhibited barrier formation and increased tracer permeability without cytotoxicity in normal human epithelial keratinocyte (NHEK) cells cultured to form a multilayered highly differentiated model of the human epidermal barrier [53]. Although the expression levels of CLDN-1 were similar between NHEK and Caco-2 cells, this antibody failed to modulate the barrier integrity of Caco-2 cells, which are representative of intestinal barrier models. These findings indicate that functional CLDN-1 could be an epidermal absorption enhancer.

Anti-CLDN-6 antibody in regenerative medicine

For successful regenerative medicine, contamination with undifferentiated cells should be avoided, because there is a risk of teratoma formation by residual undifferentiated cells remaining among the differentiated products [54]. To remove teratomas

before transplantation, recent approaches have focused on the prospective removal of undifferentiated cells by using a specific antibody, or the prospective killing of undifferentiated cells by using a targeting toxin [18,55].

As described above, CLDN-6 is not expressed in healthy differentiated cells but it is expressed in human pluripotent stem cells (hPSCs) [18]. Notably, the level of CLDN-6 mRNA is extremely high in these cells and is comparable to that of octamer-binding transcription factor 4, which is a representative pluripotency gene. Furthermore, knockdown of CLDN-6 does not affect the differentiation or morphology of hPSCs and CLDN-6 knockout mice appear phenotypically normal, indicating that CLDN-6 is dispensable for the survival and self-renewal of PSCs [56]. Anti-CLDN-6 antibody is therefore useful as a biomarker of undifferentiated hPSCs. Alternatively, anti-CLDN-6 antibody could be used as an immune toxin to eliminate undifferentiated cells from their differentiated progeny. A pioneering study has shown that treatment of a mixture of hPSCs and fibroblasts with CPE, which can bind to CLDN-6, can completely prevent teratoma formation after the transplantation of cells into immunodeficient mice, whereas all mice transplanted with untreated cells developed teratomas [18]. CPE binds to other CLDNs on differentiated cells; therefore, if a CLDN-6-specific and highly efficient immunotoxin can be developed it should be useful as a hPSC eliminator.

Concluding remarks

A series of studies over the 18 years since the discovery of CLDNs has provided us with new insights into TJs as targets for drug development in relation to drug absorption, cancer therapy and the treatment of infectious and inflammatory diseases (Table 1). Excluding the peptides derived from the extracellular domains of CLDNs [57], CLDN binders can be classified into two types: toxin and antibody. As mentioned in this review, a paradigm shift from the first-generation CLDN binders (CPE derivatives) to the second-generation CLDN binders (monoclonal antibodies) has contributed to progress in CLDN-targeted drug development. However, there are two problems in the future application of CLDN binders to pharmaceutical therapy. The first is in the need to further develop the technology for creating human monoclonal antibodies for CLDNs. The types of antibodies used pharmaceutically have changed from human–murine chimeric monoclonal antibodies to human monoclonal antibodies [58], and the technologies used to create human monoclonal antibodies, including the phage display method, are still in the development stage. The second is the technology for creating chemical CLDN binders. In terms of medical economics, chemical-type CLDN binders are promising. However, we need to establish high-throughput screening systems for chemical CLDN binders and *in silico* drug design methods. Takeda *et al.* [12] have already developed a high-throughput system for screening for the interaction of CLDN and anti-CLDN monoclonal antibody by using a liposome-based interaction assay. This system will be a novel method of screening for CLDN binders. Future determination of the complex structures of CLDNs and of anti-CLDN antibodies with pharmaceutical activity will lead us to theoretical drug design for CLDN-targeted drug development. From now on, a second paradigm shift from second-generation CLDN binders (monoclonal antibodies) to third-generation CLDN binders with high druggability (human monoclonal antibodies or chemicals) is needed. This will probably occur in the near future.

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