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The Claudin family and its role in cancer and metastasis

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TABLE OF CONTENTS

1. ABSTRACT

Tight Junctions are the most apical element of the junctional complex in epithelial and endothelial cells. Tight Junctions form a barrier to paracellular movement of substances separating the apical and basolateral fluid compartments on opposite sides of the epithelial cell layer. The Claudin family are Tight Junction proteins expressed in both endothelial and epithelial cells. They participate in the development of tissue barriers between different tissue compartments by regulating the efflux of molecules through Tight Junction complexes. At least 24 different Claudin members are known today, all of which are thought to vary in expression depending on location and cell type. Relatively little is know about Claudins and their role in carcinogenesis and progression to metastasis. Recently, this new area of research has become very promising as a result of the frequent existence of altered Claudin expression in cancer. That Claudins are pivotal in the maintenance of Tight Junctions function begs investigation into the changes that can occur during the metastatic process.

2. INTRODUCTION

Metastasis is the presence of disease at distant sites due to the spread of cancer cells which results in overwhelming mortality in patients with cancer. It is a complex, multi-staged process determined by a large number of different factors and involving a number of sequential steps and events which must be completed for the cancer cell to successfully metastasize and form a secondary tumour in a distant organ, the so called metastatic cascade.

The most widely accepted model for metastasis is the “seed and soil” hypothesis postulated by Stephen Paget in 1889. He suggested that malignant tumour cells are shed from the primary tumour and disseminated in the entire body though they will only metastases when the shed (disseminated tumour cells) and soil (secondary organ) are compatible. Ever since, the knowledge in this area has expanded significantly. However, the mechanisms underlying the whole process are still unclear and currently the available therapies are mainly palliative

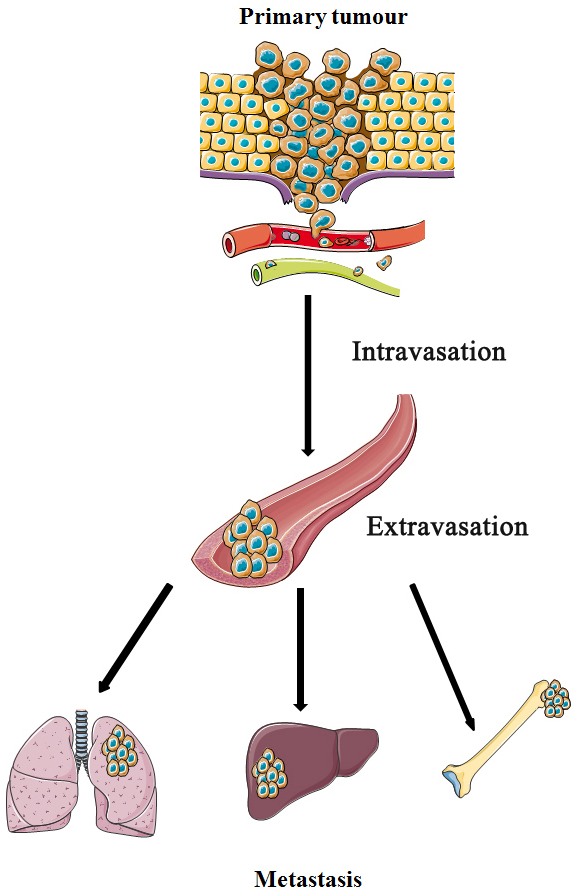

Figure 1. Schematic illustrating events in the metastatic cascade. The biological process of metastasis is a complex cascade with multiple steps: invasion, intravasation and extravasation.

The metastastatic cascade is thought to consist of the following steps: invasion, intravasation and extravasation.

Invasion occurs when tumour cells gain the ability to dissociate from the primary tumour and penetrate the surrounding tissues through degradation of the basement membrane and the extracellular matrix, leading to intravasation as the detached cells enter the circulatory or lymphatic system. Once the tumour cell arrives at a possible point of extravasation, it interacts and attaches at the new site to penetrate the endothelium and the basement membrane to produce a secondary tumour, this is called extravasation.

Extensive interactions between tumour cells and surrounding tissues during their dissemination complicates the analysis of the signalling events during this cascade. Because of its complex nature, the understanding of the cellular and molecular determinants is limited. The most important questions arising are focused to define the genetic and epigenetic changes conferring such behaviour to these cells (1).

Since Tight Junctions are located between the cancer epithelial cells themselves functioning in an adhesive manner, and between the endothelial cells as a barrier through which molecules and inflammatory cells can travel, they represent a critical barrier which the cancer cells must overcome in order to penetrate and initiate metastasis. If any microorganism or cancer cell can gain access to the systemic circulation, there exists a wealth of nutrients and an ideal environment for many to proliferate. Therefore, Tight Junction integrity is a key step in the metastatic cascade (2) (Figure 1).

3. THE TIGHT JUNCTION STRUCTURE

As the proverb states: “Good fences make good neighbours”. This humble and intelligent statement is not just applicable to the property market; it is indeed a reality seen in nature, in all species, in evolution and in development. A single cell in the ocean can exchange nutrients and waste products constantly. However, when a single cell is part of a multicellular organism this changes; moving from an easy movement of substances to a complicated and extremely organized exchange system with the external environment.

A defining characteristic of a multicellular organism is the capability of forming Tight Junctions that seal the intercellular space between neighbouring cells and transform the layer of individual cells into an effective permeability barrier. Barriers not only separate fluids, but they also perform thermodynamic work by reabsorbing solutes from compartment A to B, or secreting others from B to A, thus establishing gradients across themselves.

Tight Junctions are highly regulated areas of adhesion between epithelial and endothelial cells. They are the most apical component of the lateral plasma membrane and they are connected to the actin cytoskeleton. They create a regulated paracellular barrier to the movement of ions, solutes and immune cells between the cells and signalling pathways that communicate cell position, limit growth and apoptosis. The morphology of Tight Junctions has been intensively analysed by transmission electron microscopy, where the Tight Junctions appear as a sequence of very close points as fusions of the plasma membrane of both cells, and by freeze fracture electron microscopy where these contacts are shown as rows of intramembrane strands and complementary grooves that encircle cells (3). Physiological studies of the past decades have demonstrated that the Tight Junction barrier is not absolute, and solute permeability varies amongst different tissues. Moreover, barrier assembly and permeability characteristics are influenced by different cellular signalling mechanisms. Tight Junctions have been proposed to have two equally exclusive functions: 1. a fence function by forming an apical/basolateral intramembrane diffusion barrier which prevents the mixing of membrane proteins; and 2.a gate function by controlling the breadth and selectivity of diffusion along the paracellular pathway (4). The number of Tight Junction strands is an important factor in determining the barrier properties of the Tight Junction. There is not a linear relationship between the complexity of the Tight Junction

Table 1. Proteins involved in Tight Junction structure, function and regulation

Transmembrane protein	Cytoplasmic plaque	Associated/regulatory proteins
Occludin	Zonula Occludens (ZO-1, ZO-2, ZO-3)	Rho-GTPases
Tricellulin	AF6	Rab-13
MARVEL D3	MUPP-1	Rab-3B
Claudin 1-24	MAGI-1,-2,-3	Galphai-2
Junctional Adhesion Molecules (JAM 1-4)	Cingulin	Galpha0
	Angiomotin family Symplekin	*c-src, c-yes*
		ZONA B
		19B1
		Ponsin
		Par-3, Par-6
		Afadin
		alpha-catenin
		Pals
		PATJ
		JEAP
		Pilt
		PTEN
		ZAK
		Scrib
		ITCH
		WNK4
		Vinculin

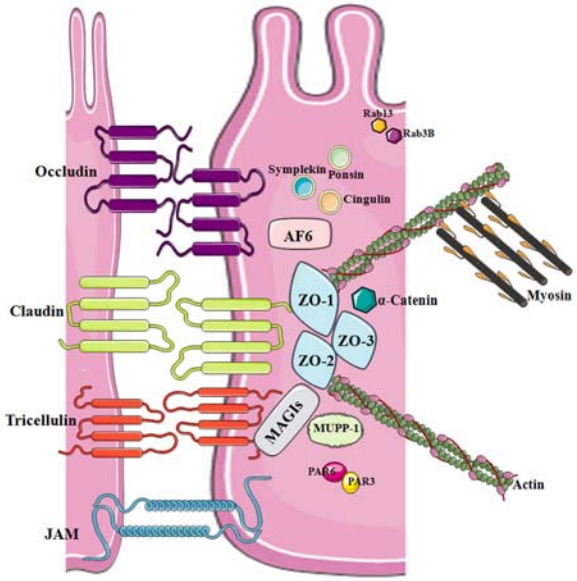

Figure 2. Molecular structure of Tight Junctions. Tight Junctions contains of two main types of transmembrane proteins: Tetraspan and Single-span proteins, Cytoplasmic plaque and Associated/regulatory proteins**.**

strand network and their measured electrical resistance (trans epithelial/endothelial resistance, or TER), in other words, the number of Tight Junction strands does not correlate to the tightness of the barrier, in fact the relationship is an exponential one (5). Such results led to the prediction that Tight Junctions must contain aqueous pores lined by proteins. It has already been reported that the strands contain aqueous pores that oscillate between open and closed states (6). Therefore, it has been accepted that the tightness of the Tight Junction is remarkably dynamic and finely regulated in individual cells.

Although the details of how intracellular signals may influence these proteins are not understood, many signalling messengers, including prostaglandins, cAMP, and protein kinase C, have been seen to regulate the actin cytoskeleton in epithelial cells (7).

Several types of proteins have been identified as components of the Tight Junction, depending on their distribution within the junction: 1. Transmembrane proteins such as the MARVEL/TAMP proteins (Occludin, Tricellulin and MARVEL D3), the Claudin superfamily and Junctional Adhesion Molecules (JAMs). These proteins span the cell membrane and are anchored into position by links to the cytoplasmic/plaque proteins.

2. Cytoplasmic plaque such as the Zonula Occludens family, ZO-1,-2,-3, AF6, MUPP-1, MAGI-1,-2,-3, Cingulin, Angiomotin family and Symplekin. These proteins link Tight Junction to the actin-cytoskeleton and the adherens junction to the regulatory proteins.

3. Associated/regulatory proteins such as the Rho subfamily proteins, Rab-13, Rab-3B, heterotrimeric G proteins like G-alpha-i-2 and G-alpha-0 etc (Table 1, Figure 2).

4. CLAUDINS, A MULTI-GENE FAMILY

4.1. Structure of Claudins

To date the Claudin family is composed of 24 members in mammals and have molecular weights ranging from 22 to 27 kDa. There have been 54 Claudins identified in the fish *Takifugu* and 15 in *Danio rerio*. Invertebrates also express Claudins despite their lack of Tight Junctions, e.g. *Drosophila Melanogaster* appears to have 6 Claudins. Claudins were originally thought to be simple sealing proteins at the Tight Junction. In fact, the name of *Claudin* derives from the latin word "claudere" which means to close.

Claudins were first identify by Furuse *et al.*, using the same isolated fraction from chicken liver from which Occludin was first identify by Tsukita's group in 1989 (8). They showed for the first time that a group of proteins existed with similar sequence to each other and with four transmembrane domains where the N- and C-terminal domains are orientated towards the cytoplasm, but with no similarity to Occludin. Claudin members have since been divided in two groups. The so called "Classic Claudins", which include members with highly similar sequence homology like Claudin-1 to -10, -14,-15, -17 and -19. And the "Non-classic" Claudins which include Claudin-11, -13, -16, -18, and -20 to -24 (9).

The cytoplasmatic C-terminal domain in Claudin varies between members in length and sequence, ranging from 21 to 63 residues. While the N-terminal domain is

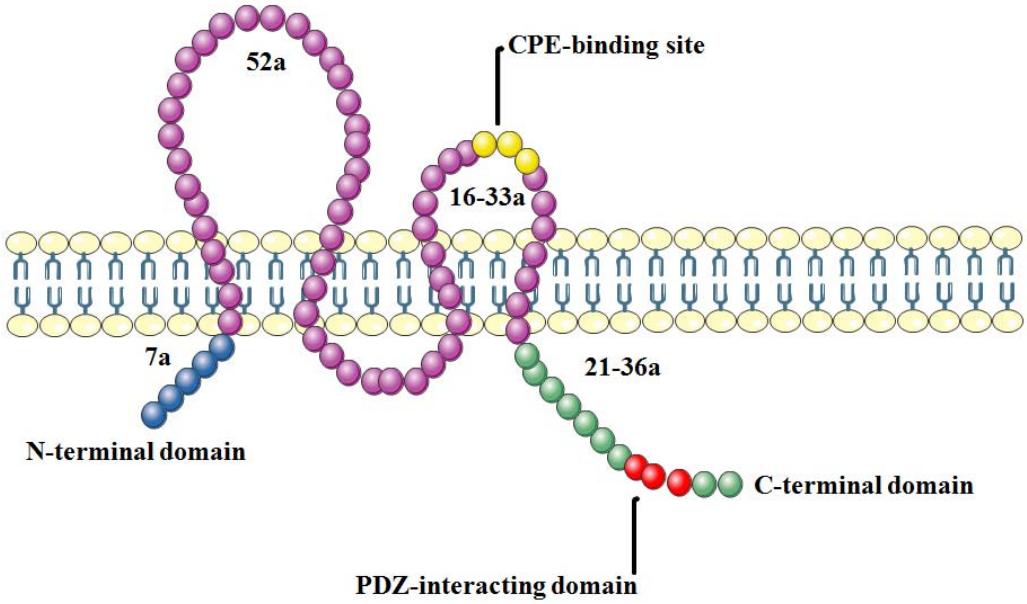

Figure 3. Topology of Claudins. Claudins have four transmembrane domains, two extracellular loops, C- and N-terminal domain and a PDZ interacting domain.

relatively short, 7 amino acid sequences. The intracellular loop is composed of 12 amino acids. The first extracellular loop is a 52 amino acid highly conserved sequence in different members with two conserved cysteines that influences parcellular charge selectivity [Gly-Leu-Trp-x-x-Cys-(8-10 aa)-Cys]. Some studies suggest that this loop determines the charge selectivity of the paracellular transport. The second extracellular loop is shorter, ranging from 16 to 33 amino acid residues. It is worth mentioning that this loop might fold in a helix-turn-helix motif which seems to participate in the Claudin-Claudin interactions (10) . It has been observed that this loop functions as a receptor for the *Clostridium perfringens* endotoxin or CPE.

The cytoplasmic tails have the most variant sequences in the topology of the Claudins, their lengths range from 21 to 63 amino acid residues, suggesting the involvement of this structure in isoform-dependent paracellular selectivity. All members have a PDZ domain in their COOH- terminal tail that allows them to interact with other proteins in the Tight Junction such as ZO-1, -2, and -3, MUPP, and PATJ. The interaction with cytoplasmic plaque proteins such as ZO-1 links Claudins to the actin cytoskeleton (11). To date, there is no information about the function of the NH_2 domain (Figure 3).

4.2. Claudin interactions

Most epithelia and endothelia express a multiple mixture of different Claudin members and more than two different Claudin members are coexpressed in a single cell (12). Consequently, these observations have raised the question of whether different Claudin proteins are co-polymerized to form Tight Junction strands as heteropolymers, and whether Claudins interact with each other in a homophilic manner, between two molecules of the same Claudin member, or heterophilic between two different Claudin members. However, little is known about the molecular mechanisms taking place during assembly and strand formation.

To assess all these questions, an early study by Furuse *et al.*, showed that when using co-expression of multiple Claudin isoforms in mouse L fibroblasts, they concentrated at cell-cell contact areas forming a well-developed network of Tight Junction strands. However, when these cells were co-cultured they found by immunoprecipitation that different Claudin members can interact within and between Tight Junction strands, but these combinations were restricted to specific combinations of isoforms (13).

These heterotypic interactions are assumed to occur in the plasma membrane of the same cell (*cis*-interaction) or between plasma membranes of opposing cells (*trans*-interaction), in a similar way these interactions were defined to cadherins (14).

Homophilic *trans*-interactions, also named homotypic interactions (15), have been seen for Claudin-1,-2,-3 (13), Claudin-5 (16), Claudin-6,-9, -14 (17), Claudin-11 (18), and Claudin-19 (19). All these Claudin isoforms were able to form Tight Junctions when transfected into Tight Junction free cells. Homophilic *cis*-interaction, or homomeric interaction (15), has been described for Claudin-5 and -5 using fluorescence resonance energy transfer (FRET) and electron microscopy (10).

Heterophilic *trans*- interactions, also termed as heterophilic interactions (15), have been observed for Claudin-1 and -3, Claudin-2 and -3 (13) and for Claudin-3 and -5 (15). The heterophilic interactions for at least these

isoforms require compatible structural features in both extracellular loops. It has not been possible to demonstrate a heterophilic trans-interaction for Claudin-1 and -2 (13), Claudin-1 and -4 , Claudin-3 and -4, and Claudin-4 and -5 (15). These results demonstrated that only specific Claudins are able to interact with each other. Heterophilic *cis*-interactions, or heteromeric interactions (15), were found for Claudin-2 and -3, Claudin-3 and -4 and assumed for Claudin-1 and -2, respectively (13).

4.3. Physiological functions of Claudins

Paracellular transport through pores in the Tight Junction differs in several important features from transcellular transport across the membrane. Firstly, it happens through the intercellular space of neighbouring cells. Secondly, this transport is passive and dependent on an electrochemical gradient. As discussed before, Tight Junctions play a central role in the intercellular space in epithelial and endothelial cells, and therefore the key factor of paracellular transport. These pores, now known to be formed by Claudins, are the major determinants of paracellular transport processes (20).

The role of different Claudin members has been studied utilising three different approaches: by overexpression or downregulation of Claudins in different cell lines, by knockdown of Claudin genes in mice and by the study of the phenotype of human diseases due to Claudin mutation (21). Taking all the data from these studies together, it is more than evident that the combination and mixing ratios of different Claudin isoforms determine the selectivity of paracellular transport across epithelia and endothelia.

To study the effect of different Claudins on conductance, monolayers of Madin-Darby canine kidney (MDCK), epithelial cells expressing single type of Claudin, have been used. There are two types of MDCK cell based on transepithelial electrical resistance, type I or "tight" cells, and type II or "leaky" cells.

A clear outline of results has been revealed, showing that the expression of Claudin-1,-4,-8,-14 and -15 significantly increases resistance when expressed in low-resistance MDCK type II cells. Whereas, the expression of Claudin-2 in high resistance MDCK type I cells decreased resistance (22). A Claudin-19 study in the same cell line has reported to increase resistance and decrease permeability to monovalent and divalent cations, but anions and urea were unaffected (23). Overexpression of Claudin-7 in the renal epithelial cell line of porcine proximal tubule LLC-PK1 has revealed an increase in resistance and a dramatic reduction of dilution potentials compared to wild type cells (24). Over expression of Claudin-4, containing a mutation simulating its phosphorylated state, in the ovarian cancer cell line OVCA433 decreases the Tight Junction strength (25). Claudin-5 transfected in the human colonic cell Caco-2, low transepithelial resistance cell line, shows a significant increase in barrier function (26).

The knockout of specific Claudin genes in mice also indicates that Claudins are the major participants of the selective size, charge, and conductance properties of the paracellular pathway. A very significant example has been seen in the Claudin-1 knockout mice, which die within 1 day of birth from dehydration (27). Claudin-11 null mice exhibit neurological and reproductive problems, showing the importance of this isoform in forming the paracellular physical barrier of the Tight Junction required for spermatogenesis and the development of a normal central nervous system (28). Whereas Claudin-14 knockout in mice showed a rapid degeneration of cochlear outer hair cells leading to deafness, suggesting the role of Claudin-14 as a cation-restrictive barrier in the maintenance of the ionic composition of this type of cell (29).

Colegio *et al.*, have shown, by swapping the first, second and both extracellular domains between Claudin -2 and -4 in MDCK type II, that charged amino acid residues on the first extracellular domain of Claudins mediates in the paracellular permeability for ions (30). However, a recent study using chimeras of Claudin-2 and -4 in MDCK type II cells suggested that the extracellular domains are sufficient to increase the permeability, but the participation of the carboxy-terminal PDZ binding motif is also necessary indicating that an interaction with other Tight Junction cytoplasmic plaque proteins like as ZO-1, ZO-2, ZO-3 or MUPP-1 are also needed in order to form pores (31).

Examples of cation pore-forming Claudins are Claudin-2 (32) as well as Claudin-16 that forms a Mg^{+2}-selective channel in the thick ascending limb of Henle (33).The confirmation for anion pore-forming Claudins is less clear, nevertheless Claudin-10 has been identified as an anion-selective paracellular (34). Using polyethylene glycol oligomers (PEGs), small uncharged solutes with increasing radii, Van Itallie *et al.*, measured the size of the pore formed by Claudin-2. This study has revealed that this pore has a high capacity for compounds charged and uncharged below 4Å and a lower capacity for larger solutes (31). To date there are no other studies on the sizes of pores formed by other Claudin isoforms.

The particular role played by a number of Claudin family members are yet to be identified. Examples are Claudin-6, -9, -12, -13,-17, -18, and 20-24. Claudin-6, -9 and -13 studies have closely related these isoforms in the maturation of the epidermis or the barrier function in different cell types such as embryonic stem cells (ES) (9). Work on Claudin-12 showed that it is expressed in endothelia and epithelia of the mouse intestine (35) as well as in the inner ear and brain endothelial cells. Claudin-18 has also been identified in the inner ear (36). For other Claudin family members such as Claudin-17, -20,-22 and -23, only the mRNA expression levels have been identified in the duodenum of rats (37). The existence of Claudin-23 and -24 have only been established from the analysis of the human genome (38).

Studies focusing on the blood-brain barrier (BBB) have proposed a "sealing" role for Claudin-5 (39) (40). BBB protects the brain from the blood surroundings within the central nervous system (CNS), and most importantly maintains homeostasis of the brain

environment, which is crucial for neural activity and function. Extremely close Tight Junctions between endothelial cells of brain capillaries prevent the passage of hydrophilic molecules from blood to brain and vice versa. Mice genetically altered to lack Claudin-5 were generated in a study by Nitta *et al.*, These mice have shown a normal development and morphology of blood vessels in the brain, however, in terms of the barrier function, these endothelial cells showed an unexpected feature: a size-selective loosening of the BBB, in other words, only small molecules (<800Da) were allowed to pass across the Tight Junction but no larger molecules were affected. Moreover, Claudin-5 deficient mice die within 10 hours of birth (40). Therefore, it appears that loss of Claudin-5 from the Tight Junction complexes in the brain can compromise barrier function making it "leakier" while keeping their structural integrity, demonstrating that Claudin-5 specifically tightens the BBB for molecules <800Da. The majority of the drugs in clinical use are included in this range: subsequently Claudin-5 might be involved in terms of drug delivery to brain tumours or neurodegenerative disease.

4.4. Regulation of Claudins

The paracellular barrier modulated by Claudin members could be affected by a wide range of physiological factors including cell signalling pathways, hormones, cytokines, and disruption of the cell-cell contacts. This field is still emerging and little is known about the mechanism that regulates the Claudin family. However, post-translational modifications, including phosphorylation, lipid modification and removal of Claudins by endocytosis, appear to be potential mechanisms for the regulation of Claudins.

A number of studies have revealed that Claudin function can be highly regulated by phosphorylation. It is widely accepted that most Claudin members have potential serine and/or threonine phosphorylation sites in their cytoplasmatic COOH-terminal domains. Results after phosphorylation contribute in some cases to increase the barrier function of the Tight Junctions and in other cases to reduce it. For example, Protein Kinase-A (PKA) phosphorylation of Claudin-5, probably at the amino acid site around Thr207, results in an increase in dependent and independent manners of the barrier function in porcine blood-brain barrier endothelial cells treated with cyclic AMP (cAMP) (41). However, PKA-dependent phosphorylation of Claudin-3, at the amino acid site Thr192 in the cytoplasmic COOH-terminal domain, in the ovarian cancer cell line OVCA433 resulted in a decrease of the Tight Junction strength (25). Yamauchi *et al.*, found in MDCK type II cells that the threonine-serine kinase WNK4, binds and phosphorylates endogenous Claudin-1,-2,-3 and -4 and that the human disease-causing mutant of WNK4 is associated with increased paracellular chloride permeability without increasing sodium permeability (42). A different study showed similar results and revealed that Claudin-7 is also a substrate to WNK4 on Ser206 in its cytoplasmatic COOH-terminal domain (43). Different studies have reported how other kinases are also linked to Claudin members such as MAPK (mitogen-activated protein kinase) phosphorylating Claudin-1 (44), Claudin-4,-7,-8 and -9 (45); Rho kinase phosphorylating Claudin-4 (46) and EphA2 phosphorylating Claudin-5 (47).

Endocystosis is a critical step in the remodelling of the Tight Junction structure. To assure the correct sealing of the intercellular space of epithelial and endothelial cells this process has to be thoroughly regulated. Live observation and electron microscopy have revealed, in confluent Eph cells, that the endocytosis of Claudins was aided when wounding the cellular sheet and that other transmembrane Tight Junction proteins such as Occludin and ZO-1 appeared to be detached from Claudins before this process occurred (48).

Another potential post translational modification is palmitoylation. Van Itallie *et al.*, shown that mutation of palmitoylation sites in MDCK type II cells alter the localization of Claudin-14 however, the stability and assembly of the Tight Junction strands were not affected indicating that alterations in transepithelial resistance in mutants might be due to the translocation of Claudin-14 (49).

When looking at the level of gene expression, the zinc finger-containing transcription factor Snail that plays a pivotal role in epithelial-to-mesenchymal transcription (EMT), emerged as a regulator of Claudin gene expression, binding directly to Claudin promoters. Claudin genes, as well as E-Cadherin and Occludin, contain E-box motifs that trigger Snail and thus repress transcription (48). A study in MDCK type II cells by Ohkubo *et al.*, reported a possible second mechanism for Snail, finding that Snail downregulates protein levels of Claudin-1 however, mRNA was unaffected, suggesting that Snail may regulate Claudin translation (50).

Further experiments using MDCK type II cells expressing Snail, showed an increase in the paracellular permeability for chloride and sodium. They also reported a slight decrease in Claudin-2 expression but a significant decrease in Claudin-4 and -7 (51). This suggests that the increase of Snail expression has different effects in different Claudin members, resulting in selective changes in the cell barrier function.

Other transcription factors have also been reported to interact with the Claudins. An early study by Niimi *et al.*, showed that one isoform lacking the C-terminal cytoplasmatic domain of Claudin-18 is regulated by the T/EBP/NKX2.1 homeodomain transcription factor which is expressed in the lung, thyroid and stomach (52). The transcription factor GATA-4 is also connected to one Claudin member, Claudin-2. GATA-4 binds to the promoter of Claudin-2 when the transcription factors CDX or HNF-1alpha are present suggesting that GATA-4 is totally necessary for the expression of Claudin-2 (53).

Hepatocyte growth factor (HGF) and epidermal growth factor (EGF) have been seen to regulate Claudin family members. Both are cytokines that have been involved in cell motility, mitogenesis and morphogenesis in a broad range of cells including cancer cells where HGF

has been seen to induce invasiveness as well as angiogenesis *in vitro* and *in vivo* (54)

Two different studies revealed that both cytokines have similar effects when MDCK type II cells are treated with HGF and EGF. Both increased the transepithelial resistance linked to a reduction in the expression of Claudin-2 and activation of the extracellular signal-kinase (ERK) 1/2. ERK 1/2 inhibitor, U0126, induced Claudin-2 expression in MDCK type I cells which showed no levels of Claudin-2, in contrast to MDCK type II, which seem to have high levels of this Claudin isoform. Adding the inhibitor U0126 the situation is inverted where Claudin-2 was not seen now it is present and transepithelial resistance is decreased (55). In addition to this, EGF, aside from increasing the transepithelial resistance, induces cellular remodelling and enhances the expression levels of Claudin-1, -3 and -4 (56). All these results indicate the importance of ERK 1/2 in determining the paracellular permeability in MDCK cells. Peter *et al.*, studied the effect of EGF in the non-small cell cancer lung (NSCLC) cell line, their results showed that after treatment with EGF levels of expression of Claudin-2 are increased however, transepithelial resistance is reduced (57).

HGF decreases transepithelial resistance and increases paracellular permeability in human endothelial cells HUVEC after treatment. The level of expression of Claudin-1 seems to be reduced overtime whereas no changes were observed in the levels of Claudin-5 (58). When breast cancer cells MDA-MB-231 and MCF-7 were treated with HGF, the transepithelial resistance was once more reduced. Claudin-1 transcripts levels were reduced in MDA-MB-123 cells through time whereas changes in MCF-7 cells were less significant. When looking at Claudin-5, significant changes in expression were seen in both cell lines (59).

Overall, it is clear that HGF and EGF modulate changes in expression of different Claudin family members therefore altering the physiological function of Tight Junctions in different cell types including breast cancer cells.

5. CLAUDINS AND CANCER

The link between altered Tight Junctions and epithelial tumour development has been confirmed by earlier studies placing Tight Junctions in the spotlight of cancer research. These studies showed dysregulation in Tight Junction structures of several epithelial cancers including breast.

Most cancers originate from epithelial tissues and are characterized by irregular growth and aberrant tissue morphology. It is absolutely necessary for tumour cells to have distinct adhesion behaviour; being significantly weaker in cancer cells. Thus the communication between cells is highly affected and disorders in the signal transduction pathways that connect cell to cell arise. This change in cell surroundings encompasses a wide spectrum of changes, revealed in early and late stages of tumour growth, when the loss of polarity and uneven growth, as well as invasion and metastasis are a reality in cancerous epithelia. As tumour epithelial cells were examined in different types of cancer, early evidence was found of disorganized structure of Tight Junctions (60) or even a lack of them in hepatocellular carcinoma, and reductions in the number of Tight Junction strands when seeing by freeze fracture in breast carcinoma (61). Decrease in transepithelial resistance (TER) and consequently increase in Tight Junction permeability has also been reported in Tight Junctions of colon tumours (62). In normal kidney epithelial cells, structural adjustments in Tight Junctions were observed during mitosis. However cell division itself does not increase epithelial Tight Junction permeability, hence inducing leakiness in the epithelial barrier. These results indicate that altered permeability may be due to disease states like cancer (63).

Taken together all this evidence of different cancer types appears to show clearly that a decrease in the epithelial barrier function and loss Tight Junction function are correlated. An extensive number of studies have shown that different protein members of Tight Junctions are directly or indirectly related to cancer progression, some of those have also been shown to correlate with staging and metastatic potential in various cancers.

In bladder cancer the expression of Claudin-1, -4 and -7 was analysed by Boireau *et al.*, Claudin-4 expression appears to be modified in 26/39 tumours compared with the exceptional modifications founded in Claudin-1 and -7. Overexpression of Claudin-4 was found in different carcinomas followed by remarkable downregulation in invasive/high grade tumours. Delocalization of Claudin-1 and -4 was seen in most human bladder tumours as well as in the bladder cell line HY-1376 (64).

In colorectal cancer Resnick *et al.*, studied the pattern of expression of Claudin-1, -4, Occludin and ZO-1 and their possible roles in prognosis of disease was evaluated in a cohort of 129 TNM stage II tumours using tissue microarray technology. They found that in that order 75%, 58%, 56% and 44% of the tumours examined displayed normal to elevated expression levels of Claudin-1, -4, Occludin and ZO-1. Low expression levels of Claudin-1 and ZO-1 were related to high tumour grade. Taking all these results together, it was confirmed that loss of Claudin-1 might be a strong candidate for disease recurrence and poor patient survival in stage II colorectal cancer (65). The levels of expression of Claudin-1and -2 were examined in adenocarcinoma tissues as well as normal mucosa by Kinugasa *et al.*, Immunohistochemistry and quantitative reverse transcription-polymerase chain reactions (RT-PCR) were used in the study. Results showed upregulation in both Claudins at mRNA level as well as at protein level linking these results to depth in tumour invasion (66). The expression of Claudin-3, -4 and -7 was identified in gastric carcinoma and gene expression was analyzed by microarrays in three oesophageal adenocarcinomas, one case of Barrett's oesophagus, and three normal oesophagi. Claudin -3 showed a marked

increase in mRNA expression compared with normal oesophagus while Claudins- 4 and -7 were moderately upregulated. Claudin-4 and -7 protein expressions were highly upregulated in Barrett's oesophagus but minimum in squamous and gastric mucosa. Claudin-3 showed high-grade dysplasia ?in adenocarcinoma, and metastases specimens. All these findings suggest that alterations in Claudin proteins are an early event in tumourigenesis of oesophageal adenocarcinoma (67). Usami *et al.*, analyzed the expression of Claudin-7 in squamous cell carcinoma of the oesophagus showing that Claudin-7 expression levels at the invasive front of the primary tumours compared to Claudin-7 levels at metastatic lymph nodes are significantly reduced in the metastatic nodes. This suggests that the reduction may be linked to tumour progression and subsequent metastatic events (68). Examination of Claudin-5, as well as Claudin-1,-3 and -4, in 118 cases of gastric carcinoma revealed that the lowest expression of all these Claudin members was seen in Claudin-5. Nevertheless, strong Claudin-5 expression was associated with levels of E-cadherin, high levels of cell proliferation and apoptosis. The results also revealed that expression of these Claudin members was lower in diffuse-type gastric carcinoma (69).

Disruptions in the Tight Junction structure have also been seen in lung cancer. Using semi-quantitative reverse transcription-PCR on 27 pairs of lung squamous cell carcinomas and normal lung tissue, Liu at al.,, confirmed differential expression of Claudin-1 in 82.1% (23/28) of lung tumour tissue (70). Paschoud *et al.*, investigated different patterns of expression of a large panel of Tight Junction proteins in lung squamous cell carcinomas and adenocarcinomas using quantitative RT-PCR. Significant changes in transcript levels were found when looking at squamous cell carcinomas in JAM-1, Occludin, Claudin-1, -3, -4, -7, Cingulin, ZO-2 and -3. Only Claudin-1 was shown to be downregulated while the other proteins were upregulated. In adenocarcinomas, transcript levels were compared to bronchial cells, they observed significant downregulation in the levels of Claudin-1, -3, -4, -7, ZO-2 and -3 (71). A similar study in human lung squamous cell carcinoma and adenocarcinoma has reported high levels of Claudin-5 in cylindric cells, pneumocytes and adenocarcinomas, and low or even undetectable levels of expression in basal cells and squamous cell carcinoma. These results indicate the possible role of Claudin-5 as a diagnostic tool to distinguish between adenocarcinomas and squamous cell carcinomas in lung cancer patients (71).

A study from Sheehan *et al.*, shows the pattern of expression of several Claudins in prostatic adenocarcinomas from 141 tissues samples. Decreased expression of Claudin-1 correlated with high tumour grade and biochemical disease recurrence, Claudin-7 appears to be decreased and also correlated with high tumour grade. However, Claudin-3 correlated with advanced tumour stage and recurrence, while Claudin-4 correlated with advanced stage (72). Examination of Claudin-5 in 48 prostate cancer patients has reported that 35% of patients showed low expression of Claudin-5 in comparison with 65% that displayed a high level of expression. From those who were classified in the low-expression group, 88% had a Gleason score of 7 or even higher and 2% had a Gleason score of 6 points or lower, whereas those classified in the high-expression group the Gleason score was 7 or higher and 48% had Gleason score of 6 points or lower. Therefore it can be concluded that Claudin-5 is associated with a Gleason score of 7 points or higher in prostate cancer patients (73).

Claudin-4 was overexpressed in epithelial ovarian cancer, although this expression did not correlate with survival or other clinical endpoints. However, Claudin-4 overexpression was correlated with changes in barrier function after treatment with *Clostridium perfringes* enterotoxin in a dose-Claudin-4 dependent non-cytotoxic manner (74). When Claudin-4 protein and transcript levels were analyzed in 110 patients with different histologic types of epithelial ovarian carcinomas, Tassi *et al.*,, found them to be significantly upregulated in both primary and metastatic tumours compared to normal human ovarian surface epithelium cell lines. At protein level, Claudin-7 appears to be significantly higher in tumours of primary and metastatic origin when compared to normal ovaries, despite grade of differentiation, histologic type and pathological state. Complementary results shown Claudin-7 to be overexpressed in all main histological types of epithelial ovarian carcinomas, in single neoplastic cells dispersed in the peritoneal cavity and pleural effusions. The data presented in this study pointed at Claudin-7 as a novel marker in the disease (75). Increased Claudin-5 expression has been associated with aggressive behaviour in serous ovarian carcinomas. Turune *et al.*, studied 85 serous ovarian cancer tissue samples. Immunostaining results revealed strong Claudin-5 staining in advanced stage and high grade carcinomas. When looking at Claudin-5 expression, only 25-30% of patients who were Claudin-5 positive were still alive at 5 years follow-up compared to 60% of patients who were Claudin-5 negative (76).

In human pancreatic endocrine tumours and ductal carcinomas, the protein and mRNA expression of different members of the Claudin family was analysed by Borka *et al.*, Claudin-1, -2, -3, -4 and -7 revealed strong staining while Claudin-2 showed diffuse staining in normal acini and ducts. Langerhans islands presented only Claudin-3 and -7 expressions. The majority of endocrine tumours were negative for Claudin- 1, -2 and -4. Claudin-2 was present in half of ductal adenocarcinomas whereas Claudin-3 was totally negative. Claudin-3 and -7 were detected in all endocrine tumours. When looking at level of expression, differences were seen between endocrine tumours and ductal adenocarcinomas, worth mentioning is the high expression of Claudin-3 in endocrine tumours and Claudin-7 in ductal carcinomas making those proteins targets for adjuvant therapy (77).

In hepatocellular carcinoma, Claudin-5 has been reported to be downregulated. Low levels of Claudin-5 and vasculobiliary invasion have been correlated with patients displaying poor prognosis. Taken together these results suggest a possible role for Claudin-5 as a prognostic factor in hepatocellular carcinoma (78).

An increasing number of studies have described the dysregulation of the Tight Junction proteins and how these changes also affect breast cancer progression. Claudin-1 protein level has been reported to be reduced in breast tumours as well as in breast cancer cell lines such as MDA-MB-231 and MDA-MB-435. In these cell lines no genetic alterations were seen in the promoters or coding sequences with no explanation for the loss of expression of Claudin-1, therefore rejecting any tumour-supressor effect for this protein (79). In similar breast cancer cell lines, Hoevel *et al.*, found no signal of expression for Claudin-1 nor Occludin, however, when Claudin-1 retroviral was transduced into these cells expression of Claudin-1 was found at the usual location at cell-cell contact sites, suggesting that other proteins might be responsible for targeting Claudin-1 in the Tight Junction. In addition the paracellular permeability was altered in the transduced cells. The authors suggested that Claudin-1 over expression might be sufficient to exert Tight Junction paracellular barrier function in metastatic breast cancer cells yet in the absence of other transmembrane proteins such as Occludin. These results aggree with the study by Kramer at al., suggesting that even though there is no evidence of any genetic changes, there must be some epigenetic or regulatory factors involved in the downregulation of Claudin-1(80). Furthermore, Claudin-1 has been seen to be a useful prognostic marker in breast cancer patients. Morohashi *et al.*, have revealed a correlation between recurrent breast tumours and low level of expression of Claudin-1 compared to primary tumours. Decreased expression of Claudin-1 also was associated with lymph node metastasis-positive groups and disease free patient groups (81).

Examination of Claudin-16 in breast cancer cells, *in vitro* and *in vivo* studies, have shown that when Claudin-16 is over expressed in the human breast cancer cell line MDA-MB-231 cells were significantly less motile and displayed reduced aggressiveness, with increase in Tight Junction function as the colonies became tighter. To complement this study, patient data revealed low expression of Claudin-16, mainly in patients who displayed high mortality (82). Studies of Claudin-7 have revealed the loss of expression in preneoplastic and invasive ductal carcinoma; this loss was mostly seen in high-grade lesions. The same situation was seen in lobular carcinoma where Claudin-7 was also absent. The authors suggested a link between Claudin-7 lack of expression and cancer progression due to the increased cellular discohesion that is frequently seen in high-grade lesions, proposing that Claudin-7 might help tumour progression and increases metastatic potential., Moreover, when the breast cancer cell lines MCF-7 and T47D, which express high levels of Claudin-7, were treated with HGF, there was a resultant loss of Claudin-7 after 24 hours of treatment as well as dissociation of these cell lines in culture, linking the loss of Claudin-7 and cell cohesion in breast cancer (83). In concordance with the above mentioned study, Sauer *et al.*, showed an inverse correlation between Claudin-7 level of expression and tumour grading. Grade 2 and 3 invasive carcinoma revealed reduced expression of protein. This data correlates with metastatic disease, including loco-regional recurrences and with heterogeneous staining pattern. However, these results do not correlate with tumour size or subtype (84). Osani *et al.*,, demonstrated that the knock down of Claudin-6 in MCF-7 cells increases cell migration and invasion (85). In agreement with Osani's results, a recently study by Wu *et al.*,, revealed that over expression of Claudin-6 in the cell line MCF-7 resulted in a decrease in cell growth rate as well as migration and invasion. However, the transepithelial resistance was increased in the transfected cells, suggesting a possible role in breast cancer progression (86).

Soini *et al*,. analysed the pattern of expression of Claudin-2, -3, -4 and -5 protein levels in breast carcinoma. The study revealed how Claudin-2 and -4 were highly expressed in non-neoplastic breast tissue whereas Claudin-3 and -5 appears to be highly expressed in ductal and acinar cells. Levels of expression were for Claudin-2 in 52%, Claudin-3 in 93%, Claudin-4 in 92% and Claudin-5 in 47%. It is important to mention that there was no correlation between level of expression of these Claudin members and tumour grade or oestrogen receptor status. Levels of expression between different Claudins were seen to be associated, strong Claudin-2 expression was linked to Claudin-5 and -3. In the same way, strong Claudin-3 expression was associated to Claudin-5 and -4 (87). A similar study, but analysing levels of mRNA compared to protein for Claudin-1, -3 and -4 in malignant breast tumours and benign lesions, was carried out by Tokes *et al.*, revealing that whereas Claudin-3 and -4 mRNA and protein levels did not show any difference in expression between invasive tumour and the surrounding normal tissue, Claudin-1 mRNA appeared to be highly downregulated when compared with the control group. However, Cl audin-3 and -4 proteins were detected in all primary breast carcinomas in one study by Kominsky *et al.*, in addition, when compared to normal epithelium, these two Claudin members were overexpressed in a 62% and 26% respectively (88). Additionally, Claudin-1 was located in the membrane of the ductal cells and in some of the ductal carcinoma *in situ*, whereas in invasive tumours it was not present or its distribution was diffuse in the tumour cells. This data showed further evidence of how Claudin-1 is involved in invasion and metastasis of breast cancer. Claudin-4 distribution was seen highly in normal epithelial cells and almost lost in mucinous, papillary, tubular breast carcinoma as well as in areas of apocrine metaplasia (89).

All these results certainly indicate the potential involvement of different Tight Junction proteins in the cascade of events related to breast cancer progression. Further studies will provide more understanding of the role of these proteins in breast cancer and will clarify their contributions to tumour development (Table 2).

5.1. Claudins as emerging targets for cancer

Due to the high specificity of expression patterns of Claudins in cancer, it has raised the possibility of Claudins being utilised as useful molecular markers. Regardless of their exact functions in cancer cells, Claudin protein expression may have a significant clinical relevance.

Table 2. Changes in level of expression of different Claudin members in human cancer

Cancer type	Claudin type	Change in expression	Ref
Breast	Claudin-16	Down-regulated	(82)
	Claudin-7	Down-regulated	(83)
	Claudin-1	Down-regulated	(79),(80, 81), (89)
	Claudin-3, -4	Up-regulated	(88)
Colon	Claudin-3,-4,-7	Up-regulated	(67)
	Claudin-1,-2	Up-regulated	(66)
	Claudin-1	Down-regulated	(65)
	Claudin-7	Down-regulated	(68)
	Claudin-1,-4	Up-regulated	(65)
Ovarian	Claudin-1,-2,-4	Down-regulated	(74)
	Claudin-7	Up-regulated	(75)
	Claudin-5	Up-regulated	(76)
Pancreatic	Claudin-4	Down-regulated	(77)
	Claudin-3,-7	Up-regulated	(77)
Prostate	Claudin-1,-7	Up-regulated	(72)
	Claudin-5	Up-regulated	(73)
Bladder	Claudin-4	Up-regulated	(64)
Lung	Claudin-1	Down-regulated	(71)
	Claudin-3,-4,-7	Up-regulated	(71)
	Claudin-5	Up-regulated	(71)
	Claudin-5	Down-regulated	(71)
Hepatocellular	Claudin-5	Down-regulated	(78)

Research in the past has been focused on the expression of members of the Claudin family in a number of cancer types. Studies have revealed how some of the Claudins were up-regulated in cancer progression while others were down-regulated. This data opens up the possibility of the potential value of Claudins as targets for therapeutic intervention. A number of key points have arisen from various studies into targeting Claudins for cancer therapy.

1. Claudins are cell surface proteins that contain two extracellular domains which are accessible as target sites for therapy.
2. Claudin members have been found to be overexpressed in a number of different cancers showing different expression patterns between normal and tumour cells.
3. Claudins could be more accessible in tumour cells due to an increase in TJ permeability compared with normal cells (62) even if Claudins are not overexpressed in that tumour type.

As already stated Claudins, as transmembrane proteins with two relatively large extracellular loops, present themselves as targets for promising antibody therapy. This therapy is based on antibodies against Claudins that will specifically recognize one of these loops and therefore induce leaky Tight Junctions or even destroy them (90); or antibodies that simply, after specific binding to the C-terminal domain of the Claudins on the surface of the cell, will provide evidence for the antibody-based therapy approach (91).

Cell tumour lysis can be achieved by attachment to the cell surface of toxins, or by stimulating a response from the immune system. The second extracellular loops of some Claudins have appeared to be a receptor for the *Clostridium perfringens* enterotoxin (CPE), usually associated with *Clostridium perfringens* type A, which is known for causing cytolysis in mammalian cells due to its effects on membrane permeability. This binding allows the formation of a large multiprotein membrane-pore complex, which alters the osmotic equilibrium in the cell causing lysis. Although several Claudins have shown binding affinity to CPE like Claudin-4, -6, -7, -3, -14, -8 (92) only Claudin-3 and -4 have been seen to form this complex. Taking these results into consideration, and based on the evidence from different studies that revealed over expression of these two Claudins members in breast, pancreatic, colon, lung as well as ovarian cancers, Claudin-3 and -4 might be perfect candidates for CPE-based therapy. More recently Kominsky *et al.*, performed similar experiments treating with CPE several breast cancer cell lines expressing Claudin-3 and -4 such as MCF-7, SKBr3 and T47D, as well as cell lines lacking both proteins such as HS578T and MDA-MB-435. Results revealed that breast cancer cell lines lacking these particular Claudin proteins were totally resistant to the cytotoxic effects caused by the enterotoxin but not the Claudin-expressing cells resulting in a complete cytolysis. To complement the *in vitro* study, they investigated the cytolytic effects of CPE on the T47D breast cancer cell line *in vivo,* resulting in a significant reduction in tumour volume as well as cell necrosis (88). Recent studies have used *Pseudomonas aeruginosa* exotoxin as a method of cancer-targeting therapy. The endotoxin binds to the cell surface, enters the cytosol by endocytosis, releasing PSIF which inhibits protein synthesis. Saeki *et al.*,, have reported that the artificial complex C-CPE- PSIF interacts with Claudin-4 through C-CPE binding domain and shows *in vivo* anti tumour activity against the 4T1breast cancer cell line, causing a significant reduction in tumour growth (93). Similar results were obtained when studying metastasis in the lung using the Claudin-4 expressing cell line B16, where the treatment with the complex C-CPE- PSIF revealed reduction in tumour growth and metastasis without any side effects in mice (94). A related study by Kakutani *et al.*, reported the fusion between C-CPE and the *diphtheria* toxin A (DTA) which also inhibits proteins synthesis in gastrointestinal L cells expressing different Claudin proteins. Results revealed specificity from the DTA-C-CDE complex to Claudin-4 expressing cells (95).

Therefore, exhaustive administration of the enterotoxin CPE to cancer cells might be a potential approach for cancer therapy. The key question to be addressed in the near future is how to avoid cellular toxicity in the surrounding normal cells by the administration of CPE, or whatever other toxin, displaying affinity to different Claudins proteins in the cell. Overall these findings indicate that further study and preclinical testing are required to assess the usefulness of Claudins as emerging targets for cancer.

6. SUMMARY AND PERSPECTIVES

Since the loss of cell-cell adhesion is a fundamental step for cancer cells to metastasise, a broad number of studies have focused on Tight Junctions as the first structure that cancer cells need to overcome to reach the blood stream followed by metastasis. As the Tight Junctions have been widely reported as not only merely intercellular seals, but also that they are key structures in

paracellular transport, gene transcription, cell signalling and cellular proliferation and differentiation, they have become a fashionable area of research. Multiple evidence for altered Tight Junction structure has been reported in the last years. The loss of Tight Junctions or even the absence of this structure alongside changes in the Transepithelial resistance appears to constitute an essential step in cancer development.

Claudin proteins are indeed unusual proteins in the Tight Junction structure as they are presented in a variety of tissues with different properties. Their mixture and different ratios between the 24 members confers specific barrier properties to each cell. Recently studies have highlighted the importance of the Claudin family as potential targets for cancer therapy due to their unusual expression patterns in different human carcinomas. Because Claudin proteins are transmembrane proteins and classically have two extracellular loops, the modulation of these proteins provides a promising method to deliver and enhance absorption of drugs to a target tissue through the paracellular pathway as 60% of these targets are located at the cell surface (96). However, little is known about the role of the Claudin proteins in cancer progression. The impact that the loss of any of the Claudin proteins or the upregulation in several carcinomas exerts on epithelial cells is starting to be unmasked. Therefore, the study of the pattern of expression of Claudins in normal and cancer human tissues might be a useful tool for the clinical prognosis of the disease. Clinical trials as well as further basic research on Claudins will be needed for providing accurate information about the promising future of the Claudin family in the treatment of cancer.

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8. REFERENCES

1. J. Yang, S.A. Mani, and R.A. Weinberg: Exploring a new twist on tumour metastasis. *Cancer Res* 66, 4549-4552 (2006)

2. T.A. Martin, G. Watkins, R.E. Mansel, and W.G. Jiang: Loss of tight junction plaque molecules in breast cancer tissues is associated with a poor prognosis in patients with breast cancer. *Eur J Cancer* 40, 2717-2725 (2004)

3. L.A. Staehelin: Further observations on the fine structure of freeze-cleaved tight junctions. *J Cell Sci* 13, 763-786 (1973)

4. M. Cereijido, R.G. Contreras, L. Shoshani, D. Flores-Benitez, and I. Larre: Tight junction and polarity interaction in the transporting epithelial phenotype. *Biochim Biophys Acta* 1778, 770-793 (2008)

5. P. Claude: Morphological factors influencing transepithelial permeability: a model for the resistance of the zonula occludens. *J Membr Biol* 39, 219-232 (1978)

6. S. Tsukita, M. Furuse, and M. Itoh: Multifunctional strands in tight junctions. *Nat Rev Mol Cell Biol* 2, 285-293 (2001)

7. J.M. Anderson and C.M. Van Itallie: Tight junctions and the molecular basis for regulation of paracellular permeability. *Am J Physiol* 269, G467-475 (1995)

8. S. Tsukita: Isolation of cell-to-cell adherens junctions from rat liver. *J Cell Biol* 108, 31-41 (1989)

9. G. Krause, L. Winkler, S.L. Mueller, R.F. Haseloff, J. Piontek, and I.E. Blasig: Structure and function of claudins. *Biochim Biophys Acta* 1778, 631-645 (2008)

10. J. Piontek, L. Winkler, H. Wolburg, S.L. Muller, N. Zuleger, C. Piehl, B. Wiesner, G. Krause, and I.E. Blasig: Formation of tight junction: determinants of homophilic interaction between classic claudins. *FASEB J* 22, 146-158 (2008)

11. C.M. Van Itallie and J.M. Anderson: Claudins and epithelial paracellular transport. *Annu Rev Physiol* 68, 403-429 (2006)

12. K. Morita, M. Furuse, K. Fujimoto, and S. Tsukita: Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. *Proc Natl Acad Sci U S A* 96, 511-516 (1999)

13. M. Furuse, H. Sasaki, and S. Tsukita: Manner of interaction of heterogeneous claudin species within and between tight junction strands. *J Cell Biol* 147, 891-903 (1999)

14. T. Ahrens, O. Pertz, D. Haussinger, C. Fauser, T. Schulthess, and J. Engel: Analysis of heterophilic and homophilic interactions of cadherins using the c-Jun/c-Fos dimerization domains. *J Biol Chem* 277, 19455-19460 (2002)

15. B.L. Daugherty, C. Ward, T. Smith, J.D. Ritzenthaler, and M. Koval: Regulation of heterotypic claudin compatibility. *J Biol Chem* 282, 30005-30013 (2007)

16. K. Morita, H. Sasaki, M. Furuse, and S. Tsukita: Endothelial claudin: claudin-5/TMVCF constitutes tight junction strands in endothelial cells. *J Cell Biol* 147, 185-194 (1999)

17. F.D. Nunes, L.N. Lopez, H.W. Lin, C. Davies, R.B. Azevedo, A. Gow, and B. Kachar: Distinct subdomain organization and molecular composition of a tight junction with adherens junction features. *J Cell Sci* 119, 4819-4827 (2006)

18. K. Morita, H. Sasaki, K. Fujimoto, M. Furuse, and S. Tsukita: Claudin-11/OSP-based tight junctions of myelin sheaths in brain and Sertoli cells in testis. *J Cell Biol* 145, 579-588 (1999)

19. T. Miyamoto, K. Morita, D. Takemoto, K. Takeuchi, Y. Kitano, T. Miyakawa, K. Nakayama, Y. Okamura, H. Sasaki, Y. Miyachi, M. Furuse, and S. Tsukita: Tight junctions in Schwann cells of peripheral myelinated axons: a lesson from claudin-19-deficient mice. *J Cell Biol* 169, 527-538 (2005)

20. S. Tsukita and M. Furuse: Pores in the wall: claudins constitute tight junction strands containing aqueous pores. *J Cell Biol* 149, 13-16 (2000)

21. S. Angelow, R. Ahlstrom, and A.S. Yu: Biology of claudins. *Am J Physiol Renal Physiol* 295, F867-876 (2008)

22. C.M. Van Itallie and J.M. Anderson: The role of claudins in determining paracellular charge selectivity. *Proc Am Thorac Soc* 1, 38-41 (2004)

23. S. Angelow, R. El-Husseini, S.A. Kanzawa, and A.S. Yu: Renal localization and function of the tight junction protein, claudin-19. *Am J Physiol Renal Physiol* 293, F166-177 (2007)

24. M.D. Alexandre, Q. Lu, and Y.H. Chen: Overexpression of claudin-7 decreases the paracellular Cl- conductance and increases the paracellular Na+ conductance in LLC-PK1 cells. *J Cell Sci* 118, 2683-2693 (2005)

25. T. D'Souza, R. Agarwal, and P.J. Morin: Phosphorylation of claudin-3 at threonine 192 by cAMP-dependent protein kinase regulates tight junction barrier function in ovarian cancer cells. *J Biol Chem* 280, 26233-26240 (2005)

26. S. Amasheh, T. Schmidt, M. Mahn, P. Florian, J. Mankertz, S. Tavalali, A.H. Gitter, J.D. Schulzke, and M. Fromm: Contribution of claudin-5 to barrier properties in tight junctions of epithelial cells. *Cell Tissue Res* 321, 89-96 (2005)

27. M. Furuse, M. Hata, K. Furuse, Y. Yoshida, A. Haratake, Y. Sugitani, T. Noda, A. Kubo, and S. Tsukita: Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. *J Cell Biol* 156, 1099-1111 (2002)

28. A. Gow, C.M. Southwood, J.S. Li, M. Pariali, G.P. Riordan, S.E. Brodie, J. Danias, J.M. Bronstein, B. Kachar, and R.A. Lazzarini: CNS myelin and sertoli cell tight junction strands are absent in Osp/claudin-11 null mice. *Cell* 99, 649-659 (1999)

29. T. Ben-Yosef, I.A. Belyantseva, T.L. Saunders, E.D. Hughes, K. Kawamoto, C.M. Van Itallie, L.A. Beyer, K. Halsey, D.J. Gardner, E.R. Wilcox, J. Rasmussen, J.M. Anderson, D.F. Dolan, A. Forge, Y. Raphael, S.A. Camper, and T.B. Friedman: Claudin 14 knockout mice, a model for autosomal recessive deafness DFNB29, are deaf due to cochlear hair cell degeneration. *Hum Mol Genet* 12, 2049-2061 (2003)

30. O.R. Colegio, C. Van Itallie, C. Rahner, and J.M. Anderson: Claudin extracellular domains determine paracellular charge selectivity and resistance but not tight junction fibril architecture. *Am J Physiol Cell Physiol* 284, C1346-1354 (2003)

31. C.M. Van Itallie, J. Holmes, A. Bridges, and J.M. Anderson: Claudin-2-dependent changes in noncharged solute flux are mediated by the extracellular domains and require attachment to the PDZ-scaffold. *Ann N Y Acad Sci* 1165, 82-87 (2009)

32. S. Amasheh, S. Milatz, S.M. Krug, A.G. Markov, D. Gunzel, M. Amasheh, and M. Fromm: Tight junction proteins as channel formers and barrier builders. *Ann N Y Acad Sci* 1165, 211-219 (2009)

33. E.E. Schneeberger: Claudins form ion-selective channels in the paracellular pathway. Focus on "Claudin extracellular domains determine paracellular charge selectively and resistance but not tight junction fibril architecture". *Am J Physiol Cell Physiol* 284, C1331-1333 (2003)

34. C.M. Van Itallie, S. Rogan, A. Yu, L.S. Vidal, J. Holmes, and J.M. Anderson: Two splice variants of claudin-10 in the kidney create paracellular pores with different ion selectivities. *Am J Physiol Renal Physiol* 291, F1288-1299 (2006)

35. H. Fujita, H. Chiba, H. Yokozaki, N. Sakai, K. Sugimoto, T. Wada, T. Kojima, T. Yamashita, and N. Sawada: Differential expression and subcellular localization of claudin-7, -8, -12, -13, and -15 along the mouse intestine. *J Histochem Cytochem* 54, 933-944 (2006)

36. S.I. Kitajiri, M. Furuse, K. Morita, Y. Saishin-Kiuchi, H. Kido, J. Ito, and S. Tsukita: Expression patterns of claudins, tight junction adhesion molecules, in the inner ear. *Hear Res* 187, 25-34 (2004)

37. N. Charoenphandhu, K. Wongdee, K. Tudpor, J. Pandaranandaka, and N. Krishnamra: Chronic metabolic acidosis upregulated claudin mRNA expression in the duodenal enterocytes of female rats. *Life Sci* 80, 1729-1737 (2007)

38. D.S. Gerhard, L. Wagner, E.A. Feingold, C.M. Shenmen, L.H. Grouse, G. Schuler, S.L. Klein, S. Old, R. Rasooly, P. Good, M. Guyer, A.M. Peck, J.G. Derge, D. Lipman, F.S. Collins, W. Jang, S. Sherry, M. Feolo, L. Misquitta, E. Lee, K. Rotmistrovsky, S.F. Greenhut, C.F. Schaefer, K. Buetow, T.I. Bonner, D. Haussler, J. Kent, M. Kiekhaus, T. Furey, M. Brent, C. Prange, K. Schreiber, N. Shapiro, N.K. Bhat, R.F. Hopkins, F. Hsie, T. Driscoll,

M.B. Soares, T.L. Casavant, T.E. Scheetz, M.J. Brownstein, T.B. Usdin, S. Toshiyuki, P. Carninci, Y. Piao, D.B. Dudekula, M.S. Ko, K. Kawakami, Y. Suzuki, S. Sugano, C.E. Gruber, M.R. Smith, B. Simmons, T. Moore, R. Waterman, S.L. Johnson, Y. Ruan, C.L. Wei, S. Mathavan, P.H. Gunaratne, J. Wu, A.M. Garcia, S.W. Hulyk, E. Fuh, Y. Yuan, A. Sneed, C. Kowis, A. Hodgson, D.M. Muzny, J. McPherson, R.A. Gibbs, J. Fahey, E. Helton, M. Ketteman, A. Madan, S. Rodrigues, A. Sanchez, M. Whiting, A. Madari, A.C. Young, K.D. Wetherby, S.J. Granite, P.N. Kwong, C.P. Brinkley, R.L. Pearson, G.G. Bouffard, R.W. Blakesly, E.D. Green, M.C. Dickson, A.C. Rodriguez, J. Grimwood, J. Schmutz, R.M. Myers, Y.S. Butterfield, M. Griffith, O.L. Griffith, M.I. Krzywinski, N. Liao, R. Morin, D. Palmquist, A.S. Petrescu, U. Skalska, D.E. Smailus, J.M. Stott, A. Schnerch, J.E. Schein, S.J. Jones, R.A. Holt, A. Baross, M.A. Marra, S. Clifton, K.A. Makowski, S. Bosak and J. Malek: The status, quality, and expansion of the NIH full-length cDNA project: the Mammalian Gene Collection (MGC). *Genome Res* 14, 2121-2127 (2004)

39. H. Wolburg, K. Wolburg-Buchholz, J. Kraus, G. Rascher-Eggstein, S. Liebner, S. Hamm, F. Duffner, E.H. Grote, W. Risau, and B. Engelhardt: Localization of claudin-3 in tight junctions of the blood-brain barrier is selectively lost during experimental autoimmune encephalomyelitis and human glioblastoma multiforme. *Acta Neuropathol* 105, 586-592 (2003)

40. T. Nitta, M. Hata, S. Gotoh, Y. Seo, H. Sasaki, N. Hashimoto, M. Furuse, and S. Tsukita: Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J Cell Biol* 161, 653-660 (2003)

41. T. Ishizaki, H. Chiba, T. Kojima, M. Fujibe, T. Soma, H. Miyajima, K. Nagasawa, I. Wada, and N. Sawada: Cyclic AMP induces phosphorylation of claudin-5 immunoprecipitates and expression of claudin-5 gene in blood-brain-barrier endothelial cells via protein kinase A-dependent and -independent pathways. *Exp Cell Res* 290, 275-288 (2003)

42. K. Yamauchi, T. Rai, K. Kobayashi, E. Sohara, T. Suzuki, T. Itoh, S. Suda, A. Hayama, S. Sasaki, and S. Uchida: Disease-causing mutant WNK4 increases paracellular chloride permeability and phosphorylates claudins. *Proc Natl Acad Sci U S A* 101, 4690-4694 (2004)

43. R. Tatum, Y. Zhang, Q. Lu, K. Kim, B.G. Jeansonne, and Y.H. Chen: WNK4 phosphorylates ser(206) of claudin-7 and promotes paracellular Cl(-) permeability. *FEBS Lett* 581, 3887-3891 (2007)

44. M. Fujibe, H. Chiba, T. Kojima, T. Soma, T. Wada, T. Yamashita, and N. Sawada: Thr203 of claudin-1, a putative phosphorylation site for MAP kinase, is required to promote the barrier function of tight junctions. *Exp Cell Res* 295, 36-47 (2004)

45. F. Carrozzino, P. Pugnale, E. Feraille, and R. Montesano: Inhibition of basal p38 or JNK activity enhances epithelial barrier function through differential modulation of claudin expression. *Am J Physiol Cell Physiol* 297, C775-787 (2009)

46. M. Tanaka, R. Kamata, and R. Sakai: EphA2 phosphorylates the cytoplasmic tail of Claudin-4 and mediates paracellular permeability. *J Biol Chem* 280, 42375-42382 (2005)

47. M. Yamamoto, S.H. Ramirez, S. Sato, T. Kiyota, R.L. Cerny, K. Kaibuchi, Y. Persidsky, and T. Ikezu: Phosphorylation of claudin-5 and occludin by rho kinase in brain endothelial cells. *Am J Pathol* 172, 521-533 (2008)

48. J. Ikenouchi, M. Matsuda, M. Furuse, and S. Tsukita: Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail. *J Cell Sci* 116, 1959-1967 (2003)

49. C.M. Van Itallie, T.M. Gambling, J.L. Carson, and J.M. Anderson: Palmitoylation of claudins is required for efficient tight-junction localization. *J Cell Sci* 118, 1427-1436 (2005)

50. T. Ohkubo and M. Ozawa: The transcription factor Snail downregulates the tight junction components independently of E-cadherin downregulation. *J Cell Sci* 117, 1675-1685 (2004)

51. F. Carrozzino, P. Soulie, D. Huber, N. Mensi, L. Orci, A. Cano, E. Feraille, and R. Montesano: Inducible expression of Snail selectively increases paracellular ion permeability and differentially modulates tight junction proteins. *Am J Physiol Cell Physiol* 289, C1002-1014 (2005)

52. T. Niimi, K. Nagashima, J.M. Ward, P. Minoo, D.B. Zimonjic, N.C. Popescu, and S. Kimura: claudin-18, a novel downstream target gene for the T/EBP/NKX2.1 homeodomain transcription factor, encodes lung- and stomach-specific isoforms through alternative splicing. *Mol Cell Biol* 21, 7380-7390 (2001)

53. F. Escaffit, F. Boudreau, and J.F. Beaulieu: Differential expression of claudin-2 along the human intestine: Implication of GATA-4 in the maintenance of claudin-2 in differentiating cells. *J Cell Physiol* 203, 15-26 (2005)

54. W.G. Jiang, T.A. Martin, C. Parr, G. Davies, K. Matsumoto, and T. Nakamura: Hepatocyte growth factor, its receptor, and their potential value in cancer therapies. *Crit Rev Oncol Hematol* 53, 35-69 (2005)

55. J.H. Lipschutz, S. Li, A. Arisco, and D.F. Balkovetz: Extracellular signal-regulated kinases 1/2 control claudin-2 expression in Madin-Darby canine kidney strain I and II cells. *J Biol Chem* 280, 3780-3788 (2005)

56. A.B. Singh and R.C. Harris: Epidermal growth factor receptor activation differentially regulates claudin expression and enhances transepithelial resistance in Madin-Darby canine kidney cells. *J Biol Chem* 279, 3543-3552 (2004)

57. Y. Peter, A. Comellas, E. Levantini, E.P. Ingenito, and S.D. Shapiro: Epidermal growth factor receptor and

claudin-2 participate in A549 permeability and remodeling: implications for non-small cell lung cancer tumour colonization. *Mol Carcinog* 48, 488-497 (2009)

58. T.A. Martin, R.E. Mansel, and W.G. Jiang: Antagonistic effect of NK4 on HGF/SF induced changes in the transendothelial resistance (TER) and paracellular permeability of human vascular endothelial cells. *J Cell Physiol* 192, 268-275 (2002)

59. T.A. Martin, G. Watkins, R.E. Mansel, and W.G. Jiang: Hepatocyte growth factor disrupts tight junctions in human breast cancer cells. *Cell Biol Int* 28, 361-371 (2004)

60. J.G. Swift, T.M. Mukherjee, and R. Rowland: Intercellular junctions in hepatocellular carcinoma. *J Submicrosc Cytol* 15, 799-810 (1983)

61. H. Robenek, C. Schopper, E. Fasske, R. Fetting, and H. Themann: Structure and function of the junctional complement of spontaneous and transplanted murine mammary carcinomas. *J Submicrosc Cytol* 13, 347-363 (1981)

62. A.P. Soler, R.D. Miller, K.V. Laughlin, N.Z. Carp, D.M. Klurfeld, and J.M. Mullin: Increased tight junctional permeability is associated with the development of colon cancer. *Carcinogenesis* 20, 1425-1431 (1999)

63. A.P. Soler, K.V. Laughlin, and J.M. Mullin: Effects of epidermal growth factor versus phorbol ester on kidney epithelial (LLC-PK1) tight junction permeability and cell division. *Exp Cell Res* 207, 398-406 (1993)

64. S. Boireau, M. Buchert, M.S. Samuel, J. Pannequin, J.L. Ryan, A. Choquet, H. Chapuis, X. Rebillard, C. Avances, M. Ernst, D. Joubert, N. Mottet, and F. Hollande: DNA-methylation-dependent alterations of claudin-4 expression in human bladder carcinoma. *Carcinogenesis* 28, 246-258 (2007)

65. M.B. Resnick, T. Konkin, J. Routhier, E. Sabo, and V.E. Pricolo: Claudin-1 is a strong prognostic indicator in stage II colonic cancer: a tissue microarray study. *Mod Pathol* 18, 511-518 (2005)

66. T. Kinugasa, Q. Huo, D. Higashi, H. Shibaguchi, M. Kuroki, T. Tanaka, K. Futami, Y. Yamashita, K. Hachimine, S. Maekawa, K. Nabeshima, and H. Iwasaki: Selective up-regulation of claudin-1 and claudin-2 in colorectal cancer. *Anticancer Res* 27, 3729-3734 (2007)

67. E. Montgomery, A.J. Mamelak, M. Gibson, A. Maitra, S. Sheikh, S.S. Amr, S. Yang, M. Brock, A. Forastiere, S. Zhang, K.M. Murphy, and K.D. Berg: Overexpression of claudin proteins in esophageal adenocarcinoma and its precursor lesions. *Appl Immunohistochem Mol Morphol* 14, 24-30 (2006)

68. Y. Usami, H. Chiba, F. Nakayama, J. Ueda, Y. Matsuda, N. Sawada, T. Komori, A. Ito, and H. Yokozaki: Reduced expression of claudin-7 correlates with invasion and metastasis in squamous cell carcinoma of the esophagus. *Hum Pathol* 37, 569-577 (2006)

69. Y. Soini, S. Tommola, H. Helin, and P. Martikainen: Claudins 1, 3, 4 and 5 in gastric carcinoma, loss of claudin expression associates with the diffuse subtype. *Virchows Arch* 448, 52-58 (2006)

70. Y. Liu, W. Sun, K. Zhang, H. Zheng, Y. Ma, D. Lin, X. Zhang, L. Feng, W. Lei, Z. Zhang, S. Guo, N. Han, W. Tong, X. Feng, Y. Gao, and S. Cheng: Identification of genes differentially expressed in human primary lung squamous cell carcinoma. *Lung Cancer* 56, 307-317 (2007)

71. S. Paschoud, M. Bongiovanni, J.C. Pache, and S. Citi: Claudin-1 and claudin-5 expression patterns differentiate lung squamous cell carcinomas from adenocarcinomas. *Mod Pathol* 20, 947-954 (2007)

72. G.M. Sheehan, B.V. Kallakury, C.E. Sheehan, H.A. Fisher, R.P. Kaufman, Jr., and J.S. Ross: Loss of claudins-1 and -7 and expression of claudins-3 and -4 correlate with prognostic variables in prostatic adenocarcinomas. *Hum Pathol* 38, 564-569 (2007)

73. K.W. Seo, Y.K. Kwon, B.H. Kim, C.I. Kim, H.S. Chang, M.S. Choe, and C.H. Park: Correlation between Claudins Expression and Prognostic Factors in Prostate Cancer. *Korean J Urol* 51, 239-244 (2010)

74. B. Litkouhi, J. Kwong, C.M. Lo, J.G. Smedley, 3rd, B.A. McClane, M. Aponte, Z. Gao, J.L. Sarno, J. Hinners, W.R. Welch, R.S. Berkowitz, S.C. Mok, and E.I. Garner: Claudin-4 overexpression in epithelial ovarian cancer is associated with hypomethylation and is a potential target for modulation of tight junction barrier function using a C-terminal fragment of Clostridium perfringens enterotoxin. *Neoplasia* 9, 304-314 (2007)

75. R.A. Tassi, E. Bignotti, M. Falchetti, M. Ravanini, S. Calza, A. Ravaggi, E. Bandiera, F. Facchetti, S. Pecorelli, and A.D. Santin: Claudin-7 expression in human epithelial ovarian cancer. *Int J Gynecol Cancer* 18, 1262-1271 (2008)

76. M. Turunen, A. Talvensaari-Mattila, Y. Soini, and M. Santala: Claudin-5 overexpression correlates with aggressive behavior in serous ovarian adenocarcinoma. *Anticancer Res* 29, 5185-5189 (2009)

77. K. Borka, P. Kaliszky, E. Szabo, G. Lotz, P. Kupcsulik, Z. Schaff, and A. Kiss: Claudin expression in pancreatic endocrine tumours as compared with ductal adenocarcinomas. *Virchows Arch* 450, 549-557 (2007)

78. T. Sakaguchi, S. Suzuki, H. Higashi, K. Inaba, S. Nakamura, S. Baba, T. Kato, and H. Konno: Expression of tight junction protein claudin-5 in tumour vessels and sinusoidal endothelium in patients with hepatocellular carcinoma. *J Surg Res* 147, 123-131 (2008)

79. F. Kramer, K. White, M. Kubbies, K. Swisshelm, and B.H. Weber: Genomic organization of claudin-1 and its assessment in hereditary and sporadic breast cancer. *Hum Genet* 107, 249-256 (2000)

80. T. Hoevel, R. Macek, O. Mundigl, K. Swisshelm, and M. Kubbies: Expression and targeting of the tight junction protein CLDN1 in CLDN1-negative human breast tumour cells. *J Cell Physiol* 191, 60-68 (2002)

81. S. Morohashi, T. Kusumi, F. Sato, H. Odagiri, H. Chiba, S. Yoshihara, K. Hakamada, M. Sasaki, and H. Kijima: Decreased expression of claudin-1 correlates with recurrence status in breast cancer. *Int J Mol Med* 20, 139-143 (2007)

82. T.A. Martin, G.M. Harrison, G. Watkins, and W.G. Jiang: Claudin-16 reduces the aggressive behavior of human breast cancer cells. *J Cell Biochem* 105, 41-52 (2008)

83. S.L. Kominsky, P. Argani, D. Korz, E. Evron, V. Raman, E. Garrett, A. Rein, G. Sauter, O.P. Kallioniemi, and S. Sukumar: Loss of the tight junction protein claudin-7 correlates with histological grade in both ductal carcinoma *in situ* and invasive ductal carcinoma of the breast. *Oncogene* 22, 2021-2033 (2003)

84. T. Sauer, M.K. Pedersen, K. Ebeltoft, and O. Naess: Reduced expression of Claudin-7 in fine needle aspirates from breast carcinomas correlate with grading and metastatic disease. *Cytopathology* 16, 193-198 (2005)

85. M. Osanai, M. Murata, H. Chiba, T. Kojima, and N. Sawada: Epigenetic silencing of claudin-6 promotes anchorage-independent growth of breast carcinoma cells. *Cancer Sci* 98, 1557-1562 (2007)

86. Q. Wu, Y. Liu, Y. Ren, X. Xu, L. Yu, Y. Li, and C. Quan: Tight junction protein, claudin-6, downregulates the malignant phenotype of breast carcinoma. *Eur J Cancer Prev* 19, 186-194 (2010)

87. Y. Soini: Claudins 2, 3, 4, and 5 in Paget's disease and breast carcinoma. *Hum Pathol* 35, 1531-1536 (2004)

88. S.L. Kominsky, M. Vali, D. Korz, T.G. Gabig, S.A. Weitzman, P. Argani, and S. Sukumar: Clostridium perfringens enterotoxin elicits rapid and specific cytolysis of breast carcinoma cells mediated through tight junction proteins claudin 3 and 4. *Am J Pathol* 164, 1627-1633 (2004)

89. A.M. Tokes, J. Kulka, S. Paku, A. Szik, C. Paska, P.K. Novak, L. Szilak, A. Kiss, K. Bogi, and Z. Schaff: Claudin-1, -3 and -4 proteins and mRNA expression in benign and malignant breast lesions: a research study. *Breast Cancer Res* 7, R296-305 (2005)

90. S. Tsukita, Y. Yamazaki, T. Katsuno, and A. Tamura: Tight junction-based epithelial microenvironment and cell proliferation. *Oncogene* 27, 6930-6938 (2008)

91. S.L. Kominsky: Claudins: emerging targets for cancer therapy. *Expert Rev Mol Med* 8, 1-11 (2006)

92. K. Fujita, J. Katahira, Y. Horiguchi, N. Sonoda, M. Furuse, and S. Tsukita: Clostridium perfringens enterotoxin binds to the second extracellular loop of claudin-3, a tight junction integral membrane protein. *FEBS Lett* 476, 258-261 (2000)

93. R. Saeki, M. Kondoh, H. Kakutani, S. Tsunoda, Y. Mochizuki, T. Hamakubo, Y. Tsutsumi, Y. Horiguchi, and K. Yagi: A novel tumour-targeted therapy using a claudin-4-targeting molecule. *Mol Pharmacol* 76, 918-926 (2009)

94. R. Saeki, M. Kondoh, H. Kakutani, K. Matsuhisa, A. Takahashi, H. Suzuki, Y. Kakamu, A. Watari, and K. Yagi: A claudin-targeting molecule as an inhibitor of tumour metastasis. J Pharmacol Exp Ther

95. H. Kakutani, M. Kondoh, R. Saeki, M. Fujii, Y. Watanabe, H. Mizuguchi, and K. Yagi: Claudin-4-targeting of diphtheria toxin fragment A using a C-terminal fragment of Clostridium perfringens enterotoxin. Eur J Pharm Biopharm

96. M. Kondoh and K. Yagi: Tight junction modulators: promising candidates for drug delivery. *Curr Med Chem* 14, 2482-2488 (2007)

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