

## Tight junctions in cancer metastasis

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## 1. ABSTRACT

Tight Junctions (TJ) are well known to function as a control for the paracellular diffusion of ions and certain molecules, it has however, become evident that the TJ has a vital role in maintaining cell to cell integrity. Loss of cohesion of the TJ structure can lead to invasion and ultimately to the metastasis of cancer cells. This review will discuss how modulation of expression of TJ molecules results in key changes in TJ barrier function leading to the progression of cancer and progression of metastasis.

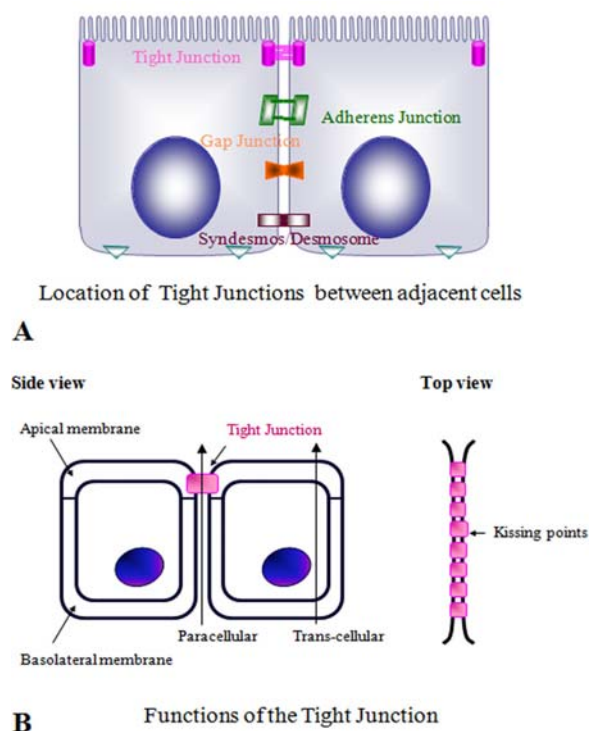
## 2. INTRODUCTION

Tight Junctions (TJ) govern the permeability of epithelial and endothelial cells and are the most topical structures of these cell types (1-3). It is a region where the plasma membrane of adjacent cells forms a series of contacts that appear to completely occlude the extracellular space thus creating an intercellular barrier and intramembrane diffusion fence (4).

An important step in the formation of cancer metastases is interaction and penetration of the vascular endothelium by dissociated cancer cells. TJ in endothelial cells function as a barrier through which molecules and

inflammatory cells can pass. In epithelial cells the TJ functions in an adhesive manner and can prevent cell dissociation (5). TJ are therefore the first barrier that cancer cells must overcome in order to metastasize, which we have previously demonstrated; TJ of vascular endothelium *in vivo* function as a barrier between blood and tissues against metastatic cancer cells (6). Since early studies suggested a link between the reduction of TJ and tumor differentiation, experimental evidence has emerged to place TJ in the frontline as the structure that cancer cells must overcome in order to metastasize (6-9). Following the early work of Martinez-Paloma (10) and others (11, 12) a considerable body of work exists on TJ and their role in a number of diseases. It is however, only in the last few years that an upsurge in interest the possible role of TJ regulation in tumorigenesis. Studies have concentrated on cell lines and to a limited degree on colorectal (13-16) and pancreatic cancers (13, 14, 17) with an increasing number of studies carried out on breast cancer (7, 18-24).

Growth factors, cytokines, regulatory mechanisms or promoter methylation modulate TJ protein expression and function. Regulatory mechanisms may be via epithelial-mesenchymal-transition (EMT), as the process of acquisition of an invasive phenotype by tumors of epithelial origin can be regarded as a pathological version EMT (25, 26). It is interesting that TJ determine



**Figure 1.** Position of TJ in epithelial and endothelial cells.

epithelial cell polarity and disappear during EMT with Snail and Slug thought to be responsible for this loss (27). The Rho GTPase family is also able to regulate TJ assembly (28). The TJ can therefore be regulated in response to physiological and tissue-specific requirements (4), with TJ being able to rapidly change their permeability and functional properties in response to stimuli. This rapid response permits dynamic fluxes of ions and solutes in addition to the passage of whole cells (29, 30).

Changes in tumor and endothelial cells are necessary for the successful growth and spread of cancer cells. An changes in cancer cells by up-regulation or down-regulation of relevant TJ proteins results in loss of cell-cell association, cell contact inhibition, leading to uncontrolled growth, loss of adhesion to and degradation of the basemen membrane. To facilitate the passage of the cancer cells through this barrier these must be a concurrent loss of cell-cell association in the endothelium and modulation of the TJ proteins involved. This review will discuss the progress that has been made in understanding how role of TJ in the invasion and metastasis of cancer via changes barrier function usually due to modulations of TJ protein expression and alterations in the structure of the TJ itself. It is evident that changes in the function and regulation of TJ in cancer is not just a consequence of cancer progression but is essential to its development and persistence, eventually enabling metastasis and secondary disease. Discovering how TJ are involved in metastasis is vital to the effort in understanding and possibly treating this terrible disease.

### 3. THE TJ MOLECULAR STRUCTURE IS A CONGLOMERATE OF PROTEINS

TJ have a characteristic structure, appearing as discrete sites of fusion between the outer plasma membrane of adjacent cells (Figure 1). They appear as continuous intramembrane particle strands in the protoplasmic face when visualized using freeze-fracture, with complimentary grooves in the extracellular face when adjacent cells are viewed in ultra-thin section electron microscopy (30). These completely circumscribe the apices of the cells as a network of intramembrane fibrils (4) appearing as what is generally described as a series of “kissing” points.

The TJ structure is representative of the conglomerate of molecules that constitute, associate with or regulate TJ (31). A number of proteins were identified in the mid 1980's, and since then the list of additional molecules has expanded considerably (Table 1). The molecular components of the TJ have been extensively investigated (1, 32).

The TJ consists of 3 regions (Figure 2):

- (1) The integral transmembrane proteins- occluding and the other TAMP proteins, claudins and junctional adhesion molecules (JAM), together with other CTX family members;
- (2) The peripheral or plaque anchoring proteins, often containing PDZ motifs- zonula occludens (ZO)-1, -2, -3, MAGI-1 *etc.*;
- (3) TJ-associated/regulatory proteins- alpha-catenin, cingulin *etc.*

The integral transmembrane proteins are the essential adhesion proteins responsible for correct assembly of the TJ structure and controlling TJ functions via homotypic and heterotypic interactions. Successful assembly and maintenance of the TJ is accomplished by anchorage of the transmembrane proteins by the peripheral or plaque proteins such as ZO-1 which act as a scaffold to bind the raft of TJ molecules together and provide the link to the actin cytoskeleton and the signalling mechanism of the cell. This is in conjunction with the associated/regulatory proteins.

### 4. EPITHELIAL AND ENDOTHELIAL TJ FUNCTION AS BARRIERS, ADHESION

Tight Junctions are structures and transducers in signaling

#### 4.1. The main functions attributed to the TJ

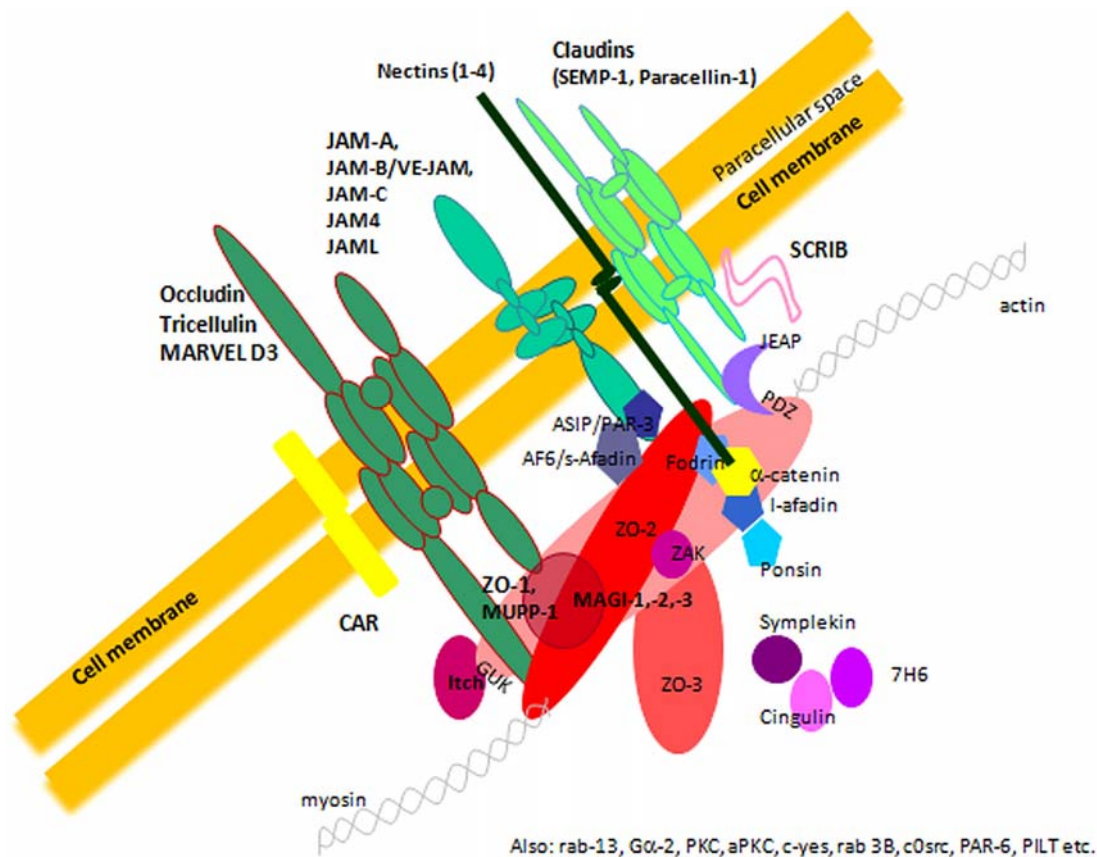
There are four main functions ascribed to epithelial/endothelial TJ (Figure 1B).

Sealing of the intercellular space and responsibility for the separation of apical and basolateral fluid compartments of epithelia and endothelia.

Acting as a reservoir for TJ molecules to act as intermediates and transducers in cell signalling, thus

**Table 1.** Proteins involved in TJ structure, function and regulation

Location	Protein
Integral transmembrane proteins	TAMP: Occludin, Tricellulin, MARVELD3
	Claudins 1-24
	Junctional Adhesion Molecules (A-C, 4, L)
	Other CTX proteins such as Cocksackie Adenovirus Receptor (CAR), ESAM, Vmp1
Peripheral plaque proteins	Zonula occludens-1 (ZO-1), ZO-2, ZO-3
	MAGI-1, -2, -3
	MUPP-1
	PAR-3/ASIP
	PAR-6
	AF-6/s-afadin
	CASK
	CAROM
Associated proteins	Cingulin, 7H6, Symplekin, ZONAB, Rab-13, 19B1, Ponsin, Rab 3B, PKC, l-afadin, c-src, Gai-2, Gai-12, alpha-catenin, Pals, PATJ, PKA, JEAP, Pilt, PTEN, ZAK, SCRIB, ITCH, Rho-GTPases, WNK4, Vinculin



**Figure 2.** Schematic of the arrangement of TJ proteins.

playing a role in the processes of polarity, cell differentiation, cell growth and proliferation.

Cell-cell adhesion.  
A barrier to cell migration.

Although cell adhesion to adjacent cells and the extracellular matrix is key to the organization of epithelium into a tissue, it is vital to the regulation of cellular processes such as differentiation, gene expression, motility and growth (33). These regulatory functions are mediated by cell adhesion molecules, transmembrane receptors and cytoskeletal proteins all of which are organized into

multimolecular complexes and the activation of signalling pathways. It has become apparent that the suppression of the malignant phenotype of cells in tumorigenesis is an additional and important function of the TJ (33). Whilst the barrier and fence functions of TJ have been well appreciated, it is only relatively recently that concept of the TJ as a complex, multiprotein structure with roles in other cellular processes such as cell polarity, proliferation and differentiation has been recognized (34). Moreover, it is becoming increasingly clear that the development of human cancer is frequently associated with the failure of epithelial cells to form TJ and to establish correct apicobasal polarity (35).

## **5. METASTASIS AS A RESULT OF CELL INVASION AND ANGIOGENESIS**

At the time of diagnosis of cancer, at least half of the patients already present clinically detectable metastatic disease (36). A higher number of patients will also have micrometastases that would be beyond conventional detection techniques. Metastasis is therefore the single event that results in the death of most patients with cancer. The metastatic process is composed of a number of sequential events termed the metastatic cascade, which must be completed in order for the tumor cell to successfully metastasize. This process contributes to the complexity of cancer as a multiplex disease. The metastatic cascade can be broadly separated into three main processes: Invasion, intravasation and extravasation .

The process of invasion occurs when malignant tumor cells dissociate from the primary tumor mass by loss of cell-cell adhesion capacity and invade the surrounding stroma. This involves the secretion of substances to degrade the basement membrane and extracellular matrix and also the expression/suppression of proteins involved in the control of motility and migration. Angiogenesis must also be initiated by the tumor, otherwise the tumor would fail to develop, as local diffusion for transport of nutrients to and removal of waste products from the tumor site will only suffice for tumors up to 2mm in diameter (37). The process of intravasation must occur for tumors to continue to grow. A connection must be made to the blood supply so that the blood vessel within the tumor's vicinity can then provide a route for the detached cells to enter the circulatory system and metastasize to distant sites (38, 39). It is obvious that interaction between the tumor cell and the surrounding stroma is extremely important in the development of tumor angiogenesis (40). The detached tumor cells must enter the circulatory system and survive the forces involved and the immune system to arrive intact at a distant site. The process of extravasation occurs once the tumor cell has arrived at a likely point of departure. It interacts with the endothelial cells by undergoing biochemical interactions (mediated by carbohydrate-carbohydrate locking reactions, which occur weakly but quickly) develop adhesion to the endothelial cells to form stronger bonds, and thus penetrate the endothelium and the basement membrane. The new tumor can then proliferate at this secondary focus. During the three processes, the TJ is the first structure impeding the path to successful metastasis of the cancer cell: TJ exist between the cancer cells themselves, the cells of the stroma and the cells of the endothelium. For the tumor cell to proceed effectively, the TJ structure must be disturbed and dismantled to enable penetration of the cancer cell.

## **6. THE ROLE OF TJ DURING METASTASIS**

The interaction and penetration of endothelium by the metastasising tumor cell is therefore a key step in the formation of metastasis (28, 41, 42). As

our knowledge and understanding of the molecular structure, mechanism of action and function of TJ is expanded the TJ can be regarded as a potentially important target for anti-cancer research and a possible area for future therapeutics.

### **6.1. Tumor cell invasion of the mesothelium due to loss of TJ integrity**

It is widely accepted that the loss of cell-cell adhesion in neoplastic epithelium is necessary for invasion of surrounding stromal elements and subsequent metastatic events (21). The association between TJ permeability of human epithelium has been investigated for some time. Tobioka *et al.* (43) have shown that the enhancement of TJ function reduced the penetration of tumor cells through mesothelial cells. Host cell signalling pathways such as phosphorylation of myosin light chain and the regulation of the TJ proteins claudin-4 and claudin-5 are the results of *Helicobacter pylori* induced chronic gastritis and may progress to gastric cancer (44). Interestingly, it was noted that DMSO (Dimethyl sulphate up to 10%) produced no significant alteration in TJ permeability or in the cell-cell TJ complex in Caco2/TC7 colon cancer cells, an unexpected result as DMSO is usually used to solubilize poorly soluble drugs in permeation assays (45).

HGF decreased trans-epithelial resistance and increases paracellular permeability of human breast cancer cell lines, MDA-MB-231 and MCF-7. Q-PCR showed that HGF modulated the levels of several TJ molecule (occludin, claudin-1 and -5, JAM-1 and -2) mRNA transcripts in MDA MB 231 and MCF-7 cells. Western blotting and immunohistochemistry also showed modulation of expression of the TJ molecule, occludin. It is suggested that HGF disrupts TJ function in human breast cancer cells by effecting changes in the expression of TJ molecules at both the mRNA and protein levels. The conclusion was that regulation of TJ could be of fundamental importance in the prevention of metastasis of breast cancer cells (24).

Increased "leakiness" of TJ in cancer has been shown to be the result of oncogene mutations. Ras mutations can modulate the expression of a number of TJ molecules (46). The abundance of claudin-2 declined to undetectable levels in ras-overexpressing cells compared with vector controls whereas levels of occludin and claudins-1, -4, and -7 increased and the abundance of claudins-3 and -5 remained unchanged. An increase in extracellular signal-regulated kinase-2 phosphorylation suggests that the downstream effects on the TJ may be due to changes in the mitogen-activated protein kinase signalling pathway and such selective changes in permeability may influence tumorigenesis by the types of solutes now able to cross the epithelial barrier. Stucke *et al.* (47) found an interaction between endogenous hDlg1 and MPP7, a previously uncharacterized MAGUK-p55 subfamily member. MPP7 targets to the lateral surface of epithelial cells via its L27N domain, through an interaction with hDlg1. Loss of either hDlg1 or MPP7 from epithelial Caco-2 cells results in a significant defect in the assembly and maintenance of functional TJ, concluding that the

formation of a complex between hDlg1 and MPP7 promotes epithelial cell polarity and tight TJ formation. It is obvious that all these factors in the tumor microenvironment predispose cells to TJ leakiness.

Soler *et al* (48) examined the permeability in normal human and rat colon epithelia and in colon tumors. TER and paracellular influx rate revealed the TJ of colon tumors – both natural and induced – were “leakier” than those of normal colon that was suggestive of an increased permeability of colon epithelium and that a decrease in epithelium barrier function precedes the development of colon tumors.

Mullin *et al* (49) have suggested that TJ leakiness is a late event in epithelial carcinogenesis but allows for growth factors in luminal fluid compartments to enter intercellular and interstitial fluid spaces for the first time, binding receptors located only on the baso-lateral cell surface, causing changes in epithelial cell kinetics, concluding that TJ leakiness is a promotional event unique to epithelial tumors. This conclusion was the result of data showing that adenocarcinomas in rat and human colon have uniformly leaky TJ, whereas most human colon hyperplastic and adenomatous polyps contain non-leaky TJ, though adenomatous polyps with dysplastic changes did possess leaky TJ. Moreover, protein kinase C activation and translocation results in increased permeability of LLC-PK1 cells and failure to regulate this activation is correlated with multilayered cell growth and persistent leakiness.

Clarke *et al* (50) furthers this by stating that TJ leakiness associated with protein kinase C activation (and its downstream effectors) suggests a potentially useful role for TJ leakiness as a marker for early cancer diagnosis. These protein kinase C activators are all tumor promoters, which supports the concept of TJ being integral to the progression of cancer. HGF/SF (hepatocyte growth factor or scatter factor), a cytokine secreted by stromal cells and key to the development and progression of cancer, particularly during metastasis has been shown to be capable of modulating expression and function of TJ molecules in human breast cancer cell lines (24).

### 6.2. Tumor cell invasion of the endothelium leads to metastatic spread

Regulation of vascular permeability is one of the most important functions of endothelial cells, and endothelial cells from different organ sites show different degrees of permeability (51). Tumor blood vessels are more permeable on macro-molecular diffusion than normal tissue vessels. However, the cause and mechanism of hyperpermeability of human vessels had not been clear (51). Tumor cells release a number of factors that can assist their transmigration through the endothelium after treating endothelial cells with conditioned media from a highly invasive and metastatic melanoma cell line (51), with TJ being irreversibly damaged (as assessed using TER-trans-epithelial resistance).

HGF has been shown to decrease TER and increase PCP (paracellular permeability) in human

endothelial cells (2). NK4, an antagonistic variant of HGF was shown to inhibit this reduction in TJ function and to inhibit HGF-stimulated invasion of endothelium by human breast cancer cells (MDA-MB-231) (6). HGF decreased the protein expression of ZO-1 and increased tyrosine phosphorylation, with no associated changes in expression of occludin, claudin-1 or claudin-5. NK4 successfully prevented HGF-derived ZO-1 expression changes.

Clark *et al*. (52) has shown that tumor necrosis factor (TNF)-induced ICAM-1 in endothelial cells promotes leukocyte adhesion with ICAM-1 also effecting barrier function. Higher levels of ICAM-1 reduced TEER, increased F/G-actin ratios, rearranged the actin cytoskeleton to cause cell elongation, and altered ZO-1 and VE-cadherin staining. Specific small-interfering RNA knockdown of ICAM-1 partially inhibited TNF-induced shape change. The authors concluded that moderately elevated ICAM-1 expression reduced endothelial cell barrier function and that expressing higher levels of ICAM-1 affected cell junctions and the cytoskeleton.

## 7. THE EXPRESSION OF TJ PROTEINS IS ALTERED DURING CANCER PROGRESSION

Most cancers originate from epithelial tissues and are characterised by loss of control of growth, differentiation and tissue architecture. It is a fundamental property of cancer cells that their mutual adhesiveness is significantly weaker than that of normal cells. Reduced cell-cell interaction allows cancer cells to disobey the social order, resulting in destruction of overall tissue architecture, the morphological hallmark of malignancy. Loss of contact inhibition, which reflects disorder in the signal transduction pathways that connect cell-cell interactions are typical of both early (loss of cell polarity and growth control) and late (invasion and metastasis) stages of tumor progression.

It has become increasingly apparent that numerous TJ components are directly or indirectly involved in cancer progression, including ZO-1, ZO-2, claudin-7, claudin-1 and occludin. Highly differentiated adenocarcinomas with well developed TJ provide an important insight into the usefulness of TJ molecules as possible prognostic indicators and future targets for therapy. In breast cancer, ZO-1 has been demonstrated to be decreased in poorly differentiated tumors and correlated with increasing Grade and TNM (tumor-nodal) status [24]. Such observations indicate that TJ molecules could be used to identify poorly differentiated tumors and hence patients with poor prognosis. An absence TJ or defective TJ has also been associated with the development of the neoplastic phenotype in epithelial cells (14, 53-56). Such observations are consistent with the accepted idea that the disruption of tight junctions leads to loss of cohesion, invasiveness, and the lack of differentiation, thereby promoting tumorigenesis (57).

The increasing number of reports demonstrating a dysregulation of transmembrane proteins in human cancers and in cell lines offers intriguing insight into the role TJ have in cancer metastasis. This dysregulation can be

the result of both up-regulation and down-regulation of expression, changes in activation and location of the proteins and epigenetic changes. The following section will discuss such dysregulation via distribution on the TJ and by and tumor type.

## 8. CHANGES IN THE EXPRESSION OF TRANSMEMBRANE PROTEINS IN CANCER

### 8.1. Breast cancer

#### 8.1.1. Claudins in breast cancer

Claudin-1 (first described by (58)) is normally expressed in mammary gland-derived epithelial cells, but is absent in most human breast cancer cell lines. Claudin-1 expression was not detectable in subconfluent MDA-MB-435 and MDA-MB-361 breast cancer cells (7). Neither of these cell lines expressed occludin protein, and MDA-MB-435 does not express ZO-1 protein. Claudin-1 retroviral transduced breast cancer cells showed expression of claudin-1 at the usual cell-cell contact sites, suggesting that other proteins may be able to target claudin-1 to the TJ in the absence of occludin and ZO-1. Moreover, paracellular permeability was reduced in these transduced cells. The authors suggest that claudin-1 gene transfer may be in itself enough to exert TJ mediated gate function in metastatic breast cancer cells even in the absence of other TJ associated proteins such as occludin. This indicates a possible tumor suppressor function. In sporadic and hereditary breast cancer, there were no genetic changes, implying that regulatory or epigenetic factors may be involved in the downregulation of the claudin-1 gene during breast cancer development (22).

Claudin-1 cDNA isolated from human mammary epithelial cells (HMECs) was highly expressed in comparison to low or undetectable levels of expression in a number of

breast tumors and breast cancer cell lines (22). This indicated a possible tumor-suppressor effect for claudin-1. In sporadic tumors and hereditary breast cancer patients, there was no evidence to support the involvement of aberrant claudin-1 in breast tumorigenesis. Likewise, in breast cancer cell lines, no genetic alterations in the promoter or coding sequences were identified to explain the loss of claudin-1 expression. It was suggested that other regulatory or epigenetic factors may be involved in the downregulation of this gene during breast cancer development.

Interestingly, activation of recepteur d'origine nantais (RON) differentially regulates tight junction function and claudin expression (59). The inhibition of claudin-1 was seen in breast cancer T-47D cells. Activation of the extracellular signal-regulated kinase 1/2 pathway was required for RON-mediated inhibition of claudin-1 expression and redistribution of claudin-3 and -4. Forced expression of claudin-1 prevented RON-mediated cell migration and restored cell morphologies to their original epithelial appearance. In conclusion, RON activation differentially regulated claudin expression in epithelial cells and Inhibition of claudin-1 expression represented a novel

mechanism contributing to RON-mediated invasive activity, leading to increased tumor malignancy.

Kim *et al.* (60) found that claudin-2 protein expression was significantly down-regulated in breast tumours compared with corresponding normal breast tissue. Down-regulation of claudin-2 was significantly associated with lymph node metastasis and with high clinical stage. The expression levels of claudin-2 mRNA in high clinical stages (stages II and III) were lower than those in low clinical stage (stage I) and normal tissue, but not significantly so. The authors concluded that claudin-2 is implicated in the progression as well as the development of breast carcinoma, indicating that claudin-2 is a possible tumour suppressor gene product.

Morohashi *et al.* (61) examined 83 breast cancer cases and demonstrated immunohistochemical expression patterns of claudin-1/-4 in recurrent and non-recurrent groups. There were significant results between the recurrent and non-recurrent group for expression of claudin-1/-4. The recurrent group (26 cases) showed decreased expression patterns of claudin-1 compared to the non-recurrent group (57 cases). Decreased expression of claudin-1 correlated with short disease-free interval. The lymph node metastasis-positive group showed decreased expression patterns. There was however, no significance between the recurrent group and non-recurrent group in claudin-4 expression and no significant difference between histological factors and claudin-4 expression. The results indicated that claudin-1 expression correlated with the recurrence status and malignant potential of breast cancer.

In a study comparing 38 estrogen (ER) and progesterone receptor (PgR) negative, HER2/neu negative, but cytokeratin 5/6 positive basal-like-mainly grade 3-breast carcinomas with 21 grade 1, 25 grade 2 and 20 grade 3 non-basal-like invasive breast (62), statistically significant differences were observable regarding claudin-4 expression in the basal-like group as compared to grade 1 and 2 cancers. Claudin-4 expression was significantly higher in the basal-like compared with the non-basal-like grade 3 carcinomas. The data suggested that basal-like carcinomas are a subset of breast cancer with high level of claudin-4 protein expression. This observation may be seen as a further proof that basal-like carcinomas represent a separable group amongst grade 3 breast carcinomas. In another cohort of breast tumors, Lanigan *et al.* (63) examined claudin-4 by Western blot analysis and found a positive correlation with tumour grade and a negative correlation with ER. Claudin-4 expression was evaluated by immunohistochemistry in a larger cohort of 299 tumours represented on a tissue microarray and claudin-4 expression correlated positively with tumour grade and Her2, and negatively with ER. High claudin-4 expression was also associated with worse breast cancer-specific survival, recurrence-free survival and overall survival. Multivariate analysis revealed that claudin-4 independently predicted survival in the entire cohort and in the ER positive subgroup treated with adjuvant tamoxifen. It can be concluded that high levels of claudin-4 protein are associated with adverse outcome in breast cancer patients,

including the subgroup of patients treated with adjuvant tamoxifen.

Tokes *et al.* (64) compared levels of protein and mRNA expression of claudin-1, -3 and -4 in malignant breast tumors and benign lesions. Altogether, 56 sections from 52 surgically resected breast specimens were analyzed by immunohistochemistry and real-time PCR. Claudins were rarely observed exclusively at TJ structures. Claudin-1 was present in the membrane of normal duct cells and in some of the cell membranes from ductal carcinoma *in situ*, and was frequently observed in eight out of nine areas of apocrine metaplasia, whereas invasive tumors were negative for claudin-1 or it was present in a scattered distribution among such tumor cells (in 36/39 malignant tumors). Claudin-3 was present in 49 of the 56 sections and claudin-4 was present in all 56 tissue sections. However, claudin-4 was highly positive in normal epithelial cells and was decreased or absent in 17 out of 21 ductal carcinoma grade I, in special types of breast carcinoma (mucinous, papillary, tubular) and in areas of apocrine metaplasia. Claudin-1 mRNA was downregulated by 12-fold in the tumor group. Claudin-3 and -4 mRNA exhibited no difference in expression between invasive tumors and surrounding tissue. The significant loss of claudin-1 protein in breast cancer cells suggests that this protein may play a role in invasion and metastasis. The loss of claudin-4 expression in areas of apocrine metaplasia and in the majority of grade I invasive carcinomas also suggests a particular role for this protein in mammary glandular cell differentiation and carcinogenesis.

Soini (65) also evaluated the expression of claudin-2, -3, -4, and -5 in 20 cases of Paget's disease (13 mammary and 7 extramammary cases), and compared the results with those of other neoplastic skin lesions, including actinic keratoses, basal cell carcinomas, and malignant melanomas. Membrane-bound claudin-3 and -4 expression was seen in all cases of Paget's disease, whereas claudin-5 was seen in 50% of cases and claudin-2 was seen in 32% of cases. However, claudins-3, -4, and -5 were not seen in the other skin lesions, and claudin 2 was seen in most of them, suggesting an inverse expression of these claudins between Paget's disease and epidermal and nevocytic lesions. Claudin expression in breast carcinomas was claudin-2 in 52%, claudin-3 in 93%, claudin-4 in 92%, and claudin-5 in 47%. Claudins-2 and -5 were found more often in ductal carcinomas than in lobular carcinomas. Expression of the claudins was frequently associated with each other. They were not associated with estrogen or progesterone receptor status or with tumor grade. No significant differences were found between claudin expression in Paget's disease and breast carcinomas. The results demonstrate that claudins could be useful in diagnosing Paget's disease and in differentiating these lesions from other epidermal lesions, such as actinic keratoses, basal cell carcinomas, and nevocytic lesions. The lack of difference in claudin expression between Paget's disease and breast tumors suggests that changes in the phenotype of claudins-2, -3, -4, and -5 are not necessary for epidermal invasion.

The claudins-1, -3, and -4 have been found to be differentially expressed in the mammary gland during pregnancy, lactation, and involution, suggesting different

roles for these proteins at different stages of mammary gland function (66). In addition, claudin-1 and -3 were detected in mammary tumors and the wide distribution of claudin-3 in particular, appears to suggest specific roles for these proteins in mammary tumorigenesis.

Based on a report that claudin-6 functions as a tumor suppressor for breast cancer, Osanai *et al.* (67) showed that suppression of claudin-6 expression resulted in increased resistance to various apoptogens, and causally enhanced anchorage-independent growth properties. Because claudin-6 expression was partially silenced by promoter CpG island hypermethylation in MCF-7 breast carcinoma cells, a synergistic effect of a demethylator and histone deacetylase inhibitor up-regulated the expression of endogenous claudin-6, which was sufficient for apoptotic sensitization and abrogation of colony-forming efficacy. In addition, decreased expression of claudin-6 promoted cellular invasiveness and transendothelial migration, accompanied by an increase in matrix metalloproteinase activity. These data suggest that the methylator phenotype of claudin-6 may at least partially contribute to enhanced tumorigenic and invasive properties of breast carcinoma cells. MCF-7 breast cancer cells transfected with the claudin-6 gene (68) grew more slowly than control cells. Anchorage-independent growth, invasive and migratory traits also decreased substantially in cells with claudin-6 expression; whereas the transepithelial electrical resistance increased in the claudin-6 transfected cells. The up-regulation of claudin-6 expression in MCF-7 cells suppressed their malignant phenotype with a correlation with the restoration of TJ integrity. Claudin-6 may function as a cancer suppressor whose down-regulation contributes to the malignant progression of certain types of breast cancers.

Human mitochondrial DNA (mtDNA) encodes 13 proteins involved in oxidative phosphorylation (OXPHOS). In order to investigate the role of mitochondrial OXPHOS genes in breast tumorigenesis, Kulawiec *et al.* (69) developed a breast epithelial cell line devoid of mtDNA (rho(0) cells). The study determined that claudin-1 and -7 were indeed downregulated in rho(0) breast epithelial cells, that the downregulation of claudin-1 or -7 led to neoplastic transformation of breast epithelial cells, and that claudin-1 and -7 were also downregulated in primary breast tumors. This suggests that mtDNA encoded OXPHOS genes play a key role in transformation of breast epithelial cells and that multiple pathways involved in mitochondria-to-nucleus retrograde regulation contribute to transformation of breast epithelial cells.

Loss of claudin-7 has been found to correlate with histological grade in both ductal carcinoma *in situ* and invasive ductal carcinoma of the breast (21). The expression of claudin-7 is lost in both preneoplastic and invasive ductal carcinoma of the breast occurring predominately in high grade lesions. Expression is also frequently lost in LCIS correlating with the increased cellular discohesion observed in LCIS. Additionally, the majority of IDC cases displaying a low claudin-7 expression have a positive lymph node status. Such



findings suggest that the loss of claudin-7 may aid in tumor cell dissemination and augment metastatic potential. Moreover, silencing of claudin-7 expression correlated with promoter hypermethylation in 3/3 breast cancer cell lines but not in invasive ductal carcinomas (0/5). In addition, HGF treatment results in disassociation of MCF-7 and T47D cells in culture, and a loss of claudin-7 expression within 24h. Sauer *et al.* (70) more recently showed that primary and recurrent/metastatic breast lesions expressed Claudin-7. 46% of cases had full expression and reduced expression was found in 54%. In cases with reduced expression, the percentage of stained cells were usually high, and no smear showed <50% stained tumor cells. The staining pattern was heterogeneous and always mixed membrane/cytoplasmic. Claudin-7 expression was significantly correlated with tumor grading local recurrences and metastatic diseases, nodal involvement and cellular cohesion in invasive carcinomas, but not with tumor size or subtype.

Claudin-16 (paracellin-1), ponsin, ZO-2, AF6, vinculin and nectin were reduced with poor prognosis of patients with breast cancer however JAM-2 does not show differences in expression (71). The levels of transcripts of claudin-16 and vinculin were significantly lower in patients that had poor prognosis (with metastasis, recurrence or mortality), compared with those that remained healthy after a median follow-up of 72.2 months. Immunohistochemistry confirmed these results, as there was a decreased level in staining for claudin-16 and AF6. In normal tissue, staining was confined to the intercellular regions whereas in the tumor tissues the staining was diffuse and cytosolic. The conclusion was that low levels of TJ molecules claudin-16 and vinculin in breast cancer are associated with poor prognosis in patients, underscoring the idea that regulation of TJ could be of fundamental importance in the prevention of metastasis of breast cancer cells.

### 8.1.2. Nectins in breast cancer

Interestingly, the nectin family has been little studied as regards TJ in cancer, being originally described as molecules involved in adherens junctions only. Recently however, it has become apparent the nectins are also involved in recruitment and maintenance of proteins within the TJ (72). Nectin-4 was not detected in normal breast epithelium. By contrast, nectin-4 was expressed in 61% of ductal breast carcinoma and in 6% in lobular type. Expression of nectin-4 strongly correlated with the basal-like markers EGFR, P53, and P-cadherin, and negatively correlated with the luminal-like markers ER, PR and GATA3. All but one ER/PR-negative tumors expressed nectin-4. The detection of nectin-4 in serum improves the follow-up of patients with MBC: the association CEA/CA15.3/nectin-4 allowed to monitor 74% of these patients compared to 67% with the association CEA/CA15.3. Serum nectin-4 was a marker of disease progression, and levels correlate with the number of metastases. Serum nectin-4 was also a marker of therapeutic efficiency and correlated, in 90% of cases, with clinical evolution. Nectin-4 appears to be a new tumor-associated antigen for breast carcinoma and possibly new bio-marker whose use could help refine breast cancer

taxonomy and improve patient follow-up. Nectin-4 emerges as a potential target for breast cancer immunotherapy (72).

### 8.1.3. Occludin in breast cancer

Osanai *et al.* (73) had previously demonstrated that epigenetic silencing of occludin resulted in the acquisition of apoptotic resistance to various apoptogenic stimuli, causally contributing to the enhanced tumorigenicity of cancer cells. In a recent study, the authors demonstrated that forced expression of occludin induced anoikis and promoted oxidative stress-induced premature senescence in breast carcinoma cells, accompanied by upregulation of negative cell cycle regulators such as p16INK4A, p21Waf1/Cip1 and p27Kip1 but not p53. Endogenous re-expression of occludin mediated by a synergistic effect with a demethylator and histone deacetylase inhibitor or retinoids that stimulate retinoic acid receptor was also sufficient for provoking the senescent phenotype. These findings suggested that the loss of occludin expression could be partially involved in the senescence-escape program during mammary tumorigenesis.

### 8.1.4. JAM in breast cancer

Naik *et al.* (74) investigated JAM-A expression in breast tumours. They showed that JAM-A was a key negative regulator of cell migration and invasion. JAM-A was robustly expressed in normal human mammary epithelium, and its expression down-regulated in metastatic breast cancer tumors. In breast cancer cell lines, an inverse relationship between JAM-A expression and the ability of these cells to migrate on a collagen matrix was observed, which correlates with the known ability of these cells to metastasize. T47D and MCF-7 cells were found to express high levels of JAM-A, whereas the more migratory MDA-MB-468 cells had lower levels of JAM-A on the cell surface. MDA-MB-231 cells, which are highly migratory, expressed the least amount of JAM-A. Overexpression of JAM-A in MDA-MB-231 cells inhibited both migration and invasion through collagen gels. Furthermore, knockdown of JAM-A using short interfering RNAs enhanced the invasiveness of MDA-MB-231 cells as well as T47D cells. The ability of JAM-A to attenuate cell invasion correlated with the formation of increased numbers of focal adhesions and the formation of functional TJ. These results showed for the first time that an immunoglobulin superfamily cell adhesion protein expressed at TJ could serve as a key negative regulator of breast cancer cell invasion and possibly metastasis. Furthermore, loss of JAM-A could be used as a biomarker for aggressive breast cancer.

### 8.1.5. CAR in breast cancer

The Coxsackie-adenovirus receptor (CAR) is the primary site for adenovirus attachment during infection and has been used as a delivery mechanism for gene therapies. Martin *et al.* (75) evaluated the expression of CAR in human breast cancers. Staining intensity of CAR was increased within tumor sections compared to background tissue. Q-PCR revealed significantly elevated levels of CAR transcript in breast tumors. CAR expression also

increased with grade of tumor. Patients who had tumor metastases also showed elevated levels of CAR expression, however those with local recurrences had reduced levels of CAR. Ductal carcinomas expressed lower levels of CAR compared to tumors of other types. Tumors with nodal involvement were also associated with higher levels of CAR. Levels of CAR were significantly correlated with long-term survival over a period of 6 years. It appears that CAR expression is elevated in primary breast cancers. This raises pertinent questions in two areas: could this provide a key to treatment using viral vectors, or, does this elevated expression result in dysregulation of barrier function? Further research is required to uncover these

### 8.2. Bladder cancer

#### 8.2.1. Claudins in bladder cancer

Recently, interest in the role of TJ in bladder carcinoma has increased. A timely review explored the current understanding on the role and regulation of TJ function in the normal and diseased bladder (76); however, few studies have materialised to further interest in this area. Boireau *et al.* (77) analyzed the expression and localization of claudins -1, -4, and -7 in human bladder carcinoma. Claudin-4 expression was significantly altered in 26/39 tumors, contrasting with the rare modifications detected in the expression of claudins 1 and 7. Overexpression of claudin-4 in differentiated carcinomas was followed by a strong downregulation in invasive/high-grade tumors, and this expression pattern was associated to the 1-year survival of bladder tumor patients. A CpG island was identified within the coding sequence of the claudin-4 gene, and treatment with a methyl-transferase inhibitor restored expression of the protein in primary cultures prepared from high-grade human bladder tumors. Claudin-4 expression also correlated with its gene methylation profile in healthy and tumoral bladders from 20 patients. Delocalization of claudins-1 and -4 from TJ was observed in most human bladder tumors and in the bladder tumor cell line HT-1376. Although the claudin-4 gene was unmethylated in these cells, inhibition of methyl transferases re-addressed the two proteins to TJ, resulting in an increase of cell polarization and transepithelial resistance. These biological effects were prevented by expression of claudin-4-specific siRNAs, demonstrating the important role played by claudin-4 in maintaining a functional regulation of homeostasis in urothelial cells. The authors concluded that the TJ barrier is disrupted from early stages of urothelial tumorigenesis and that hypermethylation was the leading to the alteration of claudin-4 expression and localization in bladder carcinoma.

### 8.3. Colorectal Cancer

Since Soler *et al.* (48) revealed the TJ of colon tumors – both natural and induced – were “leakier” than those of normal colon there have been a number of studies looking at colorectal carrier function.

Hahn-Strömberg *et al.* (78) found that the complexity of the invasive front of colon carcinomas was correlated with cell adhesion protein expression and with polymorphisms in their genes. A complexity index was constructed from 32 colon carcinomas using computer-assisted morphometry estimating fractal dimension and

tumor cell clusters followed by tree analysis. Immunohistochemical staining of beta-catenin, E-cadherin, occludin and claudin-2 was used for assessment of protein expression. Genetic screening of tissue from the tumor invasion front with laser microdissection was performed using SSCP and DNA sequencing. Adhesion protein distribution was significantly disturbed in most carcinomas. A single mutation in the gene of beta-catenin was found but there was no correlation between protein expression and genetic polymorphism. Nor was there any correlation between the complexity of the invasive border and protein distribution or genetic alterations. The authors conclude that these results indicate that the complexity of colon carcinoma invasion is not dependent on genetic derangements in the genes of adhesion proteins or the protein distribution. Indeed, aberrations in the function of other proteins related to the adhesive proteins could be responsible.

#### 8.3.1. Claudins in colorectal cancer

It has been demonstrated that there is an inverse relationship between the expression of claudin-1 and Smad4, a tumor suppressor protein, in colon cancer cell lines and in human colon cancer tissue samples (79). Smad4 expression in Smad4-deficient colon cancer cells inhibited claudin-1 expression through transcriptional regulation. Further analysis suggested the important role of h-catenin/T-cell factor (TCF)/lymphocyte enhancer factor (Lef) activity in the Smad4- dependent regulation of claudin-1 expression. In addition, the inhibition of claudin-1 expression contributes to the ability of Smad4 to inhibit invasion in colon cancer cells. Smad4-dependent inhibition of claudin-1 expression was a direct effect of Smad4 expression and not due to modulation of TGF- $\beta$  signalling. Resnick *et al.* (16) investigated the pattern of expression and prognostic value of claudin-1, claudin-4, occludin and ZO-1 in a cohort of TNM stage II colon cancer using tissue microarray technology and retrospectively analyzed samples from 129 patients with TNM stage II colonic carcinomas for claudin-1, claudin-4, occludin and ZO-1 protein expression by immunohistochemistry. Seventy-five, 58, 56 and 44% of the tumors exhibited normal to elevated expression levels of claudin-1, claudin-4, occludin and ZO-1 respectively. Low expression levels of claudin-1 and ZO-1 were directly associated with higher tumor grade. Multivariate analysis indicated that lymphovascular invasion and low levels of claudin-1 expression were independent predictors of recurrence and that reduced claudin-1 expression was associated with poor survival. This was the first study to comprehensively examine the expression of several TJ associated proteins in colonic neoplasms and to correlate their expression with disease progression. Loss of claudin-1 expression proved to be a strong predictor of disease recurrence and poor patient survival in stage II colon cancer.

Expression of claudin-1 decreases significantly in response to reduction of intracellular beta-catenin by adenovirus-mediated transfer of wild-type APC into the APC-deficient colon cancer cells, with two putative Tcf4 binding elements authors again demonstrate increased expression of claudin-1 in primary colorectal cancers.

Furthermore, immunohistochemical staining demonstrated that claudin-1 was weakly stained at apical border of lateral membrane of noncancerous epithelial cells and that it was strongly stained at all cell-cell boundaries and in the cytoplasm of cancer cells. Such results imply that claudin-1 is involved in the beta-catenin-Tcf/LEF signalling pathway. Moreover, it has been reported that there is an increased expression of claudin-1 in human primary colon carcinoma and metastasis and in cell lines derived from primary and metastatic tumors (80). There was frequent nuclear localization of claudin-1 in these samples. Genetic manipulations of claudin-1 expression in colon cancer cell lines induced changes in cellular phenotype, with structural and functional changes in markers of epithelial-mesenchymal transition. It was also demonstrated that changes in claudin-1 expression had significant effects on growth of xenografted tumors and metastasis in athymic mice. Data suggests that the regulation of E-cadherin expression and beta-catenin/Tcf signalling is a possible mechanism underlying claudin-1-dependent changes. Kinugasa *et al.* (81) also state that claudin-1 and claudin-2 were found to be over-expressed in colorectal cancer tissues. They may be useful as tumor markers and targets for the treatment of colorectal cancer.

A more recent study looked at the expression analysis of genes encoding TJ proteins to display differential gene expression on RNA and protein level and to identify and validate potential targets for colorectal cancer therapy (82). Claudin-1 and -12 are frequently over-expressed in colorectal cancer, whereas claudin-8 showed down-regulation in tumor tissue at the RNA level. Quantification of proteins confirmed the overexpression of claudin-1 in tumor tissues, whereas changes of claudin-8 and -12 were not significantly detectable on protein level. IHC confirmed the markedly elevated expression level of claudin-1 in the majority of colorectal cancer, showing membranous and intracellular vesicular staining. The authors concluded that differential expression of genes encoding claudins in colorectal cancer suggests that these TJ proteins may be associated to and involved in tumorigenesis. As claudin-1 is frequently up-regulated in large proportion of colorectal cancers and may represent potential target molecule. Added to their intrinsic properties in a correctly functioning TJ, it is becoming apparent that the over-expression or absence of expression of these proteins might provide evidence of prognostic value. In adenocarcinoma tissues the expression of claudin-1, -3 and -4 has been found to be upregulated (83). Tokunaga *et al.* (84) investigated whether or not occludin is expressed in rosette or gland-like structures in human rectal carcinoid tumors. The expression profiles of occludin in 40 carcinoid tumors were examined immunohistochemically, using an anti-occludin monoclonal antibody. In eight (20%) samples of typical carcinoid tumors, a small number of rosette-like tubular structures outlined by occludin were detected. Thus occludin might be considered to be one of the most characteristic structural markers of polarized glandular structures. The results of this study provided supportive evidence that carcinoid tumor cells are capable of glandular differentiation. The significance of claudin-4 expression in colorectal cancer and its association with

clinicopathological factors was examined (85). The levels of claudin-4 expression in a total of 129 colorectal cancers and 44 metastatic tumors were examined by immunohistochemistry. A small interfering RNA (siRNA)-mediated claudin-4 knockdown examination was also conducted to assess the biological role(s) of claudin-4 in cultured cells. Expression of claudin-4 at the intercellular membrane was well preserved at the surface of tumors, with decreased claudin-4 expression detected in colorectal cancers, particularly at the invasive front. Interestingly, decreased claudin-4 expression was detected in metastatic lesions of colorectal cancer. siRNA-mediated claudin-4 knockdown in SW480 claudin-4-positive colorectal cancer cells upregulated cell motility, whereas no significant change was detected in cell proliferation. Disruption of claudin-4-mediated TJ construction enhances cancer cell invasion and metastasis in human colorectal cancer and claudin-4 might be a good biomarker for diagnosing the risk of distant metastasis. It is interesting to note that the claudin-1 gene is regulated by beta-catenin (86).

In colorectal carcinoma it was found that mucosa of crypts and surfaces of cancers exhibited significantly elevated expression levels of claudin-1, claudin-3, claudin-4, and beta-catenin compared to intraepithelial neoplasia and normal mucosa (87). These data were confirmed by a comparative score. The expression of claudin-2, occludin, and ZO-1 showed no differences between the groups and the authors concluded that TJ proteins claudin-1, claudin-3, claudin-4, and the AJ protein beta-catenin are overexpressed in colorectal carcinoma, suggesting that these proteins may become potential markers and targets in colorectal carcinoma. In another study (88), claudin-1 expression at the mRNA and protein levels was analyzed in 41 cases of colorectal cancer and was found to be increased in the colorectal carcinoma tissue in comparison to that in the normal tissue specimens. The mRNA levels of claudin-1 were correlated with tumor depth, but not with the preoperative carcinoembryonic antigen (CEA) serum level. When T84 cells, a human colon cancer cell line, were transfected with the claudin-1 gene, the claudin-1 overexpressing cells grew as aggregates in contrast to the monolayer formation of the parental cells. The data drew the conclusion that claudin-1 plays a pivotal role in cell morphology and behavior in the colonic epithelium and that claudin-1 protein may therefore be one of the major factors involved in the tumorigenesis of colorectal carcinoma. More recently, Krishnan *et al.* (89) examined molecular mechanism(s) underlying dysregulated claudin-1 expression in colon cancer. Histone deacetylase (HDAC)-dependent histone acetylation is an important mechanism of the regulation of cancer-related genes and inhibition of HDACs induces epithelial differentiation and decreased invasion. They reported a novel post-transcriptional regulation of claudin-1 expression in colon cancer cells and showed a functional correlation between claudin-1 expression and TSA-mediated regulation of invasion. As HDAC inhibitors are considered to be promising anticancer drugs, these new findings should have implications in both laboratory and clinical settings.

Aung *et al.* (90) evaluated the specificity of the claudin-2 expression in various normal human tissues and gastrointestinal cancers by quantitative reverse

transcriptase-polymerase chain reaction and immunohistochemistry. In 14 various normal tissues, claudin-2 mRNA was expressed in the kidney, liver, pancreas, stomach, and small intestine; the highest level of which was detected in the kidney. Colorectal cancers (CRCs) expressed claudin-2 mRNA at high levels. Immunohistochemical analysis of claudin-2 in 146 gastric cancers (GCs) and 99 CRCs demonstrated claudin-2 expression in 2.1% of GCs and 25.3% of CRCs, respectively. There was no obvious correlation between claudin-2 expression and clinicopathological parameters of CRCs. These results suggested that the expression of claudin-2 may involve organ specificity, and increased expression of claudin-2 may participate in colorectal carcinogenesis.

Oshima *et al.* (91) examined the relationship between the relative expression of claudin genes and clinicopathological factors, especially invasion and metastasis, in patients with colorectal cancer. The authors studied surgical specimens of cancer tissue and adjacent normal mucosa from 205 patients with untreated colorectal carcinoma. The relative expression levels of the claudin-1, -3 and -4 genes were higher in cancer than in normal adjacent mucosa, whereas the relative expression of the claudin-7 gene was similar. An analysis of the relationship between the clinicopathological features and gene expression showed that reduced expression of claudin-7 correlated with venous invasion and liver metastasis. There was also a correlation between claudin-3 and -4 gene expression. Such results suggested that a reduced expression of the claudin-7 gene might lead to venous invasion and liver metastasis in colorectal cancer. Reduced expression of the claudin-7 gene may thus be a useful predictor of liver metastasis in patients with colorectal cancer.

In addition to this, Darido *et al.* (92) found that in healthy human colonic crypts, claudin-7 expression was found to be low in the stem/progenitor cell compartment, where Tcf-4 activity was high, but strong in differentiated and postmitotic cells, where Tcf-4 is inactive. In contrast, claudin-7 was overexpressed in areas with high Tcf-4 target gene levels in CRC samples. *In vitro*, Tcf-4 was able to repress claudin-7 expression, and the high mobility group-box transcription factor Sox-9 was identified as an essential mediator of this effect. Claudin-7 was strongly expressed in the intestine of Sox-9-deficient mice and in CRC cells with low Sox transcriptional activity. Sox-9 overexpression in these cells reinstated claudin-7 repression, and residual claudin-7 was no longer localized along the basolateral membrane, but was instead restricted to TJ. In HT-29Cl.16E CRC cell spheroids, it was found that Sox-9-induced polarization was completely reversed after virus-mediated claudin-7 overexpression. Claudin-7 overexpression in this context increased Tcf-4 target gene expression, proliferation, and tumorigenicity after injection in nude mice. These results indicated that Tcf-4 maintains low levels of claudin-7 at the bottom of colonic crypts, acting via Sox-9. This negative regulation seems to be defective in CRC, possibly due to decreased Sox-9 activity, and the resulting claudin-7 overexpression promotes a loss

of tumor cell polarization and contributes to tumorigenesis. Moreover, in Colo320 (claudin-7-negative) cells, hypermethylation at the claudin-7 promoter was detected and treatment with 5-aza-2'-deoxycytidine restored claudin-7 expression (93). In CRC tissues, decreased claudin-7 expression was detected in 62% of stage 0 CRCs and 80% of stage I-IV CRCs, compared with their adjacent adenoma lesions and non-neoplastic epithelia, which had a close correlation with the incidence of vessel infiltration and clinicopathologic stage. Hypermethylation at the claudin-7 promoter was detected in 20% of CRCs with low claudin-7 expression. However, claudin-7 expression tended to be re-expressed in their corresponding lymph node metastases. These findings suggested that the CLDN7 gene silencing by promoter hypermethylation and the resultant reduction of CLDN7 expression may play an important role in the progression of CRCs.

Patients with inflammatory bowel disease (IBD) are at increased risk of developing colorectal adenocarcinoma. The factors that result in IBD-associated carcinogenesis are not understood. Weber *et al.* (94) hypothesized that altered expression of intestinal epithelial TJ proteins might contribute to neoplastic progression. Semiquantitative immunohistochemical staining of human biopsies was used to assess expression of claudin-1, claudin-2, claudin-4, and occludin in IBD, IBD-associated dysplasia, acute, self-limited colitis (ASLC), and sporadic adenomas. Claudin-1 and claudin-2 expression was elevated in active IBD, adenomas, and IBD-associated dysplasia, but not ASLC. In contrast, claudin-4 expression was elevated in both active IBD and ASLC. Occludin expression was similar to control in all cases. Importantly, in IBD, claudin-1 and claudin-2 expression correlated positively with inflammatory activity. To investigate mechanisms underlying altered claudin expression, beta-catenin activation was assessed as nuclear localization. Like claudin-1 and claudin-2, beta-catenin was markedly activated in IBD, sporadic dysplasia, and IBD-associated dysplasia, but was only slightly activated in ASLC. Taken together, these data suggest that beta-catenin transcriptional activity is elevated in chronic injury and that this may contribute to increased claudin-1 and claudin-2 expression. The authors speculate that increased claudin-1 and claudin-2 expression may be involved at early stages of transformation in IBD-associated neoplasia.

Tanaka *et al.* (95) investigated the effects of prostaglandin E(2) (PGE(2)) treatment on AJC assembly and function. Exposition of Caco-2 cells to PGE(2) promoted differential alteration of AJC protein distribution, as evidenced by immunofluorescence and immunoblotting analysis and impairs the barrier function, as seen by a decrease in the transepithelial electric resistance and an increase in the permeability to ruthenium red marker. They demonstrated the involvement of EP1 and EP2 prostaglandin E(2) receptor subtypes in the modulation of the AJC disassembly caused by prostanoid. Furthermore, pharmacological inhibition of protein kinase-C, but not PKA and p38MAPK significantly prevented the PGE(2) effects on the AJC disassembly. These findings strongly suggest a central role of Prostaglandin E2-EP1 and EP2

receptor signaling to mediate AJC disassembly through a mechanism that involves PKC and claudin-1 as important target for the TJ-related effects in human colorectal cancer cells (Caco-2).

### 8.3.2. CAR in colorectal cancer

Modified adenoviruses represent a new approach to treatment of gastrointestinal cancer (96). However, their uptake by cells in many cases requires the major receptor for adenoviruses, CAR. Lack of CAR expression then, is a potential cause of intrinsic resistance of tumor cells to this type of treatment. To evaluate this, Korn *et al.* (96) studied the localization of CAR protein in normal and malignant gastrointestinal tissues. In normal tissues, CAR was concentrated at sites of cell-cell interaction, in particular at the apico-lateral cellular surface. Expression was particularly strong around bile and pancreatic ducts, which is in agreement with CAR's physiological function as a tight-junction protein. In GI malignancies (esophageal, pancreatic, colorectal and liver cancer), expression of the receptor varied substantially. Loss of CAR expression at cell-cell junction was evident in many samples. A significant correlation between CAR expression and histological grade was found, with moderately to poorly differentiated tumors most frequently demonstrating loss or reduction of CAR expression. These data indicate that CAR expression is frequently altered in gastrointestinal malignancy, potentially reducing the efficacy of adenovirus-based therapies.

## 8.4. Esophageal cancer

### 8.4.1. Claudins in esophageal cancer

The majority of studies on TJ in esophageal cancer have concentrated on transmembrane proteins in the claudin family. This is also reflected by the growing number of studies indicating the importance of TJ function in the precancerous predecessor to esophageal cancer, Barrett's esophagus (BE). Recently, Miyamoto *et al.* (97) examined 54 esophageal cancer cases to assess immunohistochemical expression patterns of claudin-1 with decreased expression of claudin-1 being statistically correlated with recurrence status. Decreased expression of claudin-1 was also correlated with short disease-free and overall. The results suggest that claudin-1 expression is correlated with the recurrence status and poor prognosis in esophageal cancer and claudin-1 expression may be a good indicator of recurrence in esophageal cancer.

Upregulation of claudins-3, -4, and -7 was identified in gastric adenocarcinoma Montgomery *et al.* (98). While normal gastric mucosa lacked claudin-3, -4, and -7 expression, intestinal metaplasia and dysplasia showed these proteins. The authors hypothesized that claudins would be similarly overexpressed in BE adenocarcinoma. The findings suggest that alterations in claudin proteins are an early event in tumorigenesis and may provide targets for diagnosis and directed therapy for esophageal adenocarcinoma and its precursors. Earlier studies (99) showed that reduced expression of claudin-7 at the invasive front of the esophageal cancer was significantly associated with the depth of invasion, lymphatic vessel invasion and lymph node metastasis. In

contrast, significant association was not detected between claudin-1 expression and clinicopathologic factors except for histologic differentiation of the tumor. Claudin-7 expression at the invasive front of the primary tumor and its corresponding metastatic lymph nodes revealed significant reduction in claudin-7 expression in the metastatic lymph nodes suggesting that the reduced expression of claudin-7 at the invasive front of esophageal squamous cell carcinoma may lead to tumor progression and subsequent metastatic events. Moreover, Lioni *et al.* (100) examine the expression of claudin-7 in squamous cell carcinoma (SCC) of the esophagus and its possible role in tumor progression. In this context, the claudin-7-overexpressing cells became more adhesive and less invasive associated with increased E-cadherin expression. Claudin-7 was mislocalized during the malignant transformation of esophageal keratinocytes. This demonstrated that there might be a critical role for claudin-7 expression in the regulation of E-cadherin in these cells, suggesting this may be one mechanism for the loss of epithelial architecture and invasion observed in esophageal SCC.

When examining claudin expression in BE and related adenocarcinoma, Gyorffy *et al.* (101) found that claudin-2 and -3 expression in BE was higher than in normal foveolar epithelium. Adenocarcinoma showed higher claudin-2 and -3 expression compared with normal and Barrett's epithelia. The similar claudin expression profile of BE and adenocarcinoma supported their sequential development. Gastric intestinal metaplasia showed higher expression of claudin-2, -3 and -4 as compared with normal antral foveolar mucosa. Tumors of small and large bowels exhibited higher claudin-2 expression when compared with normal epithelia. Colorectal adenoma and adenocarcinoma could not be differentiated according to their claudin profile. Intestinal metaplasias of BE and stomach show similar claudin profile to small bowel epithelium. Studies on duodenal mucosa in celiac disease in childhood demonstrated claudin-2 and -3 expression to be higher than in normal mucosa. The expression was significantly higher in the distal part of the duodenum samples. This and the serious histological findings suggested that the distal duodenum is more adequate for biopsy testing. Beside the epithelial cells, mesenchymal tumors express intercellular junctional proteins. The claudin profile was found to be representative to the individual tumor. GIST, angiosarcoma, hemangioma, leiomyosarcoma and leiomyoma showed expression of various claudins. Claudin-2 was detected in all entities, whereas claudin-1 was found positive in leiomyosarcoma only. Leiomyoma, on the other hand, expressed only claudin-2. GISTs and leiomyosarcomas showed claudin, -3, -4, -5 and -7-expression. The angiogenic tumors revealed claudin-2 and -5 expression. The similar claudin profile observable in GIST and leiomyosarcoma is apparently suggestive of a histogenetic relationship. Smooth muscle and vessel tumors of different dignity could also be separated from each other based on claudin profile.

Demura *et al.* (102) concluded from their study that transition of low- to high-grade dysplasia and to adenocarcinoma into BE was established to be

accompanied by TJ loss, as appeared as disappearance of apical staining of claudins-1, -2, -3, -4, -5, and -7, with their cytoplasmic staining increased in parallel. The lost capacity of accumulating claudins in the area of TJ in adenocarcinoma into BE led to the elimination of BE and promoted tumor progression (proliferation, invasion, and metastatic spread). The stated that as markers of cell differentiation, claudin-1, -2, -3, -4, -5, and -7 may be recommended for determination of the malignant potential of dysplasia to BE.

### 8.4.2. Occludin in esophageal cancer

Patients with BE (103) were observed to exhibit a transepithelial leak to sucrose whose mean value was threefold greater than that seen in healthy control subjects or patients with reflux but without any mucosal defect. A parallel study of claudin tight junction proteins in endoscopy biopsy samples showed that whereas BE metaplasia contains dramatically more claudin-2 and claudin-3 than is found in normal esophageal mucosa, it is markedly lower in claudins 1 and 5, indicating very different tight junction barriers. A later study of 21 claudins in BE (104) and specialized columnar epithelium (SCE) that develops as replacement for damaged squamous epithelium (SqE) in subjects with reflux disease, demonstrated that in SCE, claudin-18 was the most highly expressed at the mRNA level and this finding is paralleled by marked elevation in protein expression on immunoblots. In contrast in SqE, claudin-18 was minimally expressed at the mRNA level and undetectable at the protein level. Immunofluorescence studies showed membrane localization of claudin-18 and colocalization with ZO-1. This prompted the authors to conclude that claudin-18 is the dominant claudin in the TJ of SCE and propose that the change from a claudin-18-deficient TJ in SqE to a claudin-18-rich TJ in SCE contributes to the greater acid resistance of BE. An early study by Rendon-Huerta *et al.* (105) observed that occludin is in fact found in normal esophageal tissue, in contrast to an earlier publication reporting its absence in esophagus (105). They also observed that the amounts of occludin on a per-mg-total-protein basis are not different for biopsies from BE metaplasia compared with adjacent normal esophageal epithelium. However, the situation is very different for the claudins with claudin-1 being fairly abundant in normal esophagus but is absent in some BE metaplasia biopsies and sharply reduced in most others. Claudin-2 presented a somewhat opposite picture, that is, consistently nondetectable in normal esophageal epithelium but detectable at low-to-moderate levels in two of eight BE biopsies.

## 8.5. Liver cancer

### 8.5.1. Occludin in liver cancer

Orban *et al.* (106) analysed the expression of occludin and ZO-1 in 25 surgically removed human hepatocellular carcinomas (HCC) and 25 human colorectal liver metastases. Occludin and ZO-1 mRNAs showed significant downregulation in HCCs in comparison with normal liver and were also downregulated in the metastases when compared with normal liver. Occludin and ZO-1 proteins were weakly expressed on hepatocytes in normal

liver, while strong expression was found on bile canaliculi. In HCCs occludin and ZO-1 did not show immunopositivity on tumor cells, while colorectal metastatic tumors revealed high levels of these molecules. HCCs and metastases are characterized by markedly different protein expression pattern of occludin and ZO-1, which phenomenon might be attributed to the different histogenesis of these tumors.

## 8.6. Gastric cancer

Disruption of the TJ observed in the study by Fedwick *et al.* (44) implicate host cell signalling pathways, including the phosphorylation of myosin light chain and the regulation of tight-junctional proteins claudin-4 and claudin-5, in the pathogenesis of *Helicobacter pylori* infection. As gastric carcinoma remains one of most serious malignant tumors worldwide with *Helicobacter pylori* being the definite carcinogen this is an interesting area in determining the role of TJ barrier function (107). The *Helicobacter pylori* components, cytotoxin-associated gene A (CagA), vacuolating toxin A (VacA) and blood-group antigen-binding adhesin gene (BabA), can mimic and bind to specific receptors or surface molecules both on gastric epithelial cells and platelets, in which CagA and VacA may also be directly involved in loosening of TJ in monolayers of polarized gastric epithelial cells. It has been shown that a history of *Helicobacter pylori* infection is found in the majority of patients with GC, and that anti-CagA, anti-VacA and anti-BabA antibodies targeting both *Helicobacter pylori* components and host mimic molecules can be detected in them with increased levels. Patients with GC who are positive for *Helicobacter pylori* prospectively have a better outlook than those negative. The stimulation of mentioned autoantibodies in antigen processing and presentation and subsequent T-cell activation and proliferation improves host immune status. On the other hand, in an autoimmune response, autoantibodies can induce the cross-reaction against those localized or circulating GC cells, which are characterized by mimic or absorbed *Helicobacter pylori* antigens, and lead to the killing and even suppressing of metastasis of cancer cells (107).

### 8.6.1. Claudins in gastric cancer

A claudin-based gastric cancer classification system for gastric cancer has been proposed (108). The authors examined the expression of gastric (claudin-18) and intestinal (claudin-3 and claudin-4) claudins in non-neoplastic gastric mucosa (with intestinal metaplasia [IM], 78 cases; without IM, 88 cases) and 94 gastric cancers was analyzed immunohistochemically, as was the expression of gastric (MUC5A and MUC6) and intestinal (CD10 and MUC2) mucins. Heterogeneous expression of claudin-3, claudin-4 and claudin-18 was detected in advanced gastric cancer; however, there was no significant association between the claudins and the clinicopathological parameters. These gastric cancer tissues were also sub classified into claudin-based phenotypes: gastric claudin, 28 cases (30%); intestinal claudin, 41 cases (44%); and unclassified claudin, 25 cases (26%). Interestingly, the gastric cancers with unclassified claudin had worse malignancy grades, not only in size and invasiveness but

also in potential metastatic ability and patient outcome. Although the mucin-based gastric cancer classification was also assessed, no significant correlation was found between mucin production and clinicopathological parameters. These observations suggest that loss of claudin expression may enhance the grade of malignancy of gastric cancer *in vivo*. Classification of gastric cancers using gastric and intestinal claudins is a good biomarker for assessing the risk of poor prognosis. Quantitative real-time reverse transcriptase-polymerase chain reaction and immunohistochemistry has shown that claudin-7 is overexpressed in 10 Tff1-/- gastric dysplasia samples (109). Comparison with a serial analysis of gene expression database of human gastric cancer revealed similar deregulation in human gastric cancers. Quantitative real-time reverse transcriptase-polymerase chain reaction of human gastric adenocarcinoma samples indicated that, of these three genes, claudin-7 was the most frequently up-regulated gene. Using immunohistochemistry, both mouse and human gastric glands overexpressed claudin-7 in dysplastic but not surrounding normal glands. Claudin-7 expression was observed in 30% of metaplasia, 80% of dysplasia, and 70% of gastric adenocarcinomas. Interestingly, 82% of human intestinal-type gastric adenocarcinomas expressed claudin-7 whereas diffuse-type gastric adenocarcinomas did not. These results suggest that claudin-7 expression is an early event in gastric tumorigenesis that is maintained throughout tumor progression (109). Kuo *et al.* (110) continued to explore the roles of claudin-4 in the two histologically distinct types of gastric cancer; we selected 45 IGC (intestinal-type gastric cancer) and 48 DGC (diffuse-type gastric cancer) cases and then analyzed the expression of the protein using immunohistochemistry. The authors discovered that the overexpression of claudin-4 was greater in IGC than in DGC. A trend was observed between the overexpression of claudin-4 and lymph node metastasis, however, this association was not statistically significant. The results showed that the expression of claudin-4 was lower in DGC. Possibly it played a role in determining the diffuse phenotype and loose cohesion of cells in DGC in a similar manner as E-cadherin.

Resnick *et al.* (111) determined the expression pattern of claudins-1, -3, and -4 as well as ZO-1 in large series patients with gastric cancer and to correlate expression with clinicopathologic and prognostic variables. Tissue microarrays were created from paraffinized samples from 146 patients with distal gastric adenocarcinomas (61 intestinal and 85 diffuse or mixed subtypes). In addition, cores of normal mucosa and intestinal metaplasia were taken from most cases. The microarrays were stained for claudins 1, 3, and 4 and ZO-1, and the intensity of staining was determined using a 3-point scale. Moderate claudin 1 and ZO-1 membranous staining were present, whereas only focal weak claudin 3 and 4 membranous staining was present in normal gastric epithelium. Moderate to strong staining of claudins 1, 3, 4, and ZO-1 was disparate in intensity. Cox multivariate analysis revealed that tumor stage, diffuse subtype, and moderate to strong claudin 4 staining were associated with decreased survival. The authors state that these TJ proteins were strongly expressed

in most gastric intestinal-type adenocarcinomas but less frequently in diffuse gastric cancers. The up-regulation of claudin expression during gastric carcinogenesis suggests their potential utility as diagnostic biomarkers and possible targets for therapeutic intervention. Moreover, recent work by Jung *et al.* (112) suggests that claudin-3 and claudin-4 represent useful molecular markers for gastric cancer. Claudin-3 and claudin-4 would be the most important proteins related to the lymphatic invasion process, and claudin-4 would be useful with prognostic marker based on our results. Further investigations with a greater number of subjects are required to identify the action mechanism of claudin in gastric cancer.

Claudin-4 has been shown to activate MMP-2, indicating that claudin-mediated increased cancer cell invasion might be mediated through the activation of MMP proteins. Lee *et al.* (113) explored the roles of MMP-2, MMP-9 and claudin-4 in gastric cancer, selecting 88 cases. They found that all of MMP-2, MMP-9 and claudin-4 expressions were significantly higher in intestinal-type than in diffuse-type gastric cancer. On further analysis, testing the relationship between MMP-2 and MMP-9 expression with claudin-4 expression, claudin-4 expression was significantly associated with MMP-9 expression, but not with MMP-2 expression. The results showed that MMP-2, MMP-9 and claudin-4 expression may be phenotypic features, distinguishing intestinal-type and diffuse-type gastric cancer. Possibly, claudin-4 played a role in determining MMP-9 activity which favored intestinal-type gastric cancer to distal metastasis.

In addition to this, significant correlations have been demonstrated between the expression of claudin-4, occludin, and ZO-1 (114). In regard to claudin-4, significant correlations were seen between the expression of claudin-4 evaluated by immunohistochemistry and the expression of claudin-4 mRNA. Claudin-4 expression was significantly decreased in tumors with undifferentiated-type adenocarcinoma, advanced T stage, lymph node metastasis, and peritoneal metastasis. Occludin and ZO-1 expression was significantly decreased in tumors with undifferentiated-type adenocarcinoma. Overall survival was significantly shorter in patients with low claudin-4 expression. Cox multivariate analysis revealed that low claudin-4 expression was independently associated with significantly decreased overall survival. The authors conclude that TJ-associated proteins, particularly claudin-4, may play important roles in determining invasiveness, metastatic potential, and survival in gastric cancer.

Claudin-2 over-expression has been shown to be closely correlated to gastric carcinogenesis (115). 108 chronic superficial gastritis, 55 chronic atrophic gastritis, 109 intestinal-type metaplasia, 93 dysplasia and 52 gastric intestinal-type adenocarcinoma samples were analyzed and results indicated that the percentage of claudin-2-positive cases was 0% for chronic superficial gastritis (0/108), 0% for chronic atrophic gastritis (0/55), 0% for intestinal-type metaplasia (0/109), 35.87% for dysplasia (33/92), and 73.47% for gastric intestinal-type adenocarcinoma (36/49) respectively, primarily in the cell membrane, and gradually

increased in the multistage process of gastric carcinogenesis.

Park *et al.* (116) demonstrated that claudin-7 was up-regulated in gastric carcinoma. Claudin-7 was significantly more often expressed in intestinal metaplasia, adenoma and cancer than in normal gastric epithelium. Claudin-7 was more often unexpressed in diffuse type gastric cancer than in intestinal type. Compared to normal gastric epithelium, intestinal type gastric cancer significantly more often expressed claudin-7, but diffuse type did not. The expression pattern of claudin-7 did not change as cancer progressed. In this study we show that claudin-7 expression changed with the gastric carcinogenic process and that this is implicated in cancer characteristics.

Downregulation of claudin-18a2 is associated with gastric cancers of an intestinal phenotype; however, the mechanisms regulating its expression have not been defined. Yano *et al.* (117) analysed claudin-18 which has two alternatively spliced variants, claudin-18a1 and claudin-18a2 and are highly expressed in lung and stomach, respectively. The authors found that phorbol 12-myristate 13-acetate (PMA) treatment of MKN45 human gastric cancer cell line increased claudin-18a2 expression. They also determined to characterize the human claudin-18a2 promoter. Electrophoretic mobility shift assays and mutational analyses revealed that two activator protein (AP)-1 binding sites played an important role in the expression of claudin-18a2 in PMA-stimulated MKN45 cells. Protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) inhibitors suppressed the upregulation of claudin-18a2. These results indicated that the PKC/MAPK/AP-1 dependent pathway regulates claudin-18a2 expression in gastric cells.

In Epstein-Barr virus (EBV)-associated gastric carcinoma (GC) EBV-associated GC showed a high frequency of claudin-18 expression (84%) and a low frequency of claudin-3 expression (5%). This expression profile corresponded to that of normal gastric epithelium in adults and fetuses. Almost half of the EBV-associated GC cases demonstrated gastric mucin expression, whereas the other half lacked mucin or CD10 expression (118). In contrast, as demonstrated by the expression profiles of claudin-3 and claudin-18, EBV-negative GC comprised a heterogeneous group of four different claudin phenotypes: gastric, intestinal, mixed, and an undifferentiated type with variable expression patterns of mucins. These results indicate that EBV-associated GC is considerably homogenous with regard to cellular differentiation and that it preserves well the nature of the cells of origin. EBV-associated GC may undergo distinct carcinogenic processes, which differ from those of EBV-negative GC. Agarwal *et al.* (119) have reported that claudin-11 is silenced in gastric cancer via hypermethylation of its promoter region. Their work suggest that hypermethylation of claudin-11, leading to downregulated expression, contributes to gastric carcinogenesis by increasing cellular motility and invasiveness. A further understanding of the mechanisms underlying the role of claudin proteins in gastric carcinogenesis will likely help in the identification

of novel approaches for diagnosis and therapy of gastric cancer.

### 8.6.2. Occludin in gastric cancer

In the poorly differentiated gastric cancer cell line TMK-1, ZO-1 and occludin have been shown to be predominantly localized to the cytoplasm, although there is some weak expression at the cell-cell contact (120). Epidermal growth factor (EGF), a growth factor that is often overexpressed in gastric cancer causes ZO-1 and occludin to be rapidly translocated from the cytosol to the cell-cell contact. These effects induced by EGF were attenuated in the presence of protein kinase C (PKC) inhibitors calphostin C and bisindolylmaleimide I, but not another PKC inhibitor Gö6976, PD98059 (MAPK inhibitor), LY294002 (PI3 kinase inhibitor) or KT5720 (protein kinase A inhibitor). Yoshida *et al.* (120) suggest that EGF can rapidly alter the localization of ZO-1 and occludin via a protein kinase C signalling pathway in TMK-1 gastric cancer cells. *In situ* hybridization was used to evaluate the expression of occludin mRNA in 42 gastric carcinoma specimens obtained by surgery and 23 relatively normal gastric mucosa obtained by gastric endoscopy [86]. Occludin mRNA was found positive in the cytoplasm of gastric glandular epithelia as blue particles with intensive stain in 14 of 42 gastric carcinomas (33.3%), 23 of 42 paracancerous gastric tissues (54.8%), 14 of 23 relatively normal gastric tissues (60.9%), 9 of 16 well differentiated carcinomas (56.3%), 4 of 14 moderately differentiated carcinomas (28.6%), 1 of 10 poorly differentiated carcinomas (10.0%) and none of 2 mucosal carcinomas. There were significant differences in occludin mRNA positive rate between relatively normal gastric tissue and gastric cancer as well as between paracancerous gastric tissue and gastric cancer. The expression of occludin mRNA in moderately and poorly differentiated groups was gradually reduced when compared with well differentiated group, which suggests that there be a significant correlation between tumor differentiation and the expression of occludin mRNA. Furthermore, the positive signals of occludin mRNA distributed extensively in the cytoplasm of SGC7901/VCR cell, being vincristine resistant, derived from parental gastric cell line SGC7901. The positive signals of SGC7901/VCR were stronger than those of SGC7901 cells. The authors suggest that occludin mRNA, being mainly located in epithelial cells and its expression correlated with tumor differentiation, may be involved in the development of multi-drug resistance in gastric cancer.

### 8.6.3. Tricellulin in gastric cancer

Masuda *et al.* (121) have recently shown that abundant tricellulin expression was detected in all GC-derived cells examined. In HSC-45 cells, transduction of Snail decreased the expression levels of tricellulin and E-cadherin but increased vimentin and N-cadherin, which was accompanied by induction of EMT transcription factors such as Twist1, Twist2 and Slug. In normal gastric mucosa, tricellulin protein was localized at the tricellular TJ. In HSC-45 cells however, tricellulin protein was distributed in the cytoplasm. In GC tissues, tricellulin expression at the cellular membrane was retained in a subset of EMT-negative GCs, and it disappeared in EMT-positive GCs.



They conclude that repression of tricellulin expression may be related to Snail-induced EMT in human GCs.

### 8.7. Gynaecological cancers

#### 8.7.1. Claudins in gynaecological cancer

Cell-cell and cell-extracellular matrix interaction is crucial in tumor progression (122). The expression of occludin and claudins (and syndecan-1) in early stage cervical carcinogenesis showed that occludin and claudin-2 were found colocalized in the basal layer, while syndecan-1 and claudins-1, -4 and -7 were coexpressed in the parabasal and intermediary layers in normal epithelia. Intensity of occludin staining decreased in CIN/CIS lesions, although it was more extended towards the upper epithelial layers with inverse relation with grades, as seen in the case of claudin-2 expression. Claudin-1, -2, -4 and -7 were detected in the entire epithelium in CIN, showing decrease in CIS. The progression of CIN was associated with reduced syndecan-1 expression, in contrast to claudin-1, -4 and -7 which increased toward CIS. It is thought that significant changes occur in the composition of cell adhesion complexes even in early stages of cervical carcinogenesis (123). In ovarian cancer, laboratory generated human ovarian surface epithelial (HOSE) cells constitutively expressing wild-type claudin-3 and claudin-4 (124). Expression of these claudins in HOSE cells increased cell invasion and motility. Conversely, knockdown of claudin-3 and claudin-4 expression in ovarian cancer cell lines reduced invasion. Claudin expression also increased cell survival in HOSE cells but did not significantly affect cell proliferation. Moreover, the claudin-expressing ovarian epithelial cells were found to have increased matrix metalloproteinase-2 (MMP-2) activity indicating that claudin-mediated increased invasion might be mediated through the activation of MMP proteins. siRNA inactivation of claudins in ovarian cancer cell lines did not have a significant effect on the high endogenous MMP-2 activity present in these cells. This could be construed as malignant cells having alternative or additional pathways to fully activate MMP-2 and suggests that claudin overexpression may promote ovarian tumorigenesis and metastasis due to increased invasion and survival of tumor cells.

Claudin-7 has been found to have highly differential expression in ovarian carcinoma (124). 110 patients with various histologic types of epithelial ovarian carcinomas were studied, with claudin-7 transcript found significantly overexpressed in both primary and metastatic tumors compared to normal human ovarian surface epithelium cell lines. At the protein level, claudin-7 expression was found significantly higher in tumors of primary and metastatic origin when compared to normal ovaries, regardless of the histologic type, the grade of differentiation, and the pathologic stage of the disease. Claudin-7 was thus found to be significantly overexpressed in all main histologic types of epithelial ovarian cancer and in single neoplastic cells disseminated in peritoneal cavity and pleural effusions, suggesting its potential role as novel diagnostic marker in ovarian cancer. Claudin-4 is also overexpressed in epithelial ovarian cancer (125). Claudin-4 overexpression did not correlate with survival or other clinical endpoints and is associated with hypomethylation.

Claudin-4 overexpression did correlate changes in barrier function by treatment with the *Clostridium perfringens* enterotoxin in a dose- and claudin-4-dependent noncytotoxic manner. There is a weak or absence of expression of claudin-3 and claudin-4 in surface human ovarian surface epithelium changed to typical cell-border localisation with inclusion cysts in the normal ovarian stroma (126). Semiquantitative estimations of immunoblots showed that claudin-3 was significantly increased in ovarian adenocarcinomas compared to benign and borderline-type tumors. Claudin-4 was significantly increased in both borderline-type and ovarian adenocarcinomas compared to benign tumors, whereas no changes were found for claudin-1 or -5. Claudin-3, but not claudin-4, was significantly increased in moderately, poorly and undifferentiated adenocarcinomas compared to well differentiated and borderline-type tumors (FIGO grade). The authors concluded that both claudin-3 and -4, even though they differ in expression during ovarian malignant transformation, might be used as novel markers for ovarian tumors. A small region of the claudin-4 promoter is critical for its expression (127). This region contains two Sp1 sites required for promoter activity. However, because of the ubiquitous expression of Sp1, these sites, although necessary, are not sufficient to explain the patterns of gene expression of claudin-4 in various ovarian tissues. The claudin-4 promoter was found to be further controlled by epigenetic modifications of the Sp1-containing critical promoter region. Cells overexpressing claudin-4 exhibit low DNA methylation. The authors conclude that as claudin-4 is elevated in a large fraction of ovarian cancer, the mechanism leading to deregulation may represent a general pathway in ovarian tumorigenesis and may lead to novel strategies for therapy and an overall better understanding of the biology of this disease (127). D'Souza *et al.* (128) showed that claudin-3 and -4 can be phosphorylated in ovarian cancer cells, suggesting that claudin-3 phosphorylation by PKA, a kinase frequently activated in ovarian cancer, may provide a mechanism for the disruption of TJ in this cancer. These results have general implications for the regulation of TJ in normal epithelial cells.

Choi *et al.* (129) examined the gene expression of claudin-3 and -4 in various tumors, including 19 normal ovaries and 47 ovarian carcinomas with a further 114 ovarian serous tumors, including 10 adenomas, 20 borderline tumors and 84 carcinomas analyzed immunohistochemically. Claudin-3 and -4 transcripts were significantly up-regulated by 5-fold or more in most subtypes of ovarian epithelial carcinomas while the immunohistochemical analyses indicated that each protein was expressed in 68 (81.0%) and 72 (85.7%) of 84 serous adenocarcinomas, respectively. Borderline serous tumors and adenomas showed significantly lower expression of these proteins than the adenocarcinomas. Kaplan-Meier survival analysis showed that serous adenocarcinoma patients with high claudin-3 expression had substantially shorter survival ( $P=0.027$ ). Multivariate analysis demonstrated that claudin-3 overexpression is an independent negative prognostic factor. These findings suggest that claudin-3 overexpression can be used as a

prognostic indicator in ovarian serous carcinomas and may be a promising target for antibody-based therapy of ovarian carcinomas.

Additionally, Zhu and Sundfeldt (130) found expression for claudin-1, claudin-3, claudin-4 and ZO-1 with a typical honeycomb-staining pattern in serous adenocarcinomas indicating proper TJ formation. Clear-cell and endometrioid adenocarcinomas showed a different expression pattern. With the exception of claudin-4, claudins are upregulated in ovarian carcinoma effusions compared with solid tumors, in agreement with our previous data for cadherins and integrins in this cancer type, suggesting a prosurvival role for these surface molecules. Claudin-3 and claudin-7 expression in effusions independently predicts poor survival in ovarian cancer (131). In a recent study (132) it has been shown that the expression of claudin-3 and claudin-4 is repressed in ovarian epithelial cells in association with promoter 'bivalent' histone modifications, containing both the activating trimethylated histone H3 lysine 4 (H3K4me3) mark and the repressive mark of trimethylated histone H3 lysine 27 (H3K27me3).

During ovarian tumorigenesis, derepression of claudin-3 and claudin-4 expression correlates with loss of H3K27me3 in addition to trimethylated histone H4 lysine 20 (H4K20me3), another repressive histone modification. Although claudin-4 repression is accompanied by both DNA hypermethylation and repressive histone modifications, DNA methylation was not required for claudin-3 repression in immortalized ovarian epithelial cells. This study strongly suggests that in addition to the well-known chromatin-associated silencing of tumor suppressor genes, epigenetic derepression by the conversely related loss of repressive chromatin modifications also contributes to ovarian tumorigenesis via activation of cancer-promoting genes or candidate oncogenes (132).

Claudin-3 and claudin-4 may be differentially expressed in uterine serous papillary carcinoma (USPC) (133). The rate of claudin-3 and claudin-4 expression was significantly higher in USPC and clear-cell endometrial cancer compared to endometrioid endometrial cancer. Furthermore, expression of both TJ proteins was significantly associated with poor clinical outcome. However, both markers did not maintain prognostic independence in multivariate analyses, as their expression was tightly associated with more advanced disease stages, and higher nuclear grade. The authors conclude that these clinical observations confirm the hypothesis based on preclinical evidence that increased expression of claudin-3 and claudin-4 may contribute to the aggressive phenotype of endometrial cancer of serous papillary or clear-cell histology and suggest their potential utility as diagnostic biomarkers and possible targets for therapeutic intervention.

Pan *et al.* (134) sought to clarify the roles of claudins in endometrial tumorigenesis. Expression of claudin-3 and claudin-4 was significantly increased in the

groups of atypical hyperplasia and endometrioid adenocarcinoma compared with the groups of complex hyperplasia, simple hyperplasia, and normal cyclic endometrium at both protein and mRNA levels. The highest expression was observed in endometrioid adenocarcinoma. Although no relevance was found with regard to FIGO stage and histologic grade, overexpression of claudin-3 and claudin-4, especially claudin-4, significantly correlated with myometrial invasion. Transmission electron microscopy analysis indicated morphologic disruptions of TJs may lag behind the increase of claudins expression. These results demonstrate that claudin-3 and claudin-4 are strongly expressed in atypical hyperplasia and endometrioid adenocarcinoma, but less frequently in normal endometrium. The upregulation of claudins expression during endometrial carcinogenesis suggests their potential utility as diagnostic and prognostic biomarkers.

It has been concluded (135) that the expression data on claudins in different premalignant and malignant alterations suggest that these proteins might serve as diagnostic and prognostic markers and might be targets for future therapy.

### 8.7.2. Occludin in gynaecological cancer

Normal endometrial glands and samples of endometrial hyperplasia and endometrioid carcinoma grade 1 fully expressed occludin at the apical cell border (55). In endometrioid carcinomas grades 2 and 3, however, occludin disappeared in solid areas of the carcinomatous tissues. Occludin was also found at the apical borders of the cancer cells that formed glandular structures. Occludin expression decreased progressively in parallel with the increase in carcinoma grade, and the decreased occludin expression correlated with myometrial invasion and lymph node metastasis. These results suggest that the loss of TJ has a close relationship with structural atypia in the progression of human endometrial carcinomas and their malignant potential (136).

### 8.7.3. CAR in gynaecological cancer

Upon investigation of the 4 CAR isoforms in ovarian cancers it was found that expression levels of all CAR isoforms were elevated in ovarian carcinomas as compared with those of non-malignant controls (137). Moreover, expression of the soluble isoforms CAR 3/7 and CAR 4/7 but not that of hCAR was significantly increased in advanced ovarian cancer as revealed by a highly significant correlation with FIGO stage and residual disease. Soluble CAR isoforms 3/7 and 4/7 may play a pivotal role in ovarian cancer biology, possibly by counteracting migration- and growth-inhibitory properties of the membranous hCAR and thus favoring cancer cell dissemination throughout the peritoneal cavity.

### 8.7.4. JAM in gynaecological cancer

In human endometrial carcinoma, JAM-A immunostaining intensity was negatively correlated with histologic grade, myometrial invasion and stage (138). Low JAM-A immunostaining intensity was associated with positive vascular space involvement. Moreover, low immunostaining intensity was significantly related to low overall survival rate and progression-free survival rate.

JAM-A expression in poorly differentiated adenocarcinoma was significantly lower than that in well-differentiated adenocarcinoma. JAM-A expression seems to be reduced in high-grade or advanced endometrial carcinoma and may be a prognostic factor.

### 8.8. Prostate cancer

#### 8.8.1. Claudins in prostate cancer

There is only one reported study documenting the pattern of claudin expression in prostatic adenocarcinomas (139). Decreased expression of claudin-1 correlated with high tumor grade and biochemical disease recurrence, whereas decreased claudin-7 correlated with high tumor grade. In contrast, expression of claudin-3 correlated with advanced stage tumors and recurrence and expression of claudin-4 correlated with advanced stage. On multivariate analysis, advanced stage and decreased claudin-1 protein expression independently predicted disease recurrence. The authors conclude that immunohistochemical expression and prognostic significance of claudins are variable in prostatic adenocarcinomas, with decreased expression of claudin-1 emerging as an independent prognostic variable warranting further research. An earlier study found that occludin was also lost in polygonal (unpolarized) cells of Gleason grades 4 and 5, but remained expressed in all cells facing a lumen in all grades of cancer (140). Downregulation of occludin in prostate cancer was thus seen to be associated with loss of cell polarity and coincides with the formation of the complex glandular architecture of Gleason grade 4 pattern or complete loss thereof in Gleason grade 5 patterns.

Long *et al.* (136) investigated the expression of claudin-3 and claudin-4 in human prostate tissue as potential targets for CPE toxin-mediated therapy for prostate cancer. On human multiple-tissue Northern blot analysis, mRNAs for both claudin-3 and claudin-4 were expressed at high levels in prostate tissue. In normal prostate tissue, expression of claudin-3 was localized exclusively within acinar epithelial cells by *in situ* mRNA hybridization. Compared with expression within prostate epithelial cells in surrounding normal glandular tissue, expression of claudin-3 mRNA remained high in the epithelium of prostate adenocarcinoma (10 of 10) and prostatic intraepithelial neoplasia (5 of 5). Prostate adenocarcinoma cells metastatic to bone were obtained from a patient with disease progression during anti-androgen therapy. These metastatic cells were prostate-specific antigen-positive by immunohistochemical staining and also expressed functional CPE receptors. The persistent high level of claudin-3 expression in prostate adenocarcinoma and functional cytotoxicity of CPE in metastatic androgen-independent prostate adenocarcinoma suggests a new potential therapeutic strategy for prostate cancer.

Zheng *et al.* (141) described two forms of claudin-7, a full length form of with 211 amino-acid residues and a C-terminal truncated form with 158 amino-acid residues. These two forms of are able to regulate the expression of a tissue-specific protein, the prostate-specific antigen (PSA), in the LNCaP prostate cancer cell line. The authors also found that the expression of claudin-7 was

responsive to androgen stimulation in the LNCaP cell line, suggesting that this protein is involved in the regulatory mechanism of androgen. Both forms of claudin-7 were expressed in human prostate, kidney and lung samples, and in most samples, the full-length form of claudin-7 was predominant. However, in some prostate samples from healthy individuals, the truncated form of claudin-7 is predominantly expressed. It appeared that unlike other claudins, claudin-7 has both structural and regulatory functions, and the two forms of claudin-7 may be related to cell differentiation in organ development.

### 8.9. Lung cancer

Paschoud *et al.* (142) found a statistically significant correlation between diagnosis and positivity of tumors with either claudin-1 or claudin-5. Squamous cell carcinomas and basal cells of bronchial epithelium were positive for claudin-1 and negative for claudin-5, whereas adenocarcinomas, normal cylindrical cells and pneumocytes were positive for claudin-5 and negative for claudin-1, suggesting different pathways in tumor development and progression. Claudin-4 and ZO-1 staining were detected in both types of tumors, whereas cingulin was not detected in squamous cell carcinomas. In squamous cell carcinomas, there were statistically significant decreases in the mRNA levels of JAM-1, occludin, claudin -3, claudin -4, claudin -7, cingulin, ZO-2 and ZO-3, and an increase in claudin -1 mRNA. In adenocarcinomas, when transcript levels were compared with bronchial cells, there were also statistically significant decreases in the mRNA levels of claudin -1, claudin -3, claudin -4, claudin -7, ZO-2 and ZO-3. These results indicate that characterization of TJ protein expression in human lung tumors can be an additional diagnostic tool and provide new insights on their histogenesis. In small cell lung carcinomas, differential expression was confirmed for claudin-1 in 82.1% of lung tumor tissues, by quantitative real-time reverse transcription-PCR analysis (143).

#### 8.9.1. Claudins in lung cancer

Low-claudin-1 mRNA expression with shorter overall survival was found in 64 patients with lung adenocarcinoma (144). Affymetrix microarrays identified a panel of genes altered by claudin-1 overexpression. Claudin-1 increased expressions of cancer invasion/metastasis suppressors (e.g., connective tissue growth factor [CTGF], thrombospondin 1 [THBS1], deleted in liver cancer 1, occludin, ZO-1) and suppressed expressions of invasion/metastasis enhancers, cut-like homeobox 1, transforming growth factor alpha, solute carrier family 2 [facilitated glucose transporter] member 3, placental growth factor), supporting a role for claudin-1 as an invasion and metastasis suppressor. Claudin-1 might also be a useful prognostic predictor and potential drug treatment target for patients with lung adenocarcinoma.

Jung *et al.* (145) examined the expression of claudin-1, claudin-3, claudin-4, and claudin-5 proteins using immunohistochemical analysis in 14 normal lung tissue samples and 171 NSCLC samples. All of the claudin proteins examined were expressed in normal bronchial epithelial cells. In the normal peripheral parenchyma, only

## TJ and metastasis

claudin-5 strongly stained most of the pneumocytes. Claudin-1 expression was stronger in squamous cell carcinomas than in adenocarcinomas, whereas claudin-4 and claudin-5 expression was stronger in adenocarcinomas. Clinically, expression of claudin proteins was not found to be associated with patient survival. These data suggest that the disruption of tight junction protein might be involved in the development of these tumors.

### 8.9.2. Occludin in lung cancer

An earlier study of 68 lung carcinomas and surrounding normal lung tissues found that in normal lung tissues occludin strongly stained the apicoluminal borders of the bronchial/bronchiolar epithelia and bronchial glands as a dot or short line (56). Occludin also stained the intercellular borders of alveolar epithelia. In cancer cells that faced lumina of all adenocarcinomas, regardless of grade, including bronchioloalveolar carcinomas, occludin showed an expression pattern identical to that of the normal bronchial and alveolar epithelia. Occludin reactivity was not noted in any cases of squamous cell carcinoma, large cell carcinoma, small cell carcinoma, or large cell neuroendocrine carcinoma (56). It was suggested that occludin could serve as an immunohistochemical indicator of the “true” glandular differentiation that forms tubulo-papillary structures in human lung carcinoma tissues.

### 8.9.3. CAR in lung cancer

Yamashita *et al.* (146) examined CAR expression in lung cancer. In lung metastasis, the colony number of B16 cells stably expressing CAR (B16CAR) was significantly lower than that of the control CAR-negative B16 cells. B16 and CT26 cells transiently expressing CAR, which were transduced with adenovirus (Ad) vector expressing CAR, also reduced lung metastasis, suggesting that CAR plays a role in the early stage of metastasis. CAR expression significantly decreased the accumulation of B16 cells in the lung after i.v. injection and the migration *in vitro*. CAR expression reduced expression of  $\alpha(v)$ ,  $\alpha(4)$ ,  $\beta(3)$  and  $\beta(1)$  integrin, which play important roles in attachment to cells or basement membrane. Thus, CAR expression likely acts as a metastatic suppressor.

## 8.10. Melanoma

### 8.10.1. Claudin in melanoma

Leotela *et al.* (147) used tissue microarray technology to reveal that claudin-1 was overexpressed in melanoma, and aberrantly expressed in the cytoplasm of malignant cells, suggesting a role other than transport. Indeed, melanoma cells in culture demonstrate no TJ function. It has been shown that protein kinase C (PKC) can affect expression of claudin-1 in rat choroid plexus cells, and there was a correlation between levels of activated PKC and claudin expression. It was subsequently found that PKC activation by PMA caused an increase in claudin-1 transcription and protein. Inhibition of PKC signalling in cells with high claudin-1 expression resulted in decreased claudin-1 expression. Transient transfection of melanoma cells with claudin-1 increased MMP-2 secretion and activation, and subsequently, motility of melanoma cells. Conversely, knockdown of claudin-1 resulted in the

inhibition of motility, as well as decreases in MMP-2 secretion and activation, implicating claudin-1 in melanoma progression. Cohn *et al.* (148) also concluded that loss of claudin-1 may play a significant role in the acquisition of metastatic phenotype in cutaneous melanoma.

## 8.11. Pancreatic cancer

### 8.11.1. Claudins in pancreatic cancer

There has been found to be a correlation between TJ and cancer cell dissociation, as well as the involvement of MEK2 in regulation of TJ in cell dissociation of pancreatic cancer (149). After incubation with conditioned medium of PC-10 cells, plasma membrane distribution of claudin-1 was obviously disrupted, and expressions of MEK2 and p-MEK1/2, as well as dissociation of cell colonies, were significantly induced in PC-1 and CAPAN-2 cells. However, U0126 (a MEK1/2 inhibitor) treatment apparently induced the plasma membrane distribution of claudin-1 and aggregation of single cells in PC-1 and AsPC-1 cells, synchronously seriously suppressed MEK2 and p-MEK1/2 expression. Arrangement of expression and distribution of claudin-1 is closely related to cell dissociation status in pancreatic cancer cells through MEK2 activation.

Borka *et al.* (13) analyzed protein and mRNA expressions of different claudins in human pancreatic endocrine tumors and ductal adenocarcinomas. Normal acini and ducts showed strong claudin-1, -3, -4, and -7 and scattered claudin-2 protein expressions, while Langerhans islands revealed only claudin-3 and -7 expressions. Claudin-2 expression was found in the half of ductal adenocarcinomas, while the vast majority of endocrine tumors were negative. Claudin-1, -4, and -7 immunohistochemistry were positive in all adenocarcinomas, whereas endocrine tumors were completely negative for claudin-1 and -4. Claudin-3 and -7 proteins were detected in all endocrine tumors, while claudin-13 in ductal adenocarcinomas was negative. The mRNA expression of claudins showed differences between endocrine tumors and ductal adenocarcinomas, with high expressions of claudin-3 in endocrine tumors and claudin-4 in ductal carcinomas which make them attractive targets for adjuvant therapy. A separate study (150) compared different claudin-1, -2, -3, -4 and -7 expression patterns in normal pancreas cells, pancreatic endocrine tumors, adenocarcinomas, mucinous cystic tumors and acinar cell carcinomas. In addition to the well-known claudin-1 and -4 expression claudin-2, -3 and -7 proteins were demonstrated in ductal cells, while claudin-3 and -7 proteins showed expression in acinar cells. Expression of claudin-3 and -7 was manifest in endocrine cells. Claudin-3 and -7 showed high expression in endocrine tumors, claudin-1, -2, and -4 proteins in exocrine tumors. The level of claudin-1, -4 and -7 protein expression in borderline cystic tumors was between that of benign and malignant tumors. This supports the sequential development theory regarding mucinous cystic tumors. The authors concluded that the presence of claudin-3 refers to endocrine differentiation. The increased claudin-4 expression in adenocarcinomas and mucinous cystic tumors, as well as the high claudin-3

expression in endocrine tumors may open up new prospects in the targeted therapy of these tumors. Moreover, the claudin expression pattern in pancreas tumors may be employed in the differential diagnosis of these tumors. Solid-pseudopapillary tumor (SPT) of the pancreas is characterized by a discohesive appearance of the neoplastic cells (151). SPT shows a peculiar claudin expression profile and the highly specific pattern of claudins -5 and -7 differentiates SPT from PET, ACC, and PB.

### 8.12. Oral cancer

#### 8.12.1. Claudins in oral cancer

Oku *et al.* (152) investigated whether claudin-1 regulated invasion activity in oral squamous cell carcinoma (OSC) cells. Compared with OSC-7, both OSC-4 and NOS-2 more strongly expressed claudin-1 and possessed high activities of MMP-2 and MMP-9. Tumors formed in the tongues of SCID mice xenografted with OSC-4, NOS-2, and OSC-7 immunohistochemically revealed strong, moderate, and weak expression of laminin-5; 2 chains, respectively, and laminin-5; 2 chains were secreted in the conditioned medium of the cancer cells in parallel with the *in vivo* results. Claudin-1 siRNA largely suppressed the invasion of OSC-4 and decreased the activation of MMP-2, the expression of membrane-type MMP-1 (MT1-MMP), and the cleavage of laminin-5;2. These results suggest that claudin-1 upregulates cancer cell invasion activity through activation of MT1-MMP and MMP-2, which results in enhanced cleavage of laminin-5;2 chains.

Bello *et al.* (153) analyzed the distribution patterns of claudins-1, -4, -5, and -7 and occludin in the superficial and invasive front of squamous cell carcinoma of the tongue of 97 patients and their relationship to cause-specific (squamous cell carcinoma of the tongue) patient survival (median follow-up period of 33.5 months; range, 1-234 months). Claudins-1 and -7 were strongly expressed, claudin-4 had moderate expression, whereas claudin-5 was least expressed. The authors suggest that analyzing the immunohistologic staining levels of claudin-7 could be used for the prognostic purposes in patients with tongue squamous cell carcinoma.

### 8.13. Liver and hepatocellular cancer

VEGF induces a marked loss of pseudocanalculi and disruption of occludin-delineated tight junctions in HepG2 cells. This effect of VEGF was mimicked by phorbol-12-myristate-13-acetate (PMA) and was sensitive to protein kinase C (PKC) inhibition by Go<sup>6850</sup>. VEGF also induced the translocation of the PKC $\alpha$ -isoform to the plasma-membrane, but had no effect on the activity of Erks and p38MAPK. Sections from surgically removed human HCC showed expression of VEGF in the tumor and occludin disassembly in normal liver parenchyma next to the tumor (154). In conclusion, VEGF induced disruption of TJ in a PKC- $\alpha$  dependent manner. In addition to its known angiogenic properties, the authors suggest that VEGF may promote HCC spreading into normal liver parenchyma. The data may provide another rationale for the use of VEGF antagonists for tumor therapy.

In the liver, an array of TJ molecules is localized along the bile canaliculi forming the blood-biliary barrier, where they play pivotal roles in paracellular permeability, bile secretion, and cell polarity. In pathology, certain hepatic TJ molecules mediate virus entry causing hepatitis infection; deregulation and functional abnormality of the TJ have also been implicated in triggering liver cancer development and metastasis (155).

#### 8.13.1. Claudins in liver and hepatocellular cancer

Ip *et al.* (156) investigated claudin-10 function in two different hepatocellular carcinoma cell lines observing that overexpression of claudin-10 conferred malignant phenotypes to hepatocellular carcinoma cells, Hep3B, which lack claudin-10 expression, by promoting cancer cell survival, motility, and invasiveness. More importantly, MMP2 was up-regulated. Increase in mRNA transcription and protein expression of membrane type 1-MMP (MT1-MMP) was also observed in the claudin-10 transfectants, and in addition, claudin-1, claudin-2, and claudin-4 were up-regulated in claudin-10 overexpression transfectants, indicating that the expression of claudin-10 in cancer cells might affect the expression levels of its family members. On the contrary, small interfering RNA-based knockdown of claudin-10 in HLE, an invasive cell line with high level of claudin-10 expression, abolished invasion and strongly decreased activation of MMPs and claudin member's expression.

Claudin-1 plays a causal role in the acquisition of invasive capacity in human liver cells and c-Abl-protein kinase Cdelta (PKCdelta) signaling is critical for the malignant progression induced by claudin-1 (157). Overexpression of claudin-1 induced expression of matrix metalloproteinase-2 (MMP-2) and cell invasion and migration in normal liver cells as well as in non-invasive human hepatocellular carcinoma (HCC) cells. Conversely, small interfering RNA targeting of claudin-1 in invasive HCC cells completely inhibited cell invasion. Both c-Abl and PKCdelta are found to be activated in normal liver cell line clones that stably overexpress claudin-1. Inhibition of either c-Abl or PKCdelta alone clearly attenuated MMP-2 activation and impeded cell invasion and migration in both human HCC and normal liver cells expressing claudin-1 (157). The present observations raise the possibility of exploiting claudin-1 as a potential biomarker for the spread of liver cancer and might provide pivotal points for therapeutic intervention in HCC (157).

#### 8.13.2. Occludin in Liver and hepatocellular cancer

Persistent hepatitis C virus (HCV) infection is a primary etiological factor for the development of chronic liver disease, including cirrhosis and cancer (158). In normal human liver variable expression of alternative splice variants of occludin was observed, including two known forms (WT-OCN and OCN-ex4del) and six novel forms (OCN-ex7ext, OCN-ex3pdel, OCN-ex3del, OCN-ex3-4del, OCN-ex3p-9pdel, and OCN-ex3p-7pdel). This study suggests that the remarkable natural splicing diversity of occludin might contribute to HCV tissue tropism and possibly modify the outcome of HCV infection in humans.

**8.14. Synovial cancer****8.14.1. Claudins in synovial cancer**

Synovial sarcoma, a soft tissue sarcoma that develops in adults, is pathologically subclassified into monophasic spindle synovial sarcoma and biphasic synovial sarcoma with epithelial components (159). Expression profiles of 21 claudins in 17 synovial sarcoma tumor samples, including 9 biphasic tumors, identified claudin-4, claudin-7, and claudin-10 as biphasic tumor-related claudins, and immunohistochemical analyses demonstrated the localization of these claudins in the epithelial component in biphasic tumors, with claudin-7 the most closely associated with the epithelial component. Inhibition of ELF3 expression by small interfering RNA simultaneously down-regulated the mRNA expression of the claudin-7 gene and the introduction of ELF3 expression in claudin-7-negative cell lines induced mRNA expression of the claudin-7 gene. Therefore, the induction of claudin-7 expression by ELF3 appears critical to the formation of the epithelial structures in biphasic synovial sarcoma (159).

**8.15. Thyroid cancer****8.15.1. Claudins in thyroid cancer**

In ninety-one thyroid neoplasms (15 follicular adenomas, 15 follicular carcinomas, 26 papillary carcinomas, 16 papillary microcarcinomas, 8 medullary carcinomas, 3 poorly differentiated carcinomas, and 8 undifferentiated carcinomas) occludin was mainly expressed in the form of intracytoplasmic vesicles, whereas all claudins tested exhibited membranous immunostaining (160). Thirteen out of 15 follicular adenomas, 10/15 follicular carcinomas, 24/26 papillary carcinomas, 15/16 papillary microcarcinomas, 1/8 medullary carcinomas, 2/3 poorly differentiated carcinomas and 2/8 undifferentiated carcinomas exhibited claudin-1 expression, whereas claudin-4 was expressed in 13/15, 12/15, 23/26, 13/16, 7/8, 2/3 and 2/8 of the tumors, respectively, and claudin-7 expression was found in 67, 33, 73, 69, 25, 0 and 13% of the cases, respectively (160). Occludin was expressed in 100% follicular adenomas, 80% follicular carcinomas, 96% papillary carcinomas, 50% papillary microcarcinomas, 50% medullary carcinomas, 33% poorly differentiated carcinomas and 88% undifferentiated carcinomas. Occludin expression was reduced in papillary microcarcinomas, medullary carcinomas and poorly differentiated carcinomas. All claudins exhibited reduced expression in undifferentiated carcinomas (160). Claudin-1 was additionally reduced in medullary carcinomas and claudin-7 in follicular, medullary and poorly differentiated carcinomas. A correlation between loss of claudin-1 expression and worse disease-free survival was noted on univariate analysis. The authors suggest that dedifferentiation of the thyroid carcinomas is accompanied by reduction in claudin-1, -4 and -7 expression. A differential expression of TJ proteins in the different histologic types of thyroid gland is noted. Additionally, claudin-1 expression may be an important prognostic indicator of recurrence in thyroid carcinomas (160).

Recently a study aimed at determining the pattern of claudin-1 expression in various types of thyroid lesions at the protein level and investigating the

immunolocalization of beta-catenin reported to regulate claudin-1 expression was published (161). Samples included 19 PTCs, ten cases of corresponding regional lymph node metastasis, eight papillary microcarcinomas (PMC), 17 follicular thyroid carcinomas (FTC) and 19 follicular adenomas (FA). All cases were evaluated by quantitative immunohistochemistry. Conspicuous claudin-1 immunostaining was detected in the majority of PTC/PMC primary tumors and lymph node metastases (19/27 and 9/10, respectively). On the other hand, the authors found weak or no claudin-1 expression in any of the FA and FTC cases or peritumoral non-malignant thyroid tissues. This shows that high claudin-1 protein expression is specific for PTC and its regional lymph node metastases and that claudin-1 may be a useful tumor marker for PTC.

**8.16. Neurofibroma****8.16.1. Claudins in neurofibroma**

In a study of 16 neurofibromas from 12 patients with neurofibromatosis type 1 (NF1) cell-cell contacts with typical ultrastructural morphology of TJ were seen between adjacent perineurial cells surrounding the small nerves and between contacting perineurial cell processes embedded in tumor stroma (162). Immunohistochemistry showed expression of claudin-1, claudin-3, and ZO-1 in the intercellular junctions of a subpopulation of tumor cells. Occludin was present mainly in perineurium and claudin-5 localized to the blood vessels. Claudin-1 positive cells were also positive for type IV collagen and epithelial membrane antigen but not for S-100 protein a labelling pattern consistent with a perineurial cell phenotype. Using claudin-1 as a marker, the authors showed that clusters of perineurial cells are distributed around the rudimentary nerves within cutaneous neurofibromas and at the periphery of some neurofibromas (162).

**8.17. Renal cancer****8.17.1. Claudins in renal cancer**

Claudin-1 has been found to be expressed in 29.9% of renal cell cancer cases (163). Whereas the vast majority of clear cell carcinomas were negative for claudin-1, most papillary tumors (76-86%) were positive. Claudin-1 expression was associated with markers of unfavorable tumor biology in clear cell renal cell carcinoma, whereas the opposite was valid for papillary renal cell carcinoma. In clear cell renal cell carcinoma claudin-1 positivity was a prognosticator of shortened disease-specific patient survival in univariate analysis, which also remained significant in multivariate analyses in the clinically important subgroups of nonmetastasized or asymptomatic patients. It was concluded that claudin-1 is expressed in the majority of papillary renal cell carcinomas, suggesting a diagnostic value of this marker. Its expression is an independent prognosticator of shortened disease-specific patient survival in clinically relevant subgroups of clear cell renal cell carcinoma. Claudin-7 and claudin-8 have been considered as candidate markers to distinguish chromophobe renal cell carcinoma from other renal tumors, including oncocytoma (164). Claudin-7 protein was expressed in a membranous pattern in 10 of 11 chromophobe renal cell carcinomas and 4 of 17

oncocytomas. Claudin-8 was expressed in multiple patterns: In oncocytoma, 11 of 17 cases showed cytoplasmic, 4 of 17 membranous, and 2 of 17 negative reactions. In chromophobe renal cell carcinoma, 0 of 11 cases showed cytoplasmic, 3 of 11 membranous, and 8 of 11 negative reactions. The immunohistochemical pattern of membranous claudin-7 and negative claudin-8 was seen in 7 of 11 chromophobe renal cell carcinomas and 1 of 17 oncocytomas. Negative claudin-7 and cytoplasmic claudin-8 were observed in 10 of 17 oncocytomas and 0 of 11 chromophobe renal cell carcinomas. The distal nephron proteins claudin-7 and claudin-8 have potential use as immunohistochemical biomarkers in the differential diagnosis of chromophobe renal cell carcinoma and oncocytoma. Expression of claudin-7 and claudin-8 may reflect the relationship of chromophobe renal cell carcinoma and oncocytoma to intercalated cells of the cortical collecting duct. In addition, Li *et al.* (165) demonstrated that differential expression of claudin-7 in different types of renal cell neoplasms can be useful in their differential diagnosis, particularly when used in a panel of markers.

### 8.17.2. JAM in renal cancer

Gutwein *et al.* (166) has described JAM-A expression in distal convoluted tubule, connecting tubule, and in cells of the collecting duct of the healthy human kidney. JAM-A was weakly expressed in cells of the proximal tubule. Interestingly, treatment of HK-2 cells with IFN-gamma and TNF-alpha resulted in a metalloproteinase mediated downregulation of JAM-A. Importantly, in a tissue micro-array JAM-A protein expression was significantly downregulated in patients with clear cell renal cell carcinoma. Furthermore, knockdown of JAM-A with JAM-A specific siRNA induced the migration of RCC4 cells. It can be seen that downregulation of JAM-A is an early event in the development of renal cancer and increases the migration of renal cancer cells (166).

## 9. CHANGES IN THE EXPRESSION OF PERIPHERAL/PLAQUE PROTEINS IN CANCER

### 9.1. Breast cancer

#### 9.1.1. MAGUK's in breast cancer

MAGUKs may play a vital role in cellular functions preventing tumorigenesis as indicated by neoplastic phenotypes in *Drosophila*; Normal breast tissues have shown the expected intense staining at cell-cell junctions; however, ZO-1 staining is found to be reduced or lost in 69% of breast cancers analysed using immunohistochemistry (20). Normal tissue showed intense staining for ZO-1 at the position of the epithelial TJ, but this was lost or reduced in 69% of breast cancers analysed. In infiltrating ductal carcinomas there was a reduction in staining in 42% of well differentiated, in 83% of moderately differentiated and in 93% of poorly differentiated tumors. ZO-1 was positively correlated with tumor differentiation, and more specifically with the glandular differentiation of tumors. The ZO-1 gene *tjp-1* was mapped relative to other markers flanking the gene. There was a loss of heterozygosity in 23% of informative tumors. Loss of a *tjp-1*-linked marker suggests that genetic

loss may, in some cases, be responsible for a reduction in ZO-1 in breast cancer.

ZO-2 can be expressed in two isoforms, ZO-2A and ZO-2C, in normal epithelia. ZO-2A is absent in pancreatic adenocarcinoma of the ductal type, with none of the common mechanisms of gene inactivation responsible (19). Analysis of the ZO-2 promoters (PA and PC) showed that lack of expression of ZO-A in neoplastic pancreatic cells is caused by inactivation of the downstream promoter PA, probably due to structural or functional alterations in the regulatory elements localised outside the analysed promoter region as hypermethylation was not a convincing reason in early cancers. However, methylation of PA is responsible for the inactivation of the suppressed promoter at the late stages of tumor development (33). ZO-2 was found to be de-regulated in breast adenocarcinoma, but not in colon or prostate adenocarcinoma, both of which are considered to be of acinar rather than ductal type. Also, in 18 breast cancer cell lines, the most poorly-differentiated, fibroblastic cell lines were ZO-1 negative, and were highly invasive (167).

Martin *et al.* (23) investigated the expression of ZO-1, ZO-2 and ZO-3, and MUPP-1 in patients with primary breast cancer. Standardised transcript levels of ZO-1 and MUPP-1 were significantly lower in patients with metastatic disease compared with those remaining disease-free (median follow-up 72.2 months). Immunohistochemistry confirmed these results, with decreased levels in ZO-1 staining. For both ZO-1 and ZO-3, staining was confined to the intercellular regions in normal tissue, whereas in tumor tissues staining was diffuse and cytosolic. Q-PCR revealed a reduction in the levels of ZO-1 and MUPP-1 in patients with disease recurrence. Prognostic indicators of breast cancer were also inversely correlated with ZO-1 expression. It was concluded that low levels of TJ plaque molecules, such as ZO-1 and MUPP-1 in breast cancer are associated with poor patient prognosis.

ZO-1 is able to upregulate HER-2/neu expression *in vitro* by sequestering a repressor of the Her-2/neu gene promoter (18). ZO-1 expression was examined in a series of breast cancers: one group contained those invasive cancers scoring for HER-2/neu status and were analysed by IHC: ZO-1 expression did not correlate with HER-2/neu expression in breast carcinomas, and so other causes of HER-2/neu overexpression should be sought. Interestingly, the authors report that ZO-1 IHC stained DCIS were positive for ZO-1 in 18/20 cases, with 4/18 negative for ZO-1 in the invasive tumor.

The acquisition of a migratory/invasive phenotype by tumor cells is characterized by the loss of cell-cell adhesion contacts and the expression of degradative properties. Polette *et al.* (168) examined the effect of the disorganization of occludin/ZO-1 complexes on the expression of membrane-type 1 matrix metalloproteinase (MT1-MMP). The expression of MT1-MMP in invasive breast tumor cell lines correlated with the absence of occludin and with a cytoplasmic localization of ZO-1. In contrast, non-invasive cell lines displayed a membrane staining for both ZO-1 and occludin and did not

express MT1-MMP. Cytoplasmic ZO-1 and MT1-MMP could be detected in invasive tumor clusters of human breast carcinomas. ZO-1 small interfering RNA transfection down-regulated MT1-MMP mRNAs and proteins and subsequently decreased the ability of tumor cells to invade. The authors conclude that ZO-1 can intervene in signalling events promoting tumor cell invasion. It is apparent that not only is down-regulation of TJ proteins important in effecting an invasive phenotype, but that intracellular mislocalization can be just as important: if the TJ protein in question has not been targeted correctly, the structure and function of the TJ will be impaired.

VMP1 expression is also decreased in the invasive breast cancer cell lines HCC1954 and MDA-MB-231 as compared to the non-invasive cell lines MCF-12A, T-47D and MCF-7 (169). This study also showed for the first time that Vmp1 is a plasma membrane protein and an essential component of initial cell-cell contacts and TJ formation.

## 9.2. Colorectal Cancer

### 9.2.1. ZO-1 in colorectal cancer

Tubular gland structures of colorectal cancer have been demonstrated to undergo dedifferentiation at the primary site, with the gland structures re-formed in liver metastases. Kaihara *et al.* (14) examined the degree of differentiation of the gland structure of 48 cases of colorectal cancers (24 cases with synchronous liver metastasis, 24 cases without metastasis) by the modified Gleason grading system. The role of ZO-1, in the morphological changes (dedifferentiation and redifferentiation) at the primary site and liver metastases was also looked at. Liver-metastasized colorectal cancers showed a lower score in the modified Gleason grading system than the corresponding primary tumors. The tumor cells had undergone redifferentiation at liver metastases. ZO-1 was expressed at the apical cell borders of normal colorectal epithelium, the luminal side of which has tubular gland structures. In comparison with this normal epithelium, the ZO-1 expression level was frequently reduced in primary colorectal cancer with liver metastasis (20.8%) and ZO-1 was re-expressed in liver metastasized cancers (79.2%). Immunoprecipitation of colorectal cancers with liver metastasis showed that ZO-1 bound to epidermal growth factor receptor (EGFR) irrespective of the phosphorylation status of EGFR, and that EGFR associated ZO-1 was highly tyrosine- phosphorylated only in the primary colorectal cancers, but was dephosphorylated in the liver-metastasized cancers. The authors suggest that tyrosine phosphorylation of ZO-1 leads to down-regulation of the function of ZO-1 and dedifferentiation of the glands in colorectal cancers, and these phenomena contribute to liver metastases, and redifferentiation of the glands occurs in the liver metastases.

Chen *et al.* (170) have shown that JunD negatively regulates expression of ZO-1 and is implicated in the regulation of intestinal epithelial barrier function. Moreover, depletion of APC or Striatin affects the localization of ZO-1 and alters the organization of F-actin

(171). These results raise the possibility that the contribution of APC to cell-cell adhesion may be through interaction with Striatin in the TJ compartment of epithelial cells.

Symplekin is a ubiquitously expressed protein involved in cytoplasmic RNA polyadenylation and transcriptional regulation and is localized at tight junctions (TJs) in epithelial cells. Nuclear symplekin cooperates with the Y-box transcription factor zonula occludens 1-associated nucleic acid-binding protein (ZONAB) to increase the transcription of cell cycle-related genes and also inhibits differentiation of intestinal cells. Buchert *et al.* (2010) detected high levels of nuclear symplekin in 8 of 12 human colorectal cancer (CRC) samples. shRNA-mediated reduction of symplekin expression was sufficient to decrease significantly the anchorage-independent growth and proliferation of HT-29 CRC cells as well as their tumorigenicity when injected into immunodeficient animals. Symplekin down-regulation also was found to alter ion transport through TJ, to promote the localization of ZONAB in the membrane rather than the nucleus. Claudin-2 expression was reduced following symplekin down-regulation, an effect mimicked when ZONAB expression was down-regulated using selective siRNA. siRNA-mediated claudin-2 down-regulation increased the transepithelial resistance and decreased cyclin D1 expression and ZONAB nuclear localization, similar to observations in symplekin-depleted cells. These results suggest that nuclear overexpression of symplekin promotes tumorigenesis in the human colon and that the regulation of claudin-2 expression is instrumental in this effect (172).

## 9.3. Esophageal cancer

### 9.3.1. ZO-1 in esophageal cancer

Kimura *et al.* (15) investigated occludin expression in conjunction with ZO-1 in normal epithelia and cancers of human digestive tract. ZO-1 was expressed as a single line at the apical cell border. In the esophagus ZO-1 was expressed in the spinous layer. As for tumors, ZO-1 showed the same expression in differentiated adenocarcinoma cells as in normal epithelium, but in poorly differentiated adenocarcinomas, the expression was reduced. There was significant correlation between tumor differentiation and expression of these proteins. It was posited that ZO-1 could be involved in the formation of gland-like structures.

## 9.4. Gastric cancer

### 9.4.1. ZO-1 in gastric cancer

Similar patterns of expression have been noted for E-cadherin and claudin -4, but ZO-1 expression differed in gastric cancer tissues (123). According to the Lauren classification, the reduced expression of E-cadherin and claudin-4 was more frequent in diffuse than intestinal type tumors with the reduced expression of E-cadherin and claudin-4 correlated with poor differentiation. Western blot analysis and RT-PCR also showed decreased claudin-4 expression in diffuse type tumors and poorly-differentiated adenocarcinoma. The reduced expression of claudin-4 and E-cadherin correlates with disruption of glandular structure and loss of differentiation, which suggests that the



dysfunction of claudin-4 may play a role in the disruption of cell-to-cell adhesion in diffuse type gastric cancer and in a loss of differentiation.

### 9.5. Lung cancer

#### 9.5.1. ZO-1 in lung cancer

Disruption of intercellular adhesions, increased abundance of alpha(5)beta(1) integrin, and activation of protein kinase Cepsilon (PKCepsilon) correlate with invasion and unfavorable prognosis in lung cancer (173). It has been reported that ZO-1 preferentially interacts with alpha(5)beta(1) integrin at the lamellae of migrating cells. Disruption of ZO-1 binding to an internal PDZ-binding motif in the alpha(5) cytoplasmic tail prevented the polarized localization of ZO-1 and alpha(5) at the leading edge. Furthermore, silencing of alpha(5) integrin inhibited migration and invasion of lung cancer cells, and silencing of ZO-1 resulted in increased Rac activity and reduced directional cell motility. The formation of the alpha(5)-ZO-1 complex was dependent on PKCepsilon: Phosphorylation of ZO-1 at serine-168 regulated the subcellular localization of ZO-1 and thus controlled its association with alpha(5) integrin. In conclusion, PKCepsilon activation drives the formation of a spatially restricted, promigratory alpha(5)-ZO-1 complex at the leading edge of lung cancer cells (173).

### 9.6. Pancreatic cancer

#### 9.6.1. ZO-1 in pancreatic cancer

An early study investigating ZO-1 in pancreatic cancer (174) showed that expression of ZO-1 mRNA was increased sixfold in PDAC samples in comparison with normal samples. Confocal microscopy revealed the presence of ZO-1 in the apical and apicolateral areas of ductular cells in the normal pancreas. Similarly, in CP, ZO-1 was localized at apical and apicolateral areas of small proliferating ductular cells and large metaplastic ducts. In PDAC, however, ZO-1 expression was observed irrespective of whether the cancer cells formed duct-like structures or exhibited a diffuse infiltrating pattern. Metastatic pancreatic cancer cells within lymph nodes display variable staining patterns for ZO-1, ranging from apical and apicolateral to a diffuse membranous staining suggesting that ZO-1 is overexpressed in PDAC and raise the possibility that this overexpression may confer a metastatic advantage to pancreatic cancer cells.

A later study (175) investigated the translocation of ZO-1 and the activation of epidermal growth factor receptor (EGFR) to demonstrate the involvement and correlation of TJ protein translocation and EGFR activation in the cell dissociation and subsequent invasion of pancreatic cancer. The obvious translocation of cell-cell junction localized ZO-1 protein to the cytoplasm and nucleus, simultaneous activation of EGFR, as well as the dissociation of cell colonies of non-dissociated pancreatic cancer cells were induced by dissociation factor treatment. Translocation of TJ protein ZO-1 as thus found to be closely involved in the induction of invasion through EGFR activation in pancreatic cancer cells.

### 9.6.2. ZO-2 in pancreatic cancer

An early study (33) identified a fragment present in normal pancreatic duct cells that is not expressed in pancreatic duct carcinoma cells. Sequence analysis showed an 88% and 82% identity, respectively, to the cDNA of the canine and human TJ ZO-2 gene. Semi-quantitative RT-PCR analysis of human ZO-2 revealed a striking difference in the expression of various regions of the ZO-2 transcript in normal and neoplastic cells and the presence of an abnormality at the 5'-end of mRNA. RACE analysis identified 2 human ZO-2 mRNAs that encode proteins of different lengths, designated as ZO-2A and ZO-2C. The difference between the 2 forms of ZO-2 is the absence of 23 amino acid residues at the N terminus of ZO-2C compared with ZO-2A. Although ZO-2C was expressed in normal pancreatic cells and a majority of neoplastic tissues analyzed, ZO-2A was undetectable except in one case in all of the pancreatic adenocarcinomas analyzed.

### 9.7. Ovarian cancer

In ovarian cell lines, ET-1 (ETAR)/endothelin-1 axis (ET-1) induces loss of adherens and tight-junction protein expression, E-cadherin, b-catenin, and ZO-1, and gain of N-cadherin and vimentin expression (176).

### 9.8. Testicular cancer

#### 9.8.1. ZO's in testicular cancer

In normal seminiferous epithelium, specialized TJ between Sertoli cells constitute the major component of the blood-testis barrier (177). Sertoli cells associated with CIS exhibit impaired maturation status, but their functional significance remains unknown. In normal tubules, ZO-1 and ZO-2 immunostaining was observed at the blood-testis barrier region of adjacent Sertoli cells. Within CIS tubules, ZO-1 and ZO-2 immunoreactivity was reduced at the blood-testis barrier region, but spread to stain the Sertoli cell cytoplasm. Western blot analysis confirmed ZO-1 and ZO-2, and their respective mRNA were shown by RT-PCR. In conclusion, Sertoli cells associated with CIS show an altered distribution of ZO-1 and ZO-2 and lose their blood-testis barrier function (177).

### 9.9. Renal cancer

#### 9.9.1. ZO-1

In von Hippel-Lindau (VHL) disease, germline mutations in the VHL tumor suppressor gene cause clear cell renal carcinomas, hemangioblastomas, and pheochromocytomas (178). The VHL gene product is part of an ubiquitin E3 ligase complex and hypoxia-inducible factor alpha (HIF-alpha) is a key substrate, although additional VHL functions have been described. A genotype-phenotype relationship exists in VHL disease such that specific VHL mutations elicit certain subsets of these tumors. Bangiyeva *et al.* (178) examined VHL genotype-phenotype correlations at the cellular level, focusing on the regulation of tight junctions and cell morphology. And found that VHL has both HIF-alpha dependent and HIF-alpha independent functions in regulating TJ and cell morphology that likely impact the clinical phenotypes seen in VHL disease.

**10. TJ PROTEIN EXPRESSION IN MULTI-CANCER STUDIES****10.1. CAR**

As the coxsackie and adenovirus receptor (CAR) is involved in epithelial cell TJ (146), the author's examined CAR's role in tumor metastasis using a B16 melanoma and CT26 colon adenocarcinoma model of experimental metastasis. In lung metastasis, the colony number of B16 cells stably expressing CAR (B16CAR) was significantly lower than that of the control CAR-negative B16 cells. B16 and CT26 cells transiently expressing CAR, which were transduced with adenovirus (Ad) vector expressing CAR, also reduced lung metastasis, suggesting that CAR plays a role in the early stage of metastasis. CAR expression significantly decreased the accumulation of B16 cells in the lung after i.v. injection and the migration *in vitro*. CAR expression reduced expression of alpha (v), alpha (4), beta (3) and beta (1) integrin, which play important roles in attachment to cells or basement membrane. CAR expression likely acts as a metastatic suppressor in these cancer types.

**10.2. Claudins**

Facchetti *et al.* (179) evaluated the usefulness of claudin-4 in the diagnosis of mesothelioma and mimickers, analyzing biopsies from 454 tumors, including 82 mesotheliomas, 336 carcinomas of different origin (278 primary, 58 metastatic to serosae), 36 nonepithelial spindle cell neoplasms, as well as 97 cytological samples from reactive effusions (12), mesothelioma (23) and metastatic carcinomas (62). Claudin-4 was consistently negative in normal and reactive mesothelium, as well as in all 82 mesotheliomas. In contrast, strong reactivity was found in 57/58 serosal metastasis, and in 245/278 primary carcinomas, with uppermost expression (150/153) in those most frequently involved in the differential with mesothelioma (lung, breast, gastrointestinal tract, pancreas, ovary, primary serous papillary carcinoma of peritoneum). On effusions, reactive and neoplastic mesothelial cells were regularly negative, while metastatic tumor cells stained positively in 60/62 (96.8%) cases. Among spindle cell neoplasms, only 2/9 biphasic synovial sarcomas and 4/4 follicular dendritic cell sarcomas stained positively. Results indicate that CL-4 reacts with the majority of epithelial neoplasms that often metastasize to serous membranes, representing a pancarcinoma marker with extremely high sensitivity and specificity. It was concluded that claudin-4 may be considered a primary immunohistochemical reagent to rule out the diagnosis of mesothelioma.

**11. TJ PROTEIN EXPRESSION, MALIGNANT BRAIN TUMORS AND THE BLOOD-BRAIN-BARRIER (BBB)**

Malignant brain tumors cause cerebral edema because they have leaky endothelial TJ, which allow plasma fluid to enter the brain from the microvessel lumen (180). In order to identify molecular abnormalities in tumor endothelial TJ, occludin expression in microvessels from adult human non-neoplastic brain tissue was investigated.

The proportions of microvessels immunolabelling for occludin were >2/3 in 5/5 non-neoplastic brain tissue samples, >1/3 in 5/5 low grade (Daumas-Duport I or II) astrocytomas and <1/3 in 5/5 high grade (III or IV) astrocytomas and 6/6 metastatic adenocarcinomas. Six non-neoplastic brain tissue immunoblots gave a 55-kDa occludin band, three low-grade astrocytomas gave 55-kDa and 60-kDa bands, 13 high-grade astrocytomas gave 60-kDa or no band and four adenocarcinomas did not give an occludin band. Expression of 55-kDa occludin inversely correlated with the presence of contrast enhancement on computed tomograms. Electron microscopy showed open endothelial TJ in 0/2 non-neoplastic human brain specimens and 2/2 high-grade astrocytomas. It is thought that loss of 55-kDa occludin expression in human brain tumors may contribute to endothelial TJ opening.

The development of peritumoral edema is thought to be due to extravasation of plasma water and macromolecules through a defective blood-brain barrier (BBB), but the exact mechanism by which occurs is poorly understood (181). Biopsies of 25 patients with pathological diagnosis of astrocytic tumors were examined. Both open and close TJ were observed in the micro-blood vessels, inclusive in a same tumor. Cytoskeletal disorganization associated with disintegrated perijunctional actin filaments were seen. The paracellular space showed enlargement and commonly occupied by fluid proteinaceous, endothelial cells display oncotic and ischemic changes; basal lamina reveals enlargement, edema, vacuolization and collagen fibers disposed in irregular array. Pericytes exhibited edema and phagocytosed material, astrocytic perivascular-feet showed signs of oncosis and necrosis, cooption vessels totally surrounding by neoplastic cells also were seen (181). The ultrastructural abnormalities observed in both junctional complexes and vascular microenvironment suggest a multi-factorial pathobiology process, probably hypoxia intratumoral, calcium overload in endothelial cells, and degradative effects of metalloproteinases over the basal membrane appear as determinant factors that leading to structural modifications of junctional complexes, therefore, treatment with both HIF-1 $\alpha$  and metalloproteinases inhibitors possibly can contribute with the pharmacological handling of the peritumoral edema associated with astrocytic tumors (181).

Septic encephalopathy is associated with breakdown of the blood-brain barrier and cerebral edema (182). These features are also common properties of brain tumors. Electron microscopy revealed TJ opening in high-grade astrocytoma microvessels. Expression of the TJ protein occludin is reduced in these microvessels and this reduction is inversely correlated with the degree of cerebral edema (182). Normal astrocytes secrete factors that induce barrier properties in endothelial cells, whereas high-grade astrocytomas secrete vascular endothelial growth factor, which stimulates angiogenesis, down regulates occludin and increases endothelial cell permeability (182). The water channel protein aquaporin-4 is normally expressed in astrocyte foot processes around cerebral microvessels. Its expression is massively up-regulated in high-grade astrocytoma and around metastatic adenocarcinoma. There

## TJ and metastasis

is a significant correlation between aquaporin-4 expression and the degree of cerebral edema, but it is not clear whether increased aquaporin-4 expression enhances edema formation or clearance. These results suggest that the pathophysiology of brain edema is multifactorial, but that there may be common processes operating regardless of the aetiology (182).

The quality of the blood-brain barrier (BBB), represented mainly by endothelial TJ is now believed to be dependent on the brain microenvironment and influenced by the basal lamina of the microvessels (183). In the highly vascularized glioblastoma multiforme (GBM), a dramatic increase in the permeability of blood vessels is observed but the nature of basal lamina involvement remains to be determined. Agrin, a heparan sulphate proteoglycan, is a component of the basal lamina of BBB microvessels, and growing evidence suggests that it may be important for the maintenance of the BBB (183). This study provided the first evidence that agrin is absent from basal lamina of tumor vessels if the TJ molecules occludin, claudin-5 and claudin-1 were lacking in the endothelial cells (183). If agrin was expressed, occludin was always localized at the TJ, claudin-5 was frequently detected, whereas claudin-1 was absent from almost all vessels. Furthermore, despite a high variability of vascular phenotypes, the loss of agrin strongly correlated with the expression of tenascin, an extracellular matrix molecule which has been described previously to be absent in mature non-pathological brain tissue and to accumulate in the basal lamina of tumor vessels. These results support the view that in human GBM, BBB breakdown is reflected by the changes of the molecular compositions of both the endothelial TJ and the basal lamina (183).

Gliomas, particularly glioblastoma multiforme, perturb the blood-brain barrier and cause brain edema that contributes to morbidity and mortality (184). Cocultured glioblastoma cells and glioma-derived factors (e.g. transforming growth factor beta2) enhance the paracellular flux of endothelial cell monolayers in conjunction with downregulation of TJ proteins. Neutralizing anti-transforming growth factor beta2 antibodies partially restored the barrier properties in this *in vitro* blood-brain barrier model. The involvement of endothelial cell-derived matrix metalloproteinases (MMPs) was demonstrated by quantitative reverse-transcriptase-polymerase chain reaction analysis and by the determination of MMP activities via zymography and fluorometry in the presence or absence of the MMP inhibitor GM6001. Occludin, claudin 1, and claudin 5 were expressed in microvascular endothelial cells in nonneoplastic brain samples but were significantly reduced in anaplastic astrocytoma and glioblastoma samples. Taken together, these *in vitro* and *in vivo* results indicate that glioma-derived factors may induce MMPs and downregulate endothelial tight junction protein and, thus, play a key role in glioma-induced impairment of the blood-brain barrier (184).

## 12. TJ PROTEIN EXPRESSION IN CANCER AND ASSOCIATED ENDOTHELIUM

Regulation of endothelial barrier function is critical for vascular homeostasis, as dynamic and local control of

vascular permeability permits macromolecular transport, immune surveillance, and deposition of a fibrin barrier to contain infection at sites of inflammation (185).

Hyperpermeability triggered by inflammation or ischemia in the heart, brain, or lung promotes edema, exacerbates disease progression, and impairs recovery (185). During cancer, solid tumors release factors that promote the growth of leaky blood vessels which contribute to metastatic spread and limit targeted delivery of anticancer agents (185).

Hepatocyte growth factor (HGF) is a multi-function cytokine that has been shown to regulate the expression of cell adhesion molecules in human endothelial cells. It is also a key cytokine in the development and progression of cancer, particularly during metastasis. NK4 is a variant of HGF that has already been shown to be antagonistic to HGF. The study by Martin *et al.* (6) study shows that HGF decreased transendothelial resistance and increased paracellular permeability in human vascular endothelial cells can that such effects can be inhibited by addition of the NK4 variant. In addition, HGF-stimulated invasion of endothelium by breast cancer cells was inhibited by the addition of NK4. Western blotting revealed that HGF/SF decreased the protein level, and increased tyrosine phosphorylation of ZO-1, but did not cause a change in level of occludin or claudin-1, both molecules involved in TJ function. RT-PCR revealed that addition of HGF/SF caused no change in signal for claudin-5 or junctional adhesion molecule (JAM), but there was a decrease in the signal for claudin-1. NK4 was also able to prevent the decrease in levels of ZO-1 protein by HGF/SF indicating that TJ permeability can be modulated therapeutically.

Disruption of TJ can lead to leaky vascular bed and potentially to edema and swelling of tissues, the (186) aetiology of mastalgia. These changes may also cause vascular spread of cancer cells. In human endothelial cells GLA (gamma-linolenic acid), I (iodine), and Se (selenium) individually increased transendothelial resistance in the presence of 17beta-estradiol (17beta-estradiol), which causes leakage of endothelial cells by disruption of TJ. The combination of all three agents also had a significant effect on TER. Addition of GLA/Se/I reduced PCP of the endothelial cells. Treatment with GLA/Se/I reversed the effect of 17beta-estradiol in reducing TER and increasing PCP. Immunofluorescence revealed that after treatment with Se/I/GLA over 24 h there was increasing relocation to endothelial cell-cell junctions of the TJ proteins claudin-5, occludin, and ZO-1 (186). Interestingly, this relocation was particularly evident with treatments containing I when probing with claudin-5 and those containing Se for occludin. There was a small increase in overall protein levels when examined by Western blotting after treatment with GLA/Se/I when probed with claudin-5 and occludin. It was observable that GLA, I, and Se alone, or in combination are able to strengthen the function of TJ in human endothelial cells, by way of regulating the distribution of claudin-5, occludin, and ZO-1. Interestingly, this combination was also able to completely reverse the effect of 17beta-estradiol in these cells (186).

Koenen *et al.* (187) have demonstrated that cultured endothelial cells not only express a cellular 43-kDa variant of JAM-A but also release considerable amounts of a 33-kDa soluble JAM-A variant. This release is enhanced by treatment with proinflammatory cytokines and is associated with the down-regulation of surface JAM-A. Inhibition experiments, loss/gain-of-function experiments, and cleavage experiments with recombinant proteases indicated that cleavage of JAM-A is mediated predominantly by the disintegrin and metalloproteinase (ADAM) 17 and, to a lesser extent, by ADAM10. Cytokine treatment of mice increased JAM-A serum level and in excised murine aortas increased ADAM10/17 activity correlated with enhanced JAM-A release. Functionally, soluble JAM-A blocked migration of cultured endothelial cells, reduced transendothelial migration of isolated neutrophils *in vitro*, and decreased neutrophil infiltration in a murine air pouch model by LFA-1- and JAM-A-dependent mechanisms. Therefore, shedding of JAM-A by inflamed vascular endothelium via ADAM17 and ADAM10 may not only generate a biomarker for vascular inflammation but could also be instrumental in controlling JAM-A functions in the molecular zipper guiding transendothelial diapedesis of leukocytes (187).

Sakaguchi *et al.* (188) have shown that In the normal liver, a ubiquitous claudin-5 expression can be seen in SECs, the arteries, and portal veins but not in the central veins (188). Sinusoidal claudin-5 expression is down-regulated according to the increase of hepatic or fibrotic grade. By multivariate analysis, vasculobiliary invasion and lower claudin-5-MVD are independent factors associated with a lower postoperative overall survival rate. Attenuated claudin-5 expression in SECs may be related to SEC dysfunction in injured liver. Down-regulated claudin-5 expression in tumor vessels may serve as a potential marker for poor prognosis in HCC.

Hepatitis A virus (HAV) cellular receptor HAVcR-1, also known as KIM-1/TIM-1, is the cellular receptor for the hepatotropic picornavirus that causes acute hepatitis-A in humans. Although HAVcR-1 is expressed in every human organ, the natural function of HAVcR-1 remains unknown. Martin *et al.* (189) investigated the location, association and possible functionality of HAVcR-1 in human endothelial cells. Human endothelial cells express HAVcR-1 at low levels. The location of both endogenous and forcibly expressed HAVcR-1 can be found at the cell-cell junction, at the region of the TJ. In this study, one of the first to examine the location and binding partners of HAVcR-1, expression of this receptor was targeted to the vicinity of intercellular junctions, via ZO-1, which further was demonstrated to be at the site of the TJ by its co-localisation with ZO-2. This was true of both endogenously and forcibly expressed protein. Moreover, HAVcR-1 was co-precipitated with the regulatory factor Rho C. The authors conclude that HAVcR-1 may have a previously undiscovered role in the regulation of TJ integrity in human endothelial cells.

## 13. TIGHT JUNCTION COMPONENTS AS PROMISING NEW TARGETS FOR CANCER DIAGNOSIS AND THERAPY

Aberrant TJ function and expression in cancer indicates that individual components within the TJ complex offer intriguing and novel targets for the prognosis and detection of cancer. Moreover, they also provide possible modes of treatment for patients with cancer. Most of the work has concentrated on the use of claudins, and the reader is directed to a number of reviews showcasing this (53, 54, 83, 190).

This review has illustrated the plethora of studies that show that depending on tumor type, TJ proteins provide a vast repertoire for cancer diagnosis and progression. Not only this, TJ molecules are being increasingly viewed as potential targets for therapy. An interesting example of this is the work of Sahin *et al.* (191). The authors identified isoform 2 of claudin-18 (CLDN18.2) as a highly selective cell lineage marker. Its expression in normal tissues was strictly confined to differentiated epithelial cells of the gastric mucosa, but it is absent from the gastric stem cell zone. CLDN18.2 was retained on malignant transformation and is expressed in a significant proportion of primary gastric cancers and the metastases thereof. In addition to its orthotopic expression, they found frequent ectopic activation of CLDN18.2 in pancreatic, esophageal, ovarian, and lung tumors, correlating with distinct histologic subtypes. The activation of CLDN18.2 depended on the binding of the transcription factor cyclic AMP-responsive element binding protein to its unmethylated consensus site. Most importantly, monoclonal antibodies that bind to CLDN18.2 but not to its lung-specific splice variant and recognize the antigen on the surface of cancer cells were manufactured. Due to its highly restricted expression pattern in normal tissues, its frequent ectopic activation in a diversity of human cancers, and the ability to specifically target this molecule at the cell surface of tumor cells qualify CLDN18.2 as a novel, highly attractive pan-cancer target for the antibody therapy of epithelial tumors.

In another study, Yuan *et al.* (192) recently found that claudin-3 and claudin-4 are two of the most highly and consistently up-regulated genes in ovarian carcinomas. Because these are the naturally occurring receptors for *Clostridium perfringens* enterotoxin (CPE), the authors used the COOH-terminal 30 amino acids of the CPE (CPE(290-319)), a fragment that is known to retain full binding affinity but have no cytolytic effect, to target tumor necrosis factor (TNF) to ovarian cancers. The TNF component in CPE(290-319)-TNF was 5-fold less potent than free TNF as determined by a standard L-929 TNF bioassay. However, the CPE(290-319)-TNF was >6.7-fold more cytotoxic than free TNF to 2008 human ovarian cancer cells, which express both claudin-3 and claudin-4 receptors. shRNAi-mediated knockdown of either claudin-3 or claudin-4 expression in 2008 markedly attenuated the cytotoxic effects of CPE(290-319)-TNF. The fusion construct was efficiently delivered into target cells and located in both cytosol and vesicular compartments as

assessed by immunofluorescent staining. It was concluded that CPE(290-319) effectively targeted TNF to ovarian cancer cells and is an attractive targeting moiety for development of CPE-based toxins for therapy of ovarian carcinomas that overexpress claudin-3 and -4.

Although claudin-4 may be a promising target molecule for tumor therapy, claudin-targeting strategy has never been fully developed (193). A claudin-4-targeting molecule was prepared by fusion of the C-terminal fragment of Clostridium perfringens enterotoxin (C-CPE) with the protein synthesis inhibitory factor (PSIF) derived from Pseudomonas aeruginosa exotoxin. PSIF was not cytotoxic to claudin-4-expressing cells, whereas C-CPE-PSIF was cytotoxic. Cells that express claudin-1, -2, and -5 were less sensitive to C-CPE-PSIF. Pretreatment of the cells with C-CPE attenuated C-CPE-PSIF-induced cytotoxicity, and mutation of C-CPE in the claudin-4-binding residues attenuated the cytotoxicity of C-CPE-PSIF. TJ-undevoted cells were more sensitive to C-CPE-PSIF than TJ-developed cells. It is noteworthy that polarized epithelial cells are sensitive to C-CPE-PSIF applied to the basal side, whereas the cells were less sensitive to C-CPE-PSIF applied to the apical side. Intratumoral injection of C-CPE-PSIF reduced tumor growth. This is the first report to indicate that a claudin-4-targeting strategy may be a promising method to overcome the malignant tumors.

More recently, C-CPE was also the method of choice of Kakutani *et al.* (194). The authors genetically prepared a novel claudin-4-targeting molecule (DTA-C-CPE) by fusion of C-CPE and diphtheria toxin fragment A (DTA). Although DTA is not toxic to claudin-4-expressing L cells, even at 20 microg/ml, DTA-C-CPE is toxic to claudin-4-expressing L cells at 1 microg/ml. DTA-C-CPE-induced cytotoxicity was attenuated by pretreatment of the cells with C-CPE but not bovine serum albumin, indicating that DTA-C-CPE may bind to claudin-4-expressing L cells through its C-CPE domain. To evaluate the specificity of DTA-C-CPE, we examined its cytotoxic effects in L cells that express claudin-1, -2, -4 or -5. It was found that DTA-C-CPE was toxic to only claudin-4-expressing L cells. CPE has also been used against endometrial cancer xenografts (195). Ishida *et al.* (196) suggest that this enterotoxin may be applicable for the treatment of rectal well-differentiated endocrine neoplasms in the future in order to prevent unexpected metastatic recurrences after tumor resections, because these neoplasms have a relatively high incidence of metastases despite their small size.

All these studies show that C-CPE may be a promising ligand for the development of cancer-targeting systems. Matrix metalloproteinases (MMPs) have been implicated as possible mediators of invasiveness and metastasis in some cancers. A recent study (197) found that the inhibitory effects of sanguinarine on cell proliferation, motility and invasiveness were found to be associated with the increased tightness of the TJ, which was demonstrated by an increase in transepithelial electrical resistance (TER). Additionally, immunoblotting results indicated that sanguinarine repressed the levels of the claudin proteins, major components of TJs that play a key role in the control and selectivity of paracellular transport. Furthermore, the activities of MMP-2 and -9 in MDA-MB-231 cells were

dose-dependently inhibited by treatment with sanguinarine, and this was also correlated with a decrease in the expression of their mRNA and proteins.

Also of interest is the work carried out by Skrovanek *et al.* (198) who found that restriction of sulfur-containing amino acids (SCAA) in LLC-PK (1) renal epithelial cells resulted in reduction of methionine by 90%. Cell growth and differentiation were maintained, and both confluent cell density and transepithelial short circuit current were unaffected. Occludin and claudins-1 and -2 did not have altered expression, however, claudins-3 and -7 were significantly decreased and claudins-4 and -5 were markedly increased. The functional result of these structural changes was improved barrier function. In contrast to normal cells, tumor cells have absolute requirement for methionine. In animal models, methionine restriction limits tumor growth and reduces tumor volume. However, interruption of methionine restriction induces the regrowth of tumors. Moreover methionine restriction induces cell modifications suggesting it had a use in association with conventional chemotherapy (199). That there is a link between methionine restriction and TJ protein expression leads to interesting possibilities for future therapies.

It might be anticipated that as future work illuminates the diverse yet vital functions of the other TJ molecules, there will be increasing potential in utilising the TJ components as targets for therapy to prevent cancer metastasis.

## 14. PERSPECTIVE

It has been over 30 years since TJ were first described as having altered form and function in tumor cells and tissues (200, 201). The body of evidence now demonstrates that the TJ has a pivotal role to play during cancer metastasis. Although the claudin family group has been most explored, it is crucial that the other components attract as much attention. Recent studies have shown an increasing likelihood that TJ proteins will be proven to be true suppressor proteins and these suggest that the TJ is vital to the prevention of successful cancer cell metastasis and further research should provide answers to using TJ as an essential point for intervention during the metastatic cascade. Moreover, dysregulation of the TJ can also lead to potentially exciting markers for the prognosis of a number of tumor types.

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**Abbreviations:** BBB: blood brain barrier; BE: Barrett's esophagus; CAR: coxsackie adenovirus receptor; CIN/CIS: cervical intra-epithelial neoplasia/ carcinoma *in situ*; CP: chronic pancreatitis; CPE: Clostridium perfringens enterotoxin; CTX: cholera toxin; DGC: diffuse-type gastric cancer; DMSO: dimethyl sulfoxide; DCIS: ductal carcinoma *in situ*; EMT: epithelial-mesenchymal transition; EGFR: epidermal growth factor receptor; ELF3: E74-like factor 3; GLA: gamma linolenic acid; HGF/SF: hepatocyte growth factor/scatter factor; I: iodine; IDC: invasive ductal carcinoma; IGC: intestinal-type gastric cancer; IHC: immunohistochemistry; JAM: junctional adhesion molecule; LCIS: lobular carcinoma *in situ*; MAGI: membrane-associated guanylate kinase, WW and PDZ domains-containing; MAGUK: membrane-associated guanylate kinase homologs; MAPK: mitogen-activated protein kinase; MARVEL: MAL-related proteins for vesicle trafficking and membrane link domain; MEK: 2- MAPK kinase of ERK kinase; MMP: 2-matrix metalloproteinase-2; MT: malotilate; MT1: MMP- Membrane-type 1 matrix metallo-proteinase; MUPP-1: multi-PDZ domain protein 1; NF1: neurofibrosis type 1; Par: protease-activated receptors; PCP: paracellular permeability; PDZ: post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1); PKC: protein kinase C; PMA: paramethoxyamphetamine; PPAC: progressive pseudorheumatoid, of childhood; PSA: prostate specific antigen; Q-PCR: quantitative polymerase chain reaction; RT-PCR: reverse-transcriptase-polymerase chain reaction; RACE: remote analysis computation for gene expression data; SCC: squamous cell cancer; SCE: specialized columnar epithelium; SqE: squamous epithelium; TAMP: Tight junction-associated MARVEL proteins; TCF/LEF: T-cell factor; TER/TEER: trans-epithelial/endothelial resistance; TGF: transforming growth factor; TJ: Tight Junction/s; TNM: Tumor nodal status; VEGF: vascular endothelial growth factor.

**Key Words:** Tight Junction, Barrier function, Cancer, Metastasis, Review

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