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Alterations of the apical junctional complex and actin cytoskeleton and their role in colorectal cancer progression

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Abbreviations: AJC, apical junctional complex; TJ, tight junctions; AJ, adherens junction; ZO, zonula occludens; ERK, extracellular signal-regulated kinase; PI3K, phosphoinositide-3 kinase; CRC, colorectal cancer; Cav-1, caveolin-1; JAMs, junctional adhesion molecules; MAGUK, membrane associated guanilate kinase homolog; EGFR, epidermal growth factor receptor; CD1, cyclin D1; ZONAB, transcription factor zonula occludens 1 (ZO-1)-associated nucleic acid binding protein; MARVEL, MAL and related proteins for vesicle trafficking and membrane link; MAGI 1, membrane associated guanylate kinase inverted; EMT, epithelial mesenchymal transition; NF-κB, factor nuclear kappa B; APC, adenomatous polyposis coli; CTNNB1, catenin (cadherin-associated protein), β 1; ARP2/3, actin-related proteins 2 and 3; ROCK, Rho-associated protein kinase; MAPK, mitogen-activated protein kinase; Rap1, Ras-related protein 1; N-WASP, neuronal Wiskott–Aldrich Syndrome protein; VASP, vasodilator-stimulated phosphoprotein; GSK-3β, glycogen synthase kinase 3 β; NM II, non-muscle myosin class II; MLCK, myosin light-chain kinase; PGE_2, prostaglandin E_2; LPA, lysophosphatidic acid; FAK, focal adhesion kinase; TGF-β, transforming growth factor β; CTX, thymocyte marker for *Xenopus*

Colorectal cancer represents the fourth highest mortality rate among cancer types worldwide. An understanding of the molecular mechanisms that regulate their progression can prevents or reduces mortality due to this disease. Epithelial cells present an apical junctional complex connected to the actin cytoskeleton, which maintains the dynamic properties of this complex, tissue architecture and cell homeostasis. Several studies have indicated that apical junctional complex alterations and actin cytoskeleton disorganization play a critical role in epithelial cancer progression. However, few studies have examined the existence of an interrelation between these 2 components, particularly in colorectal cancer. This review discusses the recent progress toward elucidating the role of alterations of apical junctional complex constituents and of modifications of actin cytoskeleton organization and discusses how these events are interlinked to modulate cellular responses related to colorectal cancer progression toward successful metastasis.

Introduction

The intestinal mucosa plays a critical role in forming a barrier that separates luminal contents from the underlying interstitium. The primary structure that regulates this intestinal barrier is the apical junctional complex (AJC), which is formed by the tight junctions (TJs) and adherens junctions (AJs) that contribute to apical-basal cell polarity maintenance and to cell signaling events.[1,2] TJs and AJs are highly organized structures that are composed of transmembrane proteins, which are associated with cytoplasmic proteins that are directly or indirectly connected to the actin cytoskeleton. Transmembrane proteins and their cytoplasmic adaptor proteins work both individually and in combination as a functional module to establish and to maintain the AJC. Additionally, the proteins present in the AJC act together with the apical actin cytoskeleton to confer dynamic properties to this complex and to maintain many cellular functions.

Currently, the loss of epithelial organization is a hallmark of cancer, with neoplastic cells frequently exhibiting structural and functional deficiencies in the AJC.[3] This notion has been supported by the following findings: (a) TJ proteins play critical roles in the neoplastic process as couplers of the extracellular milieu to intracellular signaling pathways and to the cytoskeleton,[4,5] (b) alterations in TJ integrity can lead to the increased diffusion of nutrients and other factors critical for tumor growth and survival and may be an important step in developing a metastatic phenotype,[6,7] and (c) the overall down-regulation of E-cadherin, which is an important AJ protein, is related to carcinoma development.[8] However, only a few studies have shown the interrelation between the disorganization of the AJC and the actin cytoskeleton in the development of human malignancies. In the present review, we will discuss the recent progress in elucidating the roles of altered proteins that constitute the AJC and of modifications of actin organization and how these 2 events are interlinked to modulate cellular responses related to the progression of colorectal cancer (CRC), which is the fourth most common cause of cancer mortality worldwide.[9]

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Altered Expression of AJC Proteins in CRC

Previous studies in animal models and cell culture have defined essential components of apical junction formation and cell polarity and have revealed biochemical interactions among more than 40 transmembrane and cytoplasmic proteins.[10] The identified AJC proteins include integral transmembrane proteins, scaffold proteins, kinases, phosphatases, small GTPases, transcription factors, and actin binding proteins. A list of AJC proteins is provided in a recent review by Quiros and Nusrat.[11]

Many studies have described the deregulation of AJC proteins in samples of human cancers and in cell lines; this deregulation can be the result of up-regulated and down-regulated expression, epigenetic changes, and protein activation and location changes. Thus, we will discuss the causes of deregulation and their contributions to events related to CRC progression in this section.

Integral Membrane Proteins in TJs

Claudins

Claudins are primary proteins involved in TJ formation and paracellular barrier function. These proteins are composed of 4 transmembrane domains and 2 extracellular loops, with N- and C-terminal tails facing the cytoplasm. Claudins act in cell adhesion through interactions between claudins in adjacent cell membranes and between claudins within the same cell membrane.[12,13] Furthermore, these proteins can connect to other TJ transmembrane proteins, such as occludin.[14] Claudins also play important roles in cytoskeletal organization, signaling pathways, and vesicle transport. Claudins are directly associated with scaffold proteins, such as ZO1/2, during these processes.[13] The claudin family has 27 members that exhibit distinct expression patterns for each function. Alterations in the expression and localization of these proteins have been correlated to the disturbance of homeostasis, contributing to many diseases, including various types of cancer.[15] Table 1 summarizes the apical junctional proteins that are differentially expressed or mislocalized in CRC and associates this event with a functional response.

Resnick and co-workers demonstrated that low claudin-1 expression is directly associated with a higher grade of colorectal tumor. In this study, multivariate analysis indicated that lymphovascular invasion and low levels of claudin-1 expression were independent predictors of recurrence, which were also associated with poor survival of patients with colon cancer.[16] Controversially, other authors observed increased claudin-1 and decreased claudin-7 expression in SW620 colon cells, which are derived from a metastatic site. Moreover, claudin-1 redistribution of the cell-cell contacts to the nucleus was observed as compared normal mucosa with adenoma in ApcMin/+ mice.[17] Additionally, the increased expression of claudin-1, -3, and -4, which correlated with TJ disorganization and with increased cell permeability in human colon cancer samples.[18]

Significant claudin-8 downregulation and claudin-1 and -12 up-regulation were found in microdissected human colon cancer specimens compared with normal tissue, indicating that these proteins may represent potential molecular targets in colon cancer.[19] Martin and Jiang demonstrated that Smad4 inhibits claudin-1 expression and that this event contributes to inhibit the invasiveness of colon cancer cells.[20]

Darido and coworkers showed that claudin-7 was negatively regulated by the Wnt pathway in normal colon tissue and that this regulation was lost in tumor cells, possibly due to decreased Sox-9 activity leading to claudin-7 overexpression, cell polarity disruption, increased proliferation and tumorigenicity.[21] Furthermore, increased claudin-2 expression in colon cancer cells has been related to 5-FU resistance, increased cell proliferation, anchorage-independent growth, and tumor growth *in vivo*.[22] Claudin-1 expression modulates anoikis in colon cancer in a Src-Akt-Bcl$^-$2-dependent manner, which influences invasion and metastasis.[23] Recently, claudin-1 expression was able to induce colonic epithelial proliferation in a Notch-dependent manner in transgenic mice with specific overexpression of claudin-1 in the intestinal epithelium.[24] We have shown that EGF-induced claudin-3 overexpression is associated with the increased malignant potential of HT-29 cells and that the ERK1/2 and PI3K-Akt pathways are important regulators of this event. Importantly, the impaired paracellular flux of macromolecules in HT-29 cells that overexpress claudin-3 confirms the correlation between impaired epithelial barrier function and colorectal tumorigenesis.[25] Additionally, α- and β-Na,K-ATPase down-regulation and ERK1/2 activation induced by ouabain was able to increase claudin-3 levels, impairing the TJ barrier function and increasing cell viability and proliferation.[26] The presented studies show that altered expression of claudins is acting to favor initial steps of CRC progression, such as disturbed cell polarity, increased paracellular permeability, and enhanced cell migration. The mechanisms that regulate these molecular events are yet poorly understood, and future studies are needed to define the pathological relevance of claudins and exploit these proteins as potential therapeutic targets either for drug delivery or as diagnostic/prognostic markers in colorectal cancer.

Occludin

Occludin belongs to the tetramembrane-spanning TAMP family. Occludin appears to be unessential for TJ barrier formation because occludin knock-out mice showed normal TJ strand formation.[27] However, occludin, which is recruited by claudins, participates in TJ formation and in paracellular flux regulation.[28] An early study using Caco-2 cells demonstrated that oxidative stress induced by c-Src kinase caused TJ disassembly with the loss of occludin expression at the intracellular contacts.[29] Samples of hepatic metastasis from colon cancer presented downregulated occludin compared with normal liver tissue.[30] Generally, existing data show that human CRC tissues present decreased occludin expression compared with normal controls. Furthermore, this decrease also correlates with tumor grade because more advanced lesions show progressively less occludin expression compared to less advanced lesions.[31] Recently, hypoxic tumors displayed increased Cav-1 expression in Caco-2 cells, which may lead to decreased occludin expression and to increased paracellular permeability.[32]

Table 1. Altered expression/localization of AJC proteins and their roles in functional responses in CRC

Junctional Protein	Expression/ localization	Colon cancer model	Cellular function response	Reference
Claudin-1	down	Human tissue	Higher tumor grade/ predictors of tumor recurrence and poor survival	23
	up	Human tissue	Carcinoma progression and metastasis/disorganization and increased cell permeability	17–19
	up	Transgenic mouse	Colonic epithelial proliferation	24
	up	SW480 and SW620 cells	Cell differentiation inhibition and resistance to anoikis	23
Claudin-2	up	Caco-2 cells, Mouse	Resistance to 5-FU, increased cell proliferation, anchorage independent growth and tumor growth *in vivo*	22
Claudin-3	up	HT-29 cells	Increased malignant potential, cell viability and proliferation, and impaired TJ barrier function	25,26
Claudin-7	up	HT-29, SW480 cells and metastatic human tissue	Cell polarity disruption, proliferation and tumorigenicity increased	21
Claudin-8	down	Human tissue	Tumoral progression	19
Claudin-12	up	Human tissue	Tumoral progression	19
Occludin	down	Human tissue	Progression of colon lesions	31
	down	Hepatic metastasis from colon cancer	Tumoral progression	30
	down	Caco-2 cells	Increased permeability	29,32
JAM-A	down	Mouse	Permeability and proliferation increased	35
ZO-1	down	Human tissue	Tumoral progression	39–41
Symplekin	down	HT-29 cells	Decrease proliferation and down regulation of Claudin-2 cell differentiation	42,44
E-cadherin	down	Human tissue	Increase SNAIL and TWIST activity, metastasis and invasiveness	51–53
	down	HT-29 cells	Cell-cell adhesion loss/cell migration and invasion increased	26,56
	up	Human tissue	Tumor progression	54
β-Catenin	Nuclear localization	Human tissue	Tumor progression and worse prognosis/worse overall survival	59–61
	up	Human tissue/Caco-2 cells	Poor overall survival/ Increased proliferation	66,144
p-120 catenin	Nuclear localization	Human tissue	Increased proliferation/ adherens junctions loss	71
	up	Human tissue/ HT-29 cells	Proliferative cancer tissue/aberrant mitosis and polyploidy	70,145
	Cytosolic localization	HCT-116 cells	Increased migration/Rho activity increased	75
	Cytosolic localization	LIM1863 cells	EMT phenotype induction	72
Afadin	down	Mouse	Increased paracellular permeability/mislocalization of nectin-2 and -3	146

JAM

TJs include junctional adhesion molecules (JAMs) that belong to the cortical thymocyte marker for *Xenopus* (CTX) family of proteins. The JAM family encompasses 3 classical members (JAM-A, JAM-B, and JAM-C) and related molecules. Current evidence indicates that only JAM-A is expressed on mucosal epithelial cells and is directly involved in TJ formation and maintenance.[33] However, the JAM-A function is not restricted to TJ formation because the expression of this protein often correlates with functional changes such as reduced paracellular permeability and enhanced electrical resistance in colon cancer cells.[34] For instance, JAM-A deficiency enhanced colonic inflammation, permeability and epithelial cell proliferation in mice.[35] Additionally, the loss of JAM-A together with Afadin or PDZ-GEF2 lead to decreased level of activated Rap1, β1 integrin, and cell migration in human colonic epithelial cells, suggesting that JAM-A dimerization facilitates the formation of a complex Afadin/PDZ-GEF2, which activates Rap1A and in turn regulates the levels of β1 integrin and cell migration.[36] However, although the molecular mechanisms by which JAM-A controls the epithelial barrier are beginning to be understood, its role in CCR progression remains unknown.

TJ Adaptor Proteins

Zona occludens-1, -2 and -3

Zona occludens-1, -2 and -3 form a group of proteins that belongs to the MAGUK (membrane-associated guanylate kinase homolog) protein family. These proteins present a conserved modular organization of domains with homology to functionally defined signaling molecules such as a Src homology region 3 (SH3 domain), a region homologous to guanylate kinases, and PDZ domains.[37] TJ formation was delayed in ZO-1-deficient epithelial Eph4 cells, whereas deficiencies of ZO-2 and/or ZO-3 did not delay TJ formation, suggesting that ZO-1 plays a major role in the formation of belt-like TJs, unlike ZO-2 and ZO-3.[38] ZO-1 and occludin showed the same expression profile in normal epithelium of the digestive tract; however, the expression of these 2 proteins was reduced in poorly differentiated adenocarcinomas,

indicating a significant correlation between tumor differentiation and ZO-1 and occludin protein expression.[39]

In a study by using primary tumors of colorectal cancer with liver metastasis it was showed that the levels of expression of ZO-1 and E-cadherin were decreased, indicating that the decreased level of these proteins correlates with liver metastasis in this cancer type.[40] In addition, another study showed that primary colon tumors display an undifferentiated phenotype because ZO-1 was down-regulated by its association with epidermal growth factor receptor (EGFR). EGFR associated with ZO-1 was highly tyrosine-phosphorylated only in the primary CRC but was dephosphorylated in liver-metastasized cancer where cell redifferentiation occurs.[41] The importance of both ZO-2 and -3 proteins in colon cancer have not been reported.

Symplekin

Symplekin is a ubiquitously expressed protein that is enriched in the nucleus and that associates with TJs in polarized epithelial cells. At the TJ, the function of symplekin is not defined, however several studies have shown that it is involved in RNA polyadenylation and transcriptional regulation mechanisms. This protein also interacts directly with transcription factors such as, HSF1 and ZONAB/DbpA to promote the transcription of genes related to stress response or epithelial cell proliferation.[42]

A few studies have found correlations between symplekin and CRC progression. Its depletion in HT-29 cells correlates with the reduced transcription of *CD1*, which is a ZONAB target gene.[42] Buchert et al. showed that symplekin was strongly expressed in human colon cancer and that its downregulation reduced tumor growth in *in vivo* and *in vitro* models.[43] Symplekin and ZONAB silencing cooperates to down-regulate claudin-2 mRNA expression, cell proliferation and cyclin D1 expression in HT-29 CRC cells. Furthermore, the symplekin/ZONAB complex inhibits goblet cell differentiation by repressing the AML1/Runx1 transcription factor.[43,44]

Other relevant proteins that are important for tricellular contact formation, including the TJ proteins tricellulin and MARVEL D3 and the adaptor proteins MAGI 1, cingulin/paracingulin and ubinuclein, were not discussed in this review because the roles of these proteins in colon cancer have not yet been described.

AJ Proteins

The epithelial AJ core includes interactions among transmembrane glycoproteins that consist of 2 basic adhesive units: (a) the E-cadherin/catenin family and (b) nectin/afadin complexes.[10,45] Adherens junctions perform multiple functions, including the initiation and stabilization of cell-cell adhesion, as well as cell signaling.

E-cadherin

E-cadherin is a single-pass transmembrane glycoprotein that mediates Ca^{2+}-dependent intercellular adhesion with a conserved cytoplasmatic tail and with an extracellular domain formed of 5 cadherin repeats that mediate homophilic binding between cadherins of neighbor cells.[46,47] On their cytosolic tail, cadherins interact with a protein complex that is composed of α-, β- and p120-catenin, which links to the actin cytoskeleton and to several signaling pathways in a highly dynamic manner.[48,49]

E-cadherin dysregulation has been classically associated with tumor progression in several epithelial cancers, including CRC. E-cadherin down-regulation has been associated with epithelial-mesenchymal transition (EMT), as well as with the upregulation of genes such as *SNAIL* and *TWIST* during the development of colorectal adenomas in patients.[50] Immunohistochemical analysis of human colon cancer samples showed that the loss of E-cadherin (70%) was frequently observed in association with tumor progression and was considered a crucial event that favors metastasis and invasiveness.[51,52] *cKIT* gene suppression by NF-κB caused an increase in Slug activity and consequently decreased E-cadherin expression in colon cancer cells, contributing to liver metastasis.[53] However, one tissue screening study correlated increased E-cadherin expression with Rab11 expression, which was associated with colon cancer progression.[54] Our group verified that the inhibition of post-translational modifications in E-cadherin, such as *N*-linked glycosylation, induced cell-cell adhesion and decreased proliferation in CRC cells.[55] Furthermore, we found that ouabain induced both α- and β-Na,K-ATPase downregulation and ERK1/2 activation, which are interlinked events that play important roles in the cellular redistribution of E-cadherin, inducing cell-cell adhesion loss, an important step during CRC progression.[26] Recently, we also demonstrated that CRC cell progenies that are resistant to irradiation can generate more aggressive cellular progeny with a EMT-like phenotype, as well as with reduced E-cadherin expression and β-catenin overexpression.[56]

α-, β- and p-120 Catenin

The two primary functions of β-catenin are to provide dynamic adhesive connection between epithelial cells and the involvement in gene regulation interacting with transcriptional machinery in the nucleus acting as transcriptional co-factors. When β-catenin interacts with E-cadherin, AJs form and stabilize; however, when β-catenin is free in the cytoplasm, this protein may then translocate into the nucleus. Once in the nucleus, β-catenin can activate the transcription of Wnt/β-catenin target genes.[57] In CRC, 90% of all tumors have a mutation in a key regulatory factor of the Wnt/β-catenin pathway, most often in *APC* or *CTNNB*, which is the gene that encodes the β-catenin protein, resulting in the activation of the Wnt/β-catenin pathway. *CTNNB1* mutations are found more often in small colorectal adenomas than in invasive carcinomas, whereas other studies have found that *CTNNB1* mutations are associated with CRC in hereditary nonpolyposis colorectal cancer syndrome.[58] The dysfunction of the Wnt/β-catenin signaling pathway is important in CRC progression and results in the nuclear accumulation of β-catenin. β-catenin, which is an EMT-associated marker, plays a key role in CRC progression; however, the prognostic significance of β-catenin expression in patients with CRC remains controversial. For instance, some studies have shown that nuclear β-catenin expression is directly associated with high tumor budding and with a poor prognosis.[59-61] However, in other studies,

this association depends on other factors beyond β-catenin.[62-64] Additionally, a recent study revealed that β-catenin overexpression correlated with a favorable outcome to treatment but not with a predictive factor.[65] Recently, changes in β-catenin expression levels were also associated with aggressive morphological features, EMT and a poor prognosis in patients with CRC, as analyzed by immunohistochemistry.[66] Additional large-scale prospective studies will be necessary to determine the accurate prognostic significance of β-catenin.

In vitro studies using epithelial cells have shown that α-catenin is a key linker between F-actin and the AJ. α-catenin recruits actin regulatory proteins including vinculin, zyxin, Ena/VASP proteins, formins and ARP2/3 complex members. Actin polymerizing proteins control F-actin polymerization by themselves and frequently in Rho-associated protein kinase (ROCK)-independent fashion.[67] In a study using colorectal cancer cells, HCT-116 it was shown that α-catenin seems to facilitate the interaction between β-catenin and the APC complex, leading to the degradation of β-catenin. A point mutation in α-catenin prevented binding to β-catenin, which, in turn, also prevented β-catenin ubiquitylation, proteolysis and promoted β-catenin nuclear translocation. In addition, binding to APC might affect α-catenin-mediated cell adhesion and reorganization of the actin cytoskeleton leading to the misregulation of these events in cancer.[68]

p120 catenin binds to the juxtamembrane domain of E-cadherins. This protein stabilizes E-cadherin to the cell membrane and suppresses cadherin internalization, enhancing the surface abundance of this protein. The removal of p120 catenin greatly increases E-cadherin internalization in mammalian cells,[49] which could therefore result in tumor-and/or metastasis-promoting activities similar to those caused by E-cadherin down-regulation.[69]

The initial studies analyzing colon tissue samples to determine p120 expression in normal and tumor mucosa verified the correlation between p120 overexpression and high cell proliferation.[70] Subsequent studies showed the importance of the subcellular localization of p120 with AJ loss.[71] Bellovin et al. observed reduced p120-catenin-E-cadherin coprecipitation during EMT, which correlates with the formation of a p120/RhoA complex in colon cancer cells.[72] This p120/RhoA interaction seems to correlate with p120-catenin tyrosine phosphorylation in colon cancer cells.[73] K-ras gene mutation in Caco-2 cells stimulated cell migration, decreasing E-cadherin/β-catenin/p120 complex formation via MAPK signaling and increasing Rho activity.[74] p120 catenin phosphorylation in HCT-116 cells was associated with its increased internalization and with cell migration, as well as with altered Rho GTPase activity via an interaction with VAV2, which is an oncogene that controls actin cytoskeleton dynamics.[75]

AF-6/Afadin

AF-6, or afadin, is a novel intracellular AJ protein that interacts with the cytoplasmic region of nectins, which are immunoglobulin-like cell adhesion molecules at AJs, and linking nectins to the actin cytoskeleton.[76] AF-6/afadin expression level has been recently found to be adversely correlated with the prognosis and disease-free survival of breast cancer patients. Also, the loss of AF-6/afadin expression induced metastatic phenotype in breast cancer cells via activation of ERK pathway.[77] Recently, a study showed that exist an interaction between CTRF, a cAMP-activated chloride channel localized at the apical membrane of epithelial cells, and AF-6/Afadin involved in the pathogenesis of colon cancer indicating the 2 proteins as potential novel markers of metastasis and prognostic predictors for human colon cancer.[78]

GTPases

A series of studies have suggested that the E-cadherin-catenin, nectin-afadin, and claudin-ZO systems act not only as a mechanical complex to connect the actin cytoskeleton but also as a scaffold for cell signaling. Thus, the temporal and spatial regulation of contractility via Rho family protein activities is necessary for epithelial homeostasis and morphogenesis.[10] In the next section, we will discuss the interrelation between Rho GTPase members and AJC components and how alterations in this balance could be crucial in events related to cancer progression.

As discussed in this section, an understanding of the molecular structure, action mechanism and AJC component functions are potentially important targets for anti-cancer research and possible areas for future therapeutics.

Cross-Talk Between the AJC and the Actin Cytoskeleton

The correct assembly and maintenance of the AJC and its above-mentioned related proteins are essential for the preservation of tissue integrity. However, epithelial sheets are not a static unit; they constantly face different conditions and remodeling due to several cellular functions.[79] The driver of these changes is the actomyosin cytoskeleton, which provides structure and which generates force. Cellular processes require the quick and efficient reorganization of actin filaments, which is achieved by structural and regulatory proteins. The effectiveness of cell adhesion is not only determined by the strength of the connection between intercellular junction proteins but also by the intracellular links between the AJC proteins and AJC adaptors to the actin cytoskeleton.[10] The cytoskeleton, which consists of a dense and ever-changing network of filaments, is responsible for preserving cell morphology and for orchestrating processes such as cell proliferation and migration, all which, when deregulated, may trigger or favor the development of tumorigenesis. The interlink between the AJC components and the actin cytoskeleton to mediate important cellular events will be briefly discussed in the next section.

Interlink between TJ proteins and the actin cytoskeleton

The correct assembly of the 3 primary transmembrane components of TJs, claudins, occludin and JAMs, and of the cytoskeleton proteins, including actin,[80] non-muscle myosin 2A, 2B,

2C[81] and microtubules, is essential for preserving the barrier established by TJs in the epithelial sheet.[82] This linkage occurs via the adaptor proteins ZO-1, -2 and -3[83,83,84] and via certain scaffolding proteins, such as cingulin and afadin.[85] Cingulin binds directly to ZO-1 and is involved in junction formation, GTPase regulatory protein recruitment[86,87] and microtubule association.[88] Afadin is present in both TJs and AJs.[89,90] Afadin links with JAM-A and ZO-1, playing an important role in the formation and remodeling of junctions once this protein interacts with the membrane junction proteins and binds to actin filaments.[91] Actin-polymerizing proteins, such as Arp2/3,[92] N-WASP,[93] VASP[94] and cortactin,[95] have been described in close interaction to TJs.

One example of such interactions is the complex formed by JAM-A/ZO-2/Afadin/PDZ-GEF1, which regulates paracellular permeability by activating the small GTPase Rap2c. The downregulation of each of these components results in increased cell permeability. JAM-A also influences RhoA activity and non-muscle myosin phosphorylation, affecting actomyosin contraction.[96] Several actin-binding proteins also interact with ZO proteins. Inhibiting the binding between α-catenin and ZO-1 causes a disruption in the epithelial integrity, which affects ZO-1 and actin filament organization.[97] Changes in the expression levels of the tension-sensing protein vinculin[98] and α-actinin-4[99] also affect the stabilization of cell-cell junctions. Another protein, Shroom, has been shown to regulate the actomyosin cytoskeleton through an interaction with the ROCK protein[100] and with actin, myosin and microtubules, altering cell shape and junction formation.[101]

Interlink between AJs and the actin cytoskeleton

Alterations in the membrane proteins E-cadherin and nectin, which are major participants in the preservation of physical associations among cells, may lead to defective tissue organization. E-cadherin creates an adhesive force by the interaction of its extracellular domains.[102] However, E-cadherin depends on Ca^{2+} concentrations and on its linkage to other intracellular proteins to maintain its structure and localization. The core complex of E-cadherin-based cell junctions is composed of one cadherin linked by its intracellular domain to p120 catenin and β-catenin, which binds to α-catenin.[103] The α-catenin interaction with F-actin through its carboxyl-domain was thought to be an essential step in stabilizing the AJ; however, α-catenin may be unable to bind to F-actin when associated with β-catenin.[104] Despite its inability to link to F-actin when associated with the core complex of AJ, the interaction between the tension-sensing molecule vinculin (VCL) and α-catenin has suggested a possible model of this interaction. The tension created by the actomyosin cytoskeleton linked to α-catenin causes a change in its conformation, causing the M1 domain to bind to VCL.[105] Once bound, these proteins reinforce the strength through which F-actin is connected to the AJ complex. Two other actin-related proteins, vasodilator-stimulated phosphoprotein (VASP) and MENA, were found co-localized in the AJ with VCL, which is its dominant recruiting factor;[106] these proteins most likely cooperate with the E-cadherin/catenin/vinculin complex by enhancing actin polymerization.[107]

Nectins co-localize with E-cadherin in the AJ.[108] The cytoplasmic domain of nectins, more specifically their PDZ-binding motif, links with afadin, which is a protein that has been already discussed above as an actin-binding protein.[90,109] Interestingly, previous evidence indicates a direct interaction between afadin and α-catenin, suggesting the formation of a possible nectin-afadin/cadherin-catenin complex.[110] During the early AJC formation process, nectins are the first to gather at cell contacts, followed by E-cadherins,[111] possibly converging to the afore mentioned nectin-afadin/cadherin-catenin complex. E-cadherin localization at cell contact sites in Madin-Darby canine kidney (MDCK) cells is disrupted by lowering the Ca^{2+} concentration and returns to the contacts once the Ca^{2+} concentration is normalized. However, E-cadherin was unable to return to cell-cell contacts when annexin II expression was knocked down in this cell lane, which suggests a novel and important role for this protein in the formation and regulation of the AJC.[112]

Annexin II and Rho GTPases: modulators of a multi-faceted system

Annexin II is a phospholipid-binding protein with affinity for Ca^{2+} or for charged phospholipids. This protein, which is present at cellular membranes, particularly those membranes rich in phosphoinositides and cholesterol, is involved in several cellular functions, such as cell signaling, fibrin homeostasis, calcium and pH sensing, vesicle trafficking and actin binding and regulation. Both monomeric and tetrameric forms of annexin II are able to bind to F-actin and to bundle those preformed filaments. Considering its roles in actin binding and in interacting with cellular membranes, primarily the cytoplasmatic surface of the plasma membrane, annexin II may play an important role in controlling actin cytoskeleton rearrangements (for reviews, see[113]). This hypothesis has been strengthen by evidence that shows a consonant action between annexin and cofilin,[114] which is a major actin rearrangement modulator, and a pathway leading from annexin phosphorylation to small GTPase activation, more specifically, RhoA.[115] Although, annexin II is not a defined component of the AJC, it was proposed that this protein plays a role in TJ assembly possibly through linking juxtaposed exoplasmic leaflets to form a lipid platform across the intercellular space, indicating annexin II as a member of a new class of TJ proteins responsible for the long-observed convergence of adjacent exoplasmic lipid leaflets in TJ assembly.[116] However, more studies are necessary to confirm the real role that annexin II plays in actin cytoskeleton regulation and, consequently, in AJC organization.

Rho GTPases are a subset of the small GTPase Ras superfamily, which is composed of 5 families: Ras, Rho, Rab, Ran and Arf.[117] These proteins, which are responsible for the regulation of essential cellular processes (for a review see[118]), act in a wide array of mechanisms to stabilize and bundle actin filaments. The most prominent Rho GTPases related to actin regulation are members of the Rho family: RhoA (one of 3 isoforms), Rac and Cdc42. These three proteins, which are central modulators of actomyosin cytoskeleton, also play roles in microtubule dynamics, cell polarity, cell adhesion, membrane trafficking, cell proliferation, gene transcription, apoptosis, and cell survival.[118]

Although induced by the same catalysis, Rho, Rac and Cdc42 generate different effects on the cytoskeleton. When activated, Rho promotes the formation of basal F-actin stress fibers and focal adhesions, altering cell migration. When activated, Rac induces the development of lamellipodia and membrane ruffling, while Cdc42 helps to orchestrate cell polarity and filopodia formation.[119] The regulation of AJC (for a recent review, see[11]) by these 3 proteins relies on a balance between their active and inhibited states to preserve and maintain the TJ and AJ in such a manner that the epithelial barrier function is preserved.[120-122]

Taken together, these findings clearly indicate that changes in the expression of the AJC complex proteins and its adaptors and regulators of the actomyosin cytoskeleton may lead to disruptions of the epithelial function, such as higher proliferation, migration and invasion potentials and, more particularly, EMT, promoting pathogenic events such as tumorigenesis. For example, E-cadherin and p120 catenin under-expression is associated with a poor prognosis and with high invasive capacity of tumors.[123] Because of the low levels of E-cadherin, which are responsible for recruiting p120 catenin, p120 accumulates in the cytoplasm, favoring the invasive potential through Rho activation.[124,125] Lower levels of junctional E-cadherin in breast epithelial cells enhanced migration through Rho signaling,[126] and ROCK inhibition led to disorganization of the AJC complex, favoring higher cell proliferation and migration.[127]

A growing number of studies have examined the dysregulation of the AJC complex and the actin cytoskeleton in CRC, some of which will be discussed in the next section. However, the exact mechanisms by which these alterations and imbalances act still lack broader comprehension.

The Contributions of AJC and Actin Cytoskeleton Alterations in CRC Progression: Cell Signaling Involvement

Disturbances in the activities of various signaling pathways, which cause morphological and phenotypic alterations, have been observed during CRC progression. Among these alterations, we may highlight those alterations related to the loss of stability of the apical junctional complex and to the reorganization of the actin cytoskeleton, which both contribute to increased malignancy. In a recent review, Leve and Morgado-Diaz discussed the role that Rho GTPases, which are regulator proteins of the actin cytoskeleton, play in regulating both the assembly/disassembly and the function of the AJC and how subsequent cell-cell adhesion loss can trigger cell signaling pathways, leading to epithelial cancer progression.[128] However, few studies have linked alterations of AJC components and the actin cytoskeleton during CRC development, and most of these studies used *in vitro* methods, as discussed below.

The tension and contractility of actin filaments can be regulated by motor proteins of the myosin superfamily. Non-muscle myosin belonging to class II (NM-II) plays an important role in cell adhesion and cell migration and is present in non-muscle cells. NM-II activity has been related to modulating cell-cell adhesion.[129] The inhibition of this protein class can cause the dysregulation of the organization and stability of the AJC, as well as changes in TJ-regulated barrier function, as reviewed by Liu and Cheney.[81] NM-IIA knock-down in colon adenocarcinoma cell lines impairs AJC reorganization in the calcium switch assay, decreasing the presence of E-cadherin, β-catenin, occludin and ZO-1 in cell-cell contacts when the cell culture was switched from a low-calcium medium (~5 μM of Ca^{2+}) to a high-calcium medium (~1.8 mM of Ca^{2+}). These events were accompanied by the disarrangement of the cortical and perijunctional F-actin protein, which was visualized after the calcium repletion, suggesting an important role of the actin cytoskeleton in these processes.[130] Babbin and co-workers have yet reported that inhibition of NM-IIA reduces the planar migration of SK-CO15 colonic epithelial cells by increase the cell-matrix adhesion and decrease the stress fiber and mature focal adhesion formation, which are important to detachment and retraction of the cell during this process. In contrast, the NM-IIA downregulation increases the invasive potential these cells to increase in activation of the ERK1/2 pathway and calpain-2.[131] Moreover, the phosphorylation of sites in regulatory light chains or heavy chain by kinase proteins can modulate NM-II activity.[129] The activation of the PKC/ROCK-II pathway in pancreatic epithelial cells induces the phosphorylation and activation of NM-II, which causes the reorganization of the actin cytoskeleton and the disassembly of the AJC.[132] In addition, the differential regulation of myosin light chain phosphorylation, which is present in myosin class II, by MLCK and ROCK can modulate the dynamics of membrane protrusions and directional migration in fibroblasts.[133,134] Furthermore, the treatment of Caco-2 colorectal adenocarcinoma cells with the inflammatory mediator prostaglandin E_2 (PGE_2) induces AJC disassembly, barrier function dysregulation and F-actin belt disorganization, which are events modulated by PKC and MLCK.[135,136] Together, these data suggest the F-actin contractility can be modulated by expression and/or post-translational modifications of NM-II, which is involved with AJC organization and can regulate the malignant potential in colorectal cells. Specific inhibitors of these signaling pathways can be used as important tools to modulate the organization of cell adhesion and actin cytoskeleton during the development of carcinomas in an attempt to reduce the malignant potential.

Leve and coworkers have provided evidence that the PKA pathway may regulate the AJC and members of the Rho GTPase family differently in CRC cells, depending on the cell region: (1) In the basal region, PKA activation may inhibit RhoA, inducing the disruption of stress fibers, whose structures are composed of contractile bundles of actin. Furthermore, PKA activation or RhoA inhibition potentially could induce Rac activation, which would regulate the formation of lamellipodia-type membrane protrusion, which are composed of branched actin networks. (2) In the apical region, PKA activation induces AJC disassembly, causing the subcellular redistribution of AJ and TJ proteins, followed by RhoA activation and Rac inhibition. Furthermore, RhoA activation could lead to the recruitment of ROCK I or ROCK II effector proteins, which would regulate AJs or TJs and the actin cytoskeleton, respectively. Together, these events

Table 2. Signaling pathways that modulate expression/localization of apical junctional complex proteins and actin cytoskeleton regulators and their role in functional responses during tumor progression in CRC models

Signaling pathway	Apical junctional complex protein	Actin cytoskeleton regulators	Role in tumor progression	Reference
PI3K/Akt/ GSK-3β	Claudin-3, E-cadherin, β-catenin, ZO-1	?	Cell differentiation, Proliferation, migration	147
	?	Rac	Migration	148
ERK1/2	Claudin-3	?	Impaired barrier function, Proliferation, migration	25,26
	E-cadherin, β-catenin	?	Proliferation, impaired barrier function,	26
	?	Cdc42	Proliferation, migration, invasion	143
PKA	E-cadherin	Rho, Rac	Impaired barrier function	137
Src/FAK	E-cadherin, β-catenin, p120 catenin	Rho/ROCK	Migration	138
Wnt	E-cadherin, β-catenin	?	Migration, invasion	56
	β-catenin	Tiam/Rac	Proliferation, invasion	149

?, Unidentified protein.

contribute to the increased migration of CRC cells.[137] The Rho-induced increase in the migratory potential of CRC cells can also be regulated through the activation of lysophosphatidic acid (LPA) receptors. *In vitro* studies demonstrated that LPA treatment causes the activation of the Rho/Rock and Src pathways, which modulate AJ disruption. Moreover, LPA treatment in the basal region induced the activation of the Src/FAK pathway, which acts in the recruitment of Rho/Rock to modulate stress fiber and focal adhesion formation.[138] The reorganization of the actin cytoskeleton can modulate the dynamics of cell-extracellular matrix adhesion and the stability of the AJC, suggesting that cross-talk among actin-related Rho GTPases and Src/FAK pathways plays an important role in the increase in cell migration during CRC progression.

The expression of Cdc42, which is another member of the Rho GTPase family, also correlates with increased cell migration by regulating the elongation of actin filaments, which drives lamellipodia protrusions.[139] Elbediwy and co-workers have shown that SH3BP1 complex-regulated Cdc42 activity in cell-cell contacts is required to establish the AJC and the junctional actin belt in epithelial cells.[140] In contrast, Cdc42 mediates the EGFR/Src pathway activation after Ca^{2+} depletion, which induces E-cadherin degradation in MCF-7 breast cancer cells.[141] A decrease in miR-224 expression was observed in specimens of patients with CRC. This micro-RNA was able to induce the down-regulation of Cdc42 and SMAD4, which is an effector protein of the TGF-β signaling pathway, decreasing the migration of HCT-116 cells *in vitro*.[142] The selective inhibition of Cdc42 using the small-molecule inhibitor AZA197 reduced PAK/ERK pathway activation, as well as cell proliferation, migration, invasion, and increased apoptosis. Furthermore, AZA197 treatment decreased tumor growth and increased mouse survival in a preclinical xenograft model of colon cancer and has been proposed as a treatment for patients with colon cancer that overexpress Cdc42 and that present mutations in the *KRAS* gene.[143] Studies attempting to develop molecules that target Cdc42 can be promising for the treatment of patients with advanced colon cancer. However, further studies are required to determine the role of this GTPase in the regulation of the molecular mechanisms involved in the development of this cancer.

Recent evidence has supported the notion that alterations in the cell-cell adhesion system and cytoskeleton organization may increase the malignant potential of radioresistant CRC cells. Progenies of ionizing radiation survivor HT-29 cells presented actin cytoskeleton reorganization and cell spreading. Furthermore, these cells showed a mesenchymal-like phenotype, which was accompanied by an increase in cell migration and invasiveness, and these events occurred concomitant to the activation of the Wnt/β-catenin pathway. These findings suggest that CRC cells with intermediary resistance to ionizing radiation have a more aggressive phenotype than their parent cells.[56] However, the detailed molecular mechanisms that regulate the intercellular adhesion disturbance and alterations in the cytoskeleton of CRC cells surviving radiation remain to be established. Further studies in this area may identify possible targets to avoid the refractory effects of radiotherapy. The Table 2 summarizes cell signaling pathways that modulate expression/localization of AJC proteins and actin cytoskeleton regulators and the functional response during tumor progression in CRC models.

Together, these studies indicate that alterations of the organization of the AJC and the actin cytoskeleton, which are interlinked to maintain a functional cell-cell system in epithelia, may induce events related to colorectal progression, such as cell migration, proliferation and invasiveness. Thus, additional studies could to contribute to define the molecular mechanisms that govern such disorganization *in vitro* and *in vivo* in this cancer type.

Conclusions

Many studies have established that the altered expression/localization of AJC proteins and actin cytoskeleton disorganization can to contribute to human cancer development. Nevertheless, few studies have shown the interrelation between such events and their contribution in this context. In CRC, claudins, E-cadherin and β-catenin proteins are among the most-studied AJC constituents and have a vital role during CRC progression. However, understanding the functions of other important members of the AJC complex and their contributions to the development of this cancer type remains a major challenge for CRC researchers. On the other hand, despite great efforts to

understand the dynamics of the actin cytoskeleton during the events of migration and invasiveness, the key molecules that participate in the regulation of these events in cancer remain unclear. More importantly, the order in which these events occur also remains unclear. Does actin cytoskeleton reorganization cause the disruption of the AJC, or does the alteration in the expression/localization of AJC proteins lead to the reorganization of the actin cytoskeleton?. Regardless, the existing data suggest that controlling the regulation of these 2 mechanisms is crucial for preventing the metastatic process by the loss of the cell-cell adhesion system. Thus, large-scale prospective studies should provide answers that define the prognostic or diagnostic significance of the AJC components. Additionally, it is necessary to identify regulators of the actin cytoskeleton dynamics, as well as actin-binding proteins that link AJC components in CRC. We highlight the use of these components as possible therapeutic targets to prevent the increase in the malignancy of this disease. For instance, AJC proteins alone or in combination with actin cytoskeleton regulators, may represent useful biomarkers for the detection, diagnosis and prognosis of CRC, mainly through their pro-metastatic activity enhancing cell migration and invasiveness. On the other hand, the studies here discussed highlight the possibility for a target-specific systematic therapy using these proteins as therapeutic targets, specifically those that present altered expression levels.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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