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Ductal barriers in mammary epithelium

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Tissue barriers play an integral role in the biology and pathobiology of mammary ductal epithelium. In normal breast physiology, tight and adherens junctions undergo dynamic changes in permeability in response to hormonal and other stimuli, while several of their proteins are directly involved in mammary tumorigenesis. This review describes first the structure of mammary ductal epithelial barriers and their role in normal mammary development, examining the cyclical changes in response to puberty, pregnancy, lactation and involution. It then examines the role of adherens and tight junctions and the participation of their constituent proteins in mammary tumorigenic functions such as migration, invasion and metastasis. Finally, it discusses the potential of these adhesion proteins as both prognostic biomarkers and potential therapeutic targets in breast cancer.

Introduction

Breast cancer is the commonest malignancy among women worldwide, with over 1.3 million new cases diagnosed and over 450,000 deaths per annum.[1] While the prognosis of breast cancer has improved significantly in recent years, its mortality remains significant. Despite much research into targeted treatment modalities, treatment remains focused on surgical excision, chemotherapy, radiotherapy and hormone therapy, with the anti-HER2 monoclonal antibody trastuzumab currently the only targeted therapy in routine clinical use. As many of these treatment modalities are associated with significant side effects, the quest continues to discover biomarkers identifying those likely to benefit most from adjuvant treatment, and novel targets for development of anti-breast cancer agents with minimal systemic side-effects.

Proteins located in tight and adherens junctions are obvious candidates for such biomarkers and/or therapeutic targets for a number of reasons: first, cellular junctions encourage physical cell-cell associations that must theoretically be overcome to allow tumor cell shedding and distal metastasis, and second, many of the proteins in tight and adherens junctions are also involved in pro-proliferative and pro-migratory signaling cascades relevant to cancer progression (for review see[2]). As the majority of breast cancer mortality is due to metastatic disease rather than the primary tumor, the development of strategies to prevent such distal spread is particularly crucial.

This review aims to examine the role of ductal adhesion complexes and their constituent proteins in both the normal and diseased breast. We will first discuss their structure and physiology under normal circumstances such as puberty, pregnancy and lactation. Next we will examine their role in abnormal breast conditions, both inflammatory and neoplastic, but will focus on breast cancer as the most significant and best studied pathological condition of the breast. We will discuss the role of cell junctions and their proteins in such neoplastic behaviors as dysregulated proliferation, migration, invasion and metastasis. Finally, we will examine the potential use of junctional proteins both as breast cancer prognostic biomarkers and as novel therapeutic targets.

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Structure of Mammary Ductal Epithelium

The mammary gland (**Fig. 1**) is a modified apocrine sweat gland that consists of multiple pyramidal lobes each subdivided into several lobules, which in turn consist of multiple acini. These drain via a complex network of branching ducts, eventually conveying milk to the nipple. The basic functional unit is the terminal duct-lobular unit (TDLU), consisting of a lobule and its draining duct, which is supported within a network of fat and connective tissue.[3]

Microscopically, mammary ducts are lined by a single luminal layer of columnar or cuboidal epithelial cells surrounded by a discontinuous layer of contractile myoepithelial cells, in turn surrounded by basement membrane.[4] Like other epithelial layers, mammary ductal epithelial cells are joined at their cell-cell interfaces by junctional complexes; which include tight junctions, adherens junctions and desmosomes.

The tight junction forms a continuous band around the cell at the apical-most surface, effectively dividing it into apical and basolateral compartments and regulating the cellular barrier and paracellular transport.[5] Tight junction proteins can be broadly divided into integral transmembrane proteins such as occludin, claudins and junctional adhesion molecules (JAMs); peripheral or plaque adaptor proteins such as the Zona Occludens (ZO) proteins; and regulatory/signaling proteins such as cingulin and the Rho-GTPases.[2] Adherens junctions are located subjacent to tight junctions, forming a band around the cell and attaching the actin cytoskeleton to the plasma membrane.[6] Their proteins include the armadillo or armadillo-related proteins

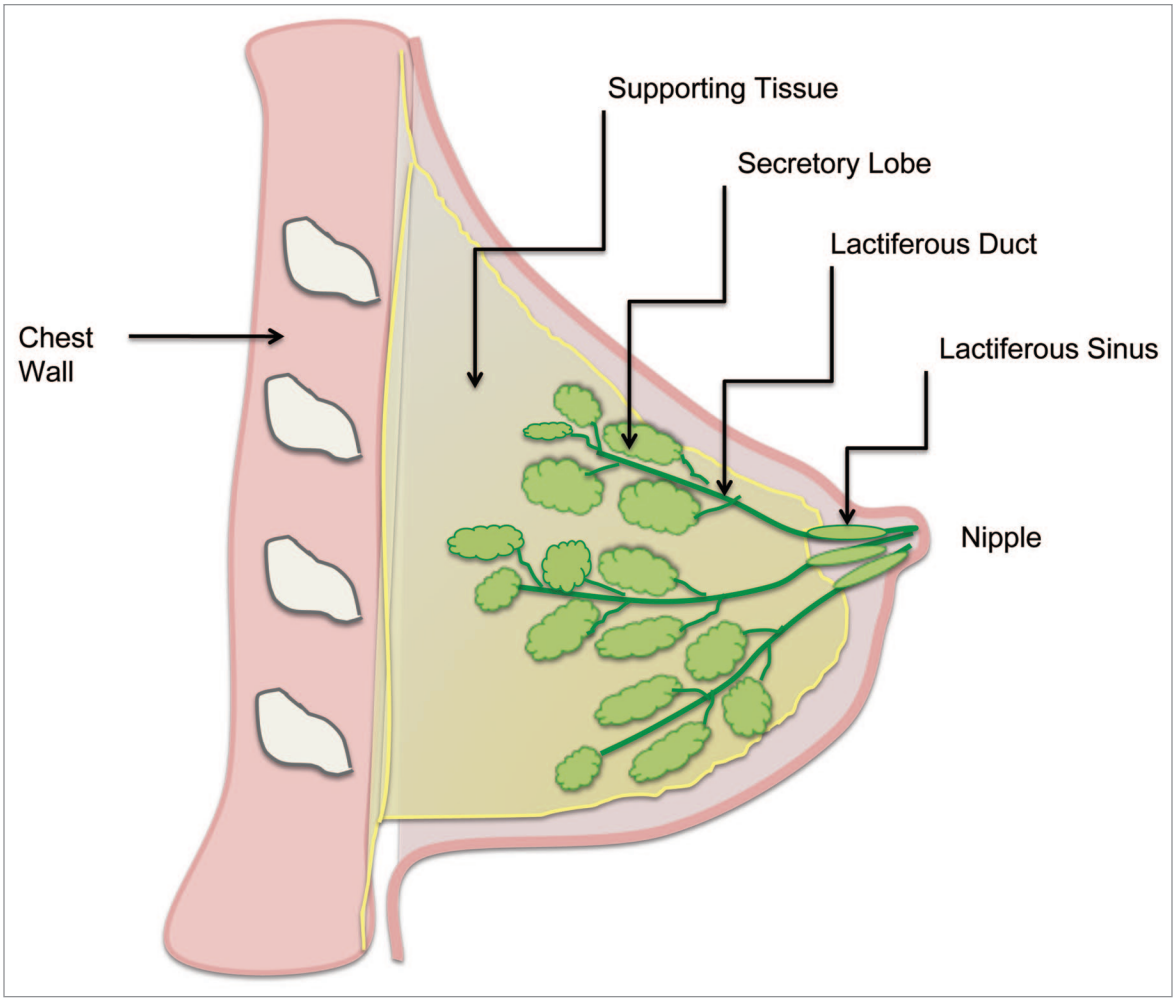

Figure 1. Normal physiology of the human breast.

(β-catenin, plakoglobin, p120); cytoskeletal adaptor proteins like α-catenin; and the cadherins (E-cadherin in normal epithelial cells, N-cadherin in mesenchymal cells).[6-8] Desmosomes appear as patches subjacent to adherens junctions. They anchor keratinous intermediate filaments to the plasma membrane, while their proteins include cadherins (desmogleins, desmocollins), armadillo proteins (plakoglobin, plakophilins) and desmoplakin.[8] The majority of this review will concentrate on the contribution of tight and adherens junction proteins to breast physiology and pathophysiology, principally because comparatively little has been published about breast desmosomal complexes. Nonetheless, desmosomal loss has recently been shown to be important for branching morphogenesis, the mammary remodeling process which ultimately permits lactation.[9] In fact desmosomal cadherins participate in epithelial-myoepithelial interactions in the normal mouse mammary gland, and selective desmosomal loss during the secretory phase of mammary development[10] likely underlies the development of myoepithelial discontinuities in lactating rat mammary glands.[11] The contribution of desmosomal alterations to pathologies such as cancer is also relatively understudied, though one might speculate that any loss of mechanical adhesion could facilitate local invasion of single cells potentially facilitating metastasis. That said, mechanically-strong cell-cell attachments might also allow multi-cell clusters to move in so-called "Indian files," a collective invasion phenomenon characteristic of some breast cancers. In support of the former speculation, loss of at least one desmosomal protein, desmoplakin, has been implicated in breast tumor progression.[12] Accordingly, it has been noted that estrogen binding to ERα, a hallmark of well-differentiated / less-invasive breast cancers, promotes adhesion via morphological enhancement of desmosome numbers and upregulation of the desmosomal proteins desmocollin, γ-catenin, plakophilin and desmoplakin.[13] Since liganded ERα also promotes transcription of the retinoic acid receptor α gene,[14] itself a positive regulator of cell-matrix adhesion in breast cancer cells,[15] this supports a model whereby hormone receptor expression, in part by promoting a pro-adhesion state, is associated with favorable differentiation status in breast cancer.

As detailed below, several of the above adhesion structures are modulated dynamically in time with the normal reproductive

and hormonal cycle, in addition to being altered in many pathological processes.

Models for Mammary Epithelial Research

As in most cell biology, research on mammary epithelial biology relies largely on in vivo and in vitro models. In vitro cell culture models, predominantly using commercially available epithelial cell lines, are the most common vehicle for researching mammary epithelial barriers. These offer the advantages of cost effective, readily available, uniform cell populations that are genetically well-characterized and grow readily and predictably in vitro. However, there are a small number of cell lines available, with only three lines (MCF 7, MDA-MB 231, T-47D) accounting for over two thirds of all abstracts reporting work on breast cancer derived cell lines appearing on Medline.[16] Given the reliance of a huge number of researchers on a small number of cell lines, cross-contamination between cell lines is an important problem, with one study suggesting 18% of cell lines may be affected.[17] Furthermore, an immortalized cell line selects only the clonal population that exhibits most proliferation under a given set of artificial conditions, most commonly derived from a metastatic cancer, and is thus inherently different from both the primary tumor and normal epithelium. Indeed, the in vitro environment, which usually consists of a clonal cell population adherent to a flat plastic surface, bathed in nutrient-rich, antibiotic-containing media; is quite different to the natural physiological milieu in which polyclonal cells proliferate in three dimensions, interacting both with other epithelial cells and with a complex supporting stroma.

In the in vivo setting, recent years have seen an explosion in the number and diversity of mouse mammary models of cancer, ranging from genetically-modified animals to mammary fat-pad injectible tumor models to patient-derived xenograft studies in the emerging era of semi-personalized medicine (reviewed in[18]). Enhanced by the recent adoption of non-invasive fluorescent tumor imaging technologies, these have collectively proven invaluable for unraveling aspects of the complex biology of breast cancer. For example, the human HER2 gene, whose amplification is linked with certain aggressive breast cancers, was originally identified as the pro-tumorigenic murine oncogene *neu*[19]; and in fact murine models still represent the gold standard pre-clinical approach for testing potential anti-cancer drugs. Interestingly however, there has been a relative scarcity of models for studying the fundamental *physiology* (rather than pathophysiology) of the mammary epithelial barrier. This may relate in part to technical difficulties in experimentally manipulating mammary tissue; although the economic importance of estimating (for example) drug accumulation in the colostrum of lactating animals has been an agricultural issue for decades. Nonetheless, important work in rodent mammary barriers has revealed several useful model systems for physiological assessment of permeability. These include measuring the relative recovery of drugs in serum vs. breast milk following oral gavage of compounds of interest in lactating rats,[20] or the assessment of basal-apical transport of radiolabeled albumin following its injection into the bloodstream of a rodent.[21] The reverse approach has utilized injection of radiolabeled sucrose or fluorescent albumin directly into mammary ducts followed by periodic blood sampling in order to determine apical-basal tight junction permeability during hormonal events such as the lactogenic switch.[22] It is, however, unclear how specific permeability coefficients in such models might relate to those in the human setting, due to an absence of comprehensive studies on substance accumulation in the breastmilk of lactating human females.

Aside from macromolecular permeability measurements, barrier function and ion transport characteristics of epithelial tissues (particularly intestine, skin and lung) have been classically assessed via electrical measurements in Ussing chambers.[23] Both semi-permeable filters (usually polycarbonate or polyester)[24,25] and collagen gels[26] have been successfully demonstrated as appropriate physical matrices for subsequent electrical measurements in primary or immortalized mammary cells. However it is interesting to note that mammary cells appear particularly sensitive to the culture microenvironment in terms of their ability to form tight, electrically-sealed barriers; as exemplified by a recent report that the non-transformed normal-like breast cell line MCF-10A can only develop measurable transepithelial electrical resistance in the absence of the adenylyl cyclase activator cholera toxin.[27] Our own unpublished observations have noted that these cells require a long period of "conditioning" to the cholera toxin-free environment (approximately one month) before a high resistance monolayer can be formed (Kieran Brennan, personal communication). These findings illustrate at least two essential points – first the complex crosstalk between electrical resistance and ion transport (via established chloride-secretory second messengers such as adenylyl cyclase), and second the importance of exercising caution in interpreting barrier function results from endocrine tissues such as the breast, whose very structure and function is heavily dependent on hormonal status and developmental stage.

Biology of Mammary Ductal Barriers

The physical structure of the mammary ductal epithelial barrier is almost identical to that of other epithelia, with the exception that intact epithelial monolayers lie on a bed of contractile myoepithelial cells (**Fig. 2**) instead of being in direct contact with the basal lamina. The myoepithelial monolayer predominantly functions as a physiological tool to expel milk into the ducts during lactation, while in pathophysiological terms myoepithelial loss is an early hallmark of ductal carcinoma in situ. However it is in its biology, particularly the dynamic alteration of mammary barrier function in response to hormonal changes, that the mammary epithelium is unique. More than most organ systems, the breast undergoes frequent changes in response to puberty, the menstrual cycle, pregnancy, lactation and menopause; and many of these are modulated via alterations in ductal junctional complexes or junctional proteins directly. Signaling via the canonical Wnt pathway involving the adherens junction protein and transcription factor β-catenin has been implicated in virtually every stage of this cycle.[28] Wnt/ β-catenin signaling is vital for the formation of the embryonic mammary placode in mice[29] and β-catenin target

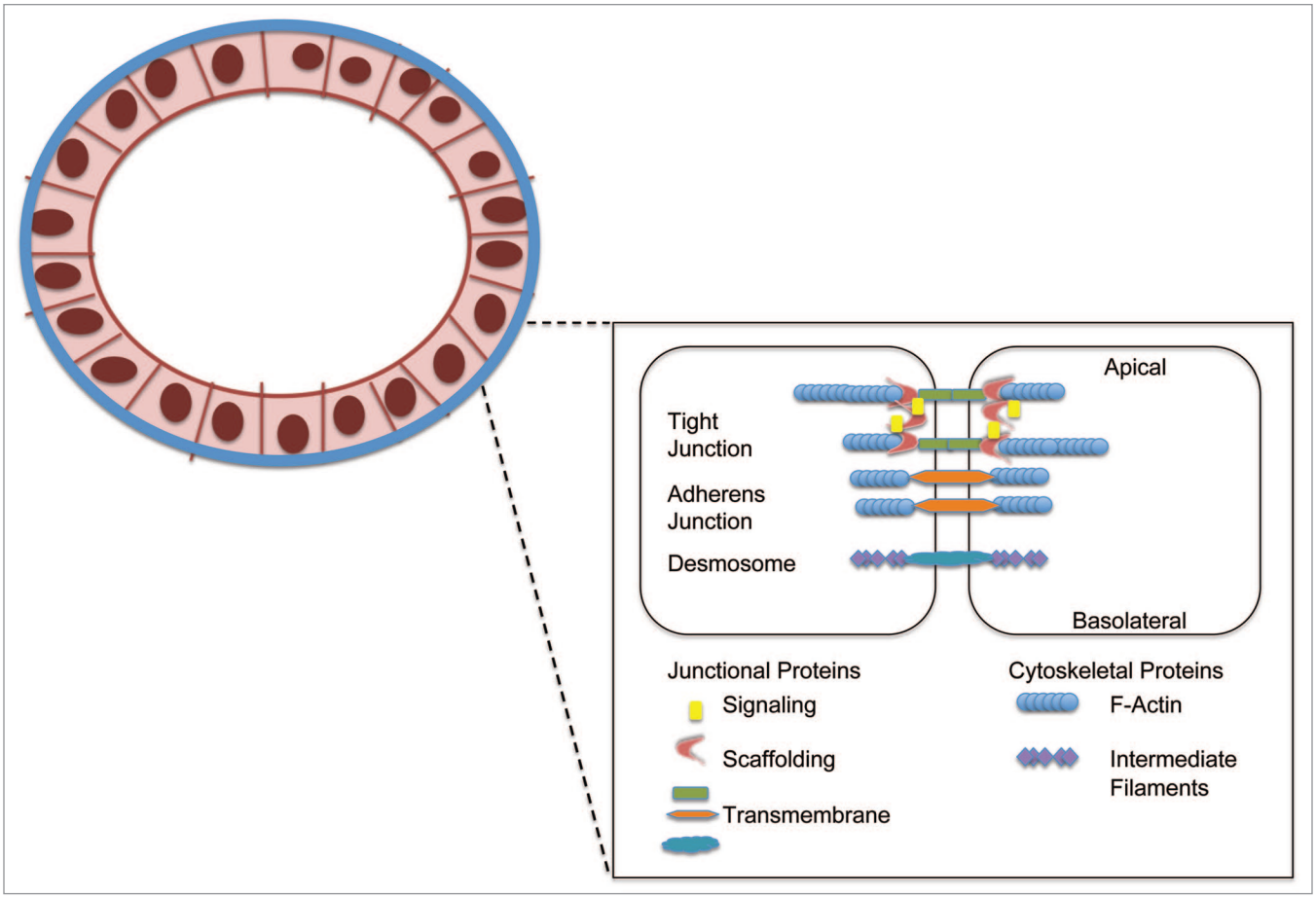

Figure 2. Cell-cell adhesion complexes in the breast epithelial barrier.

genes are upregulated in murine embryonic ductal morphogenesis.[30] Murine in vivo studies have yielded indirect evidence of low level Wnt/ β-catenin signaling occurring during mammary development at puberty, with enrichment of Wnt5a and Wnt7b mRNAs in terminal end buds and that of Wnt2 in mammary stroma.[28,31-33] During pregnancy, progesterone-induced changes such as increased ductal branching are modulated via β-catenin signaling in a mouse model.[34]

Most of our understanding of the effects of pregnancy and lactation on mammary junctions comes from the agricultural and dairy industry, with minimal research interest in human lactation. Pregnancy is characterized by increased leakiness of the mammary ductal tight junctions in particular, with numerous studies on both dairy goats[35,36] and mice[37] showing extravasation of large molecules from the pregnant duct, although to a greater extent in alveolar than ductal epithelium. This is reflected in the loss of trans-epithelial electrical resistance in the mammary epithelium of pregnant goats and mice[37,38] and the altered composition of milk pre-partum in both humans and dairy animals, with higher concentrations of the interstitial ions sodium and chloride, as well as proteins.[35,39,40] In addition, morphological alterations in tight junctions have been observed in mammary epithelium derived from pregnant sheep, with lower numbers of strands and less branching complexity exhibited.[41,42]

Mammary epithelial tight junctions are altered by several hormones including glucocorticoids,[43] prolactin,[44] serotonin[45] and progesterone, and it is the sharp fall in the latter at parturition that allows tight junction closure to provide a leak-proof duct and restore trans-epithelial resistance, thus facilitating lactation.[22] Glucocorticoids such as cortisol are raised throughout pregnancy, and decline during lactation.[46,47] There is evidence that glucocorticoid treatment reduces mammary tight junction permeability in both bovine[43,44] and rat[48,49] in vitro models, via downregulation of the small GTPase RhoA[50] and phosphorylation of GSK3 by Akt.[51] Glucocorticoid treatment has been shown to prevent rat mammary involution in vivo.[52] An in vivo study on ovariectomized mice showed that although progesterone withdrawal was the primary trigger for mammary epithelial tight junction closure in late pregnancy (as demonstrated by progesterone antagonism), low to moderate levels of both cortisol and either placental lactogen or prolactin were required for this to happen.[22] Unlike progesterone and glucocorticoids, prolactin does not appear to prevent involution in mice,[52] although several authors report that prolactin maintains mammary epithelial impermeability in late lactation in rabbits, largely indirectly by preventing apoptosis.[53-55] Interestingly, neutrophils are able to pass through these intercellular junctions to reach the lumen if necessary, with complete reconstitution of tight junctions occurring afterwards.[56]

The neurotransmitter serotonin (5-HT) is an important regulator of lactation. It is locally synthesized in mouse mammary glands.[57] In vitro work on human mammary epithelial monolayers has demonstrated that serotonin influences transepithelial resistance only when added at the basolateral membrane,[58] and directly decreases expression of ZO-1 and ZO-2.[27] It exerts biphasic effects on the mammary tight junction, promoting tight junction integrity at low concentrations via protein kinase A; while sustained exposure to higher concentrations of serotonin disrupts tight junctions via p38 MAP kinase signaling, encouraging mammary involution.[45,58] In addition, serotonin indirectly affects mammary tight junctions by stimulating prolactin secretion,[59] and is itself influenced by a prolactin-induced positive feedback mechanism.[57]

Pathobiology of Mammary Ductal Barriers – Inflammation

As in many other organ systems, inflammation affecting the breast (mastitis), which most commonly occurs in the lactating gland, causes increased permeability of the ductal epithelium. This is evidenced by increased sodium and chloride content in the milk, as well as loss of transepithelial electrical resistance.[39,60] While this may in part be due to direct epithelial injury, it has been shown in a variety of human and animal tissues that tight junction permeability is also increased as part of the inflammatory response, mediated by inflammatory cytokines such as tumor necrosis factor (TNF),[61,62] histamine[63,64] and interferon-γ in rat intestine.[65,66] While these mechanisms have yet to be demonstrated in mammary tissue, it is quite likely that they are also involved in this process and may represent an important adaptive response to allow access by immune cells.

Pathobiology of Mammary Ductal Barriers – Breast Cancer

While some of the roles of ductal barriers in normal breast physiology and benign conditions have been described above, it is the role of mammary epithelial junctions and their proteins in the pathophysiology of breast cancer that has attracted by far the most attention. We will discuss below the role of ductal barriers and their constituent proteins in the pathophysiology of breast cancer, with particular focus on core markers of neoplastic behavior such as dedifferentiation, proliferation, migration, invasion and metastasis; before discussing the potential roles of junctional proteins as tumor biomarkers and drug targets.

Cell Polarity and Dedifferentiation

Tight junctions are vital to maintaining polarity of epithelial cells, delimiting their apical and basolateral aspects via the assembly of three complexes that maintain cell polarity: CRB, PAR and Scribble.

The CRB complex, the most apically-located, includes the proteins CRB3, PALS1 and PATJ and defines the apical region of polarized epithelial cells. CRB3 (Crumbs3) has been recognized as an important regulator of tight junction formation, its overexpression delaying tight junction formation in MDCK cells, the canine kidney cell line classically used as a highly-differentiated epithelium with well-developed junctional structures.[67] Forced expression of CRB3 in MCF10A cells, a mammary epithelial cell line that expresses little CRB3 and does not form true tight junctions in culture, has been shown to promote recruitment of ZO1, occludin and claudin-1 to sites of cell-cell contact and to induce the formation of true tight junctions.[68] PATJ stabilizes the CRB complex, having been demonstrated as vital for the proper localization of CRB3, as well as claudin-1, ZO-1, ZO-3, occludin and atypical PKC in mammalian epithelial tight junctions.[69-71]

The PAR complex consists of Par3, Par6, atypical PKC (aPKC) and Cdc42/Rac1. Par3 interacts with JAM-A[72] and PTEN,[73] which in turn allows it to interact with TIAM-1, stabilizing the junction.[74] Further binding of Cdc42/Rac1 recruits aPKC to the apical surface, maintaining the integrity of the apical cellular region.[75]

The basolateral Scribble complex consists of the proteins Scribble, lethal giant larvae homolog (LGL) and discs large homolog (DLG).[76,77] Scribble has been shown to co-localize with both the tight junction protein ZO-1[78] and with E-cadherin and DLG at adherens junctions,[79] suggesting its importance in the formation of both these junctions. Loss of Scribble function in Drosophila disrupts the localization of adherens junctions and apical proteins[80] while its knockdown in MDCK cells disrupts adhesion, delaying tight junction formation and allowing more rapid migration without impairing cell polarity.[81]

Loss of cell polarity is a crucial step in epithelial-mesenchymal transformation (EMT), the process whereby epithelial-derived cancers, including breast, progressively develop an invasive mesenchymal signature and phenotype.[82] Many proteins involved in cell polarity are dysregulated in breast cancer. The transcription factor ZEB1, which is upregulated in some breast cancers and is implicated in EMT, inhibits the expression of CRB3 and PATJ.[83] Overexpression of Par6 in breast epithelial cells induces increased proliferation while maintaining cell polarity,[84] and activation of ErbB2 (the gene encoding HER2, the oncogenic receptor tyrosine kinase overexpressed in a population of aggressive breast cancers) disrupts apical-basal polarity by associating with Par6 and aPKC.[85] Scribble expression in invasive lobular carcinoma specimens has been shown to be quantitatively reduced,[79] while it is redistributed from the membrane to the cytoplasm in several invasive ductal carcinoma lines.[86] In addition, its loss in mammary epithelial cells results in abnormal morphogenesis both in vivo and in vitro and inhibits c-myc-induced apoptosis in vitro.[86]

It is tempting to speculate that alterations in the expression levels of tight junction proteins, or indeed their inappropriate localization, may also play a role in breast cellular dedifferentiation. Histological observations of deficits in the strict polarization of the breast ducts are frequently one of the earliest indicators of malignancy. Occludin is downregulated in breast cancer, and its forced expression promotes senescence in murine mammary carcinoma cells,[87] while expression of its interacting protein ZO-1 is reduced in poorly differentiated breast cancer specimens.[88] JAM-A plays an important role in regulating cell morphology

by modulating activity of the integrin-activating small GTPase Rap1.[89] Despite initial conflicts between reports of its expressional correlation with the malignant breast phenotype,[90,91] the balance of evidence from our laboratory and others now favors a model whereby JAM-A overexpression in breast cancer associates with poor prognosis.[91-94] This will be further discussed in the Migration and Invasion section.

The cadherin switch is an important precursor of EMT in breast cancer. It involves a progressive dedifferentiation, switching from expression primarily of epithelial markers such as E-cadherin and cytokeratins to mesenchymal markers such as N-cadherin, vimentin and fibronectin.[95] This may be a normal component of processes such as wound healing and development of structures such as tubules,[96] however it occurs in a dysregulated fashion in cancer. E-cadherin is underexpressed in a number of breast cancer cell lines including the highly-invasive triple negative MDA-MB 231, although E- and N-cadherin status is not fully predictive of invasiveness.[97] Immunohistochemical staining of breast cancer specimens suggests that E-cadherin is lost in the majority of lobular, but not ductal, breast carcinoma,[98-100] and occurs as early as the in situ stage in lobular carcinoma.[101] Numerous mechanisms can result in the loss or downregulation of E-cadherin, of which loss of heterozygosity and mutations in the CDH1 gene which encodes it are commonly seen in lobular carcinoma.[102,103]

A number of transcription factors can coordinate the shift from E- to N-cadherin expression, largely controlled by the transcription suppressor Snail. The latter protein activates Zeb1, which binds to the E-cadherin promoter and blocks transcription of E-cadherin.[104,105] Further repressors that have been implicated include Slug, Twist, Zeb2, E12/47, SIP1 and δEF1, in addition to hypermethylation of E-cadherin promoters.[106-109] Furthermore, E-cadherin can be targeted for endocytosis and degradation secondary to the actions of tyrosine kinases such as Src, EGFR, FGF receptor, c-Met and IGF-1R in epithelial cells[110]; and can be directly degraded by matrix metalloproteinases.[95]

The protein Twist, itself regulated by canonical Wnt1 signaling,[111] downregulates E-cadherin expression, concurrently upregulates N-cadherin expression and can induce EMT. Twist overexpression is associated with dysregulated cell growth in murine mammary tumors,[111] and with multi drug resistance in human breast cancer cells.[112,113] N-cadherin promotes fibroblast growth factor (FGF) signaling in breast cancer cells by binding to and preventing the internalization of its receptor, thus sustaining its pro-migratory and invasive effects via MAP kinase activation and matrix metalloproteinase 9 secretion.[114]

Dysregulation of Proliferation

Cancer can essentially be considered a disease of dysregulated cell growth and proliferation. A crucial point is the loss of regulation of the cell cycle, resulting in uncontrolled cell division and abnormal growth.[115] Loss of E-cadherin expression, in addition to facilitating cell detachment through its mechanical effect at adherens junctions, directly induces a number of pro-proliferative signaling pathways in breast cancer. E-cadherin interacts with the epidermal growth factor (EGF) receptor to modulate proliferation by suppressing pro-proliferative tyrosine kinase signaling.[116] E-cadherin also binds and sequesters β-catenin in adherens junctions, thus its downregulation frees β-catenin to enter the nucleus and participate in pro-proliferative canonical Wnt signaling. Increased levels of cytosolic and nuclear β-catenin have been reported in up to 68% of breast cancer patient specimens (77% of invasive lobular carcinoma, 64% of invasive ductal carcinoma), and implies a poor prognosis.[117-120] In addition, upregulation of other β-catenin signaling promoters such as Disheveled,[121] LRP 6[122] and a mutant LRP5,[123] as well as downregulation of the β-catenin inhibitor Wnt5a,[124] are commonly seen in breast cancer specimens. Several alternative pathways known to increase β-catenin expression are activated in breast cancer, including the NF-κB pathway in ER-negative, HER2-positive tumors[125]; Pin1 upregulation in breast cancer patient specimens is proportional to increasing tumor grade and associated with increased β-catenin expression[126,127]; while loss of PTEN activates Akt and β-catenin resulting in increased proliferation in breast cells.[128,129]

Placental (P-) cadherin, a junctional protein usually expressed by mammary myoepithelial cells, is expressed in approx 30% of breast cancer cell lines[97] and up to 50% of invasive ductal carcinoma specimens.[130] It is strongly expressed in the basal (classically ER, PR and HER2 triple negative) and HER2-overexpressing subtypes of breast cancer on patient tissue microarrays,[131] and has been suggested as a routine biomarker for basal-like cancers.[132,133] It is associated with increased proliferation, a reduction in ER-α signaling, increased p53 and HER2 expression; and a poorer prognosis.[131] The cadherin switch from E- to P- expression is described in embryonic development,[133] with little evidence of its occurrence in breast carcinoma, where P-cadherin is more commonly co-expressed with E-cadherin in both human and murine studies.[134,135]

Although the bulk of mechanistic work implicating dysregulated adherens junction signaling in cancer has been performed in cell line models, concordance between cell line and animal data appears to be high and thus valuable insights from a wealth of genetic ablation studies in non-breast tissues must not be overlooked. While conventional knockout animals featuring germline loss of specific adherens junction proteins are often embryonic-lethal, recent advances in tissue-targeted loss of components such as p120-catenin[136,137] have revealed interesting tendencies toward an increased risk of cancer. The precise contribution of altered adhesion vs. altered signaling to such tumorigenic events remains elusive, however contrived separation of these two functions may be unphysiological and unrealistic. As a comprehensive discussion of genetic models of adherens junction perturbation is beyond the scope of this article, interested readers are directed to a recent review on the topic.[138]

Migration and Invasion

A further hallmark of malignancy that facilitates tumor spread and thus survival is dysregulated migration and invasion. Broadly speaking, cell migration consists of five cyclical steps, reviewed in ref. 139. It begins with the formation of protrusions known as

pseudopodia at the leading edge, driven by actin polymerization controlled by the Rho GTPase Cdc42 and several downstream effectors. Small transient adhesions to extracellular matrix near the leading edge are formed by β1, β2 and α2β1 integrins and other adaptor and signaling proteins, interacting with actin; and these adhesions further develop in response to tension applied by stress fibers. Proteinases are recruited to these focal adhesions, which then cleave extracellular matrix barriers.[140,141] The cell body translocates forward driven by myosin bundles sliding along actin filaments in an energy-dependent process.[139] Release of adhesions at the rear of the cell and retraction of the rear complete the cycle.[142] Normal epithelium and well-differentiated carcinomas tend to exhibit collective migration and invasion, whereby cell-cell interactions are retained and migration occurs in single sheets or strands.[143] In contrast, inflammatory cells, mesenchymally-derived tumor cells and poorly-differentiated carcinomas with loss of strong cell-cell contacts tend to migrate individually.[144]

The tight junctional protein JAM-A, a Type I transmembrane protein belonging to the immunoglobulin superfamily and a known regulator of cell adhesion and cell migration (reviewed in[145]), has a somewhat controversial role in breast cancer. While early evidence suggested that low JAM-A expression correlated with a less migratory and invasive breast cancer phenotype,[90] an increasing body of evidence from our group and others would suggest otherwise. Specifically, JAM-A overexpression in breast cancer specimens correlates with poorer patient prognosis,[91] and its expression has been shown to correlate with HER2 expression,[92] ER negativity, higher grade, and aggressive luminal B, HER2 and basal subtypes of breast cancer. Knockdown or antagonism of JAM-A reduces migration and invasion in JAM-A-expressing breast cancer cells,[146] and JAM-A has been shown to act as an upstream regulator of various signaling pathways relevant to the promotion of migratory behavior. One such pathway has revealed a direct relationship between JAM-A expression and that of the migratory protein β1-integrin in both colonic[89] and breast[91,146] epithelial cells, and accordingly JAM-A dimerization signaling has been shown to regulate expression levels and activation status of the β1-integrin-activating small GTPase Rap1.[89,91,146] A second emerging pathway implicates JAM-A as a novel regulator of the expression levels (and therefore signaling potential) of oncogenic HER2 in breast cancer cell lines, via a mechanism regulating the proteasomal stability of HER2.[92] It is tempting to speculate that JAM-A might also regulate the signaling functions of other oncogenes or receptor tyrosine kinases by influencing their proteasomal stability, but as yet this field of investigation is in its infancy. Regarding the initial controversy over whether or not JAM-A expression levels have a positive or a negative correlation with aggressive and migratory tumor behavior,[90,91] the balance of studies supports the notion of JAM-A as a positive regulator of cancer progression at least in the breast.[94] Nonetheless it is possible that seemingly contradictory effects could be explained by JAM-A under-expression impairing cell adhesion and polarity, favoring early malignant change; while its overexpression in later stages of cancer might favor tumor progression via (among others) integrin-mediated pro-migratory signaling.[2]

Another key family of tight junction molecules is the 27-member claudin family of membrane tetra-spanning proteins (reviewed in[147]). Although originally best known for ultrastructurally driving tight junction strand formation and consequently exerting differential control over charge selectivity through various epithelia (reviewed in[148]), claudins have recently also been implicated in the control of cell migration and reported to be up- or downregulated in a variety of cancers. Claudin-4 appears to regulate migration in both normal and malignant breast cells,[149] and its expression correlates with higher grade and worse prognosis in breast cancer specimens.[150] Similarly, claudin-5 expression in breast tumor specimens correlates with worse survival and its forced expression increases breast cancer cell motility in vitro.[151] In contrast, forced overexpression of claudins -6[152] and -16[153] have been shown to decrease breast cancer cell migration and invasion in vitro, and loss of claudin-6 conversely promotes anchorage-independent survival of MCF 7 breast cancer cells.[154] Claudin 1 under-expression has been demonstrated in breast cancer specimens,[155] while conversely high levels in breast cancers have been shown to correlate with the aggressive basal-like phenotype.[156] Interestingly, a new molecular subtype of breast cancer, claudin low, has been recently described, in which tumors are characterized by low gene expression of claudins-3, -4 and -7 in conjunction with an aggressive, basal-like phenotype.[157] However there is a paucity of information on potential cross-talk mechanisms between claudins and established drivers of tumorigenicity. Intriguing recent data have demonstrated a physical interaction between claudin-7 and the potential oncogene EpCAM in various gastrointestinal cells, tissues and tumors,[158,159] while emerging evidence suggests that EpCAM regulates the lysosomal degradation of claudin-7 in addition to its localization.[160] It is likely that pursuit of knowledge regarding the mechanistic involvement of various claudins in cancer cell migration and tumor metastasis will be a lucrative area of research in coming years.

Adherens junctional proteins are also strongly implicated in breast cancer migration and invasion. The cadherin switch to expression of mesenchymal cadherins most likely facilitates migration and invasion both by increasing tumor cells' ability to detach from their normal surrounding epithelial cells, and by inducing inappropriate pro-motility signaling.[161] Transfection of N-cadherin into E-cadherin-expressing breast cancer cells induces invasion and motility,[97] while transfection of E-cadherin into highly invasive mesenchymal-like MDA-MB 231 cells reduces invasion and migration.[162]

Metastasis

Migration and invasion are particularly important for the systemic spread of tumors, acting as a key early step in the multistep cascade known as metastasis. Accordingly, cell junctions and their proteins have been reported to play an intrinsic role in preventing breast cancer metastasis. The primary event in metastasis involves detachment of cells from the primary tumor and invasion into the bloodstream, followed by extravasation at the site of metastasis. This is somewhat similar to extravasation of leukocytes in the immune response and consists primarily of

three steps; first loose attachment and rolling on the endothelial surface, second tighter attachment of the tumor cells to the endothelium, and third transmigration through the endothelium. The latter can occur either by the transcellular or paracellular route. While the loose attachment, rolling and tighter attachment steps in tumor cells are similar to leukocytes, transmigration of tumor cells differs from that of leukocytes (termed diapedesis) in that it permanently alters endothelial morphology,[163] resulting in retraction of endothelial cells and in some cases apoptosis, possibly due to loss of cell-cell contacts.[164-166] N-cadherin interactions between tumor and vascular endothelial cells appear to partly mediate tumor cell-endothelial attachment and extravasation.[167,168]

Claudin-2 expression has been shown to be increased in breast cancers that metastasize to the liver. Its ability to mediate tumor cell- hepatocyte interaction is thought to facilitate arrest in this organ.[169,170] A further study in mice has reported downregulation of claudin 4, claudin 7 and γ-catenin in liver metastases originating from breast cell lines, in conjunction with altered γ-catenin cellular localization. Interestingly, claudin 7 was also expressed by macrophage-like cells surrounding the liver metastases, and was re-expressed in large tumors, suggesting a possible interaction of with the microenvironment to promote metastasis.[171]

Breast cancer commonly metastasizes to brain, with a prevalence of approximately 30% at autopsy.[172,173] Risk factors include high grade and stage, young age, estrogen receptor negativity and HER2 overexpression.[174,175] Brain metastasis requires breach of the blood brain barrier (BBB), a unique non-fenestrated endothelial structure that prevents passage of large molecules and cells. Tight and adherens junctions are integral to the barrier function of brain microvascular endothelial cells (BMECs).[176,177] BMECs display higher transepithelial electrical resistance and lower solute permeability than other endothelial cells, while their tight junctions are more complex and passage of polar solutes via the paracellular pathway is greatly reduced. The basement membrane is relatively thicker, and the underlying astrocytes regulate flow across the barrier. Among the proteins implicated in susceptibility to brain metastasis, loss of claudins 3 and 5 is associated with increased leakiness of the BBB in vivo.[178,179] Stromal cell derived factor-1α (SDF-1 α) is a chemokine expressed by several organs including the CNS, and expression of its receptor CXCR4 in breast cancer cells may facilitate BBB penetration. SDF-1 α treatment increases permeability of BMEC monolayers to breast tumor cell invasion, activating the PI-3K/AKT signaling pathway and causing endothelial cell retraction.[180] HER2/Neu has been shown to upregulate CXCR4 expression.[181] Matrix metalloproteinases also play a role in breast to brain metastasis; with MMP-2 and -3 activity increased in vivo[182,183]; and that of MMP-1 and -9 increased in vitro.[184]

Having discussed many of the functional neoplastic processes relevant to breast cancer progression involving junctions, we will now examine some of the many proteins and membrane domains that have been implicated in junction-based signaling in breast cancer. As an exhaustive examination of all implicated proteins and domains would be beyond the scope of this short review, we will focus on a select few relevant to our own research.

Lipid Rafts and Adhesion Proteins

While the classical model of the plasma membrane describes the membrane as a liquid-disordered phospholipid bilayer with molecules such as proteins and cholesterol randomly interspersed,[185] this model is now viewed as an oversimplification. Specifically it has been recognized that cholesterol and several proteins involved in dynamic cellular processes are non-randomly segregated into liquid-ordered membrane microdomains known as lipid rafts, which form as a consequence of tight spatial packing of membrane domains predominantly composed of sphingolipids, as opposed to phospholipids. This was initially postulated based on such observations as the ability of differing phases to co-exist in lipid bilayers[186,187]; differential distribution and clustering of membrane lipids[188-190]; and the presence of sphingolipid-enriched detergent-resistant regions of cell membranes.[191] Simons et al. were among the first to define the concept of lipid rafts,[192] which have been defined as "small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes"[193].

Several adhesion proteins have been described to be associated with lipid raft domains, and it has even been suggested that tight junctions themselves constitute a subtype of raft domain.[194] Biochemical enrichment of occludin and ZO-1 have been demonstrated in detergent-resistant domains in human[194,195] intestinal cells, while claudins 1, 3, 4, 5, 7, 8[195-197] and JAM-A[197] have been retrieved from detergent-resistant domains in various epithelial cells. Cholesterol depletion with methyl β cyclodextrin has been shown to redistribute claudins 3, 4 and 7, JAM-A and occludin, but not claudin 1, out of these domains in intestinal epithelial cells.[197] The tyrosine kinases Src and EGFR both localize in breast cancer cell lipid rafts[198-200]; both regulate cadherin/catenin interactions,[201,202] and both have been strongly implicated in pro-malignant signaling in breast cancer.[202,203] An inhibitor specifically targeting raft-associated Src has been shown to inhibit cell cycle progression and cell adhesion in breast cancer cells, while the absence of a raft-targeting sequence in the inhibitor eliminated these effects.[200] Pharmacological disruption of lipid rafts using lovastatin to interfere with cholesterol biosynthesis increases the growth inhibitory effects of the EGFR inhibitor gefitinib in breast cancer cell lines that normally are resistant to this agent.[199] In addition, components of the Wnt signaling pathway may be lipid raft associated.[204,205] It has been suggested that cholesterol in lipid rafts stabilizes the protein complexes in tight junction strands.[197]

While junctions play an important role in cell migration, it has also been shown via a pharmacological raft-disruption approach that lipid rafts modulate front-rear polarity in migrating MCF7 breast cancer cells.[206] Work from our group has revealed shuttling of the hyaluronan receptor CD44 in and out of intact lipid raft domains of breast epithelial cells according to migratory status,[207] and recent site-directed mutagenesis work has revealed that genetic targeting of biochemical motifs which drive raft affiliation of CD44 is sufficient to force an EMT-like state in breast cancer cells (Babina, Donatello, Hill and Hopkins, manuscript under review). Similarly, unpublished work from our group

suggests shuttling of Na^+ K^+ ATPase in and out of raft domains in breast cancer cells lines, in a hormone receptor status-dependent manner, in response to treatment with anti-proliferative doses of the potential anti-cancer drugs cardiac glycosides (Owens and Hopkins, manuscript in preparation). It is intriguing to speculate that similar changes may occur in breast cancer with raft-associated junctional proteins in response to such junction-dependent processes as cell migration. However it is advisable to interpret the collective literature on lipid rafts in physiological/pathophysiological processes with caution due to a relative over-dependence on the pharmacological lipid raft disrupting tool, methyl-β-cyclodextrin, which has recognized limitations in terms of specificity.[208]

Clinical Application of Adhesion Molecules in Breast Cancer

A number of tight and adherens junction proteins have been suggested or investigated as potential biomarkers in breast cancer. As discussed above, claudin expression in breast cancer has been closely examined, and the aggressive claudin-low subtype is now recognized, defined by low gene expression of claudins 3, 4 and 7.[157] Thus, claudin characterization will likely become a routine part of breast cancer diagnostic and prognostic workup.

P-cadherin has been identified as a cancer stem cell marker for basal-type breast cancer,[133,209] and has been shown to be an independent marker for disease-free, but not overall, survival.[210] While the prominent role of E-cadherin downregulation in EMT would make it a tempting proposition as a prognostic indicator (and in fact E-cadherin loss has diagnostic value in lobular carcinomas), used alone its correlation with prognosis has been variable.[211-216] One study found a reduction in one of E-cadherin, β-catenin, α-catenin and plakoglobin in tumor specimens to correlate significantly with breast cancer metastasis,[217] and a recent paper has indicated a combination of E-cadherin and carcinoembryonic antigen as a useful predictor of relapse.[216] As E-cadherin sequestration of β-catenin in adherens junctions prevents the latter partaking in pro-neoplastic canonical Wnt signaling, it would be logical that a measure of β-catenin distribution might be of prognostic benefit. One study found that a novel scoring system of membrane minus cytoplasmic β-catenin correlated with worse outcome in breast cancer.[218]

Despite the obvious theoretical promise of cell junctions and their proteins as anti-metastatic therapeutic targets, junction-directed therapies are still an exciting and under-explored area. Perhaps the greatest potential lies with targeting Claudins 3 and 4, which have been recognized as the receptors for the permeability-enhancing lytic toxin *Clostridium perfringens* enterotoxin (CPE).[219] CPE may be a useful therapy in breast cancers overexpressing these proteins, as it has been shown to induce lysis of claudin 3- and 4- overexpressing breast cancer cell lines.[220]

Another exciting potential target is JAM-A, given the positive association between its overexpression and poor prognosis in breast cancer patients[91,92,94] and following a recent publication demonstrating anti-proliferative efficacy of a function-blocking JAM-A antibody in xenograft murine models of breast cancer.[221] Unpublished work from our group has also shown promising in vitro and pre-clinical in vivo efficacy of a novel small molecule inhibitor of JAM-A, which we speculate could be particularly valuable in aggressive breast cancers concomitantly overexpressing HER2 and JAM-A.

ADH-1, an anti-N cadherin protein, has shown efficacy against pancreatic and prostate cancer in preclinical studies,[222,223] in addition to promising effects on disease stabilization in early clinical trials.[224] However, it has yet to be evaluated in breast cancer.

While the targeting of junctional proteins in breast cancer is still in its infancy, the expanding roles of these proteins in driving malignant signaling processes suggest many exciting targets for future research.

Conclusion

It is clear that breast ductal adhesion complexes and their constituent proteins play vital roles in breast physiology and pathology, not just by influencing mechanical adhesion and stability but also by influencing key cell signaling and gene transcription events. While modulation of the physical properties of breast ductal barriers is essential for cyclical changes in lactation and engorgement, we have also discussed the role of junctional proteins in changes such as ductal development in embryogenesis and puberty. We have further examined how alterations in junctional integrity could potentially contribute to pathologies including breast inflammation and breast cancer invasion/metastasis. We have particularly focused upon the role of junctional proteins in dysregulated signal transduction and gene transcription events that are associated with neoplastic phenomena such as proliferation, dedifferentiation, invasion and metastasis. Finally, we have explored some of the many exciting prospects for junctional proteins as both prognostic biomarkers and as therapeutic targets. It is the latter function of junctional proteins that is currently the focus of much research, and that may yield meaningful contributions to patient care in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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