Connexin 43 in the development and progression of breast cancer: What's the connection? (Review)

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Abstract. Connexin 43 is a prominent gap junction protein within normal human breast tissue. Thus far, there have been a number of research studies performed to determine the function of connexin 43 in breast tumor formation and progression. Within primary tumors, research suggests that the level of connexin 43 expression in breast tumors is altered when compared to normal human breast tissue. While some reports indicate that connexin 43 levels decrease, other evidence suggests that connexin 43 levels are increased and protein localization shifts from the plasma membrane to the cytoplasm. In either case, the prevailing theory is that breast tumor cells have reduced gap junction intercellular communication within primary tumors. The current consensus appears to be that the loss of connexin 43 gap junction intercellular communication is an early event in malignancy, with the possibility of gap junction restoration in the event of metastasis. However, additional evidence is needed to support the latter claim. The purpose of this report is to review the connexin 43 literature that describes studies using human tissue samples, in order to evaluate the function of connexin 43 protein in normal human breast tissue as well as the role of connexin 43 in human breast tumor formation and metastatic progression.

Contents

1. Introduction

Gap junctions are intercellular channels that connect the cytoplasm of neighboring cells. These channels allow for crosstalk between cells by regulating the direct intercellular exchange of secondary messengers, small metabolites or inorganic ions, through a process known as gap junction intercellular communication (GJIC). Located at the plasma membrane of the cell, gap junctions are composed of two hemi-channels called connexons, which are assembled from six oligomerized protein subunits called connexins (Fig. 1). The connexin proteins that make up the hemi-channel subunits can be homomeric, consisting of a single protein type of connexin family member, or heteromeric/hetero-multimeric, consisting of two or more connexin family members. When the hemi-channels from two opposing cells come together to form a gap junction pore, the channels can likewise be homotypic, where both hemi-channels are composed of a group of a single type of connexin protein, or heterotypic, containing hemi-channels composed of more than one family member.

In general, connexins and gap junctions are found in numerous cell types throughout the body. To date, 21 different connexins have been identified in humans (1). Expression of these connexin proteins varies among cell types, and cells often express more than one connexin (2,3). Since the initial observation that GJIC is lost in cancer cells, the function of connexins and the role of GJIC in human cancer has been researched and debated (4-6). Since the 1960s, much of this research has centered around the hypothesis that loss of direct intercellular communication is characteristic of cancer cells and is an important driver of tumorigenesis (1).

Connexin 43 (Cx43) is a predominant connexin protein that has been studied in a variety of human cancers including prostate, lung, liver, brain, and breast. Though scientific evidence indicates that connexins, including Cx43 have a tumor suppressive role in primary tumors, their role in cancer

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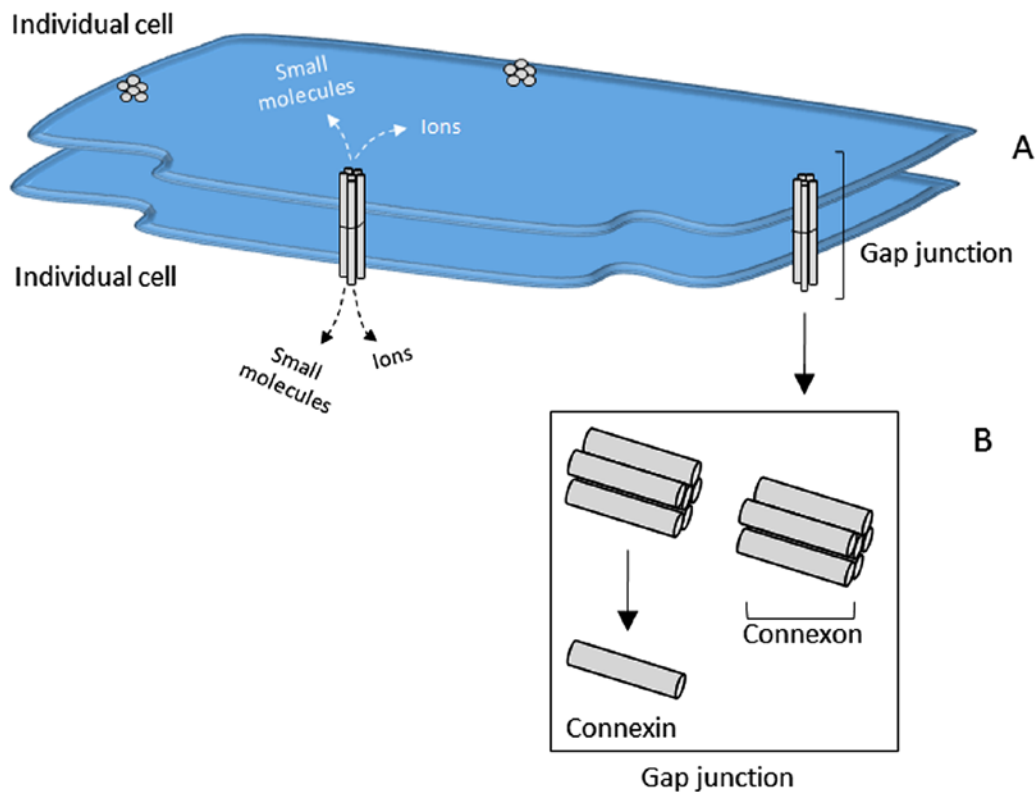E-mail: yeh@musc.edu

Figure 1. Gap junctions are the structures that allow for communication between individual cells via the direct intercellular exchange of secondary messengers, small metabolites and inorganic ions. Gap junctions are located at the plasma membrane and connect neighboring cells (A). Each gap junction is composed of two hemi-channels known as connexons and each connexon is comprised of six oligomerized protein subunits called connexins (B).

progression and metastasis is quite controversial. Studies that examine human breast cancer tissue suggest that levels of Cx43 either increase or decrease with cancer stage (7-10). Studies also suggest that Cx43 localization to gap junctions could play a part in determining disease severity, suggesting that preserving Cx43 gap junctions could be an important distinction between normal and malignant breast epithelial cells (7,10,11,23,25-28). However, evidence supports both pro- and anti-metastatic roles for Cx43 and many reports indicate that levels of Cx43 are both elevated and reduced in breast cancer (7,9,11-24). Consequently, there has been prominent debate in breast cancer regarding whether Cx43 promotes or inhibits breast cancer progression and metastasis because experimental findings have been somewhat contradictory (11-21).

　　To date, it appears that in primary breast tumors, overall Cx43 expression is either decreased or increased, with a shift from the protein's normal localization in the plasma membrane within gap junctions to a primarily cytoplasmic localization (7,10,22,23,25-28). In the majority of studies, the consensus is that GJIC is lost within primary tumors. However, in the event of breast cancer metastases, it has been suggested that Cx43 may become upregulated, which may or may not coincide with a restoration of GJIC (9). Although some consensus, particularly with primary tumors, has been reached, the exact role of Cx43 in breast cancer, particularly with metastasis, remains elusive. This report seeks to review the role of Cx43 in human breast and to present the current literature on clinical observations of Cx43 from studies that focus solely on human breast and breast cancer tissue samples.

2. Connexin 43 expression in the normal human breast

The normal, non-malignant, human breast contains a ductal epithelial tree that is composed of a contiguous layer of luminal epithelial cells that is surrounded by an outer layer of basal, myoepithelial cells. In the human breast, two predominant connexins have been described, Cx26 and Cx43 (7,22,24,25,29). Studies to decipher which connexins are expressed in human breast have indicated that Cx26 and Cx43 are expressed in individual compartments of the epithelial tree (1-5). Evidence suggests that Cx26 appears to be predominantly expressed in the luminal epithelium and Cx43 appears to be predominantly expressed in the myoepithelium (7,22,24,29,30).

　　An initial study by Wilgenbus *et al* evaluated Cx26, Cx32, and Cx43 in non-malignant and malignant human tissues, including breast, by immunohistochemical (IHC) analysis and immunoblotting with antibodies directed to each of the connexin proteins (25). Although no discernable expression of Cx26 and Cx32 was reported, Cx43 was found to be expressed in normal breast and the connective tissue of invasive ductal carcinomas of the breast. Following, a study by Pozzi *et al* evaluated Cx43 by IHC and reverse transcriptase PCR (RT-PCR) in human breast samples from archived tissues that were obtained from non-pregnant women who had undergone reductive mammoplasty (29). This group identified Cx26, Cx32, and Cx43 in human breast using RT-PCR. However, only Cx43, localized to myoepithelial cells, was detected by IHC. It is of potential interest to note that Cx43 expression appears to be predominant when the method of detection for each connexin relies on protein detection using antibodies.

A follow-up study by Monaghan *et al*, also using human breast obtained from reduction mammoplasties, confirmed the expression of Cx43 in human breast (24). In this study, IHC, immunoblotting, and RT-PCR were used to evaluate connexins including Cx26 and Cx43 in normal breast tissue. IHC staining indicated that Cx43 is predominantly expressed in the basal myoepithelium of the ducts whereas Cx26 was detected among the luminal epithelial cells of the ducts. Additional studies by Jamieson *et al* (22) and Kanczuga-Koda *et al* (7) confirmed the observation by IHC analysis that Cx43 is expressed in myoepithelial cells in normal, non-malignant human breast. Furthermore, consensus suggests that Cx43 is localized primarily to the plasma membrane at the lateral surface of myoepithelial cells, presumably at sites of gap junction formation between the myoepithelial cells (10,22,24,26).

In the study by Monaghan *et al*, normal breast samples were also evaluated for Cx43 by RT-PCR, which confirmed that Cx26 and Cx43 mRNA are present (24). This observation confirms the expression of Cx26 and Cx43, but it does not rule out the possibility that the expression signal from either Cx26 or Cx43 could have originated in the epithelial cells and/or other stromal tissue found in the human breast. However, while Cx43 expression has been reported in the stromal compartment of different types of breast malignancies, it has not been extensively reported in the non-epithelial cell compartment of non-malignant, normal human breast tissue (22,25).

In the study by Monoaghan *et al*, the authors also purified populations of luminal and basal cells from the normal human breast samples (24). These cells were cultured *in vitro* and were further evaluated by immunofluorescent (IF) staining and RT-PCR. Isolated basal cell populations showed an increase in Cx43 staining by IF as a function of time in culture. The isolated luminal cell cultures were shown to primarily express Cx26, similar to the *in vivo* tissue analysis done in this study. By western blotting, Cx43 but not Cx26 was present in purified luminal and basal cells. By RT-PCR, Cx43 was more abundant than Cx26 but still present. Certainly, cell culturing techniques and antibody sensitivity toward individual connexins are among the possibilities suggested for the differences in observations between the studies described here. Additionally, if Cx43 is localized to the plasma membrane of basal myoepithelial cells that border luminal cells, it is potentially logical to presume that both luminal and basal cell types would be positive for Cx43 protein. Furthermore, multipotent cells may have been present in isolated cell populations, consistent with the observation that Cx43 expression increased with time. None-the-less, these findings suggest a potential role for Cx43 in normal human breast function and it follows that a disruption of normal Cx43 expression and function, potentially between luminal and basal cell populations or the surrounding stroma, could play a role in the development of human breast malignancy.

3. Connexin 43 expression and breast cancer prognosis

Since Cx43 protein expression is relatively abundant in normal human breast myoepithelial cells, studies to determine whether Cx43 expression increases or decreases in breast cancer relative to normal tissue and in relation to cancer stage is a prominent area of investigation. A study by Jamieson *et al*, sought to evaluate the pattern of expression of Cx26 and Cx43 in human breast tumors (n=27, 12 grade 2 tumors and 15 grade 3 tumors) relative to their expression in normal human breast (22). The study authors confirmed Cx43 in normal human breast tissue localizes to myoepithelial cells and further observed that the stroma of invasive breast carcinomas contained Cx43-positive cells, unlike the stroma of normal breast tissue. These findings were consistent with earlier studies from Wilgenbus *et al* who also observed stromal staining of Cx43 in samples of invasive ductal carcinomas (25). Together, these studies indicate that there is an increase in Cx43 protein expression in the stroma of invasive breast carcinoma.

Interestingly, the majority of the carcinomas examined at in the study by Jamieson *et al* showed upregulation of Cx26 and/or Cx43 in carcinoma cells (22). The authors suggest that this marked increase in Cx43 protein expression levels goes against the hypothesis that GJIC suppresses tumorigenesis. However, if Cx43 protein is elevated but not localized or functional at the site of gap junctions, then this would suggest either gap junction independent functions for Cx43 exist or that cancer cells upregulate Cx43 protein as a compensatory mechanism. It is important to note that 4 out of 12 of the grade 2 tumors and 10 out of 15 grade 3 tumors exhibited carcinoma cells that were Cx43-positive and the observed staining was mainly diffuse and cytoplasmic. The authors speculate that the ductal epithelial cells of normal human breast express connexins other than Cx26 or Cx43 and, that the upregulation of Cx26 and/or Cx43 by the carcinoma cells could alter GJIC between hyperplastic or malignant cells and normal non-cancerous cells (22).

A study by Laird *et al* reported observations that Cx43 protein expression is reduced or lost in breast tumors compared to normal breast tissue (23). However, the authors did not observe increased Cx43 staining in the stromal compartment. In the study by Laird *et al*, 32 surgical specimens from breast cancer patients who did not receive pre-operative neoadjuvant chemotherapy or radiation therapy were evaluated and compared to adjacent normal tissue from corresponding patient samples (23). Similar to the observations made by Jamieson *et al*, the non-malignant breast tissue specimens showed moderate to high levels of Cx43 staining (22). The 32 specimens evaluated by Laird *et al* included malignant samples from ductal carcinoma *in situ* (DCIS), invasive lobular carcinoma (ILC), and invasive ductal carcinoma (IDC) but no difference in Cx43 expression was observed between the different histological types and almost all the samples were negative for Cx43 staining (23). The study authors also used IHC to evaluate estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) in relation to Cx43 expression but no correlation to any of the three receptors was observed. Taken together, these observations suggest that loss of Cx43, and presumably GJIC regulated by Cx43, is a common feature of breast malignancies regardless of histological or receptor defined subtype.

A later study by Kanczuga-Koda *et al* evaluated a cohort of samples from normal breast (n=25), samples with evidence of dysplasia (n=40), and breast cancer samples including intraductal and invasive carcinomas (n=29) (7). Consistent with earlier observations, the majority of intraductal carcinomas showed little to no immunoreactivity with Cx43 antibodies suggesting that Cx43 expression is lost or reduced

during malignancy (22,23). However, in many of the cases of invasive breast carcinomas that were evaluated in the study by Kanczuga-Koda *et al*, diffuse cytoplasmic staining was observed suggesting a noticeable amount of Cx43 protein is present (7). The authors suggest that their findings are consistent with the study by Jamieson *et al*, who also observed increased Cx43 staining in invasive carcinomas (22). However, it should be noted that in the study by Jamieson *et al*, the increased Cx43 staining was also associated with increased Cx43 in the tumor stroma (22). Whether or not this distinction is significant, remains to be determined.

A follow-up study by Kanczuga-Koda *et al* evaluated 71 breast cancer patient samples that only included invasive ductal carcinomas of histological grade 2 or grade 3 (10). While the authors found no association between Cx43 expression with age, tumor size, or lymph node status, there was a significant positive correlation with Cx43 expression and histological grade 3 tumors. The authors also evaluated Bak and Bcl-2 expression to follow-up on findings from a study that implicated Cx43 in apoptosis and found a significant association between Cx43 expression and the pro-apoptotic protein, Bak (10,31). No association with Cx43 and the anti-apoptotic protein, Bcl-2 was found (10).

A more recent study by Teleki *et al* sought to evaluate the expression of Cx26, Cx32, Cx43, and Cx46 in breast cancer patient samples prior to and after neoadjuvant chemotherapy (27). In this study, the authors used IHC to evaluate each connexin in samples taken from patients before and after treatment with a variety of chemotherapeutic agents including docetaxel, epirubicin, cyclophosphamide, doxorubicin, viorelbine, fuorouracil, and trastuzumab. These samples were also correlated with Ki67 expression staining and information regarding ER, PR, and HER2 status was available for most of the patient samples. The pre-chemotherapy levels of Cx43 correlated with ER status where high Cx43 expression was more likely to be found in ER-positive samples both before and after chemotherapy treatment. The observation that Cx43 is associated with ER status was also made in an earlier study by Shipitsin *et al* (30). Conversely, Cx43 expression negatively correlated with HER2 expression pre-chemotherapy (27). Since previous reports indicate that expression of Cx43 is hormone responsive, the finding that Cx43 expression positively correlates with ER status seems reasonable (32-35). However, this finding is contradictory to the study by Laird *et al* which reported no association between Cx43 and ER or HER2 (23). No relationship between Cx43 and Ki67 was reported as Ki67 staining was not provided for the Cx43 pre-chemotherapy treatment samples for comparison (27). Together, the observations made in this study by Teleki *et al* indicate that elevated Cx43 expression corresponds with ER expression but not HER2. Furthermore, Cx43 expression is not changed due to chemotherapy treatment.

An additional observation that can be deduced from the Teleki *et al* study is that the presence of Cx43-positive cells in both pre-chemotherapy and post-chemotherapy samples suggest that breast cancer cells are positive for Cx43 (27). This observation is consistent with the study by Kanczuga-Koda *et al* which reported a significant level of Cx43 staining in invasive breast carcinoma samples (7). However, no additional measures were taken to distinguish between epithelial and stromal components in breast cancer samples in the former (27). As this study also evaluated Cx26, Cx32, and Cx46, it seems pertinent to discuss the overall conclusions that the authors made was that a reduced level of Cx26 post-chemotherapy suggested a better prognosis. Likewise, high levels of Cx46, regardless of whether expression was observed before or after chemotherapy, suggested a better prognosis. These additional observations open up the opportunity for discussion that changing the mixture of connexins expressed in the breast could have significant impact on malignancy and bring into question the potential roles of heterotypic channels as well as hemi-channels in breast cancer.

Almost all of the studies that evaluated Cx43 expression have focused on protein expression detection by IHC. Therefore, Teleki *et al* performed a study to evaluate connexin gene expression using mRNA platforms (26). The authors also performed an additional protein based analysis on a cohort of tumor microarray (TMA) samples which represented all histological grades of breast cancer. The mRNA analysis was performed separately on data mined from Affymetrix and Illumina platforms but both analyses yielded similar results.

From the Affymetrix analysis, high Cx43 (*GJA1*) mRNA expression was associated with a reduced relapse-free survival (RFS) and overall survival (OS) in ER-negative patients (26). A study from Stoletov *et al* confirmed this observation using analysis from the Oncomine database (www.oncomine.org) (28). The analysis by Stoletov *et al* indicates that higher Cx43 expression correlated with increased patient death and recurrence. However, in the study by Teleki *et al* the opposite was true when evaluating distant metastasis-free survival (DMFS) (26). In this case, elevated Cx43 was associated with longer DMFS in the whole patient cohort, in lymph node-negative patients, and ER-positive patients. Similar to these findings, the Illumina-based analysis showed a significant positive association between elevated Cx43 and OS in the whole patient cohort, the ER-positive group, the luminal A group, and the ER-positive cohort that received endocrine therapy. A negative association was reported for OS between elevated Cx43 and ER-negative as well as triple-negative patients.

Interestingly, the Cx43 protein analysis using TMAs also indicated that elevated Cx43 expression (scored as 1-3+ vs. 0 for no expression) associated with increased RFS in the whole patient cohort and in histological grade 2 tumors (26). Further analysis of Ki67 staining, as a marker of proliferation, showed low Cx43 stain in high proliferating tumor cells but prominent cytoplasmic Cx43 staining in low proliferating tumor cells. Though not discussed, it would be interesting to determine if the same Cx43 protein expression patterns were present for the stromal compartment or if there was any association between Cx43 expression in the stroma and survival or histological grade. Similar to the previous study by Teleki *et al*, other connexins also held significant prognostic value (27). Whether or not there is a relationship between each connexin, GJIC, and prognosis remains to be determined.

4. The relationship between connexin 43 and tumor initiating cells

Breast tumors are composed of a heterogeneous mixture of cells. Many avenues of investigation have argued for the

existence of a defined subgroup of cancer cells called tumor initiating cells, which are characterized by stem cell-like properties (36-39). These tumor initiating cells, often referred to as cancer stem cells, are defined by their capacity to maintain stem cell phenotypes including self renewal, asymmetrical division, and an ability to promote migration and invasion. Several groups have isolated tumor initiating cells using cell surface markers specific for the normal stem cells of the same organ, and for breast, these cells can be defined by a CD44$^+$, CD24$^-$ population (36-39).

A study by Shipitsin et al sought to identify genes that are expressed in CD44$^+$ (stem-like cells) and CD24$^+$ (differentiated) cells (30). The study authors isolated different cell populations, representing CD44$^+$ and CD24$^+$ cells, from human breast tissue obtained from reduction mammoplasty as well as breast tumor samples and then performed gene expression profiling to determine if specific genes were enriched in each population. Interestingly, they found that Cx43 was significantly expressed in the CD44$^+$ subset. Consequently, the study authors defined Cx43 as a CD44$^+$ specific gene.

A follow-up study from the same group by Park et al expanded these findings by looking at breast cancer samples from 4 histological subgroups defined as IDC, IDC with DCIS, DCIS with microinvasion, and DCIS only (40). Consistent with previous findings, Cx43 expression correlated with CD44 expression. The findings from this study indicated that Cx43 is low in DCIS with microinvasion but significantly higher in IDC with DCIS. This observation was also true for CD44$^+$CD24$^-$-positive cells. Therefore, the correlation of Cx43 with these two distinct histological subtypes corresponds with findings from the CD44$^+$CD24$^-$ cell populations within these same histological subgroups. Altogether, these findings imply that Cx43 gene expression is enriched in CD44$^+$ cell populations, which further corresponds with specific histological IDC subtypes, implicating Cx43 in breast cancer progression.

5. Cytoplasmic localization of connexin 43 points to decreased GJIC in human breast cancer

Many studies have questioned if the loss of GJIC between a potential cancer cell and normal cells is an alteration that leads to a cancer cell transformation (1). There are two methods that have been used as a surrogate for GJIC in static, clinical samples: localization and expression. As discussed, studies evaluating expression have led to findings suggesting both increased and decreased expression levels of Cx43 protein or mRNA (7,10,22,23,25-27). However, expression levels are not necessarily reflective of protein function, particularly if protein expression is increased. Therefore, Cx43 localization can also be informative when trying to determine if GJIC is compromised.

In the previously referenced study by Jamieson et al, the localization of Cx26 and Cx43 in human breast tumors was evaluated in addition to total expression levels (22). The study confirmed that Cx43 in normal human breast tissue localizes to the myoepithelial cells with the localization of Cx43 predominantly on the lateral aspect of the plasma membrane (10,22,24,26). Contrary to this observation, Cx43 localization in 4 of the 12 grade 2 tumors showed

predominantly cytoplasmic staining, suggesting that Cx43 was localized away from sites of gap junctions (22). A similar observation was made in the grade 3 tumors where 10 out of 15 samples showed predominantly cytoplasmic staining of Cx43. Somewhat consistent with this observation, the study by Laird et al determined that Cx43 expression was predominantly lost, reporting no Cx43 expression in breast tumors, and consequently no Cx43-positive gap junctions (23).

In the earlier study by Kanczuga-Koda et al, IHC analysis showed primarily cytoplasmic expression of Cx43 but only in 3 out of 11 intraductal carcinomas (7). The remaining 8 samples were negative for Cx43, consistent with the Laird et al study (23). Samples of invasive carcinoma (n=29) showed mainly diffuse cytoplasmic staining of Cx43 in the majority of the samples (26/29) (7). This finding could suggest an upregulation of Cx43 as tumors progress to an invasive phenotype or that a dominant cell type within the tumor that expressed cytoplasmic Cx43 made up the bulk of invasive tumors. The cytoplasmic localization of Cx43 suggests that gap junctions are lost in invasive breast carcinomas.

A later study by Kanczuga-Koda et al, also confirmed cytoplasmic expression of Cx43 but in a larger subset of samples, consistent with their assertion that Cx43 expression is increased in invasive carcinomas (10). The study authors observed cytoplasmic Cx43 staining in 55 out of 71 (~80%) invasive ductal breast carcinoma samples evaluated. In the remaining 16 samples, 6 samples showed punctate staining of Cx43 in the plasma membrane of the carcinoma cells, presumably representing sites of gap junctions. A punctate plasma membrane staining for Cx43 was found in a small number of samples (1-2 tumors) and in a small fraction of cells within a total IHC section; a phenomenon that has been reported in other studies as well (7,22). The remaining 10 samples were negative for Cx43 (10). A more recent study by Teleki et al similarly alluded to the idea that Cx43 becomes more cytoplasmic in tumors with higher levels of proliferation, suggesting a more invasive phenotype (26). All together, these studies confirm the observation that Cx43 is predominantly cytoplasmic in breast carcinomas, which likely correlates with invasiveness as well as a loss of Cx43 GJIC.

6. Phosphorylated connexin 43 in the progression of breast cancer

A major regulatory feature of Cx43 is that the protein contains at least 16 reported phosphorylation sites (41). Studies indicate that the phosphorylation of Cx43 regulates the Cx43 lifecycle including assembly of Cx43 into gap junctions and formation of gap junction plaques at the plasma membrane as well as trafficking of Cx43 via the endolysosomal and autophagosomal systems. The phospho-mediated regulation of Cx43 can result in increased or decreased GJIC (1). A study by Gould et al, sought to determine whether Cx43 phosphorylation correlated with breast cancer stage (42). The researchers developed an antiserum (SA226P) that strongly and selectively reacts with Cx43 phosphorylated on Serine (S) residues at amino acid positions 279 and 282 (S279/S282). Previous studies showed that S279 and S282 are phosphorylated by MAPK signaling through ERK1/2 (43,44). Additional studies indicated that phosphorylation of these residues can either enhance or
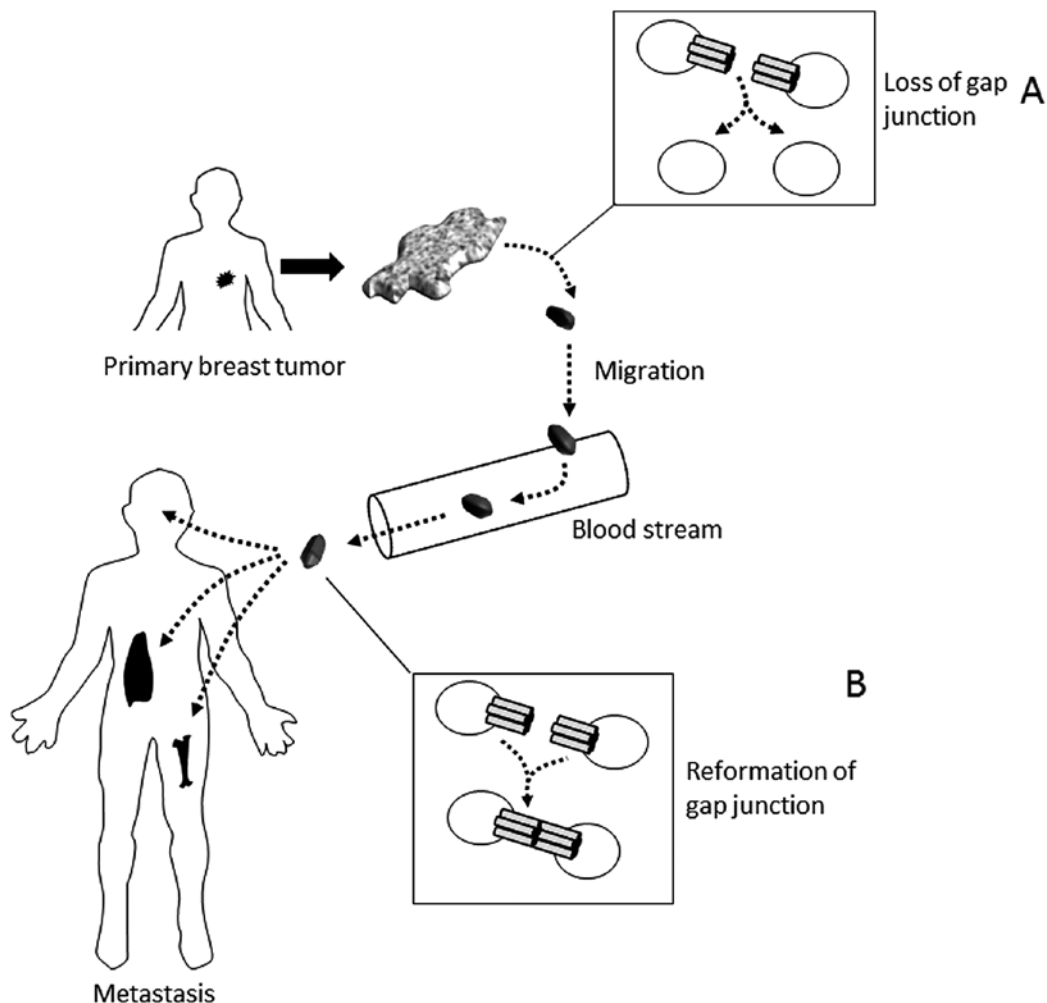
Figure 2. Early stages of breast cancer are thought to be marked by a loss of gap junctions leading to the development of a primary tumor (A). Migration of primary tumor cells into the circulation is part of the inital steps of metastasis. It is thought that gap junction intercellular communication is lost during this initial process and following, a possible reformation of gap junction intercellular communication may occur (B).

inhibit GJIC, depending on the particular experimental cell type (45-53).

In the study by Gould *et al*, a total of 98 breast samples were examined, which included tissue from normal adult breast tissue, fibrocystic disease (FCD), fibroadenomas (FA), ductal and lobular carcinomas *in situ* of all grades, infiltrating ductal carcinomas, infiltrating lobular carcinomas, and infiltrating carcinomas with mixed ductal and lobular features (42). The study authors evaluated phosphorylated Cx43 using the SA226P antiserum directed at Cx43 phosphorylated at S279/S282 by IHC and compared these findings with IHC staining of total pan-Cx43. In the normal breast, FCD and FA samples, the SA226P antiserum stained myoepithelial cells in all samples, which was predominantly cytoplasmic and diffuse. Very few cells showed membrane related staining with SA226P. No pan-Cx43 staining was observed in normal breast, which is largely inconsistent with prior studies (7,10,22,24,26,29). Interestingly, staining for pan-Cx43 was also not reported in malignant samples. However, the phospho-Cx43 signal varied depending on the grade of malignancy. While it is difficult to form any conclusions regarding the pan-Cx43 IHC results, the SA226P IHC results suggest that phosphorylation of Cx43 could vary between normal and malignant breast.

The authors went on to confirm their findings by immunoblotting for pan-Cx43 and phosphorylated Cx43 using the SA226P antiserum (42). These results showed that the intensity of the bands that reacted with the SA226P antiserum were high in normal breast but were somewhat decreased in papilloma and DCIS samples. A clear increase in phosphorylated Cx43 levels was observed in infiltrating ductal carcinomas, infiltrating lobular carcinomas, and infiltrating carcinomas when compared to papilloma and DCIS samples, suggesting that the amount of phosphorylated Cx43 increases with increasing disease severity. A similar pattern was observed using the pan-Cx43 antibody. It seems worthwhile to note that because no pan-Cx43 was observed in any of the IHC samples, it may be interesting to revisit these studies using alternative pan-Cx43 antibodies to independently confirm the IHC findings.

7. Increased connexin 43 expression and membrane localization in breast cancer metastasis

Studies that evaluated Cx43 expression and localization uniformly suggest that GJIC is lost during breast malignancy whether it be due to loss of protein expression

or a predominant localization of Cx43 in the cytoplasm of tumor cells, away from plasma membrane sites of gap junction formation (7,10,22,23,25). Additional studies have also argued that Cx43 expression at the mRNA and protein level correlates in a statistically significant manner with poor prognosis (26-28). As a result, many have questioned whether Cx43 plays a role in metastasis and furthermore, whether Cx43-regulated GJIC is required for the metastatic process. Studies using breast cancer and mammary tumor cell lines have presented conflicting findings indicating both pro-metastatic and anti-metastatic roles for Cx43 (11-21). Therefore, evaluation of clinically relevant samples would be informative.

A later study by Kanczuga-Koda *et al* evaluated 51 samples from primary breast cancers that were matched with metastatic lymph node samples taken from the same patient (9). Cytoplasmic Cx43 staining by IHC was observed in ~50% of the primary tumors and 42 of the 51 (82.4%) tumors exhibited some membrane associated Cx43 staining. Interestingly, all the lymph node metastases were positive for Cx43, even if the primary tumors were negative for Cx43 staining. The localization of Cx43 in the lymph node metastases was heterogeneous with cytoplasmic and membrane associated staining. Moreover, the study authors observed a statistically significant increase in Cx43 levels in 18 of the 51 (35.3%) metastases. Cx26 was also evaluated in this study and while Cx26 was mainly cytoplasmic in both the primary tumor and their metastases, Cx26 levels were higher in the metastases compared with the primary tumor.

As a whole, these findings suggest that Cx43 likely plays a role in breast cancer metastasis to lymph node and that Cx43-positive tumor cells in lymph node metastases are likely able to localize to sights of gap junctions. These observations are consistent with prior experimental studies that suggest Cx43-mediated GJIC promotes metastasis (12,14,19). It is not known whether the plasma membrane localized Cx43 in the lymph node metastasis samples evaluated in the Kanczuga-Koda *et al* study are in functional gap junctions, whether the proposed channels are homotypic or heterotypic, or whether hemi-channels could be implicated (9). It is also worth mentioning that it is possible that the tumor cells in the lymph node metastases could be a specific tumor cell subtype that selects for Cx43 expression, which is a potential explanation for why 100% of the lymph node metastasis samples were positive for Cx43 despite some primary tumor samples staining negative for Cx43.

Additional observations support a role for Cx43 in mediating interaction with the vasculature during breast cancer metastasis (28,42). The previously described study by Gould *et al*, whose goal was to evaluate Cx43 phosphorylation in human breast malignancies, showed a strong staining pattern for phosphorylated Cx43, using their SA226P antiserum, in capillary endothelial cells within tumors (42). Along the same lines, Stoletov *et al* observed small populations of Cx43-positive tumor cells that are in direct contact with blood vessel surfaces (28). While the exact nature of Cx43 in the vasculature is largely unclear, these studies point to a potential interaction between tumor cells and the vasculature that may be Cx43-dependent. It is reasonable to speculate that Cx43 could play a role in intravasation or extravasation during

metastasis, but based on these minimal observations, that conclusion seems largely premature.

8. Conclusions

Since the expression of Cx43 was observed in the myoepithelial cells of the human breast over two decades ago, researchers continue to investigate the role of Cx43 in the development and progression of breast cancer. Though not completely understood, Cx43 gap junctions have a potential mechanistic connection to the development of malignancy in human breast. Some debate still exists regarding the levels of expression of Cx43 in the malignant progression of breast cancer, however, it seems evident from the literature that loss of GJIC is integral to breast tumor formation. As reviewed here, a general trend seems to be that loss of Cx43 GJIC is an early event in malignancy. Based on at least one study, it may be reasonable to speculate that Cx43 also plays a role during intravasation or extravasation during metastasis (28). Consequently, we put forth a possible model that is thus far consistent with published reports (Fig. 2), however at this point, the conclusions, in particular with metastasis, may be premature. Though some connection seems apparent, the true role of Cx43 in breast cancer development and progression remains unclear.

Efforts to therapeutically modulate Cx43 have largely centered around chemicals that indirectly target Cx43 through changing membrane dynamics or through direct inhibition of Cx43 gap junction activity (54,55). Since restoring Cx43 gap junction intercellular communication is preferable, at least at early stages of breast cancer development, agents to restore rather than inhibit Cx43 gap junction communication are needed. At least one such agent is available; a therapeutic peptide called α-connexin carboxyl-terminal peptide, which has been tested in several breast cancer cell lines (56). This 25-amino acid length peptide mimics the c-terminal cytoplasmic domain of Cx43. However, clinical application of peptide agents is somewhat limited due to certain caveats including instability and poor oral availability (57). Furthermore, a highly studied area of Cx43 research is the non-canonical (i.e., non-gap junction related) functions of Cx43. Numerous reports demonstrate that the carboxy-terminus (c-terminus) regulatory domain of Cx43, that is located in the cytoplasm, is a primary site for protein-protein interaction and phosphorylation (58). The c-terminal domain has been reported to contribute to intracellular functions including proliferation, apoptosis, migration, and transcription (58-62). Consequently, two significant issues remain: deciding exactly how to modulate Cx43 activity and identifying agents that can directly target Cx43 to restore, rather than inhibit, gap junction intercellular communication. As detection techniques and reagents are refined, the analyses and information gained will continue to improve. Targeted studies to evaluate discreet stages of the metastatic process are needed to further our understanding of Cx43's role in breast cancer progression. Ideally, evaluating individual breast cancer subtypes (e.g., ER⁺, HER2⁺, and triple-negative) is required for gaining clear insight. Further insight may also be gained as the function of heterotypic gap junctions and hemichannels in breast cancer is revealed, or if non-gap junction functions for Cx43 and other connexins are identified. Additional studies are

needed to elucidate the fundamental workings of this protein, which could lead to an enhanced understanding of malignant progression and reveal better potential therapeutic strategies for breast cancer patients.

Acknowledgements

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