**Connexin 43, Breast Cancer Tumor Suppressor: Missed Connections?**

Christina L Grek[1]

Email: grek@firststringresearch.com

J. Matthew Rhett[2]

Email: rhettj@musc.edu

Jaclynn S Bruce[3]

Email: scheboth@musc.edu

Gautam S Ghatnekar[1]

Email: ghatnekar@firststringresearch.com

Elizabeth S Yeh[3, 4,*]

[*] Corresponding author

Email: yeh@musc.edu

[1] FirstString Research, Inc., 300 W. Coleman Blvd., Suite 203, Mount Pleasant, SC, USA

[2] Department of Surgery, Division of General Surgery, Medical University of South Carolina, Charleston, SC, United States

[3] Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, 173 Ashley Ave, BSB358, MSC509, Charleston, SC 29425, USA

[4]Hollings Cancer Center, Medical University of South Carolina, Charleston, SC 29412, USA

**Abstract**

Connexins are a family of transmembrane proteins that are characterized by their capacity to form intercellular channels called gap junctions that directly link the cytoplasm of adjacent cells. The formation of gap junctions by connexin proteins facilitates intercellular communication between neighboring cells by allowing for the transfer of ions and small signaling molecules. Communication through gap junctions is key to cellular equilibrium, where connexins, and the gap junction intercellular communication that connexins propagate, have roles in cellular processes such as cell growth, differentiation, and tissue homeostasis. Due to their importance in maintaining cellular functions, the disruption of connexin expression and function underlies the etiology and progression of numerous pathologies, including cancer. Over the past half a century, the role of connexins and gap junction intercellular communication have been highlighted as critical areas of research in cellular malignancies and much research effort has been geared toward understanding their dysfunction in human cancers. Although ample evidence supports a role of connexins in a variety of human cancers, detailed examination in specific cancers, such as breast cancer, are still lacking. This review highlights the most abundant gap junction connexin isoform in higher vertebrate organisms, Connexin 43, and its role in breast cancer.

**Key words**

Breast cancer, Connexin 43, Gap Junctions, Metastasis

**Abbreviations**

- Cx43 – Connexin 43
- Cx – Connexin
- GJIC – Gap Junction Intercellular Communication
- WT – Wildtype
- ErbB2 – Epidermal Growth Factor Receptor 2
- ZO-1 – Zona Occludens-1
- ZO-2 – Zona Occludens-2
- TCDD ─ 2,3,7,8-Tetrachlorodibenzodioxin
- PQ1 – Substituted quinolones
- Akt – Protein kinase B
- MAPK – Mitogen Activated Protein Kinase
- αCT1 – α–connexin carboxyl-terminal

## Introduction

The complexity of multicellular organisms necessitates an efficient, coordinated route of cell-to-cell communication. Connexins make up a family of transmembrane proteins that are the primary component in intercellular gap junction pores and have roles in propagating intercellular communication; a property called Gap Junction Intercellular Communication (GJIC) [1-3]. Each gap junction is composed of a hexagonally arrayed lattice of intercellular aqueous channels spanning the two membrane bilayers of opposing cells. Each of these channels is made up of 2 hexameric connexin oligomers termed connexons or hemichannels; one each contributed by the two contacting cells. In addition to forming gap junctions, connexin hemichannels have their own

physiological roles in regulating cell-to-extracellular communication and purinergic signaling [4].

There are as many as 21 connexin family members in mammals which can combine to form heteromeric and/or heterotypic channels. Studies examining connexin mutations show that the disruption of connexin protein conformation, turnover, and channel function lead to disease phenotypes and reveal the importance of connexins and gap junctions in maintaining tissue homeostasis [5]. A critical role for connexins, gap junctions, and GJIC in tumorigenesis and metastasis was documented as early as 1966 in studies that revealed impaired intercellular electrical coupling in chemically-induced and xenografted rat hepatocarcinomas [6, 7]. Expression and functional studies have identified connexins as potential tumor suppressors, where restoration of depressed connexin expression and thus restoration of GJIC inhibits tumor cell growth [8, 9]. However, this story has been made more complex by recent studies that report aberrant increased expression of connexins in a variety of carcinomas and sarcomas as well as the more recently discovered transcriptional functions of connexins that are independent of their gap junction function [10, 11].

Connexin 43 (Cx43) is one of the most highly expressed and widely studied connexins and has been found to be aberrantly expressed in several tumor types including liver, prostate, and breast. While recent studies support the therapeutic potential of targeting Cx43 as a treatment in breast cancer [12-15], the complexities that are associated with the disseminating roles of Cx43 in the processes of tumorigenesis, tumor cell migration and metastasis in breast cancer remain unresolved. Evidence suggests that Cx43-directed GJIC is critical for normal cell function and loss of this feature promotes malignant transformation of breast/mammary epithelial cells [16-20]. These studies have yielded interesting but early stage information suggesting that Cx43 has a role in breast cancer cell proliferation, differentiation, and migration. The exact nature of this role is complicated by studies examining human breast cancer tissue that suggest that levels of Cx43 change with cancer stage [16-19]. These studies have also revealed that, regardless of expression level, atypical (e.g., cytoplasmic) expression of Cx43

and impaired GJIC could play a part in determining disease severity and act as an early sign of malignancy, suggesting that preserving Cx43 gap junctions could be an important distinction between normal and malignant breast epithelial cells [16, 19, 20]. Consequently, maintaining Cx43 gap junctional activity could be a mechanism of Cx43-dependent tumor suppression. A schematic of this concept is shown in **Figure 1A** where it is represented that loss of GJIC maintained by Cx43 in normal mammary gland results in breast cancer malignancy. Furthermore, it is a common feature for breast cancer cells to exhibit Cx43 localized away from the plasma membrane where gap junctions would normally form. This mis-localization is exemplified in **Figure 1B**, which shows examples of Cx43 immunofluorescence staining in MCF7 and BT474 breast cancer cell lines where Cx43 is localized away from the plasma membrane, likely in endocytic vesicles in the cytoplasm. Here, we review the current literature on Cx43 and breast cancer in order to highlight a possible tumor suppressor role for Cx43 in breast cancer, what circumstances have been observed where Cx43 acts as a tumor suppressor or does not, and the potential for therapeutic targeting of this protein for treatment of primary and metastatic disease (summarized in **Table 1**).

## Materials and Methods

*Immunofluorescence*

The anti-Cx43 antibody used for immunofluorescence detection of Cx43 in Figure 1B was purchased from Sigma-Aldrich (C-terminal directed antibody). Wheat Germ Agglutinin (WGA) stain was purchased from Life Technologies (Thermo-Fisher). Images were obtained using a Leica L5 scanning confocal microscope.

*Crystal Violet Stain Analysis*

For Figure 2B, equal numbers of cells were plated and treated the following day with α–connexin carboxyl-terminal peptid**e** (αCT1), a negative control peptide containing a reverse

sequence of the peptide used for αCT1 (R-pep), or Vehicle (Veh; $H_2O$) for 24 hr. When indicated, cells were also treated with DMSO (vehicle) or tamoxifen (Sigma). Following drug treatments, cells were fixed in 4% paraformaldehyde and stained with crystal violet. Cells were subsequently washed in deionized $H_2O$ and crystal violet was extracted with methanol. A540 was read using Benchmark Plus plate reader (Biorad).

**Connexin 43 in Mammary Gland Development and Relation to Breast Cancer**

Cx43 is a major connexin in breast tissue. Several studies have implicated Cx43 in mammary gland development [21-24] and have investigated Cx43 expression in breast cancer cells or human breast cancer tissues [16, 18, 25-31]. Northern analyses, immunohistochemistry, and quantitative PCR have shown the expression of Cx43 throughout mammary gland development and differentiation in mammary epithelial cells, myoepithelial cells, and mammary fibroblasts [8, 21, 32-35]. Studies suggest that Cx43 expression is high during developmental stages in mammary gland development when cells are most plastic including during puberty, early pregnancy, and early involution [36]. These studies show a role for Cx43 in regulating expansion of the mammary tree, which is consistent with a proliferative role for this gap junction protein [36]. Cx43 expression is subsequently downregulated during mid-pregnancy followed by increased protein phosphorylation leading up to and at the onset of lactation. Cx43 may also have regulatory roles in early involution, a mammary gland developmental stage that is marked by the onset of gland reorganization regulated by apoptosis. Cx43 expression remains dramatically reduced until the end of involution, at which time expression increases as the mammary gland returns to the pre-pregnant state [8, 21, 32-37].

Many parallels have been drawn between protein function during mammary gland development and deregulation in breast cancer. A regulatory role for Cx43 in ductal elongation during puberty and early pregnancy could suggest that Cx43 is responsive to hormones, including estrogen, which has known roles in cell proliferation. Interestingly, studies using

myometrial cell culture systems or tissues indicate an inverse relationship between estrogen receptor (ER) expression and Cx43 [38, 39], which is contradictory to the finding in mammary gland that Cx43 levels are high during times of proliferation when estrogen signaling is predominant. Consequently, additional studies are needed to define the relationship between Cx43 and estrogen receptor signaling in the breast and mammary gland. Furthermore, downregulation of Cx43 expression during involution brings into question what the role of Cx43 is in regulating mammary epithelial cell survival, since involution is marked by significant levels of apoptosis that are required for mammary gland reorganization to a pre-pregnancy-like state. These associations could mean that Cx43 is proliferation and survival promoting and may give credence to why some experimental results indicate a conditional tumor suppressive role for Cx43. Whether a proliferative or survival function for Cx43 requires its gap junctional activity, or conversely is independent of Cx43 gap junction activity, also requires clarification.

Consistent with the aforementioned expression studies, findings from knockout and transgenic mouse models indicate that both Cx43 expression and function at gap junction channels is tightly regulated during mammary gland development and differentiation. While Cx43 knockout mice die at birth [40] transgenic mice containing an autosomal dominant Cx43 mutation ($Gja1^{jrt/+}$) that mimics the disease oculodentodigital dysplasia (ODDD) and knock-in heterozygous mouse models that replace Cx43 with Cx32 (Cx43KI32) have been useful in disseminating Cx43's role in mammary gland development and function [22, 41]. In $Gja1^{jrt/+}$ transgenic mice there is a delayed development of the mammary gland due to loss of GJIC as well as an impairment in milk delivery [22]. Similarly, Cx43KI32 mice have defects in pup feeding [41]. Taken together, the studies in these transgenic models suggest that Cx43 has roles in mammary epithelial cell differentiation. Additional studies using GJIC blocking agents on mammary epithelial cells grown *in vitro* support this idea [42]. Interestingly, these findings are at odds with the expression studies described above, which suggest a role for Cx43 during times of cellular plasticity, again pointing toward "conditional" roles for Cx43 in the mammary gland.

**Connexin 43 and Breast Cancer Metastasis**

Cancer metastasis is a multistep process involving invasion, intravasation and extravasation where connexin expression and intercellular communication have roles in cell-cell adhesion, how neoplastic cells interact with cellular microenvironment, and metastatic potential. Several studies indicate that Cx43 expression is upregulated during breast cancer progression and expression levels correlate with breast cancer metastasis [16-20]. Evidence using overexpression of wildtype (WT) and dominant negative forms of Cx43 have shown that Cx43 can promote metastatic behavior of 4T1 cells [43]. These studies suggest that Cx43 supports the development and growth of breast tumors as well as the persistence of metastatic lesions.

Conversely, studies have also shown that forced expression of Cx43 in breast cancer cell lines reduced migration and angiogenesis [29, 44-46] whereas downregulation of Cx43 or loss of GJIC correlated with increased migration and expression of angiogenesis promoting factors [29, 30, 44, 47]. Consistent with this idea, studies in mouse models suggest that Cx43 overexpression and maintenance of GJIC are important for inhibiting metastasis *in vivo* [45, 47]. Taken together, these findings support the idea that deregulation of Cx43 is an important metastatic driver in breast cancer. However, given that reports show that levels of Cx43 are both elevated and reduced in breast cancer [8, 14, 16, 18, 20, 35, 43, 45-53] it is possible that pro- as well as anti-tumorigenic and metastatic roles for Cx43 exist. Consequently, the functional relevance of Cx43 localization and activity at gap junctions in breast cancer remains to be distinctly clarified.

During metastasis, the loss of Cx43 expression at gap junctions has been suggested to allow cells to physically detach and lead to invasion and metastatic disease progression [54-57]. At least one study suggests that Cx43 and GJIC contribute to breast cancer cell adhesion and migration through a mechanism utilizing OB-cadherin, thereby regulating breast cancer cell metastatic potential [45]. Cx43 has also been found to be upregulated in established breast

cancer metastatic lesions suggesting that this protein could play roles in late metastatic steps involving extravasation and tissue colonization [18, 43, 58, 59]. Furthermore, Cx43 function during metastasis may not always be tumor-cell intrinsic. Evidence shows that Cx43 expression is evident in stromal compartments during cancer progression suggesting that Cx43 may be regulating invasion and metastasis through interactions between epithelial tumor cells and the stroma [60]. Additionally, heterocellular communication between breast carcinoma cells and vascular endothelia has been confirmed during metastatic tumor invasion [50, 51]. Further possibilities include that Cx43 expression is restricted to minor populations of cancer stem cells characterized by invasive potential [61, 62] and consistent with this idea, metastatic breast cancer cells selected for their ability to home to the brain showed increased Cx43 expression [63]. Therefore, if Cx43 is differentially expressed by highly metastatic breast cancer cells when compared to less metastatic breast cancer cells, as well as between primary versus disseminating tumor cells; Cx43 expression has the potential to be investigated as a prognostic biomarker [34].

The development and evaluation of therapeutic interventions aimed at targeting Cx43 in breast cancers is further complicated due to evidence that connexins have differential and dynamic mechanistic functions during tumor cell dissemination. Typically, the loss of GJIC corresponds with the initial stages of malignant phenotype progression in neoplastic mammary tissue and may be related to changes in cell-cell adhesion. Research suggests that Cx43 expression is downregulated in primary breast cancer tumors and restoration of GJIC by the upregulation of Cx43 restores normal cellular phenotypes *in vitro* and reduces tumor growth *in vivo* [16-18, 20, 25, 28]. However, conflicting data suggests that loss of Cx43 expression can also occur in late stage breast cancers [20, 64]. Additional experimental evidence using mouse models shows that, when crossed with the ErbB2 overexpressing mice, mice with Cx43 inactivating mutations show delayed onset of palpable mammary tumors while displaying increased pulmonary metastases [47]. Additional context-dependent breast cancer related

effects of Cx43 are evidenced in a recently completed series of studies in breast cancer cell lines that demonstrate that the tumor suppressor effects of Cx43 expression depend on culture conditions (i.e. 3D vs 2D) and the assembly of gap junction complexes with associated proteins including α-catenin, β-catenin, and ZO-2 [65]. While the role of Cx43 in tumor initiation and later stages of tumorigenesis including tumor cell dissemination are not always clear, it is important to acknowledge that these contradictory observations likely represent differences in experimental approaches, the cellular heterogeneity of tumors, the overlapping roles of other connexin family members whose expression can vary depending on the experimental model, and the complexity of the metastatic process. Moving forward, careful experimental validation of specific stages of tumorigenesis as well as use of consistent and appropriate experimental models to study each stage of breast/mammary carcinogenesis should assist in clarifying Cx43's role in these processes.

**Gap junction dependent role of Cx43 in breast cancer carcinogenesis**

There is ample evidence that correlates the loss of GJIC with metastatic potential in breast cancers [52, 66]. In examining Cx43 as a tumor suppressor it can be assumed that the downregulation of Cx43 and the concurrent loss of GJIC in tumor cells associate with increased cell heterogeneity and the disruption of the homeostasis that characterizes normal, healthy tissue. Furthermore, loss of GJIC may also support carcinogenesis by preventing the spread of growth-inhibitory or pro-apoptotic stimuli from neighboring cell via the bystander effect.

Several reports directly support a role for GJIC and Cx43 expression in regulating metastatic potential. Expression of the anti-metastatic gene, BRMS1, results in the upregulation of Cx43 expression resulting in GJIC restoration [50, 52]. Depressed homotypic GJIC between MDA-MD-435 cells as well as MCF7 cells and the human osteoblastic cell line hFOB1, perhaps due to changes in connexin expression profiles, associated with increased tumorigenesis and metastatic potential [50]. However, the authors emphasize that it is important to consider the

degree of heterotypic GJIC with hFOB cells relative to homotypic GJIC rather than the absolute degree of homotypic or heterotypic GJIC. Unfortunately, full correlation of these studies to breast cancers is unfortunately lost as they were performed in MDA-MD-435 cells, a melanoma cell line that has been routinely misused as a breast cancer line. Furthermore, comparison of these observations with cell migration or metastatic phenotypes was not directly tested in these studies and thus, this aspect remains to be determined.

Alternatively, studies using GJIC inhibitors and GJIC deficient Cx43 mutants support a tumor facilitator role for Cx43 GJIC during extravasation. Using HBL100 cells Pollmann et al found that preventing heterocellular GJIC between breast tumor cells and endothelial cells reduced cellular diapedesisa and this may be an important regulatory step during breast cancer metastasis [51]. In co-culture experiments breast cancer cells cultured with endothelial cells induced a rapid and transient loss of GJIC attributed to increased tyrosine phosphorylation of Cx43 [67]. The authors suggest that altering GJIC between endothelial cells may serve to facilitate movement through the vasculature. However, because studies using different cell lines have yielded conflicting results [65] additional *in vivo* studies in appropriate metastatic animal models are needed.

GJIC-dependent mechanisms may also have a role in mediating breast cancer metastasis to secondary organs. Co-culturing breast cancer cells with bone marrow stroma suggests that during the formation of metastatic tumors Cx43 GJIC-dependent mechanisms mediate the proliferation of breast cancer cells in contact with stroma via the exchange microRNAs that target the chemokine CXCL12 [68]. Eliciting tumor cell quiescence may permit an adaptive advantage whereby breast cancer cells ‘wait’ until appropriate growth conditions and evade therapeutic targeting.

The cancer promoting properties of Cx43 could also be regulated through signaling processes such as ubiquitination, glycosylation, S-nitrosylation, and phosphorylation [8, 18, 26, 29, 31, 45, 46, 69, 70]. Cx43’s role in malignancy could be dependent upon upstream

phosphorylation events that direct Cx43 signaling as well as GJIC, many of which are driven by common oncogenic pathways including epidermal growth factor receptor (EGFR), which activates Akt and ERK1/2 signaling, but has not been studied extensively in the context of Cx43 related breast cancer model systems [8, 18, 26, 29, 31, 45, 46, 69, 70]. Future studies to delineate the role of growth factor signaling in the regulation of Cx43 in breast cancer could be of critical importance as deregulation of growth factor signaling through receptors, such as EGFR and its family member human epidermal growth factor receptor 2 (HER2), which are commonly deregulated in breast cancers. Moreover, the phosphorylation status of Cx43 plays a critical role in the transport of Cx43 to the membrane [71], as well as incorporation and retention in gap junctions [72, 73]. Progressive Cx43 phosphorylation corresponds to enhanced Cx43 incorporation into gap junctions and the maintenance of non-junctional hemichannels in a low-open-probability state [74, 75].

The activation of tumor promoters such as PKC, ERbB2, EGF and/or Src pathways may contribute to the reversible loss of connexins and GJIC in human breast cancer and carcinogen-induced breast tumors [48]. However, despite evidence that loss of Cx43-mediated GJIC is due to a reduction in both protein and RNA levels, a number of studies link reduced GJIC to deficiencies in Cx43 trafficking, resulting in aberrant Cx43 localization in the cytoplasm. The rate of connexin degradation and turnover may also contribute to connexin stability, assembly and function [76], thus providing an additional method to mediate GJIC.

An additional layer of complexity regarding connexin regulation is bestowed by the fact that two or more connexins can co-assemble to form mixed gap junction channels. Heterotypic and heteromeric channels have unique channel properties where the type of connexin forming gap junction channel influences channel selectivity and the specificity of GJIC [77-79]. Expression studies showed that forced co-expression of Cx26 and Cx43 did not result in formation of heteromeric channels, but rather a reduction in the total junctional conductance to around 10% of that in cells expressing a single connexin [80]. Heyman et al showed that while

protein kinase C (PKC) phosphorylation of either Cx40 or Cx43 alone does not alter charge selectivity of either homomeric channel, cellular co-expression of the two connexins introduced PKC mediated charge selectivity. Their data indicate that PKC regulation of multi-connexin junctions relies on the Cx43 content of the junction and present an *in vivo* mechanism for cells to finely regulate permeability by altering relative expression levels and phosphorylation status [81]. It is thus possible that deregulation in the cellular ratios of connexins and the unique functions of heterotypic and/or heteromeric channels might contribute to the mechanisms underlying tumor cell potential for invasion and metastasis.

It should also be taken into consideration that Cx43 has a number of binding partners that have functions involving intracellular trafficking, gap junction formation and size regulation, channel gating, and possibly signaling and cell cycle regulation [82]. One classical example is the zonula occludens-1 (ZO-1) protein, whose PDZ2 domain interacts with the carboxy-terminal tail of Cx43 to modulate gap junctional plaque size and activity [83]. The clinical potential of therapeutics that target the interaction of Cx43 with ZO-1 has recently been elucidated in clinical and preclinical studies that evaluate peptide therapeutics modeled after key regulatory domains of Cx43 [15, 84, 85]. When considering Cx43's role in breast cancer, it will be useful to consider how ZO-1 protein, and other Cx43 interacting partners, is being modified post-translationally to direct its function as well as which interacting partners are regulating its gap junctional versus non-junctional activities.

**Gap junction independent role of Cx43 in breast cancer carcinogenesis**

Connexin mutants that do not form functional gap junction channels have been useful in revealing Cx43 as tumor suppressor via GJIC-independent mechanisms. Inhibition of tumor growth was seen in *in vivo* xenograft models using MDA-MB-321 cells expressing defective Cx43 that was not assembled into junctional plaques [31]. In these studies DNA array and

Western blot analysis revealed a down-regulation of fibroblast growth factor receptor-3 (FGFR-3), suggesting a putative mechanism via reducing the effects of paracrine pro-tumorigenic growth signals. Along the same lines, in three-dimensional cultures of MDA-MB-231 cells overexpression of Cx43 decreased the malignant properties of breast cancer cells by stimulating proper organoid polarity as assessed by the localization of β1 integrin and collagen IV, and promoting mesenchymal to epithelial transition, as well as revealing a role in inhibiting angiogenesis *in vitro* and *in vivo* by regulating the transcriptional and translational expression of a number of pro- and anti-angiogenic molecules [29, 30]. In these studies Cx43 expression appeared to be diverted to lysosomes and did not localize to the cell-cell interface nor rescue GJIC, suggesting a GJIC independent mechanism of mammary tumor growth and angiogenesis suppression.

Recent reviews highlight the channel-independent roles of connexin-associated proteins that regulate junctional assembly, indicating that the functional interaction of connexin with enzymes, cytoskeletal components, and other junctional proteins have key roles in regulating normal tissue development, differentiation and function [86, 87]. Furthermore, connexin protein-protein interactions as well as hemichannel function are linked to Cx43's role in cell proliferation and growth [88-90]. Co-staining breast cancer tissue arrays with proliferation markers suggests a role for Cx43 in regulating breast cancer cell proliferation [91] putatively via GJIC-independent mechanisms. In support of this hypothesis, overexpression of Cx43 in GJIC-deficient breast cancer cell lines resulted in Cx43 selectively associating with α-catenin, β-catenin and ZO-2 in breast cancer cells depending on culture conditions [65]. This study suggests that Cx43 exerts tumor suppressive effects in a context-dependent manner where junctional assembly with α-catenin, β-catenin and ZO-2 may be implicated in reducing growth rate, invasiveness, and, malignant phenotype of breast cancer cells independent of GJIC. Furthermore, the correlation of cytoplasmic Cx43 with the pro-apoptotic factor Bak suggests a GJIC-independent role in

signaling apoptotic pathways [19].

Phosphorylation and dephosphorylation events have been linked to connexin endocytosis and degradation [92]. Because the endocytic pathway plays a seminal role in receptor downregulation and signal potentiation, critical connections between Cx43 signaling are missing without further insight that could be provided with additional investigation. Furthermore, endocytosis is a major mechanism for transporting Cx43 away from the plasma membrane, the sight of GJIC. Engulfment of Cx43 gap junctions into enodocytic vesicles results in the formation of annular gap junctions, where Cx43 can then be degraded through lysosomal degradation [93]. However, given that endocytic vesicles can serve as signaling platforms in addition to feeding into a lysosomal degradation pathway, dependent on their route of trafficking, it will be interesting to determine whether Cx43 annular gap junctions serve a defined signaling role.

**Purinergic signaling, Cx43 hemichannels, and breast cancer**

In addition to the role of GJIC in cancer, there are emerging functions for Cx43 hemichannels and extracellular adenosine and adenosine nucleotide signaling (aka purinergic signaling) in cancer [94, 95]. Breast cancer is no exception, and several lines of evidence support the hypothesis that increased purinergic signaling through Cx43 hemichannels affects mammary tumor growth and metastasis. Breast cancers display increased expression of purinergic receptors [96]. The pro-metastatic effects of purinergic signaling appear to be primarily enacted by adenosine [95], which is the end product of a cascade where ecto-apyrases convert ATP to ADP and AMP, and ecto-nucleotidases hydrolyze AMP to adenosine [97]. Importantly, the ecto-nucleotidase, CD73, has been found to increase the adhesion, migration and invasion of T-47D and MB-MDA-231 cells through generation of adenosine [98]. Furthermore, therapeutic application of CD73 antibodies in an animal model inhibited breast cancer tumor growth and metastasis [99].

Alterations in the ATP-ADP-AMP levels, which influence adenosine content in cells has also been shown to alter breast cancer cell function. Zhou et al have shown that extracellular ATP has a biphasic effect on growth and migration of MDA-MB-231 cells. Specifically, at low concentrations extracellular ATP displayed an inhibitory effect, while at high concentrations purinergic signaling through adenosine enhanced migration [100]. Importantly, nerve growth factor (NGF) expression in breast cancer has been shown to be oncogenic [101], and treatment of PC12 cells with NGF increases Cx43 hemichannel-mediated ATP release [102]. In addition, oxidative stress and hypoxia (such as found in the tumor microenvironment [103]) are known to increase connexin-mediated hemichannel function and ATP release [104-107]. Taken together, these data suggest a mechanism by which purinergic signaling through Cx43 hemichannels in breast cancer cells could enhance the malignancy of mammary tumors. Namely, oncogenic signaling factors and the tumor microenvironment are proposed to increase ATP released through Cx43 hemichannels, which is then converted to adenosine by ecto-apyrases and nucleotidases leading to activation of purinergic receptors by adenosine, enhancing tumor growth and metastasis. Consequently, this pathway would indicate that Cx43 hemichannels are a potent target for therapeutics – either by down regulation, or upregulation in combination with other therapies, such as targeting CD73 ecto-nucleotidase.

## Methods of Targeting Cx43

Multiple opportunities are present for targeting Cx43 in breast cancer and these areas of potential intervention are described in **Figure 2A**. A primary mechanism that has been suggested for therapies that foster connexin-mediated GJIC, pertains to a phenomenon called the “bystander effect.” One potential benefit of modulating Cx43-directed GJIC in breast cancer cells is the ability to elicit a “bystander effect”, where increases in the number, size, or stability of gap junctions result in increased diffusion of cytotoxic substances, in particular chemotherapeutic agents [108]. The outcome of this effect is an amplification of therapeutic

response by promoting tumor cells to pass-on cytotoxic signals instigated by chemotherapeutics that have successfully targeted accessible neoplastic cells. Given the previously discussed reports that indicate that Cx43 is deregulated in breast cancer patient samples, resulting in the loss of gap junctional incorporation and increases in levels of cytoplasmic expression [16, 18, 35],, a major therapeutic approach in the treatment of metastatic breast cancer may be to restore Cx43 expression or activity in gap junctions [49]. This approach includes re-establishing any signaling aspects of Cx43, such as modification by phosphorylation that may have occurred due to loss of appropriate localization or function in breast cancer cells. Practically speaking, these approaches go hand-in-hand as restoring Cx43 gap junctional function would propagate cytotoxic signaling supported by the bystander effect.

A number of agents exist that reportedly target Cx43 and GJIC [109, 110]. These agents include a quinolone derivative and an organochlorine compound that have specifically been used to modulate GJIC in breast cancer cell lines and models, as well as a peptide mimetic that has shown preliminary efficacy in inhibiting breast cancer cell proliferation. While the organochlorine and quinolone compounds are able to modulate Cx43 and GJIC, like the majority of connexin-targeting therapeutics, they lack specificity and do not necessarily directly target Cx43 [12-14, 26]. The peptide agent is based on the nine carboxy terminal-most amino acids of Cx43 and has completed Phase II clinical trials for wound healing (CTRI/2011/09/001984, CTRI/2011/09/001985, CTRI/2011/09/002004) [85, 111, 112]. A fourth agent, an unmodified Cx43 antisense oligonucleotide that downregulates Cx43 is also being tested in clinical trials for wound healing (NCT01199588, NCT00820196) [113] but has not been evaluated as a potential cancer agent. Alternative therapeutic strategies may exist in targeting the kinases that mediate Cx43 phosphorylation [114, 115] or through the post-transcriptional restoration of connexin expression and functional gap junctions thorough internal ribosomal entry site-dependent synthesis [116]. However, the potential of any of these agents in the

treatment of human cancers, including breast cancer is still at an early stage. Several of these compounds are discussed in more detail below.

The organochlorine compound, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin **(**TCDD), was shown to alter phosphorylation of Cx43 and GJIC [117, 118]. Studies in MCF7 breast cancer cells show that TCDD decreases GJIC and therefore, may not have the desired modulatory effect of Cx43 in breast cancer since it is potentially more preferable to restore GJIC [26]. Consistent with this, it was noted that female factory workers exposed to TCDD had a 2-fold increase in breast cancer incidence [26]. Similarly, the Cx43 antisense oligonucleotide, which downregulates Cx43, may also not have the desired anti-cancer effect being sought. Indeed, the targeted inhibition of Cx43 expression in cancer cells has been shown to prevent GJIC and promote tumor aggressiveness [119].

The quinolone agent, PQ1, restores gap junction activity and has been tested in at least one *in vivo* mammary tumor model. Using the MMTV-Polyoma-Middle-T genetically engineered mouse model of spontaneous mammary tumorigenesis, it was determined that treatment of mice prior to tumor formation as well as at early and at late stages of tumor formation was able to reduce tumor growth. The authors suggest that the mechanism of action of PQ1 is to increase Cx43 expression during early stages of tumor formation and also prevents an increase in Cx46 expression during late stage tumor formation [14]. While these results suggest therapeutic potential for PQ1 in the treatment of breast cancer, in addition to targeting multiple connexins, PQ1 also alters the phosphorylation status of Akt and MAPK and therefore, has the potential to induce off-target effects [12, 13, 120]. More thorough investigation into how Cx43 and Cx46 collaborate or work independently in regulating gap junction activity during specific stages of mammary tumor formation is required to validate the observations suggested by this study.

Recently, evidence showed that a Cx43 mimetic peptide agent based on linkage of an antennapedia internalization domain to the carboxy-terminal PDZ binding domain of Cx43,

called α–connexin carboxyl-terminal (αCT1 or ACT1) peptide [121, 122], was able to inhibit the proliferation of breast cancer cells in multiple breast cancer cell lines and induced cell death in human epidermal growth factor receptor-2 (HER2)-positive breast cancer cells [15]. In support of these findings, recent reports have elucidated a distinct role for the carboxy-terminal domain of Cx43, independent of full-length Cx43, in mediating the proliferation of breast cancer cells [123]. Furthermore, combining αCT1 with the targeted inhibitors tamaxofen or lapatinib, enhanced the effects of these approved breast cancer therapies [15], thus establishing that αCT1 has therapeutic potential in the treatment of breast cancer. An example of αCT1 enhancement of tamaxofen activity is shown in **Figure 2B** where treatment of ER+ MCF7 breast cancer cells with the combination of αCT1 and tamaxofen has the greatest impact on cell viability. However, in order to translate these findings for clinical use the effect of αCT1 *in vivo* needs to be tested using preclinical mouse models and these studies remain to be evaluated.

## Conclusions and Future Directions

Targeting Cx43 in breast cancer by restoring its ability to localize to gap junction and propagate GJIC is a potential therapeutic strategy. However, additional studies to tease apart the mechanism of Cx43 regulation in breast cancer as well as whether regulation of Cx43 protein levels are important during different stages of breast cancer progression are required to determine if this strategy will be effective. Furthermore, studies that investigate the functions and impact of hemichannels in carcinogenesis and tumor progression are lacking. Studies characterizing hemichannel expression in cancerous compared to non-cancerous cells as well as the functional consequence of targeting hemichannels in malignant cells would be useful in fully elucidating the therapeutic potential associated with targeting Cx43 in breast cancer. Given that Cx43 targeting agents have already been developed, clinical translation of these agents has near term potential.

## References


1. Grek CL, Rhett JM, Ghatnekar GS: **Cardiac to cancer: connecting connexins to clinical opportunity**. *FEBS letters* 2014, **588**(8):1349-1364.
2. Kumar NM, Gilula NB: **The gap junction communication channel**. *Cell* 1996, **84**(3):381-388.
3. Makowski L, Caspar DL, Phillips WC, Goodenough DA: **Gap junction structures. II. Analysis of the x-ray diffraction data**. *The Journal of cell biology* 1977, **74**(2):629-645.
4. Rhett JM, Fann SA, Yost MJ: **Purinergic Signaling in Early Inflammatory Events of the Foreign Body Response: Modulating Extracellular ATP as an Enabling Technology for Engineered Implants and Tissues**. *Tissue engineering Part B, Reviews* 2014.
5. Pfenniger A, Wohlwend A, Kwak BR: **Mutations in connexin genes and disease**. *European journal of clinical investigation* 2011, **41**(1):103-116.
6. Loewenstein WR, Kanno Y: **Intercellular communication and the control of tissue growth: lack of communication between cancer cells**. *Nature* 1966, **209**(5029):1248-1249.
7. Loewenstein WR, Kanno Y: **Intercellular communication and tissue growth. I. Cancerous growth**. *The Journal of cell biology* 1967, **33**(2):225-234.
8. McLachlan E, Shao Q, Laird DW: **Connexins and gap junctions in mammary gland development and breast cancer progression**. *The Journal of membrane biology* 2007, **218**(1-3):107-121.
9. El-Saghir JA, El-Habre ET, El-Sabban ME, Talhouk RS: **Connexins: a junctional crossroad to breast cancer**. *The International journal of developmental biology* 2011, **55**(7-9):773-780.
10. Cronier L, Crespin S, Strale PO, Defamie N, Mesnil M: **Gap junctions and cancer: new functions for an old story**. *Antioxidants & redox signaling* 2009, **11**(2):323-338.
11. Vinken M, Decrock E, Leybaert L, Bultynck G, Himpens B, Vanhaecke T, Rogiers V: **Non-channel functions of connexins in cell growth and cell death**. *Biochimica et biophysica acta* 2012, **1818**(8):2002-2008.
12. Ding Y, Nguyen TA: **PQ1, a quinoline derivative, induces apoptosis in T47D breast cancer cells through activation of caspase-8 and caspase-9**. *Apoptosis : an international journal on programmed cell death* 2013, **18**(9):1071-1082.
13. Ding Y, Prasain K, Nguyen TD, Hua DH, Nguyen TA: **The effect of the PQ1 anti-breast cancer agent on normal tissues**. *Anti-cancer drugs* 2012, **23**(9):897-905.
14. Shishido SN, Delahaye A, Beck A, Nguyen TA: **The anticancer effect of PQ1 in the MMTV-PyVT mouse model**. *International journal of cancer Journal international du cancer* 2014, **134**(6):1474-1483.
15. Grek CL, Rhett JM, Bruce JS, Abt MA, Ghatnekar GS, Yeh ES: **Targeting connexin 43 with alpha-connexin carboxyl-terminal (ACT1) peptide enhances the activity of the targeted inhibitors, tamoxifen and lapatinib, in breast cancer: clinical implication for ACT1**. *BMC cancer* 2015, **15**:296.
16. Kanczuga-Koda L, Sulkowska M, Koda M, Reszec J, Famulski W, Baltaziak M, Sulkowski S: **Expression of connexin 43 in breast cancer in comparison with mammary dysplasia and the normal mammary gland**. *Folia morphologica* 2003, **62**(4):439-442.
17. Kanczuga-Koda L, Sulkowska M, Koda M, Rutkowski R, Sulkowski S: **Increased expression of gap junction protein--connexin 32 in lymph node metastases of human ductal breast cancer**. *Folia histochemica et cytobiologica / Polish Academy of Sciences, Polish Histochemical and Cytochemical Society* 2007, **45 Suppl 1**:S175-180.
18. Kanczuga-Koda L, Sulkowski S, Lenczewski A, Koda M, Wincewicz A, Baltaziak M, Sulkowska M: **Increased expression of connexins 26 and 43 in lymph node metastases of breast cancer**. *Journal of clinical pathology* 2006, **59**(4):429-433.

19. Kanczuga-Koda L, Sulkowski S, Tomaszewski J, Koda M, Sulkowska M, Przystupa W, Golaszewska J, Baltaziak M: **Connexins 26 and 43 correlate with Bak, but not with Bcl-2 protein in breast cancer**. *Oncology reports* 2005, **14**(2):325-329.
20. Laird DW, Fistouris P, Batist G, Alpert L, Huynh HT, Carystinos GD, Alaoui-Jamali MA: **Deficiency of connexin43 gap junctions is an independent marker for breast tumors**. *Cancer research* 1999, **59**(16):4104-4110.
21. Monaghan P, Moss D: **Connexin expression and gap junctions in the mammary gland**. *Cell biology international* 1996, **20**(2):121-125.
22. Plante I, Laird DW: **Decreased levels of connexin43 result in impaired development of the mammary gland in a mouse model of oculodentodigital dysplasia**. *Developmental biology* 2008, **318**(2):312-322.
23. Plante I, Wallis A, Shao Q, Laird DW: **Milk secretion and ejection are impaired in the mammary gland of mice harboring a Cx43 mutant while expression and localization of tight and adherens junction proteins remain unchanged**. *Biology of reproduction* 2010, **82**(5):837-847.
24. Stewart MK, Gong XQ, Barr KJ, Bai D, Fishman GI, Laird DW: **The severity of mammary gland developmental defects is linked to the overall functional status of Cx43 as revealed by genetically modified mice**. *The Biochemical journal* 2013, **449**(2):401-413.
25. Bier A, Oviedo-Landaverde I, Zhao J, Mamane Y, Kandouz M, Batist G: **Connexin43 pseudogene in breast cancer cells offers a novel therapeutic target**. *Molecular cancer therapeutics* 2009, **8**(4):786-793.
26. Gakhar G, Schrempp D, Nguyen TA: **Regulation of gap junctional intercellular communication by TCDD in HMEC and MCF-7 breast cancer cells**. *Toxicology and applied pharmacology* 2009, **235**(2):171-181.
27. Hirschi KK, Xu CE, Tsukamoto T, Sager R: **Gap junction genes Cx26 and Cx43 individually suppress the cancer phenotype of human mammary carcinoma cells and restore differentiation potential**. *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research* 1996, **7**(7):861-870.
28. Kandouz M, Bier A, Carystinos GD, Alaoui-Jamali MA, Batist G: **Connexin43 pseudogene is expressed in tumor cells and inhibits growth**. *Oncogene* 2004, **23**(27):4763-4770.
29. McLachlan E, Shao Q, Wang HL, Langlois S, Laird DW: **Connexins act as tumor suppressors in three-dimensional mammary cell organoids by regulating differentiation and angiogenesis**. *Cancer research* 2006, **66**(20):9886-9894.
30. Shao Q, Wang H, McLachlan E, Veitch GI, Laird DW: **Down-regulation of Cx43 by retroviral delivery of small interfering RNA promotes an aggressive breast cancer cell phenotype**. *Cancer research* 2005, **65**(7):2705-2711.
31. Qin H, Shao Q, Curtis H, Galipeau J, Belliveau DJ, Wang T, Alaoui-Jamali MA, Laird DW: **Retroviral delivery of connexin genes to human breast tumor cells inhibits in vivo tumor growth by a mechanism that is independent of significant gap junctional intercellular communication**. *The Journal of biological chemistry* 2002, **277**(32):29132-29138.
32. Tomasetto C, Neveu MJ, Daley J, Horan PK, Sager R: **Specificity of gap junction communication among human mammary cells and connexin transfectants in culture**. *The Journal of cell biology* 1993, **122**(1):157-167.
33. Pozzi A, Risek B, Kiang DT, Gilula NB, Kumar NM: **Analysis of multiple gap junction gene products in the rodent and human mammary gland**. *Experimental cell research* 1995, **220**(1):212-219.
34. Teleki I, Szasz AM, Maros ME, Gyorffy B, Kulka J, Meggyeshazi N, Kiszner G, Balla P, Samu A, Krenacs T: **Correlations of differentially expressed gap junction connexins Cx26, Cx30, Cx32, Cx43 and Cx46 with breast cancer progression and prognosis**. *PloS one* 2014, **9**(11):e112541.

35. Jamieson S, Going JJ, D'Arcy R, George WD: **Expression of gap junction proteins connexin 26 and connexin 43 in normal human breast and in breast tumours**. *The Journal of pathology* 1998, **184**(1):37-43.
36. Lambe T, Finlay D, Murphy M, Martin F: **Differential expression of connexin 43 in mouse mammary cells**. *Cell biology international* 2006, **30**(5):472-479.
37. Talhouk RS, Elble RC, Bassam R, Daher M, Sfeir A, Mosleh LA, El-Khoury H, Hamoui S, Pauli BU, El-Sabban ME: **Developmental expression patterns and regulation of connexins in the mouse mammary gland: expression of connexin30 in lactogenesis**. *Cell and tissue research* 2005, **319**(1):49-59.
38. Yu W, Dahl G, Werner R: **The connexin43 gene is responsive to oestrogen**. *Proceedings Biological sciences / The Royal Society* 1994, **255**(1343):125-132.
39. Andersen J, Grine E, Eng CL, Zhao K, Barbieri RL, Chumas JC, Brink PR: **Expression of connexin-43 in human myometrium and leiomyoma**. *American journal of obstetrics and gynecology* 1993, **169**(5):1266-1276.
40. Reaume AG, de Sousa PA, Kulkarni S, Langille BL, Zhu D, Davies TC, Juneja SC, Kidder GM, Rossant J: **Cardiac malformation in neonatal mice lacking connexin43**. *Science* 1995, **267**(5205):1831-1834.
41. Plum A, Hallas G, Magin T, Dombrowski F, Hagendorff A, Schumacher B, Wolpert C, Kim J, Lamers WH, Evert M *et al*: **Unique and shared functions of different connexins in mice**. *Current biology : CB* 2000, **10**(18):1083-1091.
42. El-Sabban ME, Sfeir AJ, Daher MH, Kalaany NY, Bassam RA, Talhouk RS: **ECM-induced gap junctional communication enhances mammary epithelial cell differentiation**. *Journal of cell science* 2003, **116**(Pt 17):3531-3541.
43. Elzarrad MK, Haroon A, Willecke K, Dobrowolski R, Gillespie MN, Al-Mehdi AB: **Connexin-43 upregulation in micrometastases and tumor vasculature and its role in tumor cell attachment to pulmonary endothelium**. *BMC medicine* 2008, **6**:20.
44. Wang WK, Chen MC, Leong HF, Kuo YL, Kuo CY, Lee CH: **Connexin 43 suppresses tumor angiogenesis by down-regulation of vascular endothelial growth factor via hypoxic-induced factor-1alpha**. *International journal of molecular sciences* 2015, **16**(1):439-451.
45. Li Z, Zhou Z, Welch DR, Donahue HJ: **Expressing connexin 43 in breast cancer cells reduces their metastasis to lungs**. *Clinical & experimental metastasis* 2008, **25**(8):893-901.
46. Li Z, Zhou Z, Donahue HJ: **Alterations in Cx43 and OB-cadherin affect breast cancer cell metastatic potential**. *Clinical & experimental metastasis* 2008, **25**(3):265-272.
47. Plante I, Stewart MK, Barr K, Allan AL, Laird DW: **Cx43 suppresses mammary tumor metastasis to the lung in a Cx43 mutant mouse model of human disease**. *Oncogene* 2011, **30**(14):1681-1692.
48. Carystinos GD, Bier A, Batist G: **The role of connexin-mediated cell-cell communication in breast cancer metastasis**. *Journal of mammary gland biology and neoplasia* 2001, **6**(4):431-440.
49. Kandouz M, Batist G: **Gap junctions and connexins as therapeutic targets in cancer**. *Expert opinion on therapeutic targets* 2010, **14**(7):681-692.
50. Kapoor P, Saunders MM, Li Z, Zhou Z, Sheaffer N, Kunze EL, Samant RS, Welch DR, Donahue HJ: **Breast cancer metastatic potential: correlation with increased heterotypic gap junctional intercellular communication between breast cancer cells and osteoblastic cells**. *International journal of cancer Journal international du cancer* 2004, **111**(5):693-697.
51. Pollmann MA, Shao Q, Laird DW, Sandig M: **Connexin 43 mediated gap junctional communication enhances breast tumor cell diapedesis in culture**. *Breast cancer research : BCR* 2005, **7**(4):R522-534.

52. Saunders MM, Seraj MJ, Li Z, Zhou Z, Winter CR, Welch DR, Donahue HJ: **Breast cancer metastatic potential correlates with a breakdown in homospecific and heterospecific gap junctional intercellular communication**. *Cancer research* 2001, **61**(5):1765-1767.
53. Monaghan P, Clarke C, Perusinghe NP, Moss DW, Chen XY, Evans WH: **Gap junction distribution and connexin expression in human breast**. *Experimental cell research* 1996, **223**(1):29-38.
54. Locke D: **Gap junctions in normal and neoplastic mammary gland**. *The Journal of pathology* 1998, **186**(4):343-349.
55. Laird DW: **Life cycle of connexins in health and disease**. *The Biochemical journal* 2006, **394**(Pt 3):527-543.
56. Naus CC, Laird DW: **Implications and challenges of connexin connections to cancer**. *Nature reviews Cancer* 2010, **10**(6):435-441.
57. Langlois S, Cowan KN, Shao Q, Cowan BJ, Laird DW: **The tumor-suppressive function of Connexin43 in keratinocytes is mediated in part via interaction with caveolin-1**. *Cancer research* 2010, **70**(10):4222-4232.
58. Ito A, Watabe K, Koma Y, Kitamura Y: **An attempt to isolate genes responsible for spontaneous and experimental metastasis in the mouse model**. *Histology and histopathology* 2002, **17**(3):951-959.
59. Chao Y, Wu Q, Acquafondata M, Dhir R, Wells A: **Partial mesenchymal to epithelial reverting transition in breast and prostate cancer metastases**. *Cancer microenvironment : official journal of the International Cancer Microenvironment Society* 2012, **5**(1):19-28.
60. Solan JL, Hingorani SR, Lampe PD: **Changes in connexin43 expression and localization during pancreatic cancer progression**. *The Journal of membrane biology* 2012, **245**(5-6):255-262.
61. Lawson JC, Blatch GL, Edkins AL: **Cancer stem cells in breast cancer and metastasis**. *Breast cancer research and treatment* 2009, **118**(2):241-254.
62. Visvader JE, Lindeman GJ: **Cancer stem cells in solid tumours: accumulating evidence and unresolved questions**. *Nature reviews Cancer* 2008, **8**(10):755-768.
63. Bos PD, Zhang XH, Nadal C, Shu W, Gomis RR, Nguyen DX, Minn AJ, van de Vijver MJ, Gerald WL, Foekens JA *et al*: **Genes that mediate breast cancer metastasis to the brain**. *Nature* 2009, **459**(7249):1005-1009.
64. Teleki I, Krenacs T, Szasz MA, Kulka J, Wichmann B, Leo C, Papassotiropoulos B, Riemenschnitter C, Moch H, Varga Z: **The potential prognostic value of connexin 26 and 46 expression in neoadjuvant-treated breast cancer**. *BMC cancer* 2013, **13**:50.
65. Talhouk RS, Fares MB, Rahme GJ, Hariri HH, Rayess T, Dbouk HA, Bazzoun D, Al-Labban D, El-Sabban ME: **Context dependent reversion of tumor phenotype by connexin-43 expression in MDA-MB231 cells and MCF-7 cells: role of beta-catenin/connexin43 association**. *Experimental cell research* 2013, **319**(20):3065-3080.
66. Nicolson GL, Dulski KM, Trosko JE: **Loss of intercellular junctional communication correlates with metastatic potential in mammary adenocarcinoma cells**. *Proceedings of the National Academy of Sciences of the United States of America* 1988, **85**(2):473-476.
67. Cai J, Jiang WG, Mansel RE: **Gap junctional communication and the tyrosine phosphorylation of connexin 43 in interaction between breast cancer and endothelial cells**. *International journal of molecular medicine* 1998, **1**(1):273-278.
68. Lim PK, Bliss SA, Patel SA, Taborga M, Dave MA, Gregory LA, Greco SJ, Bryan M, Patel PS, Rameshwar P: **Gap junction-mediated import of microRNA from bone marrow stromal cells can elicit cell cycle quiescence in breast cancer cells**. *Cancer research* 2011, **71**(5):1550-1560.
69. Krutovskikh VA, Troyanovsky SM, Piccoli C, Tsuda H, Asamoto M, Yamasaki H: **Differential effect of subcellular localization of communication impairing gap junction protein connexin43 on tumor cell growth in vivo**. *Oncogene* 2000, **19**(4):505-513.

70. Lampe PD, Lau AF: **The effects of connexin phosphorylation on gap junctional communication**. *The international journal of biochemistry & cell biology* 2004, **36**(7):1171-1186.
71. Maass K, Shibayama J, Chase SE, Willecke K, Delmar M: **C-terminal truncation of connexin43 changes number, size, and localization of cardiac gap junction plaques**. *Circulation research* 2007, **101**(12):1283-1291.
72. Solan JL, Marquez-Rosado L, Sorgen PL, Thornton PJ, Gafken PR, Lampe PD: **Phosphorylation at S365 is a gatekeeper event that changes the structure of Cx43 and prevents down-regulation by PKC**. *The Journal of cell biology* 2007, **179**(6):1301-1309.
73. Solan JL, Lampe PD: **Key connexin 43 phosphorylation events regulate the gap junction life cycle**. *The Journal of membrane biology* 2007, **217**(1-3):35-41.
74. Evans WH, De Vuyst E, Leybaert L: **The gap junction cellular internet: connexin hemichannels enter the signalling limelight**. *The Biochemical journal* 2006, **397**(1):1-14.
75. Saez JC, Berthoud VM, Branes MC, Martinez AD, Beyer EC: **Plasma membrane channels formed by connexins: their regulation and functions**. *Physiological reviews* 2003, **83**(4):1359-1400.
76. Musil LS, Le AC, VanSlyke JK, Roberts LM: **Regulation of connexin degradation as a mechanism to increase gap junction assembly and function**. *The Journal of biological chemistry* 2000, **275**(33):25207-25215.
77. Koval M, Molina SA, Burt JM: **Mix and match: investigating heteromeric and heterotypic gap junction channels in model systems and native tissues**. *FEBS letters* 2014, **588**(8):1193-1204.
78. Cottrell GT, Wu Y, Burt JM: **Cx40 and Cx43 expression ratio influences heteromeric/ heterotypic gap junction channel properties**. *American journal of physiology Cell physiology* 2002, **282**(6):C1469-1482.
79. Ayad WA, Locke D, Koreen IV, Harris AL: **Heteromeric, but not homomeric, connexin channels are selectively permeable to inositol phosphates**. *The Journal of biological chemistry* 2006, **281**(24):16727-16739.
80. Gemel J, Valiunas V, Brink PR, Beyer EC: **Connexin43 and connexin26 form gap junctions, but not heteromeric channels in co-expressing cells**. *Journal of cell science* 2004, **117**(Pt 12):2469-2480.
81. Heyman NS, Kurjiaka DT, Ek Vitorin JF, Burt JM: **Regulation of gap junctional charge selectivity in cells coexpressing connexin 40 and connexin 43**. *American journal of physiology Heart and circulatory physiology* 2009, **297**(1):H450-459.
82. Palatinus JA, Rhett JM, Gourdie RG: **The connexin43 carboxyl terminus and cardiac gap junction organization**. *Biochimica et biophysica acta* 2012, **1818**(8):1831-1843.
83. Rhett JM, Jourdan J, Gourdie RG: **Connexin 43 connexon to gap junction transition is regulated by zonula occludens-1**. *Molecular biology of the cell* 2011, **22**(9):1516-1528.
84. Grek CL, Prasad GM, Viswanathan V, Armstrong DG, Gourdie RG, Ghatnekar GS: **Topical administration of a connexin43-based peptide augments healing of chronic neuropathic diabetic foot ulcers: A multicenter, randomized trial**. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* 2015, **23**(2):203-212.
85. Ghatnekar GS, Grek CL, Armstrong DG, Desai SC, Gourdie RG: **The effect of a connexin43-based Peptide on the healing of chronic venous leg ulcers: a multicenter, randomized trial**. *The Journal of investigative dermatology* 2015, **135**(1):289-298.
86. Dbouk HA, Mroue RM, El-Sabban ME, Talhouk RS: **Connexins: a myriad of functions extending beyond assembly of gap junction channels**. *Cell communication and signaling : CCS* 2009, **7**:4.
87. Zhou JZ, Jiang JX: **Gap junction and hemichannel-independent actions of connexins on cell and tissue functions--an update**. *FEBS letters* 2014, **588**(8):1186-1192.

88. Harris AL: **Connexin channel permeability to cytoplasmic molecules**. *Progress in biophysics and molecular biology* 2007, **94**(1-2):120-143.
89. Goodenough DA, Paul DL: **Beyond the gap: functions of unpaired connexon channels**. *Nature reviews Molecular cell biology* 2003, **4**(4):285-294.
90. Wei CJ, Xu X, Lo CW: **Connexins and cell signaling in development and disease**. *Annual review of cell and developmental biology* 2004, **20**:811-838.
91. Conklin C, Huntsman D, Yorida E, Makretsov N, Turbin D, Bechberger JF, Sin WC, Naus CC: **Tissue microarray analysis of connexin expression and its prognostic significance in human breast cancer**. *Cancer letters* 2007, **255**(2):284-294.
92. Saffitz JE, Laing JG, Yamada KA: **Connexin expression and turnover : implications for cardiac excitability**. *Circulation research* 2000, **86**(7):723-728.
93. Jordan K, Chodock R, Hand AR, Laird DW: **The origin of annular junctions: a mechanism of gap junction internalization**. *Journal of cell science* 2001, **114**(Pt 4):763-773.
94. Schalper KA, Carvajal-Hausdorf D, Oyarzo MP: **Possible role of hemichannels in cancer**. *Frontiers in physiology* 2014, **5**:237.
95. Burnstock G, Di Virgilio F: **Purinergic signalling and cancer**. *Purinergic signalling* 2013, **9**(4):491-540.
96. Li S, Huang S, Peng SB: **Overexpression of G protein-coupled receptors in cancer cells: involvement in tumor progression**. *International journal of oncology* 2005, **27**(5):1329-1339.
97. Burnstock G: **Introductory overview of purinergic signalling**. *Frontiers in bioscience* 2011, **3**:896-900.
98. Wang L, Zhou X, Zhou T, Ma D, Chen S, Zhi X, Yin L, Shao Z, Ou Z, Zhou P: **Ecto-5'-nucleotidase promotes invasion, migration and adhesion of human breast cancer cells**. *Journal of cancer research and clinical oncology* 2008, **134**(3):365-372.
99. Stagg J, Divisekera U, McLaughlin N, Sharkey J, Pommey S, Denoyer D, Dwyer KM, Smyth MJ: **Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis**. *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107**(4):1547-1552.
100. Zhou JZ, Riquelme MA, Gao X, Ellies LG, Sun LZ, Jiang JX: **Differential impact of adenosine nucleotides released by osteocytes on breast cancer growth and bone metastasis**. *Oncogene* 2015, **34**(14):1831-1842.
101. Dolle L, Adriaenssens E, El Yazidi-Belkoura I, Le Bourhis X, Nurcombe V, Hondermarck H: **Nerve growth factor receptors and signaling in breast cancer**. *Current cancer drug targets* 2004, **4**(6):463-470.
102. Belliveau DJ, Bani-Yaghoub M, McGirr B, Naus CC, Rushlow WJ: **Enhanced neurite outgrowth in PC12 cells mediated by connexin hemichannels and ATP**. *The Journal of biological chemistry* 2006, **281**(30):20920-20931.
103. Grek CL, Tew KD: **Redox metabolism and malignancy**. *Current opinion in pharmacology* 2010, **10**(4):362-368.
104. Retamal MA, Cortes CJ, Reuss L, Bennett MV, Saez JC: **S-nitrosylation and permeation through connexin 43 hemichannels in astrocytes: induction by oxidant stress and reversal by reducing agents**. *Proceedings of the National Academy of Sciences of the United States of America* 2006, **103**(12):4475-4480.
105. Contreras JE, Sanchez HA, Eugenin EA, Speidel D, Theis M, Willecke K, Bukauskas FF, Bennett MV, Saez JC: **Metabolic inhibition induces opening of unapposed connexin 43 gap junction hemichannels and reduces gap junctional communication in cortical astrocytes in culture**. *Proceedings of the National Academy of Sciences of the United States of America* 2002, **99**(1):495-500.

106. Bodin P, Burnstock G: **Purinergic signalling: ATP release**. *Neurochemical research* 2001, **26**(8-9):959-969.
107. Orellana JA, Froger N, Ezan P, Jiang JX, Bennett MV, Naus CC, Giaume C, Saez JC: **ATP and glutamate released via astroglial connexin 43 hemichannels mediate neuronal death through activation of pannexin 1 hemichannels**. *Journal of neurochemistry* 2011, **118**(5):826-840.
108. Spray DC, Hanstein R, Lopez-Quintero SV, Stout RF, Jr., Suadicani SO, Thi MM: **Gap junctions and Bystander Effects: Good Samaritans and executioners**. *Wiley interdisciplinary reviews Membrane transport and signaling* 2013, **2**(1):1-15.
109. Evans WH, Bultynck G, Leybaert L: **Manipulating connexin communication channels: use of peptidomimetics and the translational outputs**. *The Journal of membrane biology* 2012, **245**(8):437-449.
110. De Vuyst E, Boengler K, Antoons G, Sipido KR, Schulz R, Leybaert L: **Pharmacological modulation of connexin-formed channels in cardiac pathophysiology**. *British journal of pharmacology* 2011, **163**(3):469-483.
111. http://ctri.nic.in/.
112. Grek CL, Prasad GM, Viswanathan V, Armstrong DG, Gourdie RG, Ghatnekar GS: **Topical administration of a Connexin43-based peptide augments healing of chronic neuropathic diabetic foot ulcers: A multicenter, randomized trial**. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* 2015.
113. clinicaltrials.gov.
114. Solan JL, Lampe PD: **Connexin43 phosphorylation: structural changes and biological effects**. *The Biochemical journal* 2009, **419**(2):261-272.
115. Nakamura Y, Chang CC, Mori T, Sato K, Ohtsuki K, Upham BL, Trosko JE: **Augmentation of differentiation and gap junction function by kaempferol in partially differentiated colon cancer cells**. *Carcinogenesis* 2005, **26**(3):665-671.
116. Lahlou H, Fanjul M, Pradayrol L, Susini C, Pyronnet S: **Restoration of functional gap junctions through internal ribosome entry site-dependent synthesis of endogenous connexins in density-inhibited cancer cells**. *Molecular and cellular biology* 2005, **25**(10):4034-4045.
117. Andrysik Z, Prochazkova J, Kabatkova M, Umannova L, Simeckova P, Kohoutek J, Kozubik A, Machala M, Vondracek J: **Aryl hydrocarbon receptor-mediated disruption of contact inhibition is associated with connexin43 downregulation and inhibition of gap junctional intercellular communication**. *Archives of toxicology* 2013, **87**(3):491-503.
118. Bager Y, Lindebro MC, Martel P, Chaumontet C, Warngard L: **Altered function, localization and phosphorylation of gap junctions in rat liver epithelial, IAR 20, cells after treatment with PCBs or TCDD**. *Environmental toxicology and pharmacology* 1997, **3**(4):257-266.
119. Forster T, Rausch V, Zhang Y, Isayev O, Heilmann K, Schoensiegel F, Liu L, Nessling M, Richter K, Labsch S *et al*: **Sulforaphane counteracts aggressiveness of pancreatic cancer driven by dysregulated Cx43-mediated gap junctional intercellular communication**. *Oncotarget* 2014, **5**(6):1621-1634.
120. Ding Y, Nguyen TA: **Gap Junction Enhancer Potentiates Cytotoxicity of Cisplatin in Breast Cancer Cells**. *Journal of cancer science & therapy* 2012, **4**(11):371-378.
121. Hunter AW, Barker RJ, Zhu C, Gourdie RG: **Zonula occludens-1 alters connexin43 gap junction size and organization by influencing channel accretion**. *Molecular biology of the cell* 2005, **16**(12):5686-5698.
122. Ghatnekar GS, O'Quinn MP, Jourdan LJ, Gurjarpadhye AA, Draughn RL, Gourdie RG: **Connexin43 carboxyl-terminal peptides reduce scar progenitor and promote regenerative healing following skin wounding**. *Regenerative medicine* 2009, **4**(2):205-223.

123. Maqbool R, Rashid R, Ismail R, Niaz S, Chowdri NA, Hussain MU: **The carboxy-terminal domain of connexin 43 (CT-Cx43) modulates the expression of p53 by altering miR-125b expression in low-grade human breast cancers**. *Cellular oncology* 2015.
124. Lee SW, Tomasetto C, Paul D, Keyomarsi K, Sager R: **Transcriptional downregulation of gap-junction proteins blocks junctional communication in human mammary tumor cell lines**. *The Journal of cell biology* 1992, **118**(5):1213-1221.
125. Conklin CM, Bechberger JF, MacFabe D, Guthrie N, Kurowska EM, Naus CC: **Genistein and quercetin increase connexin43 and suppress growth of breast cancer cells**. *Carcinogenesis* 2007, **28**(1):93-100.
126. Wilgenbus KK, Kirkpatrick CJ, Knuechel R, Willecke K, Traub O: **Expression of Cx26, Cx32 and Cx43 gap junction proteins in normal and neoplastic human tissues**. *International journal of cancer Journal international du cancer* 1992, **51**(4):522-529.
127. Stoletov K, Strnadel J, Zardouzian E, Momiyama M, Park FD, Kelber JA, Pizzo DP, Hoffman R, VandenBerg SR, Klemke RL: **Role of connexins in metastatic breast cancer and melanoma brain colonization**. *Journal of cell science* 2013, **126**(Pt 4):904-913.
128. Bellahcene A, Bachelier R, Detry C, Lidereau R, Clezardin P, Castronovo V: **Transcriptome analysis reveals an osteoblast-like phenotype for human osteotropic breast cancer cells**. *Breast cancer research and treatment* 2007, **101**(2):135-148.
129. el-Sabban ME, Pauli BU: **Cytoplasmic dye transfer between metastatic tumor cells and vascular endothelium**. *The Journal of cell biology* 1991, **115**(5):1375-1382.
130. Gakhar G, Ohira T, Shi A, Hua DH, Nguyen TA: **Antitumor effect of substituted quinolines in breast cancer cells**. *Drug Development Research* 2009, **69**(8):526-534.
131. Kong H, Liu X, Yang L, Qi K, Zhang H, Zhang J, Huang Z, Wang H: **All-trans retinoic acid enhances bystander effect of suicide gene therapy in the treatment of breast cancer**. *Oncology reports* 2015.

## Acknowledgements and Funding

Research in the Yeh Lab is supported by R01- CA187305-01A1 from the NCI (to E.S.Y), a grant from the Concern Foundation (to E.S.Y), as well as previous pilot funds from an American Cancer Society Institutional Research Grant (IRG-97-219-14) awarded to the Hollings Cancer Center at MUSC and from a Department of Defense grant (W81XWH-11-2-0229) at MUSC. This study used the services of the Morphology, Imaging and Instrumentation Core, which is supported by NIH-NIGMS P30 GM103342 to the South Carolina COBRE for Developmentally Based Cardiovascular Diseases.

## Competing and Conflicting Interests

FirstString Research has an exclusive, worldwide license for all fields of use for αCT1 (a.k.a. ACT1). GSG is president and CEO of FirstString Research. CLG is an employee of FirstString Research. GSG and CLG have stock options issued by the company. ESY, JMR, and JSB do not hold any financial interest in FirstString Research Inc. The authors declare that they have no competing interests.

## Figure Legends

**Figure 1. Gap junctional intercellular communication and breast cancer malignancy.**

**A)** Normal mammary gland structure shown on the left is maintained by mammary epithelial and myoepithelial cells that have functional Cx43 gap junctions. Loss of the GJIC mediated by Cx43 is thought to promote the development of breast cancer malignancies that contain cells that no longer have functional Cx43 gap junctions either due to loss of Cx43 expression or mis-localization of Cx43 away from the plasma membrane. Restoration of Cx43 activity is proposed to reverse the malignant phenotype driven by loss of Cx43 GJIC. **B)** Cx43 immunofluorescent staining in MCF7 and BT474 cells demonstrates localization of Cx43 to cytoplasmic areas not associated with gap junction or hemichannel formation at the plasma membrane. Cx43 staining in green. Wheat germ agglutinin staining to show the plasma membrane in red.

**Figure 2. Restoration of GJIC for enhancing cancer treatment.**

**A)** Current literature supports differential and dynamic mechanistic functions for Cx43 during tumor cell dissemination. During early stages of malignancy and in the primary tumor Cx43 is frequently downregulated and associated with loss of gap junctional intercellular communication GJIC. Cx43 expression during the processes of intravasation and extravasation remains controversial and is likely cell and context dependent. Transient upregulation of Cx43 has been associated with metastatic progression and the formation of secondary tumors. The red colored

X represent points during the metastatic process where targeting Cx43 offers therapeutic opportunity. Therapeutic opportunity for targeting Cx43 in breast cancer may exist in the prevention of : (***1***) Primary Tumor formation [16, 19, 20, 29-31, 47, 65, 124-126], (***2***) Tumor cell detachment/Metastatic progression [18, 46, 47, 127], (***3***) Intravasation [51, 67] (***4***) Extravasation/Formation of secondary lesions [8, 18, 43, 59, 63, 67, 127-129].

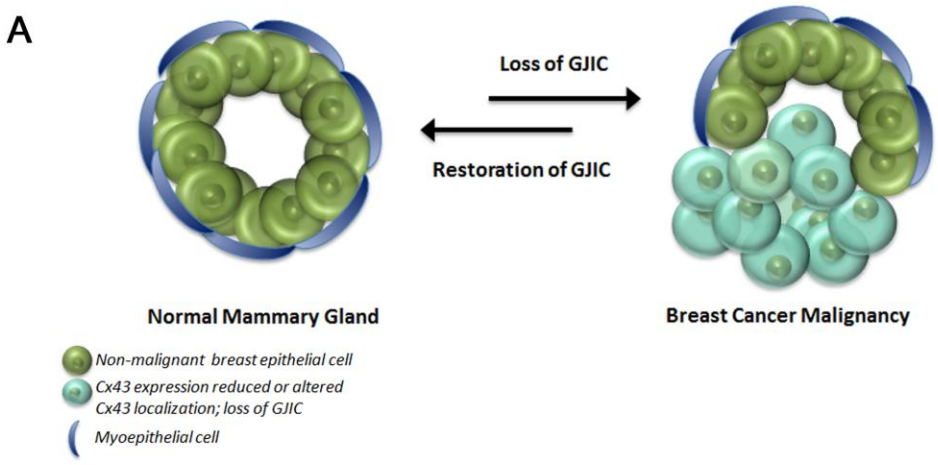
**B**) Treatment of MCF7 breast cancer cells with the gap junction restoring agent, αCT1, enhances tamoxifen cytotoxicity. Cellular toxicity was measured using crystal violet staining analysis.

| **Cx43 Targeting Mechanism** | **Study results** | **Reference** |
|---|---|---|
| *Cx43 over-expression*<br>MCF-7 and MDA-MB-231 | • MCF-7 proliferation decreased (2D and 3D culture)<br>• MDA-MB-231 proliferation decreased (3D culture). | [65] |
| *Cx43 gene knockdown*<br>Hs578T cells | • GJIC impaired<br>• Proliferation increased<br>• Cell migration enhanced; decreased expression of thrombospondin-1, and increased VEGF expression. | [30] |
| *Cx43 overexpression*<br>MDA-MB-231 and HBL100 | • Inhibited tumor growth *in vivo* (xenograft)<br>• Tested GJIC and proliferation but observed no effect | [31] |
| *Cx43 overexpression; Carbenoxolone treatment (inhibits GJIC)*<br>HBL1000 | • GJIC increased<br>• Tumor cell diapedesis increased<br>• GJIC and diapedesis blocked with carbenoxolone | [51] |

| | | |
|---|---|---|
| *Cx43 overexpression*<br>MDA-MET | • GJIC increased<br>• Cell invasion decreased; decreased ability to adhere to hFOB and HUV-EC-C cells | [46] |
| *Cx43 overexpression*<br>MDA-MB-231 | • Invasive potential impaired (MC3T3-E1 model)<br>• Tested growth, adhesion, and regulation of osteogenic gene expression but observed no effect | [8] |
| *Cx43 WT and Cx43 G138R overexpression*<br>4T1 | • Increased adhesion of 4T1 cells to the pulmonary endothelium with overexpression of Cx43 WT<br>• Decreased adhesion of 4T1 cells to the pulmonary endothelium with overexpression of Cx43 G138R | [43] |
| *Cx43 overexpression*<br>MDA-MB-231 | • Proliferation decreased<br>• Anchorage-independent cell growth decreased<br>• Partial re-differentiation of 3D organoids<br>• Tested GJIC but observed no effect | [29] |
| *Transgenic expression of Cx43 G60S*<br>MMTV-ErbB2 GEMM | • Onset of palpable with 7,12-dimethylbenz[alpha]anthracene (DMBA)-treatment was delayed in Cx43 G60S;MMTV-ErbB2 mice<br>• Pulmonary metastases increased in Cx43 G60S;MMTV-ErbB2 mice | [47] |
| *RNAi depletion of Cx43; Carbenoxolone treatment (inhibits GJIC); Twist expression (increases Cx43 expression)*<br>Zebrafish and chicken embryo models | • Tumor cell extravasation blocked by RNAi and carbenoxolone<br>• GJIC increased by Twist expression<br>• Tumor cell extravasation increased by Twist expression | [127] |
| *PQ1 treatment (binds to Cx43 hemichannel)*<br>T47D breast cancer cells; MMTV-PyVT GEMM | • GJIC increased<br>• Tumor growth decreased (T47D xenograft and MMTV-PyVT) | [14, 120, 130] |
| *Genistein and quercetin (increases Cx43 expression)*<br>MDA-MB-231 | • Cell proliferation inhibited<br>• Tested GJIC but observed no effect independent of GJIC<br>• Cx43 localized to the plasma membrane and perinuclear region following genistein treatment and quercetin treatment, respectively. | [125] |
| *ACT1 therapeutic peptide (enhances GJIC via Cx43 binding)*<br>MCF-7, MDA MB 231, BT474 | • GJIC increased (MCF-7, MDA MB 231, BT474)<br>• Proliferation decreased (MCF-7, MDA MB 231, BT474)<br>• Survival impaired (BT474)<br>• Augments the chemotherapeutic activity of lapatinib and tamoxifen (BT474 and MCF-7 respectively) | [15] |

| | | |
|---|---|---|
| *ATRA (increases Cx43 expression)*<br>MCF-7 | • GJIC increased when combined with a VEGFP-TK/CD gene suicide system<br>• Survival decreased<br>• Bystander effect increased | [131] |
| A number of studies have incorrectly used MDA-MB-435 cells to investigate the role of Cx43 in breast cancer tumorigenesis and metastasis | | [27, 45, 52] |

Figure 1


A
Loss of GJIC
Restoration of GJIC
Normal Mammary Gland
Breast Cancer Malignancy
Non-malignant breast epithelial cell
Cx43 expression reduced or altered
Cx43 localization; loss of GJIC
Myoepithelial cell

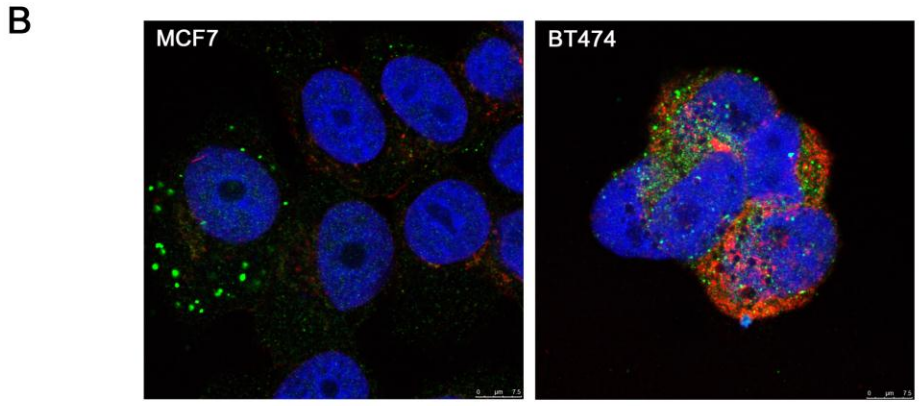
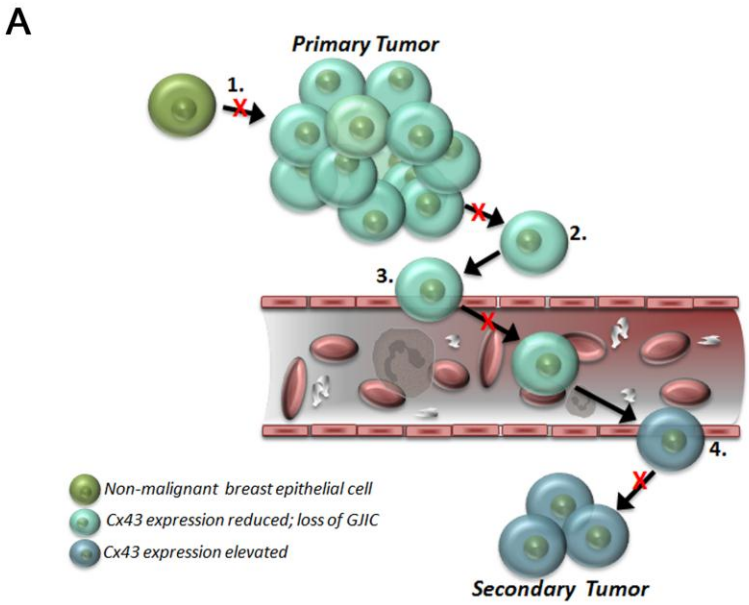
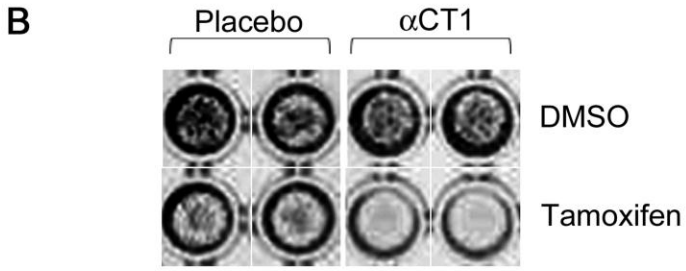

B
MCF7
BT474

Figure 2


A
Primary Tumor
1.
2.
3.
4.
Non-malignant breast epithelial cell
Cx43 expression reduced; loss of GJIC
Cx43 expression elevated
Secondary Tumor


B
Placebo
αCT1
DMSO
Tamoxifen