Cx26 inhibits breast MDA-MB-435 cell tumorigenic properties by a gap junctional intercellular communication-independent mechanism

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It has been well established that the restoration of connexin expression in tumor cells often leads to a partial reversion of tumor cell phenotypes and increased growth control. In this study, a less-aggressive variant of MDA-MB-435 cells obtained from the MDA-MB-435 cell line was engineered to express gap junctional intercellular communication (GJIC)-competent Cx26, a GJIC-incompetent cell surface transported GFP-Cx26 chimera, or a Golgi apparatus-localized, disease-linked Cx6 mutant (D66H). Collectively, these cell lines were designed to establish whether Cx26 regulates tumor properties, such as migration, invasion and growth, by (i) a GJIC-dependent pathway; (ii) a mechanism requiring Cx26 transport to the cell surface; or (iii) a mechanism where Cx26 expression alone was sufficient. The expression of Cx26 and green fluorescent protein (GFP)-Cx26 decreased cell proliferation while all three Cx26 variants inhibited anchorage-independent cell growth. All three Cx26 variants also altered the distribution of filamentous actin and significantly reduced cell migration, while only the D66H mutant failed to inhibit cell invasion through matrigel. Furthermore, expression of all the Cx26 variants reduced the levels of total β1 integrin, and decreased the activity of matrix metalloproteinase-9 (MMP-9) while increasing tissue inhibitors of MMP-1 (TIMP-1) activity. Interestingly, the expression of Cx43 regulated the same gene products without significantly affecting the tumorigenic properties of the MDA-MB-435 cells. Together, these results suggest that Cx26 expression, independent of the necessity for gap junctional intercellular communication, partially reverted MDA-MB-435 cell properties associated with tumorigenesis, and regulated the expression of genes important in cell migration and invasion.

Abbreviations: Cx, connexin; D66H-GFP, deafness and skin disease-linked Cx26 mutant; GFP, green fluorescent protein; GJIC, gap junctional intercellular communication; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of MMPs.

Introduction

Gap junctions are clusters of membrane spanning channels that link neighboring cells and allow for the direct intercellular exchange of ions, small metabolites and second messengers (1). A single gap junction channel consists of two hemi-channels called connexons, and each connexon is composed of six transmembrane proteins known as connexins (2). Connexins are a family of proteins composed of 21 members that are differentially expressed in almost all tissues (3). Gap junctional intercellular communication (GJIC) provides a mechanism for individual cells to communicate directly with each other and regulate important cellular functions ranging from homeostasis to highly specialized cell activities (2). Important to the current study are the numerous reports that have linked the loss of GJIC and/or connexin expression to tumor cell growth (see ref. 4 for a review). Cx26 and Cx43 re-introduction into many rodent and human breast tumor cell lines has reverted the tumor phenotype, exemplified by the establishment of significant levels of growth control both in vitro and in vivo (5–17). Pertinent to this study, both Cx26 and Cx43 restored the differentiation potential and growth control of MDA-MB-435 cells (16).

In earlier studies, it was assumed that connexins would exert their tumor growth suppression effect through their primary role in establishing GJIC and the direct exchange of secondary messengers and other small molecules. However, the number of possible mechanism(s) responsible for connexin-mediated tumor suppression has grown with recent insights into the possible functional roles of hemichannels (18), the cross-talk between gap junctions and other adhesion-based junctional complexes (19–22) and the discovery of novel connexin-binding proteins (23–25). Thus, there are several putative mechanisms by which connexins may participate in regulating cell growth and differentiation that are GJIC-independent (26–29). Furthermore, connexins have been shown to regulate a variety of molecules involved in growth and proliferation (30), tumor angiogenesis (8) and cell cycle control (31).

Although many studies have examined the role of connexins in regulating cell growth within the primary tumor, far less is known about the role of connexins and gap junctions during the various stages of metastasis. Reports have suggested that connexin expression reduces the likelihood and instances of tumor cell metastasis (32–34). Weakly metastatic rat mammary clones were shown to have higher incidences of GJIC and more gap junction plaques than highly metastatic clones (35). In addition, quite interesting is the evidence that under the influence of metastasis suppressive proteins such as breast metastasis suppressor-1 (BRMS-1) (34) or tissue inhibitors of MMPs-1 (TIMP-1) (36), there was decreased metastasis as well as a restoration of connexin content and/or GJIC. Relevant to our study, the expression of BRMS-1 was shown to cause an increase in Cx43 expression...
in MDA-MB-435 cells (34). However, other reports have documented that gap junctions form between tumor cells and endothelium (32,37) and one report has suggested that the expression of Cx26 may facilitate melanoma cell metastasis in vivo (38). Thus, a relationship between connexin expression and metastatic potential is beginning to emerge but remains controversial owing to the complexity of the multiple stages and environments tumor cells must pass before colonizing in a distinct organ. In addition, it is also becoming more clear that different members of the connexin family as well as different tumor cell types may engage different mechanisms to either inhibit or assist the molecular processes needed for cells to metastasize.

In the current study, we used a variant of the human breast tumor cell line, MDA-MB-435, to examine if the expression of functional and GJIC-incompetent species of Cx26 could change cell growth characteristics, migration and invasion potential of the cells and regulate genes important in cancer progression. To this end, MDA-MB-435 cells were engineered to express (i) functional wild-type Cx26; (ii) a GJIC-incompetent variant of Cx26 that is transported to the cell surface; and (iii) a Cx26 mutant found in human populations with Vohwinkels syndrome, which resides within the Golgi apparatus and is therefore also non-functional in terms of GJIC.

## Materials and methods

### Cell culture

MDA-MB-435 and NRK (normal rat kidney) cells were cultured in Dulbecco’s modified Eagle’s high glucose medium obtained from Invitrogen (Burlington, ON) and MDA-MB-231 cells were cultured in RPMI 1640 medium from Sigma-Aldrich (St Louis, MO). All cell lines were originally obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained at 37°C in an environment of 95% air and 5% CO₂ in the recommended medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine.

### Constructs and retroviral infection

Generation of AP2 retroviral vectors containing cDNA for Cx43, Cx26, green fluorescent protein (GFP)-Cx26 or D66H-GFP were described previously (7,39,40). All four recombinant retroviral vectors, as well as an empty retroviral vector, were transferred into the 293GPG packaging cell line to produce replication-defective virus-containing supernatant, and the retroviral infection of MDA-MB-435 cells was accomplished as described previously (41). Five cell lines were created: MDA-MB-435 cells expressing a functional Cx26 (Cx26), a GJIC-incompetent GFP-tagged Cx26 (GFP-Cx26), a mutant Cx26 (D66H-GFP), a functional Cx43 (Cx43) and an empty viral vector (v) control. In each case, the expression of recombinant connexins exceeded 90% of the cultured cells as revealed by immunofluorescent labeling for connexins or intrinsic GFP fluorescence. The functional status of MDA-MB-435 cells that expressed wild-type Cx26 and GFP-tagged Cx26 was assessed in a previous study through the use of gap junction permeable dyes (8).

### Immunofluorescent labeling and confocal microscopy

Cells were immunolabeled as described previously by Laird et al. (42). Antibodies used in this study included a polyclonal anti-Cx43 (Sigma-Aldrich) (1:500), a polyclonal anti-Cx26 (Zymed Laboratories Inc., San Francisco, CA) (1:200), or a polyclonal anti-giantin (BabCo, Richmond, CA) (1:1000). Secondary antibodies conjugated to either Texas Red (1:200) or FITC (1:100) (Jackson ImmunoResearch Lab, West Grove, PA) were used to detect antibody binding. Phalloidin conjugated to Texas Red was used to label filamentous actin at a dilution of 1:50 (Molecular Probes, Eugene, OR). In many instances cells were labeled with Hoechst 33342 nuclear stain, a Chameleon multi-photon laser set at 730 nm was used for further incubations with anti-mouse, or anti-rabbit horseradish peroxidase (HRP) (1:10000) (Pierce Biotechnology, Rockford, IL) and chemiluminescence (Pierce Biotechnology) To assess equal protein loading, nitrocellulose membranes were stripped in Reblot (Chemicon International, Temecula, CA), or replica gels were probed for GAPDH (1:10000) (Cedarlane Laboratories, Hornby, ON, Canada). Immunoblots were scanned and quantified using Kodak Digital Science 2.0 (Eastman Kodak, Rochester, NY). Data are expressed as mean ± SD and parametric analysis was performed using an unpaired Student’s t-test.

### Trans-well migration and invasion assays

Cells were prepared in serum-free cell culture media to a final concentration of 1 x 10⁶ cells/ml. Culture media containing 10% fetal bovine serum (FBS), as chemo-attractant, was added to all the wells of the 24-well plate. A FluorBlok insert with a porous membrane (0.8 μm pore size) (BD Bioscience Discovery Labware, Canaan, CT) was transferred to each well containing the chemo-attractant, creating an upper and lower chamber. Cells (5 x 10⁴) were placed into the upper chamber and incubated for 24 h at 37°C. Invasion studies were completed as stated above over a 48 h period using FluorBlok membranes pre-coated with 62.5 μg/ml of Matrigel (Bioscience, Canaan, CT) diluted in cell culture media. Insert membranes were pre-coated and allowed to set for 1 h in a tissue culture incubator before the addition of the cells and media.

To assess the extent of cell migration or invasion, cells adhering to both the top and the bottom of the membrane were rinsed with Hank’s balanced salt solution (HBSS) (Invitrogen, Burlington, ON). Cells were fixed with HemaColor solution 1 fixative (EM Science Harleco, Kansas City, MO). Nuclei on both the top and bottom of each membrane were stained with 10 μg/ml Hoechst 33342. Random digital images (10 from the top and 10 from the bottom of the filter) were acquired using Leica microscope equipped with a digital CCD camera (Hamamatsu Photonics, Japan). Acquired images were density sliced and Open Lab software (Improvision, Lexington, MA) was programmed to calculate the total area occupied by stained nuclei per image. This area was divided by the average area of a single nucleus (~0.4 μm²), to estimate the number of cells in each image. The experiment was repeated four times, with three internal replicates. Data are expressed as mean ± SD and parametric analysis was performed using an unpaired Student’s t-test.

### SDS-PAGE and western blot

SDS-PAGE and western blotting was performed as described previously (42). The blots were labeled with anti-Cx43 (Sigma-Aldrich) (1:5000 dilution), rabbit polyclonal anti-Cx26 (Zymed Laboratories, Z-28 (1:200) or UM214 (1:1000), or anti-β1 integrin (Santa Cruz Biotechnology, Santa Cruz, CA) (1:200) antibodies. Primary antibody binding was detected by further incubations with anti-mouse, or anti-rabbit horseradish peroxidase (HRP) (1:10000) (Pierce Biotechnology, Rockford, IL) and chemiluminescence (Pierce Biotechnology).
Zymography and reverse zymography

Equal numbers of cells were grown to 80% confluence, and then incubated with 2 ml of serum free culture media. After 48 h, media were collected, centrifuged at 10,000 g for 10 min at 4°C, and the supernatant was assessed for protein concentration using the BCA protein assay. Protein (40–50 μg) was diluted in non-reducing sample buffer [0.5 M Tris (pH 6.8), 20% glycerol, 4% SDS and 0.005% bromophenol blue]. Zymography was carried out as described previously with the following changes (43,44). Equal concentrations of proteins were separated by 10% SDS–PAGE containing 1 mg/ml gelatin substrate (ICN Biomedical, Aurora, OH). Upon completion of electrophoresis, gels were washed twice in 2.5% Triton X-100 solution and rinsed in distilled, deionized H2O. The gels were incubated for up to 36 h at 37°C in a developing buffer [50 mM Tris (pH 7.4), 0.2 M NaCl, 5 mM CaCl2 and 0.02% Brij 35]. Gels were stained in Coomassie Brilliant Blue (Bio-Rad Laboratories, Hercules, CA) and zymographs were analyzed and quantified for densitometry using Kodak Digital Science 2.0 (Eastman Kodak, Rochester, NY). The experiment was repeated four times. Data are expressed as mean ± SD and parametric analysis was performed using an unpaired Student’s t-test. Reverse zymography was performed as described above with a 12% SDS–PAGE where the gel included 1 ml of purified MMP-9 and included a control lane of purified TIMP-1 (Dr Kevin Leco, UWO, London, ON, Canada).

Results

Expression of connexin variants in MDA-MB-435 cells

In order to assess the mechanistic role of connexins in the regulation of cell migration, growth and invasion, it was necessary to generate a series of connexin-positive cell lines on a genetically identical background. To that end, we initiated these studies by characterizing the growth and phenotypic properties of an MDA-MB-435 human breast tumor cell line. Surprisingly, these cells were found to be far less aggressive than reported in other studies (16) exhibiting minimal ability to form tumors in nude mice (data not shown) and they exhibited reduced invasive properties when grown on or within matrigel-coated filters as compared with other aggressive human breast tumor cell lines such as MDA-MB-231 (Figure 4B). In addition, although MDA-MB-435 cells have been reported to express both Cx43 and Cx32 (34) we found no evidence for Cx43 protein and only a small subpopulation of cells expressed any detectable Cx32 protein. Nevertheless, this less aggressive ‘variant’ of the MDA-MB-435 cell line was devoid of Cx26, GJIC-deficient and sufficiently migratory and invasive to assess the impact of the overexpression of functional and GJIC-incompetent connexin species. Importantly, these cells were efficient at trafficking overexpressed connexins to the cell surface for the formation of functional gap junctions, properties not exhibited by MDA-MB-231 cells (7).

Immunolabeling (Figure 1A) and western blots (Figure 1B) revealed that GJIC-incompetent MDA-MB-435 (WT) and wild-type cells infected with an empty viral vector (v) are devoid of detectable Cx26 (Figure 1A) and Cx43 (Figure 1B). Cells expressing wild-type Cx26 or Cx43 (Figure 1A, arrows) formed gap junction-like structures at the cell surface and appropriately sized protein bands were detected by western blot (Figure 1B). It is notable that multiple phosphorylation species of Cx43 were identified by western blot at 41–44 kDa but since Cx26 is not phosphorylated, only one protein band was detected as expected at ~21 kDa. When functionally inactive GFP-tagged Cx26 was expressed in MDA-MB-435 cells, apparent gap junction plaques were observed at the cell surface (Figure 1A, arrows) and immunoblotting revealed an appropriately sized fusion protein band at ~48 kDa (Figure 1B). Conjugation of the GFP tag to the N-terminal of Cx26 resulted in the formation of non-functional plaques in MDA-MB-435 cells (8). The deafness and skin disease-linked Cx26 mutant, D66H-GFP, was localized to the Golgi apparatus as revealed by the co-localization between D66H-GFP and resident Golgi protein, giantin (Figure 1A, insert, note yellow color). Since the Cx26 antibody used in this study did not bind human Cx26, the D66H-GFP mutant was not detected on the western blot (Figure 1B). Consequently, cell lysates were probed with a second anti-Cx26 antibody to reveal the presence of the GFP-tagged Cx26 and D66H mutant (Figure 1C). This study revealed that, although the D66H mutant was expressed in >90% of the cell population, it was far less abundant than GFP-Cx26 assuming the antibody had equal affinity for both rat and human Cx26. As controls, Cx43 was visualized within gap junction plaques in NRK cells (Figure 1A) but predominantly found in intracellular structures in MDA-MB-231 cells as described previously (Figure 1A) (8).

Expression of functional and GJIC-incompetent Cx26 decreases the tumorigenic characteristics of MDA-MB-435 cells

In vitro cell growth analysis revealed that cells expressing Cx26 or GFP-Cx26 experienced a lag in proliferation and by Day 9, a significant suppression of growth rate was observed as both cell lines had ~40% less cells than the vector control (Figure 2A). Surprisingly, neither Cx43 nor D66H-GFP expressing cells inhibited growth in vitro (Figure 2B), suggesting that the suppression of tumor cell growth was restricted to Cx26 when expressed at the cell surface and assembled into apparent gap junction plaques. Interestingly, upon the expression of Cx26, GFP-Cx26 and D66H-GFP, MDA-MB-435 cells had a 76, 68 and 38% reduced frequency of colony formation in soft agar, respectively, when compared with the vector control cells. The expression of Cx26, and GFP-Cx26, also had an effect on colony size, rarely forming colonies with ≥10 cells.

MDA-MB-435 cells expressing connexins exhibited altered morphology but only cells expressing Cx26 variants were less migratory and invasive

Phalloidin-staining of actin microfilaments revealed that wild-type and cells infected with an empty vector exhibited a flattened phenotype, with dispersed and periphery-localized stress fibers (Figure 3). However, upon the expression of Cx26, GFP-Cx26, D66H-GFP or Cx43, MDA-MB-435 cells exhibited a more compact shape, with many overlapping processes and filamentous actin staining was primarily perinuclear (Figure 3, arrows). Interestingly, Cx26, GFP-Cx26 or D66H-GFP overexpression significantly inhibited MDA-MB-435 migration compared with wild-type or empty vector control cells (P < 0.01) (Figure 4A). The percentage of migrating cells seen in all three Cx26-expressing cell lines was similar to that seen in both normal mammary epithelial cells, MCF10A, which revealed minimal migration after a 24 h period (data not shown), and non-transformed, non-motile normal rat kidney (NRK) cells. Notably, there was no significant reduction in Cx43 expressing MDA-MB-435 cell migration. To examine whether Cx26 expression reduced the ability of MDA-MB-435 cells to digest and pass through a thin layer of matrigel, invasion assays were performed in vitro. First, it is notable that MDA-MB-435 cells were about one-third as efficient in invading though the matrigel
coating as compared with MDA-MB-231 cells. Our results revealed that MDA-MB-435 cells expressing Cx26 or GFP-Cx26 were significantly less invasive than the controls ($P < 0.05$) (Figure 4B). Although D66H-GFP cells also trended toward a reduced capacity for invasion, this inhibition was not statistically significant (Figure 4B).

Fig. 1. Expression patterns of untagged and GFP-tagged connexins in mammalian breast tumor cell lines. MDA-MB-435 wild-type (WT) cells, cells infected with an empty retroviral vector (v) or MDA-MB-435 cells expressing Cx26 (Cx26) or Cx43 (Cx43) were immunolabeled and immunoblotted for Cx26 or Cx43 (A and B). Note that wild-type or empty vector control cells lacked Cx26 but cell surface gap junction plaques were observed in cells overexpressing Cx26 or Cx43 (A, arrows). Similarly, gap junction plaques were readily observed in MDA-MB-435 cells expressing GFP-tagged Cx26 (GFP-Cx26) (A, arrows) whereas a disease-linked Cx26 D66H mutant (D66H-GFP) was retained within a perinuclear compartment that co-localized with the resident Golgi protein, giantin (A, insert, yellow). Cx43 was retained within an intracellular compartment in MDA-MB-231 cells (A) but assembled into gap junctions at cell–cell interfaces in normal rat kidney (NRK) cells (A). All cells, except NRK, were counterstained with Hoechst 33342 to denote the nuclei. Bar = 10 μm. Cx26 and GFP-Cx26 were identified by western blots in appropriate cell types whereas GAPDH was used as a loading control (B). A second anti-Cx26 antibody that recognizes both human and rat Cx26 was used to probe cell lysates obtained from cells overexpressing GFP-Cx26 or D66H-GFP (C).

β1 Integrin expression, TIMP-1 and MMP-9 activity were regulated in connexin-expressing cells

Our previous Affymatrix array studies suggested that β1 integrin was downregulated in cells expressing Cx26 or GFP-Cx26 compared with control cells whereas TIMP-1 expression was increased (Qin et al., unpublished data).
Growth curves were expressed as mean ± SE. Cx26, GFP-Cx26 or D66H-GFP exhibited a reduction of cell growth and 2532

In accordance with this array data, western blots revealed that D66H-GFP (closed squares) or Cx43 (open squares) (circles) cells containing the empty vector (open circles) or cells expressing triangles) or GFP-Cx26 (open triangles) but not in wild-type cells (closed 2532

soft agar. Growth suppression was observed in cells expressing Cx26 (closed 2532

by which connexins act as tumor suppressors. In principle, 2532

The purpose of this study was to elucidate the mechanism(s) 2532

Connexins as tumor suppressors

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there are four basic mechanisms by which connexins may 2532

GJIC appears unnecessary for Cx26-based growth control

It has already been established that connexins exert an inhibitory effect on tumor growth when re-introduced in 2532

Discussion

Connexins as tumor suppressors

The purpose of this study was to elucidate the mechanism(s) 2532

MDA-MB-435 cell growth further suggesting that the mere delivery of ‘any’ connexin is not sufficient to induce growth control.

Fig. 2. Cx26-induced inhibition of MDA-MB-435 cell growth and growth in soft agar. Growth suppression was observed in cells expressing Cx26 (closed triangles) or GFP-Cx26 (open triangles) but not in wild-type cells (closed circles) cells containing the empty vector (open circles) or cells expressing D66H-GFP (closed squares) or Cx43 (open squares) (A). Cells expressing Cx26, GFP-Cx26 or D66H-GFP exhibited a reduction of cell growth and colony formation in soft agar compared with empty virus-infected cells (B). Growth curves were expressed as mean ± SE (n = 4) (**P < 0.05, 2532

***P < 0.001). Bar = 10 μm.

In accordance with this array data, western blots revealed that β1 integrin expression was ~3-fold lower in cells engineered to express functional, GJIC-incompetent or mutant Cx26 as well as Cx43 (Figure 5A and B).

Zymography and reverse zymography were carried out in order to determine the activity level of both the inhibitor TIMP-1, and the enzyme, MMP-9, in MDA-MB-435 cells expressing Cx26, GFP-Cx26 or D66H-GFP as compared with control cells. Gelatin zymography revealed a significant reduction in MMP-9 activity in cells expressing Cx26, GFP-Cx26, D66H-GFP or Cx43 (Figure 6A and C). Coordinate reverse zymography revealed an increase in TIMP-1 activity in cells expressing Cx26, GFP-Cx26, D66H-GFP or Cx43 as seen by increased substrate digestion by these cell lines (Figure 6B). These results suggest that both TIMP-1 and MMP-9 are regulated by all functional and GJIC-incompetent connexin variants used.

Discussion

Connexins as tumor suppressors

The purpose of this study was to elucidate the mechanism(s) by which connexins act as tumor suppressors. In principle,
Cx26 inhibits migration and invasion independent of its cell surface transport and assembly into functional gap junctions

In our study, we present evidence that the functional status or subcellular localization of Cx26 appears to be of minimal significance for Cx26 to inhibit MDA-MB-435 cell migration and invasion or to regulate genes important in these processes. In this regard, all overexpressed Cx26 variants exhibited the ability to significantly inhibit the migration of MDA-MB-435 cells. At this stage it is impossible to determine if they regulate migration by identical mechanisms and given that the D66H mutant has a steady-state profile that reflects its localization to the Golgi apparatus (40), it is possible that the bolus of mutant protein may act to indirectly affect the secretion of proteins important in migration. Given

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**Fig. 3.** Expression of connexin variants induced a perinuclear distribution of F-actin as revealed by phalloidin staining. Wild-type (WT) MDA-MB-435 cells, cells infected with an empty retroviral vector (v) or cells expressing Cx26, Cx43, GFP-Cx26 or D66H-GFP were stained with phalloidin conjugated to Texas Red. The perinuclear arrangement of F-actin revealed a spindle shaped cytoskeletal structure in cells expressing Cx26, GFP-Cx26, D66H-GFP or Cx43. WT and v cells had a flattened appearance with actin stress fibers in the cortical regions and in association with the cell surface. Bar = 10 µm.
the immense capability of cells to secrete, this seems unlikely but all protein overexpression strategies remain subject to such critique. It is also difficult to completely eliminate the possibility that Cx26 variant overexpression may upregulate other compensatory connexins family members in these MDA-MB-435 cells. However, we have shown previously that Lucifer yellow failed to transfer between MDA-MB-435 cells that overexpressed GFP-Cx26 (8). It is notable that the D66H mutant caused a non-statistically significant trend toward a reduction in cell invasion. At first approximation, this could suggest that it is a far less potent mutant (or even incompetent) at inhibiting cell invasion but this may also reflect the fact that it is expressed at lower levels than either Cx26 or GFP-Cx26. It is also important to note that we examined the migratory or invasive properties of cells that have disrupted their junctional contact with neighboring cells before movement through the pores of uncoated or matrix-coated filters. Given the well understood principle that tumor cell lines are typically heterogeneous, it is possible that cells that retain junctional contact with their neighbors, represent a second population that are more resistant to Cx26-induced changes in cell migratory and invasive properties.

In culture, MDA-MB-435 cells exhibited characteristics of integrin-induced, actin-dependent cytoskeletal remodeling (50,51) and cell spreading as evidenced by prominent stress fibers. Cells expressing functional, GJIC-incompetent and mutant Cx26 appeared more compact with actin staining being mainly perinuclear. In prostate cells, drug inhibition of Cx26-based GJIC was found to inhibit invasion and migratory properties of PC-3 cells, and furthermore, evidence was presented for a direct interaction between Cx26 and focal adhesion kinase (52). This study would argue for a link between Cx26 and focal contacts through a mechanism that may not require Cx26 to be functional in cell–cell

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**Fig. 4.** Overexpression of Cx26 variants decreased cell migration and invasion. Empty virus control cells (v) show no statistically significant difference in migration or invasion from WT cells, whereas cells expressing Cx26, GFP-Cx26 or D66H-GFP were less migratory compared with empty vector control cells ($P < 0.01$) (A). Cx26 and GFP-Cx26 expressing cells were less invasive compared with vector control cells ($P < 0.05$) (B). D66H-GFP also showed a considerable reduction in cell invasion although this change was not statistically significant ($n = 4$) ($^{*}P < 0.05$, $^{**}P < 0.005$).

**Fig. 5.** β1-Integrin was downregulated in MDA-MB-435 cells expressing wild-type connexins and GJIC-incompetent Cx26 variants. Western blots revealed that β1-integrin was downregulated in cells expressing Cx43, Cx26, GFP-Cx26 or D66H-GFP in comparison with wild type (WT) or empty viral vector controls (v) (A). Quantification of three independent experiments revealed a significant reduction in β1-integrin expression in cells engineered to express any of the wild-type or connexin variants. No statistical difference was seen between WT and v cells ($B$) ($n = 3$) ($^{**}P < 0.005$, $^{***}P < 0.001$).
communication. Moreover, other studies have shown that interactions with the extracellular matrix induce GJIC and promote mammary epithelial cell differentiation (53). Interestingly, a recent study demonstrated a potential role for β1-integrin in the development of breast cancer in conditional transgenic mouse model (54). In our study, western blot analysis revealed that the expression of functional, GJIC-incompetent and mutant Cx26, as well as Cx43, downregulated β1-integrin expression seen in MDA-MB-435 cells when compared with controls. Changes in MMP-9 activity levels in combination with the levels of TIMP-1, which bind and inactivate MMPs, would be expected to affect the ability of MDA-MB-435 tumor cells to invade surrounding extracellular matrix. In breast cancer cells there are increased levels of MMP-1, -2 and -9 in both the cancerous and surrounding non-cancerous cells (57). The activation and expression of MMP-9 has been shown to be related to a number of different molecules, that include β1-integrin (58) and TIMP-1 (56), both of which have been demonstrated in this study, to be regulated by Cx26 and Cx43 expression in MDA-MB-435 cells.

Only Cx26 variants regulated MDA-MB-435 tumorigenic cell properties yet common genes were regulated by both Cx26 and Cx43! The lack of a significant Cx43-linked inhibition of growth, migration and invasion in MDA-MB-435 revealed that not all connexins exhibit the same properties in vitro. The obvious conclusion form these studies is that β1-integrin, MMP-9 and TIMP-1 represent an incomplete set of genes regulated by Cx26 and other genes must be coordinately altered by Cx26 that are not regulated by Cx43. In many ways this is not unexpected given the multitude of differences between connexin family members related to binding proteins (24) and channel permeability characteristics (1). Previous studies using GJIC-deficient cervical HeLa cells showed that although GJIC was restored upon the overexpression of a variety of connexins including Cx40, Cx43 and Cx26, only Cx26 expressing cells demonstrated decreased growth rates in vitro and reduced tumor formation in vivo (59). It is also notable that some connexins are poor tumor suppressors in vitro but potent inhibitors of tumor cell growth in vivo (7).

How does GJIC-incompetent Cx26 signal to regulate gene expression levels?

Mechanistically, it would seem plausible that Cx26 and its GJIC-incompetent variants regulate cell proliferation, migration and invasion by putative binding partners. It has already been established that more than one member of the connexin family of proteins interacts with ZO-1, ZO-2, Src, tubulin and several protein kinases (24,25). However, only a ubiquitin ligase subunit, OCP1, found in the organ of Corti (60) and focal adhesion kinase (52) have been reported to interact with Cx26. Cx26 has a relatively short polypeptide domains exposed to the cytoplasm and lacks any nuclear localization sequences. Thus, it is most unlikely that any Cx26 proteolytic fragment or intact molecules extracted from the membrane would signal directly to the nucleus. Given the surge of new connexin-binding partners being identified it is likely that additional Cx26-associated signaling molecules will be identified in the near future that may shed some insight into how Cx26 is linked to tumor growth, progression and metastasis.

In summary, these data indicate that Cx26 plays a pivotal role in reducing the tumorigenicity of a non-aggressive variant of the MDA-MB-435 breast tumor cell line. Cx26-induced tumor suppression relies on a unique GJIC-independent mechanism, although it still remains possible that GJIC will augment the tumor suppressive effect or that GJIC will play a more central role in other cell types. Interestingly, the overexpression of Cx26 variants, whether intracellular localized or at the cell surface, reduced the migration and invasion of MDA-MB-435 cells. A unifying feature of the regulation evoked by these Cx26 variants was their consistent regulation of β1-integrin, MMP-9 and TIMP-1.

Fig. 6. Decreased MMP-9 activity and a corresponding increase in TIMP-1 activity were observed in cells expressing wild-type connexins and Cx26 variants. Gelatin zymography of cultured media collected from wild-type (WT) cells and cells expressing the empty vector (v), or cells expressing Cx26, GFP-Cx26, D66H-GFP or Cx43 was used to assess changes in MMP-9 activity (A). Although, proMMP-9 (92 kDa) and active MMP-9 (94 kDa) were detected in all cells, a decrease in MMP-9 activity was seen in cells expressing Cx26, GFP-Cx26, D66H-GFP or Cx43 (n = 4). Reverse gelatin zymography of the same cultured media was completed in order to determine if there was an increase in TIMP-1 activity in connexin-expressing cells. Increased TIMP-1 (migrating at 28 kDa) activity (note the reduction in band intensity within the red box area) was seen upon the expression of functional, GJIC-incompetent and mutant Cx26 as well as Cx43 (B) (n = 3) (**p < 0.05, ***p < 0.005).

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Cx26 regulation of tumorigenic properties
TIMP-1 potentially providing insight into how Cx26 may regulate both MDA-MB-435 cell migration and invasion. The observed molecular changes may represent only a few of a vast number of molecules that are regulated by the expression of Cx26 in breast tumor cells.

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