Down-regulation of Cx43 by Retroviral Delivery of Small Interfering RNA Promotes an Aggressive Breast Cancer Cell Phenotype

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Abstract
Connexins are gap junction proteins that assemble into channels that mediate direct intercellular communication. Connexins are well-documented tumor suppressors and are thought to regulate both cell growth and differentiation. As previously reported, most human breast tumors and cell lines down-regulate gap junctions or have defective gap junctional intercellular communication. Furthermore, over-expression of connexins in breast cancer cells inhibits tumor growth in vivo. In this study, we hypothesize that controlled Cx43 down-regulation would induce breast tumor cells to acquire a more aggressive phenotype. Here we report that Cx43 was down-regulated in both normal rat kidney (NRK) cells and human breast cancer cell lines (MDA-MB-231 and Hs578T) by transfection with chemically synthesized small interfering RNA (siRNA) or short hairpin RNA generated from a retroviral infection. Furthermore, we show that retroviral delivery and expression of siRNA directed to different coding regions of Cx43 resulted in differential levels of Cx43 silencing and impaired gap junctional intercellular communication. Cx43-silenced Hs578T cells grew faster and were more migratory. Finally, Western blot analysis revealed that down-regulation of Cx43 resulted in decreased expression of thrombospondin-1, an antiangiogenesis molecule, and increased expression of vascular endothelial growth factor. Taken together, these results suggest that Cx43 is required for maintaining cell differentiation and the regulation of molecules important in angiogenesis. (Cancer Res 2005; 65(7): 2705-11)

Introduction
Connexins are a 20-member family of transmembrane proteins that oligomerize early in the secretory pathway into hemichannels containing six connexin subunits (1). Upon reaching the cell surface, two hemichannels pair to complete an intercellular gap junction channel and these channels cluster into gap junction plaques (2). Gap junctional intercellular communication (GJIC) established by these channels allows for the passage of second messengers, ions, and other small molecules of <1,000 Da. Gap junctions play important roles in various physiologic functions such as regulation of cell proliferation, cell differentiation, tissue development, and cell apoptosis. Importantly, connexins have repeatedly been shown to be tumor suppressors and to play a crucial role in carcinogenesis. However, the mechanism by which connexins can act as a tumor suppressor is not well understood as both GJIC-dependent and GJIC-independent mechanisms have been proposed (3-9).

Connexin overexpression has commonly been used to examine the role of gap junction proteins in tumorigenesis. However, this approach can lead to erroneous conclusions resulting from gross overexpression levels that may far exceed any physiologic environment. To overcome this limitation, connexin gene ablation mouse models (10) and antisense technologies (11-14) are commonly used. Unfortunately, connexin ablation can lead to premature animal death as is the case for the Cx43 null mouse (15). Antisense approaches to reduce Cx43 expression have met with greater success as Cx43 was stably down-regulated in rat-1 fibroblasts (11) and used to examine the influence of Cx43 on foci formation of cocultured transformed cells. Likewise, a Cx43 antisense approach was used to transiently down-regulate Cx43 in skin as a means of examining its role in wound repair (12). Nevertheless, the efficacy of repressing Cx43 expression using antisense technology remains challenging. Recently, RNA interference has become available and has been proven to be a powerful tool for studying gene function (16). RNA interference approaches are thought to closely approximate disease states where gene products may persist at low levels. Short 21- to 23-nucleotide interfering RNAs (siRNA) have been successfully used to provide a strong and specific suppression of gene expression in mammalian cells. However, chemically synthesized siRNA is expensive, requires high transfection efficiency, and the gene silencing effects are transient in nature. To overcome these limitations, viral systems can be used to deliver siRNA via short hairpin constructs (shRNA), thus providing more efficient and stable gene silencing (17).

Cx43 is thought to be important in epithelial differentiation and breast carcinogenesis (3, 18, 19) and, furthermore, the level of GJIC may be critical in determining how cells respond to therapeutic drugs such as tamoxifen (20). In the human breast epithelium, only Cx43 and Cx26 have been unequivocally identified (7, 21–25), whereas rodents also express Cx32 (26). Interestingly, in human breast cancer, the expression of both Cx43 and Cx26 or their assembly into gap junctions are abnormal (7, 18, 22, 23, 25, 26). Importantly, not only has the overexpression of Cx43 or Cx26 been found to restore growth control in human breast tumor cells, but connexin-expressing tumor cells partially revert to a less malignant phenotype (7). Whereas connexin overexpression has desirable effects on re-differentiating tumor cells, it has not yet been established whether connexin silencing would be causal in promoting an aggressive tumor cell phenotype. In addition, the downstream mechanisms that are activated by connexin overexpression or silencing remain poorly defined. In the present study, we use a siRNA retroviral approach to stably silence Cx43 in human breast cancer cells. Our studies revealed that in Cx43-silenced Hs578T cells, cells grew faster, were more aggressive, and genes related to angiogenesis were regulated. These results suggest that Cx43 is acting as a tumor...
suppressor by mechanisms related to cell proliferation, migration and angiogenesis, supporting a causal relationship between physiologic changes in Cx43 levels and aggressive malignant breast tumor cell phenotypes.

Materials and Methods

Cell culture. Normal rat kidney (NRK) cells (American Type Culture Collection, Manassas, VA) were grown in DMEM (Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine. Human breast cancer cell lines (MDA-MB-231) and Hs578T cells derived from a carcinoma of the human breast (American Type Culture Collection) were grown in RPMI 1640 (Invitrogen) containing 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine. The cells were maintained at 37°C with 5% CO₂. A Cx43-overexpressing MDA-MB-231 cell line (MDA-MB-231/Cx43) was used as described previously by Qin et al. (27). HEK293 packaging cells for retroviral infection were cultured in DMEM (Invitrogen) supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine (3, 27).

Engineering of the Cx43 small interfering RNA constructs and retroviral short hairpin RNA vector. The coding regions of rat and human Cx43 genes were used for target gene sequence templates (Fig. 1). For simplicity, we will refer to all RNA interference reagents as siRNA. We selected four siRNA sequences from various regions of Cx43 for evaluation: (a) Cx43 siRNA-1 (intracellular loop) 5'-GAAGTTCAGTACCGG-GATT-3' , rat Cx43 from 398 to 416; (b) siRNA-2 (second extracellular loop to the third transmembrane domain) 5'-CCATCTTCATCATCTTCAT-3', rat Cx43 from 617 to 637; (c) siRNA-3 (first transmembrane domain) 5'-GGTGTTGCCTGTCAGTACTT-3', human Cx43 from 68 to 87; and (d) siRNA-4 (third transmembrane domain) 5'-TGCTGGCAGAACCTACATCAT-3', human Cx43 from 451 to 469. All sequences were selected by cross-checking and reaching consensus with three companies that offer siRNA design: GenScript Corporation (Scotch Plains, NJ), Qiagen (Mississauga, ON), and Ambion (Austin, TX). siRNA-1 and siRNA-2 oligonucleotides were chemically synthesized by Qiagen with a rhodamine modification on the 3'-terminal. As a control, the nonsense sequence AAATTCGGCCAGGTT-CAGCTC tagged with rhodamine was used (nonsilencing sequence). For retroviral vector constructs, small DNA inserts encoding a short hairpin targeting the Cx43 gene were synthesized and cloned into the retroviral vector pH1.1-QCXIH retroviral vector. The coding regions of rat and human Cx43 genes were used for target gene sequence templates (Fig. 1). For simplicity, we will refer to all RNA interference reagents as siRNA. We selected four siRNA sequences from various regions of Cx43.

Transfection and infection of small interfering RNAs. Chemically synthesized siRNAs were transfected according to the manufacturer's specifications using TransMessenger transfection reagent (Qiagen). Briefly, cells were subcultured into six-well plates containing glass coverslip inserts and incubated under their normal growth conditions. On the day of transfection, cells were washed with Opti-MEM (Invitrogen) medium twice, then Opti-MEM medium and 3.2 μg of siRNA complex mixed with TransMessenger transfection reagent were applied to the cells and incubated for 3 hours. Cells were then changed to regular cell culture media and after 48 hours, cells were either fixed for immunocytochemical study or the cell lysates were collected and prepared for Western blot analysis.

The recombinant siRNA (shRNA) retroviral vectors were transfected using LipofectAMINE 2000. Briefly, packaging cells were cultured in 60 mm culture dishes and 2 μg of retroviral vectors were mixed with LipofectAMINE 2000 reagent in Opti-MEM and incubated for 4 to 6 hours. Cells were then cultured in regular medium for 48 hours. Medium was collected for 7 to 10 days and filtered with a 0.45 μm filter and stored at −80°C until use. Filtered retroviral supernatant was applied to NRK, MDA-MB-231, or Hs578T cells for infection as described (3, 27). After three rounds of infection (cell culture media was replaced with retroviral supernatant every 24 hours), cells were further cultured into selection media containing 500 μg/mL hygromycin and antibiotic-resistant cells were passed a minimum of three times prior to further experimentation.

Immunocytochemistry and confocal microscopy. Wild-type, control, transfected or infected cells were cultured on glass coverslips and fixed in 80% methanol/20% acetone at 4°C for 20 minutes. Cells were immunolabeled with an anti-Cx43 (1:500) polyclonal antibody (Sigma, Oakville, ON) or anti-ZO-1 (Zonula occludin-1; 1:100) antibody (Hybridoma Developmental Bank, Iowa City, IA) as previously described (28). The images were captured on a Zeiss LSM 510 inverted confocal microscope (28).

Western blot analysis. Control and Cx43-silenced cells were rinsed briefly in PBS and harvested by scraping. Cells were pelleted by centrifugation (4,000 × g, 2 minutes) resuspended in lysis buffer containing 50 mmol/L Tris–Cl (pH 8.0), 150 mmol/L NaCl, 0.02% sodium azide, 100 μg/mL phenylmethylsulfonyl fluoride and 1% NP40. 50 mmol/L NaF, 2 mmol/L EDTA and a protease inhibitor cocktail from Roche (Mississauga, ON; ref. 27), and ruptured by sonication. Protein concentrations were determined using the...
bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL) and 50 μg per lane were separated by 10% SDS-PAGE except in the case of gels destined for anti-Cx43 immunoblotting where 10 μg were loaded per lane. In some cases, proteins were transferred to nitrocellulose membranes and subsequently immunoblotted with anti-Cx43 antibody (1:1,000; Sigma). Antibody binding was detected using the enhanced chemiluminescence system (Pierce). Fresh membranes, or after the membranes were stripped as described previously (27), were probed with a vimentin-specific monoclonal antibody (1:5,000; Zymed, Markham, ON), an anti–vascular endothelial growth factor (VEGF) monoclonal antibody (1:1,000; Sigma), a polyclonal anti-ZO-1 antibody (1:1,000; Zymed) or an anti-thrombospondin-1 (TSP-1) monoclonal antibody (1:100; NeoMarkers, Fremont, CA). The membranes were stripped and re-probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to ensure equal loading. To account for any minor variations in gel loading, the expression of Cx43, ZO-1, vimentin, VEGF, and TSP-1 were normalized to GAPDH. The relative intensity of signals was quantified using SigmaScan Pro software (Sigma) and expressed as a percentage of control.

**Cell growth and migration assays.** To examine cell growth in vitro, 1 × 10^5 cells/mL were plated in six-well tissue culture dishes and after 2, 4, 6, or 8 days, cells were collected and counted using a Coulter Z1 particle counter (Beckman-Coulter, Miami, FL).

To assess cell migration, 2,000 cells were plated on top of FluoroBlok transwell (BD Biosciences) filters. After 24 hours, cells were fixed in Harleco solution (27, 33–34) for 5 minutes, stained with 0.1% Hoechst 33342 (Molecular Probes, Eugene, OR) for 10 minutes, and the number of cells on the bottom and top of the filters from 10 different fields was counted using OpenLab software. The results were presented as the percentage of total cells that migrated to the bottom of the filter within the 24-hour period.

**Soft agar and dye coupling assays.** Cell growth in soft agar was assessed as previously described (8). Briefly, cells were seeded within 0.2% soft agar medium layered on top of 12-well dishes precoated with 0.3% soft agar medium. After 2 weeks at 37°C, the number of colonies that exceeded 10 cells was counted.

To assess GJIC in siRNA-treated cells, preloading dye coupling assays were done as described by Bani-Yaghoub et al. (29). Briefly, cells were cultured in 60 mm dishes containing 12 mm coverslips until confluent. Coverslips were transferred to a new dish and the medium was replaced with 1 mL of isotonic glucose solution (29) containing 0.1% calcein-AM and 0.1% DiI (Molecular Probes) for 15 minutes at room temperature. The cells were subsequently washed twice with isotonic glucose solution and trypsinized. Finally, 50 to 100 μL of preloaded cells resuspended in a total

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**Figure 2.** Chemically synthesized siRNA and retroviral vectors encoding siRNA (shRNA) suppress Cx43 expression in NRK cells. A, NRK cells were transfected with rhodamine-modified control siRNA, siRNA-1, or siRNA-2 and after 48 hours the cells were immunolabeled for Cx43. Red fluorescence, cells transfected with rhodamine-modified siRNAs; green fluorescence, Cx43 (arrows); asterisks, untransfected cells. Cx43 and GAPDH expression in control and siRNA-transfected cells were analyzed by Western blots (inserts). The fastest migrating band represents GAPDH, whereas the higher molecular bands represent Cx43 and its well documented phosphorylated species (48); B, NRK cells were infected with retroviral vectors encoding siRNA and immunolabeled for Cx43 or ZO-1. Note that siRNA-1- and siRNA-2-expressing cells had a dramatically reduced number of gap junctions; C and D, Cx43 and ZO-1 immunoblots from control cells and cells expressing siRNA-1 or siRNA-2 revealed a reduction in total Cx43, whereas multiple repeats revealed no statistically significant change in ZO-1 expression. The graph summarizes combined Western blot data from at least three separate experiments where the expression level of Cx43 was quantified and normalized to the house keeping protein GAPDH. Bar, 10 μm.
Results

Cx43-targeted small interfering RNA silenced Cx43 expression in normal rat kidney and breast tumor cells. NRK cells were transiently transfected with rhodamine-modified Cx43-targeted siRNAs and nonsilencing siRNA controls (Fig. 2A). After 48 hours, cells were either immunolabeled with anti-Cx43 antibodies (Fig. 2A) or cell lysates were analyzed by Western blotting for Cx43 expression (Fig. 2A, inserts). In control NRK cells, Cx43 gap junctions were readily identified in rhodamine-positive cells (Fig. 2A, arrows), and all untransfected cells (Fig. 2A, asterisks). However, when siRNA-1 or siRNA-2 were used to silence Cx43 expression, gap junction plaques at cell-cell interfaces were less prevalent and this reduction in Cx43-positive plaques coincided with a reduction in Cx43 expression as revealed by Western blots (Fig. 2A, inserts). Thus, these results suggested that chemically synthesized siRNA targeting Cx43 downregulated endogenous Cx43 expression and gap junction plaque formation in NRK cells. These same siRNA constructs reduced Cx43 gap junction number in the Cx43-positive breast tumor cell line, Hs578T (data not shown).

Whereas the introduction of siRNA by transfection was effective in identifying suitable Cx43 nucleotide sequences for silencing Cx43 expression, this silencing approach was transient in nature. To overcome this limitation, we engineered retroviral vectors encoding siRNA (shRNA) sequences known to silence Cx43 expression. Both siRNA-1 and siRNA-2 were effective in silencing Cx43 in NRK cells to ~40% of control (Fig. 2B-D). Silencing of Cx43 revealed a modest redistribution of the Cx43 binding protein, ZO-1, to intracellular locations (Fig. 2B) with no significant change in total ZO-1 expression (Fig. 2C). Since ZO-1 also binds to protein constituents of adherens and tight junctions (30, 31), Cx43 silencing was not expected to dramatically change its expression or distribution.

Since we chose Cx43 nucleotide sequences that were identical to rat and near identical to human (Fig. L4), we proposed that siRNA-1 and siRNA-2 would be effective in silencing both human and rat Cx43. Consequently, upon retroviral-based delivery of both siRNA-1 and siRNA-2 to MDA-MB-231 human breast cancer cells that coexpressed endogenous human Cx43 and exogenous rat Cx43, siRNA-1 expression was found to reduce total Cx43 levels by >70%, whereas siRNA-2 was somewhat less effective (Fig. 3A). Likewise, silencing of Cx43 in the metastatic human breast tumor cell line Hs578T was most notable with siRNA-1 (Fig. 3B). Together, these studies revealed that both endogenous and exogenously expressed rat and/or human Cx43 can be effectively and stably silenced by retrovirally introduced Cx43-targeted siRNAs.

Stable silencing of Cx43 in Hs578T cells promotes tumor cell growth and migration. To rigorously examine if other domains of Cx43 might be effectively targeted to silence Cx43 expression, we expanded our study to include siRNA-3 and siRNA-4 (Fig. 1A and C). Importantly, siRNA-1 shared 100% identity with rat and mouse sequences with only one nucleotide difference from human Cx43. On the other hand, siRNA-2, -3, and -4 were all directed to human Cx43 with siRNA-2 possessing sequence identity with rat Cx43 (Fig. 1A). Western blots of stable Hs578T cells lines established through hygromycin selection revealed that siRNA-1 and siRNA-3 reduced Cx43 expression by ~70% (Fig. 5). Consistently, when we used dye transfer studies to assess GJIC, reduced GJIC was most apparent in cell lines expressing siRNA-1 and -3 (Table 1).

To examine the consequences of Cx43 silencing on Hs578T cell growth, we evaluated growth curves for cells lines exhibiting differential silencing of Cx43 (Fig. 4A). Cell lines exhibiting the greatest reduction in Cx43 expression and GJIC (siRNA-1 and siRNA-3) grew statistically faster than controls. Likewise, these same two cell lines exhibited increased migration potential when cultured on FluoroBlok transwell inserts (Fig. 4B).

To assess anchorage-independent growth of Cx43-silenced Hs578T cells, cells were grown in soft agar and colony formation was evaluated. Again siRNA-1- and siRNA-3-expressing cells that exhibited the greatest reduction in Cx43 and GJIC were found to more readily form colonies in soft agar (Table 2).

Figure 3. siRNA-1 and -2 down-regulate both endogenous and exogenous Cx43 expression in human breast tumor cells. Western blotting was used to analyze Cx43 protein expression levels in control cells (lane a) or after expression of siRNA-1 (lane b) or siRNA-2 (lane c) in (A) MDA-MB-231 cells, which express both endogenous and exogenous Cx43, and (B) Hs578T cells, which endogenously express Cx43. Graphs represent the quantification of Cx43 in three independent Western blot experiments normalized to GAPDH. The vast majority of Cx43 expressed in these control MDA-MD-231 cells had previously been shown to be due to exogenous Cx43 expression (3, 27).
Thrombospondin-1 and vascular endothelial growth factor were differentially regulated in Cx43-silenced Hs578T cells. In order to determine the mechanism(s) by which Cx43 regulates tumor growth and progression, we examined potential Cx43-regulated molecules. Our previous study using overexpression models revealed that one connexin-regulated mechanism involved reducing cell growth and migration as well as anchorage-independent growth in soft agar. Importantly, Cx43 regulates the expression of key genes linked to angiogenesis and epithelial-mesenchymal transition.

This is the first report where RNA interference technology is used to silence the expression of either endogenous or exogenous Cx43 expression in mammalian cells. Both siRNA-1 and siRNA-2 duplexes were near equally effective in reducing Cx43 expression suggesting that chemical syntheses of siRNA is a quick and relatively efficient method to down-regulate Cx43, similar to antisense technology (11). However, chemically synthesized siRNAs are relatively unstable, transient, and are dependent on high transfection efficiencies. To overcome these limitations, shRNA constructs targeting Cx43 were engineered in the pHL1-QCXIH retroviral vector (34). NRK cells were used to establish proof-of-principle and two retroviral constructs were shown to significantly reduce the endogenous expression of rat Cx43. As expected, the localization of the Cx43-binding protein, ZO-1, was modestly affected and the retention of ZO-1 at the cell surface may reflect localization of the Cx43-binding protein, ZO-1, was modestly affected and the retention of ZO-1 at the cell surface may reflect

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of cells examined</th>
<th>No. of cells expressing dye transfer</th>
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<tbody>
<tr>
<td>Control</td>
<td>206</td>
<td>71</td>
</tr>
<tr>
<td>siRNA-1</td>
<td>143</td>
<td>21</td>
</tr>
<tr>
<td>siRNA-2</td>
<td>158</td>
<td>32</td>
</tr>
<tr>
<td>siRNA-3</td>
<td>151</td>
<td>27</td>
</tr>
<tr>
<td>siRNA-4</td>
<td>160</td>
<td>32</td>
</tr>
</tbody>
</table>

NOTE: Cx43 siRNA (shRNA) partially inhibits GJIC in Hs578T cells. GJIC was assessed by dye coupling assays (mean number of cells exhibiting dye transfer evaluated over six experiments containing three replica each) as described in Materials and Methods.

Table 1. Cx43 siRNA partially inhibits GJIC in Hs578T cells as assessed by preloading dye transfer assays

Discussion

In this study, we use Cx43 silencing to examine the mechanism by which Cx43 acts as a tumor suppressor in human breast tumor cells. Our studies conclusively show that Cx43 is a tumor suppressor in Hs578T cells and functions effectively in reducing cell growth and migration as well as anchorage-independent growth in soft agar. Importantly, Cx43 regulates the expression of key genes linked to angiogenesis and epithelial-mesenchymal transition.

Since our retroviral vectors were constructed with sequences targeting the coding region of Cx43, we found that both

Table 2. Cx43 suppresses Hs578T cell colony formation in soft agar

<table>
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<tr>
<th>Treatment</th>
<th>No. of colonies formed, mean ± SD (n = 7)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.4 ± 2.1</td>
</tr>
<tr>
<td>siRNA-1</td>
<td>24.4 ± 6.7*</td>
</tr>
<tr>
<td>siRNA-2</td>
<td>22.7 ± 4.9</td>
</tr>
<tr>
<td>siRNA-3</td>
<td>24.9 ± 4.1*</td>
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<tr>
<td>siRNA-4</td>
<td>25.7 ± 7.6</td>
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NOTE: Hs578T colony formation in soft agar is enhanced in Cx43 siRNA (shRNA)-expressing cells. Quantification represents the mean values from seven independent experiments.

*P < 0.01.

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endogenous and exogenously expressed Cx43 could be silenced in human breast tumor cells regardless of whether they were driven by the native Cx43 promoter or the constitutively active cyclo-
megalovirus promoter. In order to ensure specificity of biological
readouts with regards to Cx43 silencing, we engineered four unique
Cx43 sequences and found that two of these were significantly
more effective. Interestingly, siRNA-3 was one of the more effective
sequences for silencing Cx43 expression and it targets within the
first 100 nucleotides of the Cx43 coding region. This challenges the
working model that the gene targeting sequence should avoid
the first 50 to 100 nucleotides located downstream of the start
codon where binding sequences for regulatory proteins may affect
the accessibility of RNA target sequence to the RISC complex. The
fact that siRNA-1 had one nucleotide different than the human
sequence (100% identity to rat) also suggests that the selection of
the specific target region may be more, or equally as important as
using 100% complementary target sequences.

Since the early 1990s, several studies have reported that different
members of the 20-member connexin family are tumor suppressors
(9, 35–38). In most cases, this conclusion was established by
examining the GJIC status of tumor cells and the growth and
differentiation characteristics of tumor cells in culture and animal
models upon the overexpression of connexin genes (9, 36, 39). The
limitations of this approach are reflected by the nonphysiologic levels
of connexin expression that are evaluated and assessed. Arguably, a
more physiologic assignment of connexins as tumor suppressors was
uncovered from studies using mice that lacked Cx32. These mice
were found to be more sensitive to chemical-induced liver (40) and
lung tumors (41). Since gene ablation of Cx43 is fatal in newborn
mice (15), a similar evaluation of tumor onset cannot be done.

Our studies and other studies have clearly shown that Cx43 is a
major connexin in the rodent mammary gland and in human breast
(18, 21, 23, 42). Cx43 was shown to be down-regulated and/or gap
junctions were poorly formed in human breast tumors in situ (18)
and in a variety of human breast tumor cell lines (18). Thus, the
present study addresses the protective role of Cx43 in regulating cell
growth, migration, and angiogenesis-linked molecules in human
breast tumor cells, Hs578T, that retain a modest level of Cx43. Since
most tumor cells are thought to contain minimal levels of GJIC (42)
we chose to use cells that retained some level of Cx43 expression for
our studies. We found a tight correlation between cell growth and
migration that was dependent upon the degree of Cx43 silencing.
Likewise, as a measure of epithelial-mesenchymal transition (33),
vimentin was up-regulated in the cells that exhibited the greatest
degree of Cx43 silencing.

Our earlier results revealed that Cx43 suppressed breast tumor
growth in vivo (3) raising possibilities that Cx43 may be regulating
factors that are important in the vascularization of the developing
tumor (3, 32). Previously, Huang et al. (43) identified that the
tumor-promoting cytokine, monocyte chemotactic protein-1, was
down-regulated in Cx43 overexpressing cells and this cytokine
regulated glioblastoma cell growth. Here we found that the
angiogenesis factor, TSP-1, was significantly down-regulated in
Cx43-silenced cells and correspondingly, VEGF was up-
regulated. Consistently, the regulation of both of these key
angiogenic factors was tightly correlated with the degree of Cx43
silencing in the various Hs578T cell lines. Previously, we showed
that TSP-1 was up-regulated in MDA-MB-435 as a consequence of
Cx26 overexpression (32), suggesting that several connexin family
members regulate TSP-1 expression. Often human breast tumors

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**Figure 5.** Stable silencing of Cx43 regulates angiogenesis-related molecules in Hs578T cells. Western blot analysis of control Hs578T cells (lane a) and cells expressing siRNA-1 (lane b), siRNA-2 (lane c), siRNA-3 (lane d), and siRNA-4 (lane e). Immunoblots revealed that Cx43 was differentially down-regulated by siRNAs targeting different Cx43 sequences. Vimentin, a mesenchymal marker, was significantly up-regulated in cells with reduced Cx43 expression. TSP-1, an antiangiogenesis factor, was significantly down-regulated in cells with reduced Cx43 expression, whereas VEGF, a proangiogenesis factor, was up-regulated. The control cells used for comparison depict cells infected with the empty viral vector. All graphs were normalized to GAPDH.
overexpress the oncogene ErbB2 which has been shown to decrease TSP-1 expression levels further supporting the key role of TSP-1 in tumor progression (44). It has been well-established that VEGF can regulate the expression of Cx43 and block GJIC (45, 46) but the reverse cross-talk between Cx43 and VEGF has not previously been documented. The fact that VEGF is up-regulated by >50% in Cx43-silenced cells strongly suggests that Cx43 dramatically regulates VEGF. Interestingly, members of the ErbB family are potent enhancers of VEGF expression (47), further suggesting that VEGF may act in combination with other angiogenic molecules to promote tumor growth and progression by vascularization of the primary tumor. It is likely that the number of molecules that are regulated by Cx43 will be extensive lending to potential cross-talk and converging pathways that collectively suppress tumor growth and inhibit tumor cell progression into aggressive and invasive phenotypes.

In summary, these studies are the first to report the effective use of siRNA to silence Cx43 expression. Using this novel approach, we were able to rigorously address the role of Cx43 as a tumor suppressor in a physiologic-like situation where Cx43 reduction is tightly regulated and correlated with cell growth, cell motility, epithelial-mesenchymal transition, and expression of key genes important in angiogenesis. Collectively, these data indicate that Cx43 expression levels strongly influence cell differentiation and provide protective properties to tumor cells acquiring aggressive phenotypes.

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