# *Connexin43* pseudogene in breast cancer cells offers a novel therapeutic target

**Andrew Bier,[1] Irene Oviedo-Landaverde,[1] Jing Zhao,[1] Yael Mamane,[2] Mustapha Kandouz,[1] and Gerald Batist[1]**

[1]Departments of Oncology and Experimental Medicine, Segal Cancer Centre and Lady Davis Institute for Medical Research, Jewish General Hospital, and [2]Department of Biochemistry and McGill Cancer Centre, McGill University, Montreal, Quebec, Canada

## Abstract

***Connexin43* (*Cx43*) is often deregulated in breast cancer tissue compared with normal adjacent tissue. Stable reexpression of *Cx43* in cancer slows growth and renders the cells more sensitive to cytotoxic chemotherapeutics. Pseudogenes are often considered nonfunctional copies of DNA. The *Cx43* pseudogene (*ΨCx43*) possesses all the features of an expressed gene and is exclusively transcribed in breast cancer cell lines and not in normal cells. *ΨCx43* can be translated *in vivo*, and its protein exhibits growth-suppressive behavior similar to *Cx43*. We showed that *ΨCx43* binds to the polyribosomes in breast cancer cells and that exogenous expression of *ΨCx43* induces translational inhibition of *Cx43*. Furthermore, *ΨCx43* is translated and binds more efficiently to the translational machinery than does *Cx43* in an *in vitro* system. Following knockdown of *ΨCx43* in breast cancer cells, we observed an increase in *Cx43* RNA and protein. This results in increased cellular sensitivity to cytotoxic chemotherapy. Our results show that *ΨCx43* acts as a posttranscriptional regulator of *Cx43* in breast cancer cells, and that this represents an example of the regulation of genes by pseudogenes with potential therapeutic implications in cancer. [Mol Cancer Ther 2009;8(4):786–93]**

## Introduction

Pseudogenes are often considered nonfunctional copies of genes (1, 2). They arise either as processed pseudogenes resulting from reverse transcription of mRNA transcripts or as nonprocessed pseudogenes from gene duplication (3). Processed pseudogenes are intronless and often accumulate genetic alterations such as deletions, frameshift mutations, and insertions compared with their genes of origin. These changes prevent them from producing an mRNA transcript and a functional protein. However, there are many exceptions of pseudogenes that are capable of being transcribed and translated (4–8). Pseudogenes were originally thought of as a cluster of genes capable of being used during critical events such as mutation conditions and/or microenvironmental stress (9, 10). However, increasing evidence has suggested a greater involvement of pseudogenes with the onset of tumorigenesis (11–15).

Gap junctions are composed of two hemichannels (or connexons) located in the membranes of opposing cells and directly mediate the transport of small molecules between cells. Each connexon consists of a hexamer of proteins called connexins, which are important in processes such as development, cell growth, and metabolism(16). To date, there are more than 20 different types of connexins that have been identified. Mutated connexins have been implicated in various human diseases (17–19), including an evidently increasing role in cancer development (16, 20–22). *Connexin43* (*Cx43*) is the most widely studied connexin due to its abundant expression. It has been established that *Cx43* plays an integral role in tumorigenesis, and we have shown a loss of *Cx43* protein in breast cancer compared with normal adjacent tissue (23). Furthermore, an increase in sensitivity to common chemotherapeutic agents has been shown following the reexpression of *Cx43* in human cancers (24, 25), which suggests that understanding the regulation of *Cx43* is of importance for future cancer treatment.

When *ΨCx43* was first discovered, it was considered to be nonfunctional like most other pseudogenes (26, 27). Although similar in sequence, *ΨCx43* was shown to contain several base substitutions and an amino acid deletion, but it still maintained a fully intact open reading frame. We showed that *ΨCx43* mRNA transcripts are easily detected in breast cancer cell lines, particularly in those that express minimal *Cx43* transcript and very little *Cx43* protein (Table 1). *ΨCx43* also has the capability of being translated *in vitro* when fused to green fluorescent protein. We also showed that *ΨCx43* inhibited cell growth, similarly to *Cx43* but in a gap junction–independent manner (28).

In this article, we further characterize the role of *ΨCx43* in relation to *Cx43*. In using breast cancer cell lines with varying degrees of *Cx43* and *ΨCx43* expression (Table 1), we report that the *ΨCx43* transcript is preferentially transcribed in a cell-free protein synthesis system and is associated with polyribosomes in a variety of cell types. Using short interfering RNA (siRNA) directed against *ΨCx43*, we showed increased *Cx43* levels in different cell types, which increased

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**Requests for reprints:** Gerald Batist, Segal Cancer Centre, Sir Mortimer B. Davis Jewish General Hospital, 3755 Cote-Ste-Catherine Road, Montreal, Quebec, Canada H3T 1E2. Phone: 514-340-8222, ext. 5418; Fax: 514-340-8708. E-mail: gerald.batist@mcgill.ca



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**Table 1. Expression of *Cx43* and *ΨCx43* in breast cancer cell lines**

| Cell lines | *ΨCx43* | *Cx43* RNA | *Cx43* protein |
|---|---|---|---|
| Hs578T | – | ++++ | ++++ |
| MDA 231 | + | ++++ | +++ |
| MCF7 | ++ | + | – |
| MDA 435 | +++ | + | – |

cellular sensitivity to the chemotherapy drugs paclitaxel and doxorubicin. Together the data show that *ΨCx43* can antagonize *Cx43* protein expression, and inhibition of its transcript can be a potential therapeutic strategy in breast cancer chemotherapy.

## Materials and Methods

### Cell Cultures and Stable Clones

The MCF7 and MDA 231 cell lines (American Type Culture Collection) were maintained in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum and 100 μg/mL of penicillin/streptomycin. The Hs578T and MDA 435 cell lines (American Type Culture Collection) were maintained in DMEM supplemented with 10% fetal bovine serum, 100 μg/mL of penicillin/streptomycin, and 10 μg/mL of insulin. All cell lines were grown at 37°C in a humidified atmosphere containing 95% air and 5% carbon dioxide.

### Generation of Hs578T and MDA 231 Stable Cell Lines

Hs578T and MDA 231 cells were transfected with both pcDNA3.1 and pCX43P (28) using Lipofectamine transfection reagent (Invitrogen). Clones were selected using G418 resistance and were characterized by reverse transcription-PCR (RT-PCR).

### RT-PCR

RT-PCR was done using a one-step RT-PCR kit (QIAGEN) according to the manufacturer's instructions. Briefly, total RNA was isolated from cells using Trizol (Invitrogen) and *Cx43*-specific primers (forward, 5′-ATGAGCAGTCTGCCTTTCGT-3′; reverse, 5′-AAGGGTCGCTCTTTCCCTTA-3′; Invitrogen); 10 ng of total RNA were synthesized into cDNA and amplified by PCR as mentioned previously (28). The total cycle number for the reaction was increased to 35 cycles. The reaction yielded a product ~500 nucleotides long, which corresponded to similar regions from *Cx43* and *ΨCx43*. Following the reaction, the products were digested with *Nco*I and separated on a 1.2% agarose gel to separate *Cx43* from *ΨCx43*.

### Polysome Profile Analysis of *Cx43* mRNA

Cells were treated with 100 μg/mL cycloheximide for 15 min at 37°C before being harvested. Cells were then lysed on ice for 10 min in lysis buffer [150 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.4), 0.5% NP40, 10 mmol/L $MgCl_2$, 100 μg/mL cycloheximide, 2 mmol/L DTT, 100 /mL RNA Guard] followed by a 10-min spin at maximum speed at 4°C. Equal volumes of cytoplasmic extracts (800–900 μL) were then overlaid onto a linear sucrose gradient [10–45% (w/w) 50 mol/L Tris-HCl (pH 7.4), 50 mmol/L KCl, 10 mmol/L $MgCl_2$, 3 mmol/L DTT, RNA Guard] and centrifuged at 36,000 rpm for 2 h at 4°C in SW40 rotor. Using an ISCO UA-6, fractions were collected with continuous UV monitoring at 254 nm. RNA was extracted using Trizol (Invitrogen) from each fraction and analyzed by RT-PCR with the primers mentioned above.

### Cloning of Cx43V5, ΨCx43Myc, and ΨCx43-AS

To fuse *Cx43* and *ΨCx43* in frame to the $NH_2$ terminus of V5 and Myc, respectively, in pcDNA3.1/V5-His and pcDNA3.1/Myc-His (Invitrogen), we amplified *Cx43* and *ΨCx43* from *Cx43* and *ΨCx43* cDNAs (28) by PCR with the following primers: forward, 5′-CTTTTAAGCACTCGAGTGGTGCCC-3′; reverse, 5′-AGCCTGTCTAGAAGATCTCCAGGTC-3′. In these primers, *Xho*I and *Xba*I sites were created (restriction sites underlined) and *Cx43* and *ΨCx43* stop codons were destroyed. PCR products were digested with the indicated restriction enzymes and ligated into their respective vectors. To create ΨCx43-AS, *ΨCx43* was cloned into pcDNA3.1/zeo(–) vector (Invitrogen) from Cx43P (28) using *Bam*HI and *Xho*I restriction enzymes. All cDNA clones were verified by sequencing (BioS&T).

### *In vitro* Competition Assay

*Cx43* cDNA and *ΨCx43* cDNA were cloned into pcDNA 3.1 V5 and pcDNA 3.1 Myc, respectively. Each construct was *in vitro* transcribed using RiboMAX Large Scale RNA Production System (Promega) reaction to produce cRNA (RNA) and were capped according to the manufacturer's protocol (Promega). Varying quantities and combinations of both cRNAs were translated *in vitro* using the Rabbit Reticulocyte Lysate system (Promega). Proteins were then resolved by SDS-PAGE and blotted with Myc and V5 antibodies. To determine transcript affinity to the translational machinery, equal amounts of Cx43V5 and ΨCx43Myc cRNA (0.2 μg of each) were simultaneously translated while varying the concentration of KCl in the reaction. Protein amount was visualized as mentioned previously. All blots were quantified using Scion Image 3.0.

### Western Blot

Cells were lysed in Tris-Cl buffer (pH 8.0) containing 0.5 mmol/L phenylmethylsulfonyl fluoride, 60 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L DTT, 0.5% NP40, 0.01 mg/mL leupeptin, and 0.01 mg/mL aprotinin as previously indicated (25). Equal amounts of protein were subjected to SDS-PAGE. A rabbit polyclonal antibody to *Cx43* (Sigma) was used at 1:2,000 dilution. V5 (Invitrogen) and Myc (Sigma) antibodies were used at 1:1,000 dilutions. Signals were visualized via ECL Western Blotting Detection agent (Amersham).

### siRNA Preparation and Transfection

Duplexes for siRNA expression contained 19-nucleotide sequences specifically targeting *Cx43* (GGATCGGGTTAAGGGAAAG) and *ΨCx43* (GGATTCCCTTAAGGGAAAG), in both sense and antisense orientations,

separated by a 9-nucleotide (TTCAAGAGA) spacer sequence. The 5′ and 3′ ends correspond to the *Bgl*II and *Hin*dIII sites, respectively. Forward and reverse oligonucleotides were annealed to produce double-stranded RNA, digested with *Bgl*II and *Hin*dIII, and cloned into pSUPER.retro.puro (OligoEngine); correct insertion and insert sequence were verified by sequencing (BIOS&T). Stable transfection of MCF7 and MDA 231 cells with viral supernatant from Phoenix packaging cells containing *Cx43* and *ΨCx43* siRNAs was done according to the manufacturer's instructions (OligoEngine). Clones were selected with puromycin (1 μg/mL) and assessed for knockdown by RT-PCR. The 25-nucleotide modified synthetic RNAs (stealth RNAi) were custom synthesized (Invitrogen). Primer sequences were as follows: *Cx43* siRNA sense, 5′-GGAUCGGGUUAAGGGAAAGAGCGAC-3′; antisense, 5′-GUCGCUCUUUCCCUUAACCCGAUCC-3′; *ΨCx43* siRNA sense, 5′-GGAUUCCCUUAAGGGAAAGAGCGAC-3′; antisense, 5′-GUCGCUCUUUCCCUUAAGGGAAUCC-3′. Transient transfection of synthetic siRNA was achieved using Lipofectamine 2000 transfection reagent (Invitrogen).

### Cell Viability

MDA 231 PSR and *ΨCx43* siRNA cells were plated at a density of $2 \times 10^5$/mL in a 96-well plate. Twenty-four hours later, paclitaxel (Biolyse Pharma Corporation) and doxorubicin (Mayne Pharma, Inc.) were added to the cells in increasing doses for 48 h. Alamar Blue assay was used to assess cell viability following paclitaxel treatment. Briefly, medium was aspirated from cells at the end of treatments and replaced with 10% (v/v) Alamar Blue (BioSource) in PBS. Plates were returned to the incubator for 2 h at 37°C before measuring the absorbance at 544 nm (lower wavelength) and 590 nm (upper wavelength) using a spectrophotometric plate reader (FLUOstar OPTIMA, BMG LABTECH). Results are presented as mean ± SE of experiments done in quadruplicates. *P* values were calculated using *t* test. Differences were considered significant (*) if $P < 0.05$.

## Results

### *ΨCx43* Cosediments with Polysomes

Given our previous observation of inverse levels of *ΨCx43* and *Cx43* mRNA (28), we investigated the distribution of *ΨCx43* RNA in the cytoplasm of Hs578T and MDA 435 cells by attempting to identify *ΨCx43* RNA bound to polyribosomes. Figure 1 shows the differential digestion of RT-PCR products by *Nco*I, which illustrates the polyribosomal distribution of *Cx43* and *ΨCx43* RNAs. In Hs578T cells, which endogenously express *Cx43* RNA and protein, *Cx43* RNA is distributed primarily in the polysome fractions with minimal amounts detected in the monosome fractions. In MDA 435 cells, which endogenously express both *Cx43* and *ΨCx43* RNAs but lack *Cx43* protein, both *Cx43* and *ΨCx43* RNAs are distributed among the polysome profile. *ΨCx43* RNA seems to be mostly present within the polysome fractions, whereas *Cx43* RNA is expressed in greater amounts in the monosomes compared with the polysomes. Endogenous *ΨCx43* RNA does associate with the polyribosomes, and in cells with high levels of *Cx43* protein (Hs578T), *Cx43* RNA is primarily linked with the polyribosome fractions. In cells lacking *Cx43* protein (MDA 435), *Cx43* RNA is primarily associated with the monosomes.

### Evidence for Competitive Translation of *Cx43* and *ΨCx43* mRNAs

*Cx43* and *ΨCx43* mRNAs were synthesized and capped *in vitro*, and then competitively translated over a range of relative ratios using the Rabbit Reticulocyte Lysate system. Equal amounts of *Cx43* and *ΨCx43* proteins are produced even when the *Cx43*/*ΨCx43* mRNA ratio is ~2:1 (Fig. 2A). The stability of *Cx43* versus *ΨCx43* mRNA binding to eukaryotic translation initiation factor 2 (eIF2), analyzed by exposure to increasing KCl concentrations, is shown in Fig. 2B. Equal amounts of both RNA transcripts were translated in the same reaction, and protein production in response to increasing salt concentrations was determined. Binding of *Cx43* mRNA to eIF2 decreased sharply above 50 mmol/L KCl and was inhibited by 50% at ~60 mmol/L KCl. On the other hand, *ΨCx43* mRNA bound more tightly to eIF2 as is evident from its resistance to increasing salt concentrations.

### Exogenous *ΨCx43* Shifts the Polysomal Distribution of *Cx43* mRNA

To determine the direct affect of expressing exogenous *ΨCx43* RNA on the polysomal distribution of *Cx43* mRNA, we generated stable *ΨCx43* cell lines from two cell types that highly express *Cx43* protein, Hs578T and MDA 231. We then subjected the cell lysate of these cells to sucrose density gradient fractionations and observed whether the presence or absence of *ΨCx43* transcripts correlated with the sedimentation properties of *Cx43*. *Cx43* and *ΨCx43* mRNAs were extracted from every second sucrose gradient fraction for Hs578T cells and from pooled fractions for MDA 231 cells, reverse transcribed, and differentially digested. In both wild-type and *ΨCx43*-overexpressing cells, Hs578T neo versus Hs578T *ΨCx43* (Fig. 3A) and MDA 231 neo versus MDA 231 *ΨCx43* (Fig. 3B), *Cx43* transcripts are associated with polysome fractions. However, in both Hs578T *ΨCx43* and MDA 231 *ΨCx43* cells, the *Cx43* transcript is shifted toward the monosome fractions. To further evaluate these shifts, we semiquantitatively analyzed the gel band intensity using Scion Image and plotted the results using GraphPad software. In Hs578T *ΨCx43* cells, there is an increase in *Cx43* transcript levels in the monosomes (fractions 2 and 4) and a decline in the early polysomes compared with Hs578T neo (Fig. 3A). A similar trend is observed in MDA 231 *ΨCx43* cells (Fig. 3B), with a decrease in *Cx43* transcript levels in the early polyribosomes (fractions 7 and 8) and an increase in the monosomes (fraction 5). Although these shifts seem to be relatively small quantitatively, they are consistently observed in various experimental settings. *ΨCx43* results in a shift of *Cx43* mRNA toward the monosomes thereby impeding translation of the *Cx43* transcript.

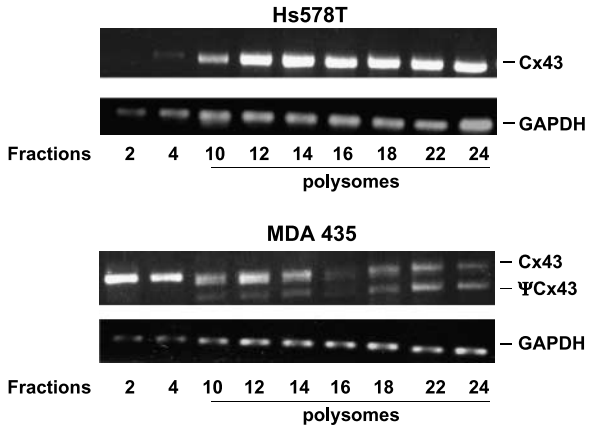



**Figure 1.** Sedimentation properties of *ΨCx43* RNA. Hs578T and MDA 435 cell lysates were fractionated by sucrose gradient centrifugation and collected while continuously monitoring the absorbance at 254 nm. Analysis of *Cx43/ΨCx43* mRNAs was done using semiquantitative RT-PCR followed by *Nco*I digestion.

### Exogenous *ΨCx43* Decreases *Cx43* Total RNA Levels in MDA 231 Cells

Figure 4A shows the effects of exogenous *ΨCx43* on *Cx43* RNA levels in MDA 231 neo and MDA 231 *ΨCx43* cells. In cells expressing *ΨCx43*, we observe overexpression of the pseudogene along with a corresponding decrease (65%) in *Cx43* RNA compared with wild-type cells. Figure 4B shows an immunoblot for *Cx43* using cell lysates from both cell lines. In contrast to the results we obtained with RNA analysis, the *ΨCx43*-overexpressing cells displayed greater amounts of *Cx43* protein. Given the high degree of homology between *Cx43* and *ΨCx43* along with our previous evidence that *ΨCx43* is translated, this observation is likely due to the fact that both *ΨCx43* and *Cx43* proteins are being recognized by the antibody.

### *ΨCx43* siRNA Increases *Cx43* RNA and Protein Expression in MCF7 Cells

Figure 4C shows the analysis of the effect of *Cx43* and *ΨCx43* siRNAs on endogenous *Cx43* and *ΨCx43* expression using a retroviral vector system. There is a decrease in *Cx43* and *ΨCx43* RNA levels in *Cx43* and *ΨCx43* knockdown cells (MCF7 *Cx43* siRNA and MCF7 *ΨCx43* siRNA), respectively, compared with the control (MCF7 PSR). Furthermore, the decrease in *ΨCx43* RNA we observed in MCF7 *ΨCx43* siRNA cells results in a parallel increase in *Cx43* RNA levels. This is not observed for *ΨCx43* RNA in MCF7 *Cx43* siRNA. Figure 4D shows the result of immunoblotting for *Cx43* protein levels in the knockdown cell lines. As seen previously, no signal for *Cx43* was detectable in MCF7 PSR cells. When comparing the control with MCF7 *Cx43* siRNA and MCF7 *ΨCx43* siRNA cells, there is detectable *Cx43* protein in the MCF7 *ΨCx43* siRNA cells. As well, transient transfection of *in vitro* synthesized double-stranded siRNAs to *Cx43* and *ΨCx43* in MCF7 cells was done, and a decrease in *ΨCx43* RNA also resulted in a corresponding elevation in *Cx43* RNA expression as seen in the retroviral vector system (data not shown).

### *ΨCx43* siRNA Sensitizes MDA 231 Cells to Paclitaxel and Doxorubicin

We explored whether we could further sensitize MDA 231 cells to paclitaxel and/or doxorubicin by inhibiting *ΨCx43* in these cells. Figure 5A shows that there is more *Cx43* protein in the *ΨCx43* knockdown cells compared with *Cx43* knockdown cells. Figure 5B shows the results of cells exposed to paclitaxel and doxorubicin for 48 hours, measuring cell mortality using Alamar Blue. There is a statistically significant increase in the sensitivity of *ΨCx43* knockdown cells when compared with control and *Cx43* knockdown cells. The level of toxicity (measured as $IC_{50}$) induced by paclitaxel and doxorubicin in MDA 231 *ΨCx43* siRNA cells was about 1.6- and 1.5-fold higher, respectively, than that of MDA 231 PSR cells.

## Discussion

The biological relevance of pseudogenes remains largely uninvestigated because they are often considered to be neither

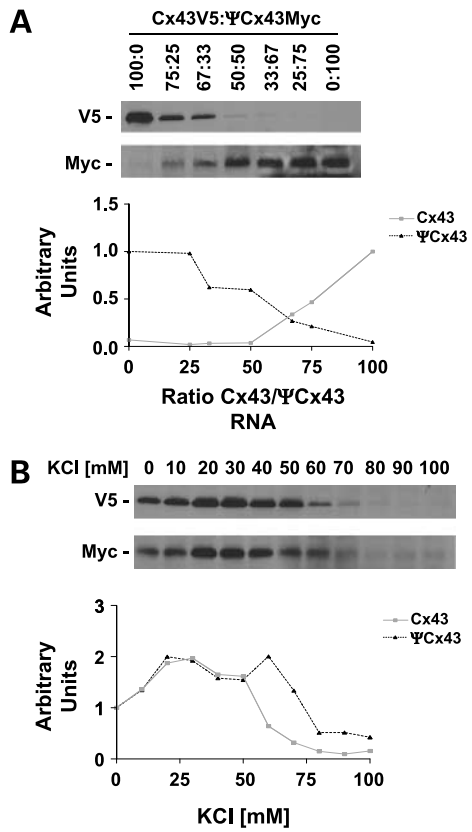



**Figure 2.** *ΨCx43* is preferentially translated and binds with higher affinity to the translational machinery than *Cx43*. Cx43V5 and ΨCx43Myc cRNAs were *in vitro* translated in a cell-free system. Translation products were analyzed by electrophoresis on a 12% SDS-PAGE, transferred, and immunoblotted with V5 and Myc antibodies, respectively. Band intensities were quantified using Scion Image and results were plotted in GraphPad Prism 3.0. **A,** ratios of concentrations of Cx43V5/ΨCx43Myc were varied from 100 to 0. **B,** cRNAs were added to the translation reaction with increasing amounts of KCl.

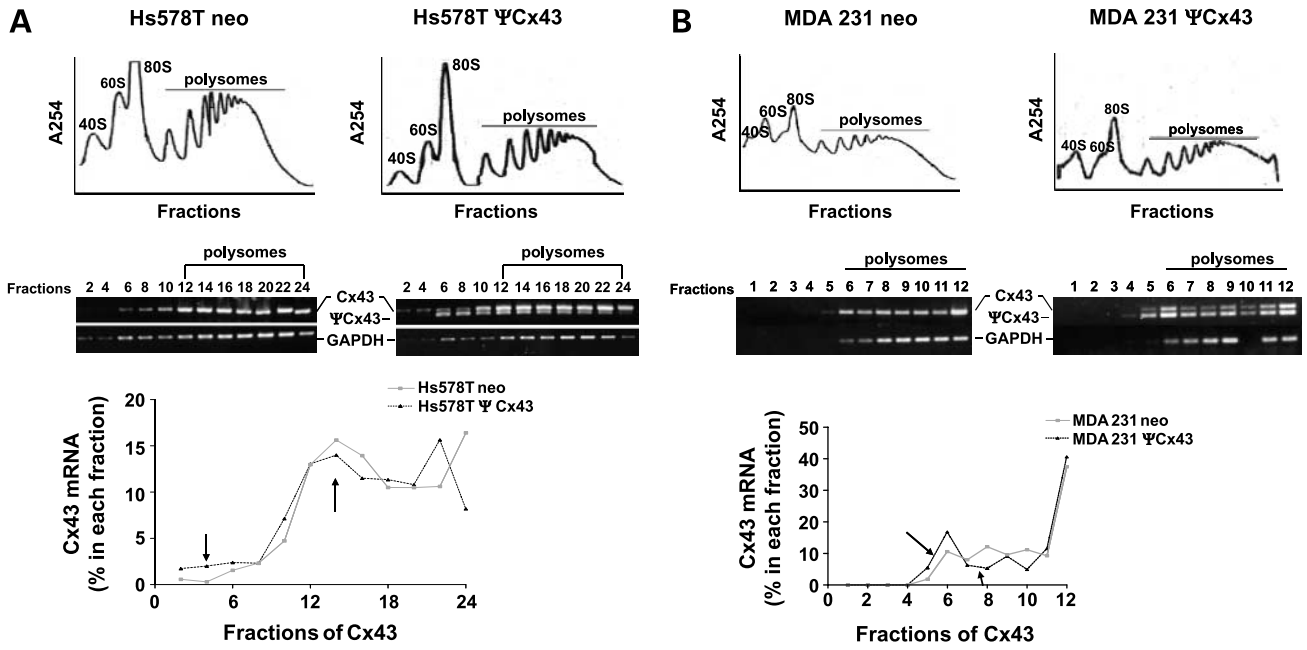



**Figure 3.** Polyribosome profile analysis of *Cx43* mRNA following the addition of *ΨCx43* mRNA. *Cx43* and *ΨCx43* mRNAs were extracted and analyzed as mentioned in Materials and Methods. **A,** polysome profile for Hs578T neo and Hs578T *ΨCx43* cells. **B,** polysome profile for MDA 231 neo and MDA 231 *ΨCx43* cells. Band intensities were quantified using Scion Image and results were plotted in GraphPad Prism 3.0. *Cx43* mRNA levels in each fraction were expressed as a percentage of total levels summed over all fractions.

transcribed nor translated (4–6, 8). However, there is increasing evidence for the role of pseudogenes in cellular processes such as tumorigenesis (13–15). *ΨCx43* is not only transcribed but is also capable of inhibiting cell growth and forming a functional protein when fused to green fluorescent protein *in vivo* (28). Detection of *ΨCx43* in breast cancer cell lines and its inverse relationship with *Cx43* protein led us to suspect a potential regulatory role of *ΨCx43* on its homologue *Cx43*. Because it had previously been documented that the pseudogene of the *Makorin1* gene, *Makorin1-p1*, had the ability to regulate the stability of Makorin1 (29), we examined this hypothesis for *Cx43* and its pseudogene. The mechanism of this regulation is unknown, but we did not detect a significant effect on *Cx43* mRNA stability.

Previous findings indicated that the 5′ and 3′ ends of *ΨCx43* align closely with that of *Cx43* mRNA and that the *ΨCx43* open reading frame, which contains a deletion and several point mutations, is capable of encoding a full-length protein (27, 30). Due to the high homology of *ΨCx43* to *Cx43* and the inability of all available antibodies to discriminate between the two proteins (28), we examined *ΨCx43* translation at the level of polysomes. Contrary to prior reports that indicate the incapability of pseudogenes to be translated, *ΨCx43* cosediments with polysomes, supporting our earlier evidence that *ΨCx43* can be translated *in vitro* (28).

Both *Cx43* and *ΨCx43* bind to the translational machinery, and our observations indicate that *ΨCx43* RNA is preferentially translated. KCl dissociation experiments also showed tighter binding to the translational machinery by *ΨCx43* compared with *Cx43*. These features have been seen before for two homologous genes, *α-globin* and *β-globin*. During erythroid development, protein synthesis of *β-globin* mRNA is initiated more frequently than *α-globin* because the former competes more efficiently for critical components in translation (31, 32). As well, it was determined that *β-globin* binds with higher affinity to eIF2 during translation than does *α-globin* (33, 34). Protein translation is a complex process wherein the majority of the regulatory mechanisms occur at the initiation step because it requires the function and cooperation of a large number of proteins. One protein, eIF2, is required to bind and deliver the initiator met-tRNA to the translational machinery. The eIF2 α-subunit becomes phosphorylated as a result of environmental stresses, which in turn inhibits translation initiation and halts global protein synthesis (35). Our data show a competitive advantage of *ΨCx43* over *Cx43* RNA for translation.

Given the preferential translation of *ΨCx43* over *Cx43* in a cell-free system, we looked at the effect of the pseudogene on the polysome profile of two cell lines that have elevated *Cx43* protein levels, Hs578T and MDA 231. Recruitment of mRNAs into polysomes could either increase or decrease protein production depending on subsequent posttranslational initiation events such as translation elongation, peptide chain termination, protein folding, and protein or RNA stability. It has previously been shown that a shift of mRNA away from polysome fractions correlated well with a reduction of protein synthesis (36). We indeed found that the presence of *ΨCx43* transcript in these cell lines resulted in

a shift of *Cx43* mRNA levels from the polysome fractions toward the monosomes in Hs578T cells and to an overall decrease in *Cx43* mRNA in the polysomes of MDA 231 cells. These findings suggest that *ΨCx43* inhibits both the translational initiation and efficiency of *Cx43* mRNA (37, 38).

The most frequent and rate-limiting step of translational control occurs at the initiation stage (35, 39). There are two mechanisms used to explain the efficient utilization of *Cx43* mRNA. The first mechanism is by mobilization of *Cx43* mRNA from translationally inactive messenger ribonucleoprotein (mRNP) particles to actively translating polysomes, whereas the second entails increased rate of ribosome initiation on mRNA in the polysome pool (35, 38, 40–42). Our findings suggest an inhibition of *Cx43* at or before the initiation stage of translation. Indeed, we observed a decrease in *Cx43* total RNA in the presence of *ΨCx43* in MDA 231 cells. The increase in *Cx43* protein is most likely due to the ability of the antibody to recognize both the *Cx43* and *ΨCx43* proteins being produced. Therefore, as a result of competition for the translational machinery and a shift of the *Cx43* polysome profile toward inactive mRNPs in the presence of *ΨCx43*, we conclude that *Cx43* and *ΨCx43* are coordinately regulated at the level of translation and are differentially regulated at the level of transcription.

Studies using siRNAs targeted to *Cx43* and *ΨCx43* showed that *Cx43* knockdown caused no change in *ΨCx43* RNA levels, but *ΨCx43* knockdown results in a marked increase in *Cx43* RNA and protein in MCF7 cells. These findings add further support to the proposition that *ΨCx43* is a regulator of *Cx43* synthesis, which occurs at the posttranscriptional stage of RNA synthesis. This also suggests a potential therapeutic strategy to enhance *Cx43* and sensitize cancer cells to cytotoxic chemotherapy. The expression of *Cx43* in gap junction–deficient tumor cell lines has been well documented as a means of reestablishing several aspects of growth control (43). More recently, it has been shown that increased *Cx43* enhances chemosensitivity in several cancer models (24, 25). These studies used a variety

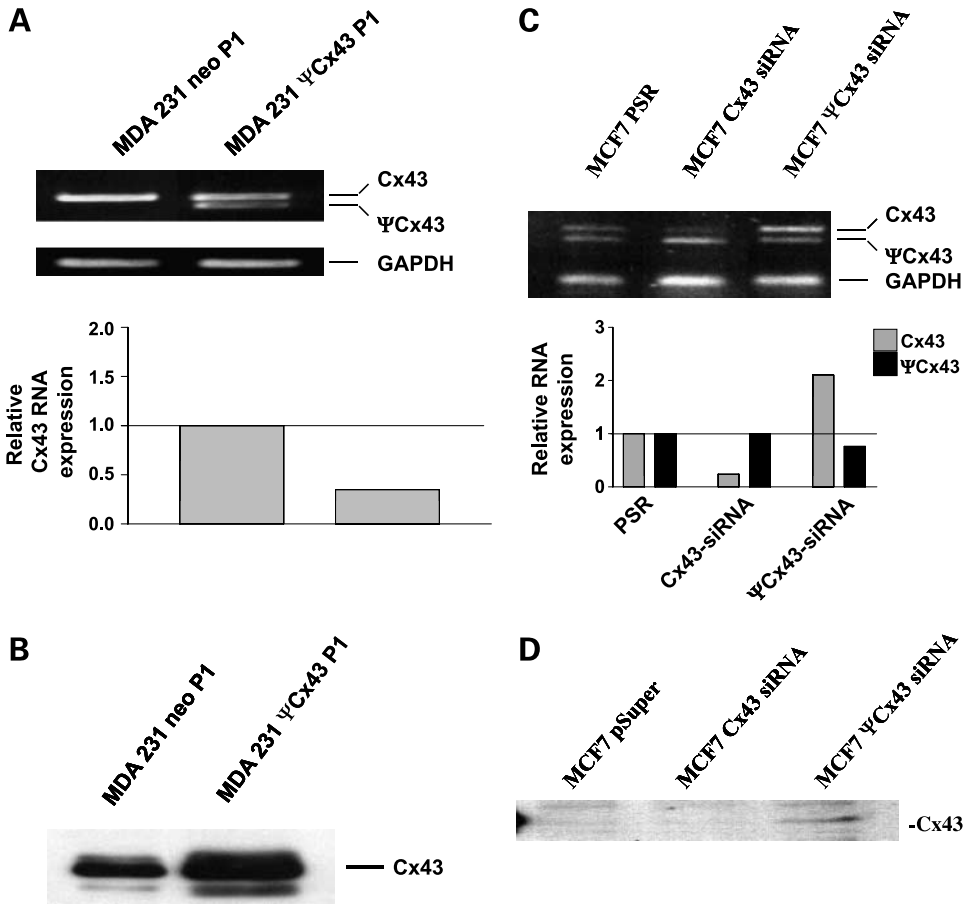
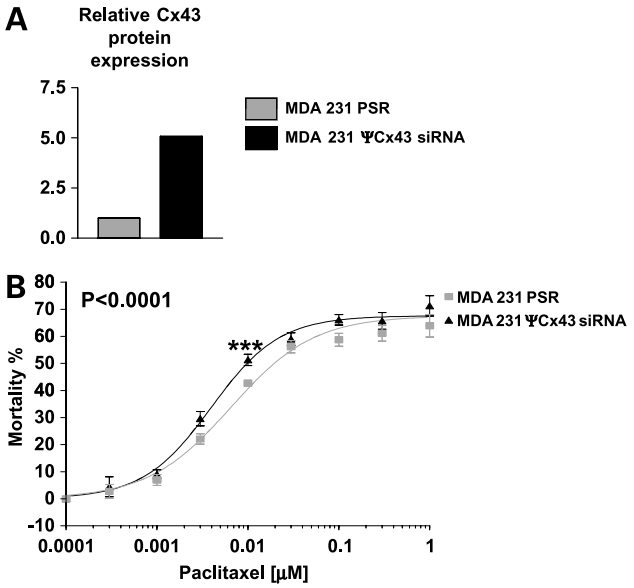



**Figure 4.** Effects on *Cx43* RNA and protein expression in response to exogenous *Cx43* and *ΨCx43* siRNAs. **A,** RT-PCR products digested with *Nco*I from MDA 231 neo and *ΨCx43* cells. *Cx43* RNA decline was quantified using Scion Image and results were plotted in GraphPad Prism 3.0 as the decline in MDA 231 *ΨCx43* cells relative to the control (neo cells). **B,** *Cx43* protein levels were determined by Western blot detection of *Cx43* as described in Materials and Methods. **C,** expression of *Cx43* and *ΨCx43* RNAs was determined by RT-PCR followed by *Nco*I digestion in MCF7 cells stably expressing *Cx43* and *ΨCx43* siRNAs. Band intensities were quantified using Scion Image and results were plotted in GraphPad Prism 3.0. *Cx43* and *ΨCx43* expression levels were analyzed relative to the control cells (MCF7 PSR). **D,** *Cx43* expression was determined by Western blot of protein lysates from MCF7 cells expressing *Cx43* and *ΨCx43* siRNAs using *Cx43* antibody.




| Cells | IC50 | |
|---|---|---|
| MDA 231 | Paclitaxel (nM) | Doxorubicin (nM) |
| PSR | 6.538 | 28.69 |
| ΨCx43 siRNA | 3.977 | 18.95 |

**Figure 5.** *ΨCx43* knockdown sensitizes cells to chemotherapeutics. **A,** *Cx43* expression levels analyzed by Western blot in MDA 231 cells stably expressing *ΨCx43* siRNA. Band intensities were quantified using Scion Image and results were plotted in GraphPad Prism 3.0. *Cx43* and *ΨCx43* expression levels were analyzed relative to the control cells (MDA 231 PSR). **B,** $IC_{50}$ values were estimated from dose-response curves obtained after 48 h of exposure to different doxorubicin and paclitaxel concentrations as indicated in Materials and Methods. *Points,* mean of four independent experiments; *bars,* SE. ***, $P < 0.0001$.

of pharmacologic approaches to increase *Cx43*. We found that treatment of MDA 231 breast cancer cells with siRNA targeted to *ΨCx43* also effectively increased *Cx43* protein levels and rendered the cells more sensitive to paclitaxel and doxorubicin treatment.

Regulation of a gene by its pseudogene remains a controversial issue (44, 45). Evidence is mounting not only for pseudogene transcription but also for the transcription of pseudogenes as an antisense (46, 47). The discovery of pseudogene antisense transcripts suggests that a pseudogene can act to regulate its gene of origin through an antisense mechanism (48). Furthermore, the recent identification of pseudogene-derived endogenous siRNAs regulating gene expression in mouse oocytes (49) underlines the importance and regulatory capacity of these so-called "useless" genomic sequences. The data presented here highlight the relevance of *ΨCx43* in *Cx43* inhibition. At a translational level, *ΨCx43* seems to compete more effectively for the translational machinery. However, the primary mechanism of action seems to be at the level of posttranscription, where *ΨCx43* shifts *Cx43* mRNA toward nonactive mRNPs and inhibits overall *Cx43* RNA synthesis. Regulating *Cx43* in *trans* makes *ΨCx43* a desirable target for siRNA knockdown, which could be further used as a potential and attractive new anticancer therapeutic approach.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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