# Connexin 43 interacts with Bax to regulate apoptosis of pancreatic cancer through a gap junction-independent pathway

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**Abstract.** Connexins play important roles in many physiological and pathological processes. Although connexins are considered as tumor suppressors in several types of cancer, their roles in pancreatic cancer are unknown. In this study, we found that connexin 43 (Cx43) caused apparent apoptosis and growth inhibition in pancreatic cancer cells. The tumor-suppressive role of Cx43 was independent of the canonical gap junction pathway. In the context of apoptosis, Cx43 translocated to the mitochondria, where it interacted with Bax to initiate the mitochondrial apoptotic pathway. Following further examination of the Cx43 protein, we found that the 241-382aa region of Cx43 was required for interaction with Bax. Furthermore, this region was responsible for permeabilizing mitochondrial membrane potential. The results from the present study elucidate a novel mechanism of the Cx43-mediated regulation of apoptosis in pancreatic cancer.

## Introduction

Pancreatic cancer is the fourth most frequent cause of cancer related mortality, with a 5-year overall survival of less than 5%. In the United States, approximately 42,470 individuals are diagnosed with this condition and 35,240 die from the disease each year (1). To date, tumor resection has remained the only curative therapy for pancreatic carcinoma. Irradiation and chemotherapy do not have a significant therapeutic effect on pancreatic cancer (2). Therefore, pancreatic cancer still represents a therapeutic challenge in oncology.

Connexins (Cxs) are encoded by a multigene family; to date, 21 different Cx genes have been identified. Gap junctions (GJs), which are composed of Cx proteins, allow the direct exchange of small molecules between adjacent cells (3). A previous study showed that Cxs exert their function through a GJ-independent pathway. The underlying mechanisms of a GJ-independent pathway mainly involve protein interactions (4). The dysregulation of Cx expression has been associated with carcinogenesis of the lung, breast, prostate, liver, stomach and colon. Cxs are involved in the regulation of tumor proliferation, apoptosis, invasion and metastasis (5). Connexin 43 is the major isoform in the pancreas, yet few studies have addressed its role in pancreatic cancer (6). We performed this study in order to unravel the mechanism of the Cx43-regulated apoptosis of pancreatic cancer.

## Materials and methods

*Cell culture, plasmids and materials.* Human pancreatic cancer cells (BxPC-3, SW1990, PaTu8988, PANC-1, AsPC-1 and CFPAC-1) were grown in high glucose DMEM or RPMI-1640, supplemented with 10% (v/v) FBS at 37˚C with 5% $CO_2$. The siRNAs against Cx43 oligonucleotides were synthesized by Ambion (Grand Island, NY, USA) with the sequence 5'-GAUGAUAACCAGAA UUCTA-3'. Non-silencing control siRNA was synthesized using scrambled sequence as the negative control (NC). The dominant-negative Cx43 mutant (Cx43N) was constructed by deletion of amino acids 130-136 from the cytoplasmic loop. This mutant is effective as a dominant-negative inhibitor of GJs. The transfection of siRNA or plasmids was applied using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

*Assay for GJ intercellular communication.* The GJ assay protocol was employed based on a modification of the methods

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*Abbreviations:* Cx43, connexin 43; GJ, gap junction; β-GA, 18-β-glycyrrhetinic acid; MMP, mitochondrial membrane potential; Cyt c, cytochrome c

*Key words:* pancreatic cancer, gap junction, connexin 43, Bax, apoptosis

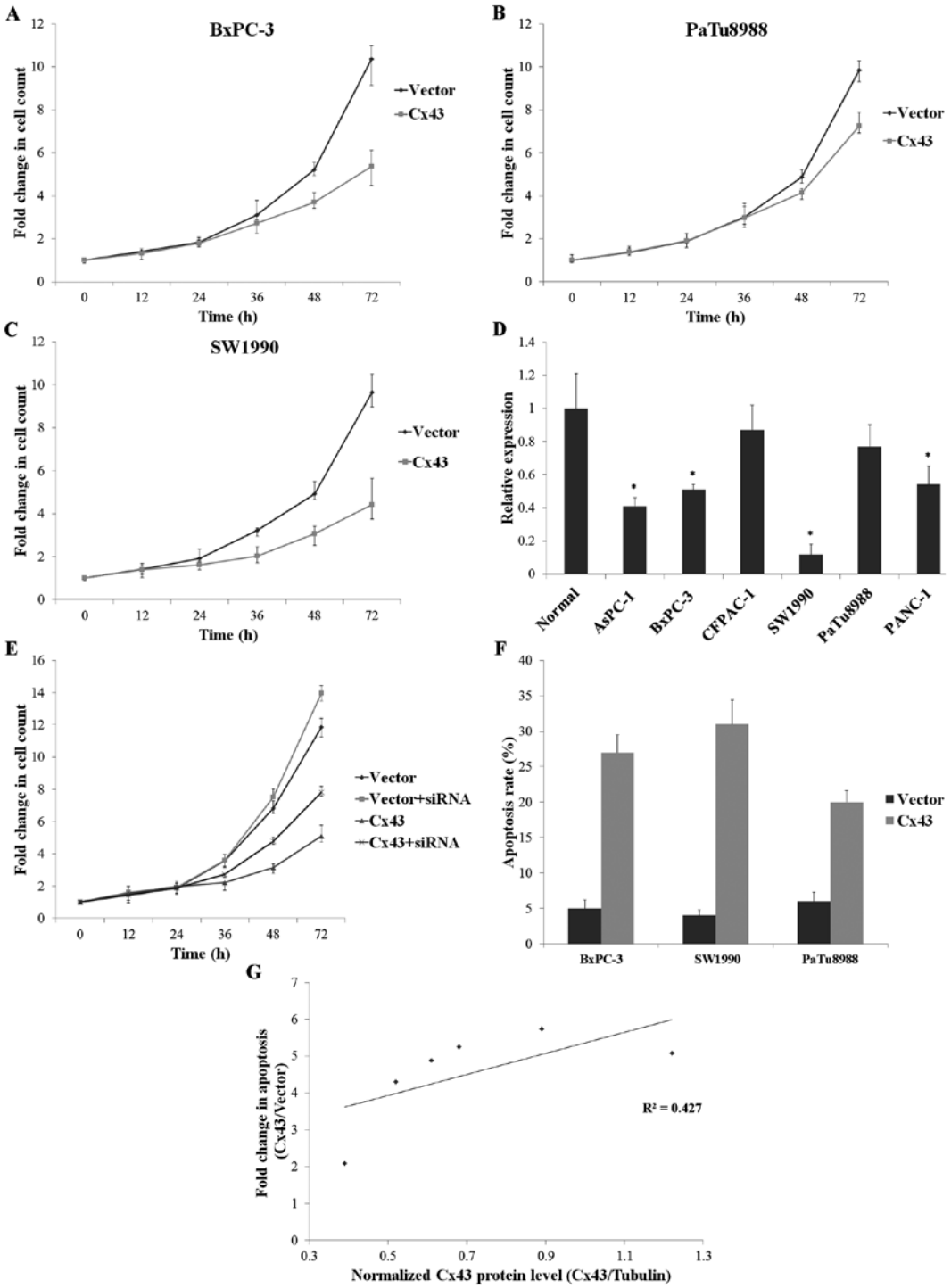


Figure 1. Cx43 inhibits the growth of human pancreatic cancer cells. (A-C) Cell proliferation was analyzed in BxPC-3, PaTu8988 and SW1990 cells. Cells were transfected with Cx43 or the control plasmid. Cell numbers were recorded for 3 days. (D) Analysis of relative Cx43 expression in the pancreatic cancer cell lines, AsPC-1, BxPC-3, CFPAC-1, SW1990, PaTu8988 and PANC-1. (E) Cell proliferation was analyzed in BxPC-3 cells with Cx43 plasmid or siRNA. (F) Apoptotic rate was assessed in BxPC-3, SW1990 and PaTu8988 cells by Annexin V/PI assay. (G) The ordinate shows changes in apoptotic rate. The abscissa was normalized to Cx43 protein levels. Dots represent the average changes in AsPC-1, BxPC-3, CFPAC-1, SW1990, PaTu8988 and PANC-1 cells. Data represent the means ± SEM values from at least 3 separate experiments.

described by Goldberg *et al* (7). Donor and recipient cell populations were differentially labeled for 30 min with the GJ-permeable dye, calcein AM, and the lipophilic dye, $DiIC_{18}$ (Molecular Probes, Eugene, OR, USA), in DMEM containing 10% FBS, respectively. After washing, donor cells were trypsinized and added to acceptor cells at a 1:5 (donor: acceptor) ratio for 3 h at 37˚C. Co-cultures were harvested and subjected to FACS analysis. If GJ intercellular communication (GJIC) occurred, calcein was transferred from donor cells (green) to recipient cells (red). Thus, double-labeled cells represented the communicating cells in this assay. Cytometric data were expressed in terms of the coupling index (coupled acceptor cells/total potential acceptor cells) per donor cell and were plotted as the means ± SEM of 3 experiments.

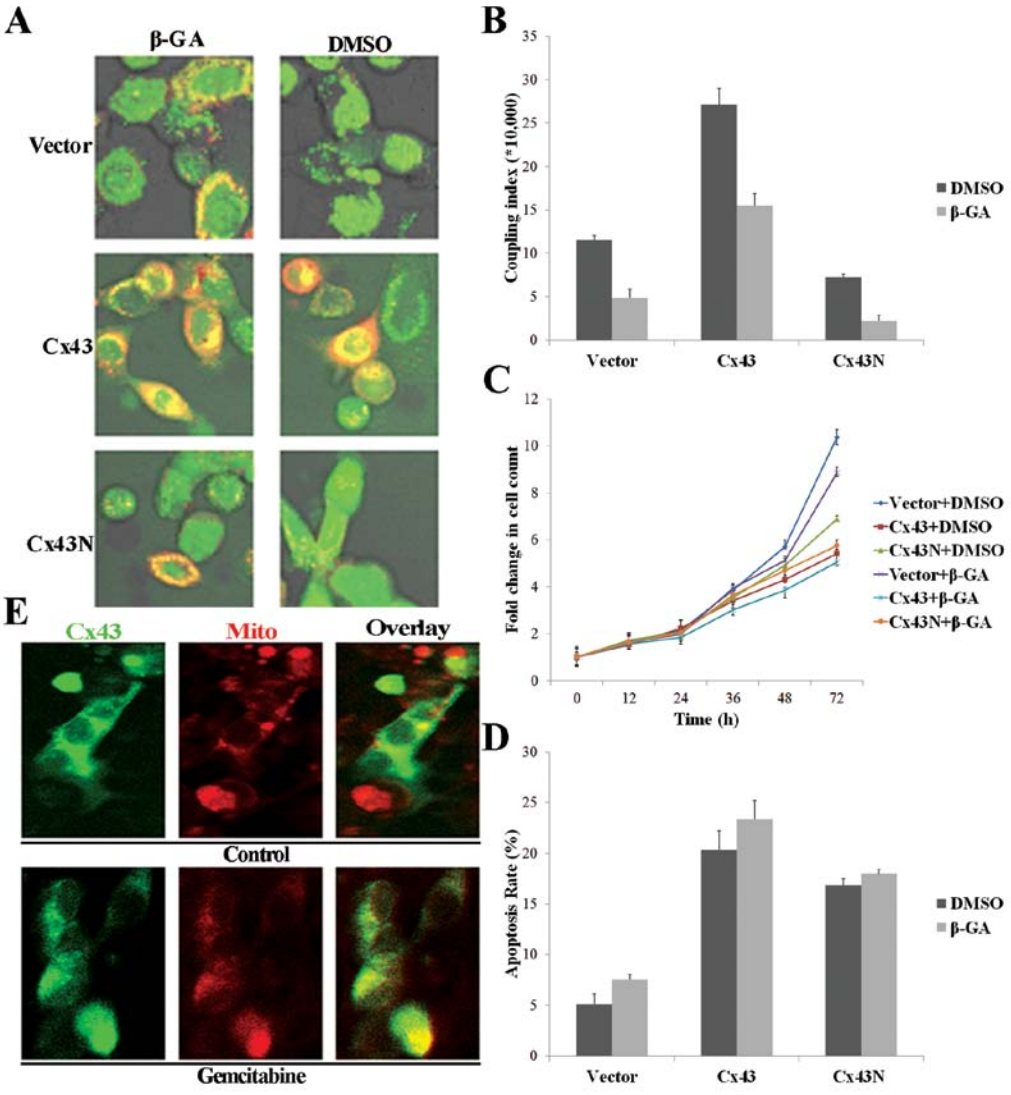


Figure 2. The tumor-suppressive role of Cx43 is independent of GJ. (A) GJs were analyzed by confocal microscopy in cells transfected with Cx43, Cx43N or control plasmid. Calcein was transferred from donor cells (green) to recipient cells (red). Double-labeled cells (yellow) represent the communicating cells. (B) Coupling index was calculated according to the ratio of communicating cells per recipient cells. After cells were transfected with Cx43, Cx43N or control plasmid, they were further treated with or without β-GA. The apoptotic rate (C) and growth curve (D) were also analyzed in these cells. (E) Cx43 (green) subcellular localization was analyzed by confocal microscopy in BxPC-3 cells with or without gemcitabine. Mitochondria were labeled by MitoTracker dye (red). The overlay images (yellow) reveal the co-localization of Cx43 with the mitochondria. Data represent the means ± SEM values from at least 3 separate experiments.

*Analysis of mitochondrial depolarization*. JC-1 (Invitrogen) was employed to measure mitochondrial depolarization in BxPC-3 cells. Mitochondrial depolarization was indicated by a decrease in the red/green fluorescence intensity ratio. Cells were incubated with 5 $\mu$g/ml of JC-1 at 37˚C for 15 min and then analyzed by fluorescence-activated cell sorter.

*Co-immunoprecipitation*. For immunoprecipitation, 200 $\mu$g of cell lysate were incubated with 25 $\mu$l each of agarose A and G (Invitrogen) in 500 $\mu$l total volumes for 1 h. Immunoprecipitating antibody was added with an additional 3 h of incubation at 4˚C with constant rotation. The complex was washed 3 times. It was then resuspended in SDS loading buffer. After boiling, the supernatant was loaded for western blot analysis. The total protein in the input lysate was approximately 1/10 of the amount used for immunoprecipitation. The antibodies used were: Cx43 (Sigma, St. Louis, MO, USA), Bax, Bcl-2 and GAPDH (Cell Signaling Technology, Danvers, MA, USA).

*Statistical analysis*. All experiments were performed in triplicate and the results are expressed as the means ± SEM. The data were analyzed with the Student's t-test or by one-way analysis of variance (ANOVA) and $P \leq 0.05$ denoted a statistically significant difference.

## Results

*Cx43 induces apoptosis of pancreatic cancer cells*. We examined the proliferation rates in pancreatic cancer cells transfected with Cx43. Cell growth was significantly inhibited in the presence of Cx43. Of note, growth inhibition was more apparent in SW1990 and BxPC-3 cells, but not in PaTu8988 cells (Fig. 1A-C). The possible reason was that PaTu8988 cells had a relatively higher level of Cx43 (Fig. 1D). We then tried to use siRNA to rescue the growth inhibition induced by Cx43. Growth inhibition was recovered in the cells co-transfected with DNA and Cx43 siRNA. The Cx43 knockdown promoted

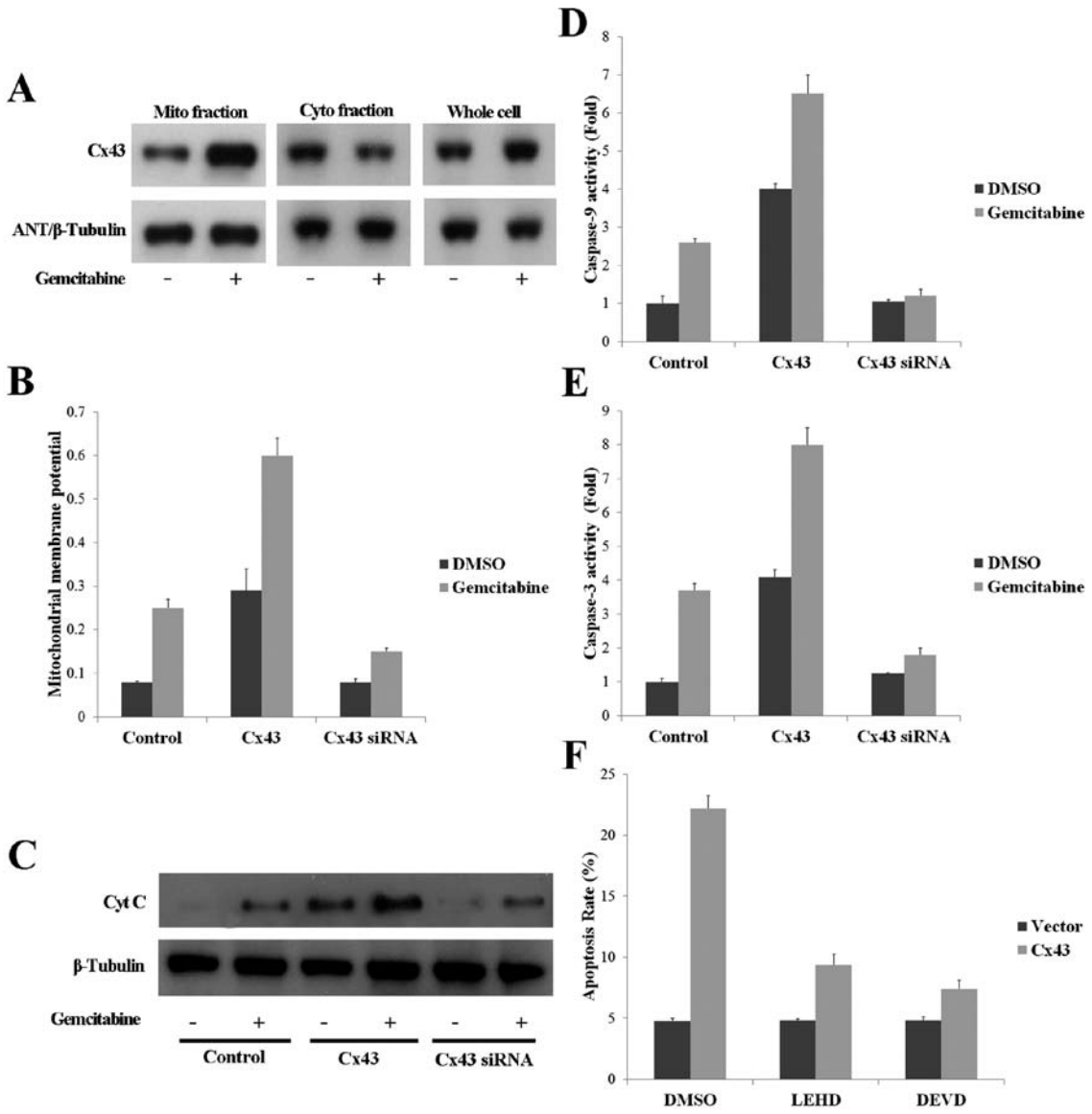


Figure 3. Cx43 regulates apoptosis via the mitochondrial apoptotic pathway. (A) Gemcitabine-induced Cx43 mitochondrial localization was analyzed by immunoblotting of mitochondrial fraction, cytosolic fraction and whole cell lysates. ANT was used as the loading control of mitochondrial fraction, and β-tublin for cytosolic and whole cell lysates. Mito fraction, miochondrial fraction; Cyto fraction, cytosolic fraction. (C) Release of Cyt c was assessed by western blot analysis of cytosolic fraction. After expression of Cx43 or knockdown of Cx43, the mitochondrial membrane potential (B), caspase-9 activity (D), caspase-3 activity (E) and apoptotic rate (F) were analyzed. Data represent the means ± SEM values from at least 3 separate experiments.

cancer cell growth (Fig. 1E). As Cx43 is involved in regulating apoptosis in multiple cancers, we speculated that Cx43 may be a pro-apoptotic gene in pancreatic cancer. The apoptotic rates were approximately 20-30% when the pancreatic cancer cells were transfected with Cx43 (Fig. 1F). Gemcitabine is a widely-used drug for the first-line therapy of pancreatic cancer. In this study, we found that level of Cx43 positively correlated with the apoptosis induced by gemcitabine (Fig. 1G). These results suggest that Cx43 plays an important role in regulating the apoptosis of pancreatic cancer cells.

*Tumor-suppressive function of Cx43 occurs via a GJ-independent pathway*. It is well known that Cx43 exerts its function through a canonical GJ-mediated pathway. We therefore investigated whether GJ is necessary for Cx43 tumor-suppressive function. 18β-glycyrrhetinic acid (β-GA) was used for GJ inhibition (Fig. 2A and B). With β-GA treatment, Cx43 still inhibited the growth and induced the apoptosis of BxPC-3 cells (Fig. 2C and D). As β-GA inhibits GJs formed by any Cx protein besides Cx43, the Cx43 mutant, Cx43N, constructed by the deletion of amino acids 130-136 from the cytoplasmi loop, was used for inhibiting the Cx43-meditated GJ (8). GJ formation in Cx43N-expressed BxPC-3 cells was dramatically decreased (Fig. 2A and B). However, the Cx43 mutant preserved tumor-suppressive function without forming GJs (Fig. 2C and D). These results suggest that a GJ is not the key mechanism involved in the anti-tumor effect of Cx43 in pancreatic cancer cells.

*Cx43 induces apoptosis through mitochondrial apoptotic pathway*. The idea of a GJ-independent pathway was further confirmed by Cx43 subcellular localization. While BxPC-3 cells were exposed to gemcitabine, Cx43 translocated from the

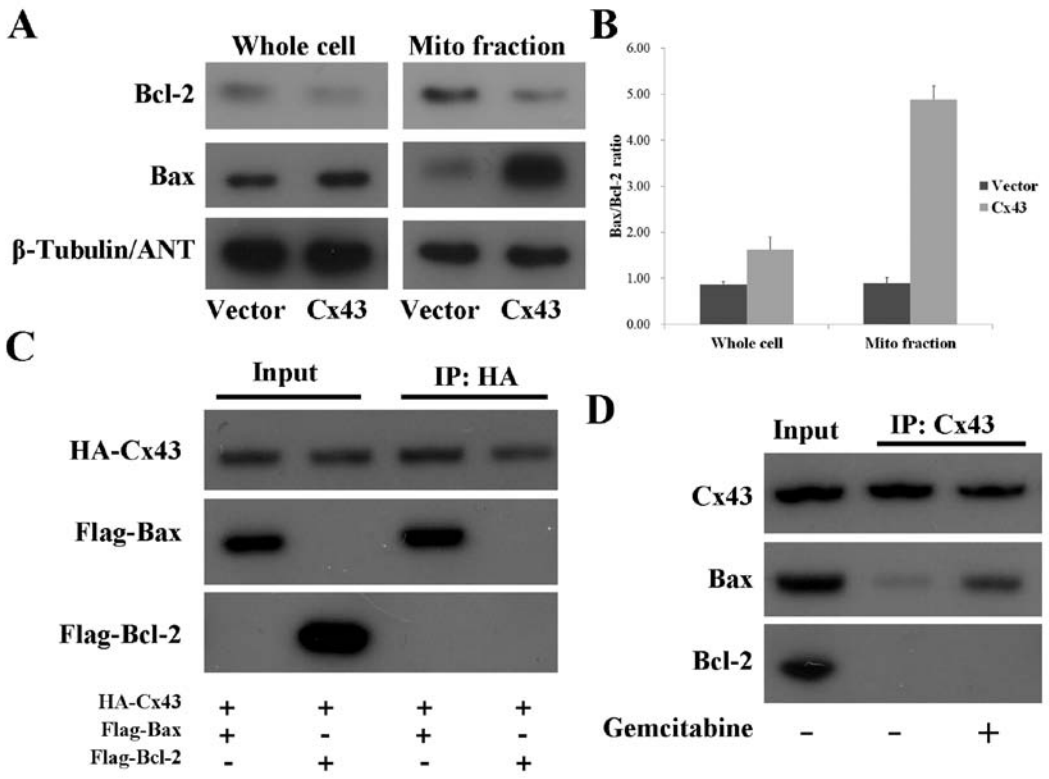


Figure 4. Cx43 interacts with Bax to regulate the apoptosis of pancreatic cancer cells. (A) Bax and Bcl-2 expression was assayed in both whole cell lysates and mitochondrial fractions. ANT was used as the loading control of mitochondrial fraction, and β-tubulin for whole cell lysates. (B) The Bax/Bcl-2 ratio was calculated by densitometric analysis of 3 separate experiments. The association of Cx43/Bax or Cx43/Bcl-2 was analyzed by co-immunoprecipitation. Mito fraction, miochondrial fraction (C) HA-Cx43 was immunoprecipitated by HA antibodies. Immunoblotting was performed with Flag antibody. (D) BxPC-3 cells were treated with or without gemcitabine. Endogenous Cx43 was immunoprecipitated by Cx43 antibodies. Then Bax or Bcl-2 antibodies were used for immunoblotting. IP, immunoprecipitation.

cytoplasm to the mitochondria (Fig. 2E). The characteristic of mitochondrial sublocalization was also assessed by cellular fractionation. Mitochondrial Cx43 expression was dramatically increased with gemcitabine treatment (Fig. 3A). We presumed that the Cx43 localization was related to the mitochondrial apoptotic pathway. The mitochondrial membrane potential (MMP) was increased when BxPC-3 cells were transfected with Cx43. The increase of MMP was more dramatic in the cells treated with gemcitabine simultaneously (Fig. 3B). The knockdown of Cx43 itself did not cause a significant change in MMP. However, the gemcitabine-induced decrease of MMP was compromised by the Cx43 knockdown (Fig. 3B). Another indicator of mitochondria-mediated apoptosis was the release of cytochrome c (Cyt c) (Fig. 3C). The activities of caspase-9 and caspase-3 were increased with Cx43 expression (Fig. 3D and E). When the cells were treated with the caspase inhibitors, LEHD (against caspase-9) and DEVD (against caspase-3), the Cx43-induced apoptosis was compromised (Fig. 3F). Overall, the mitochondrial apoptotic pathway was shown to be involved in the Cx43-induced apoptosis.

*Cx43/Bax interaction is required to induce apoptosis of pancreatic cancer cells*. Bcl-2 family proteins are master regulators of the mitochondrial apoptotic pathway. The Bax/Bcl-2 ratio serves as a rheostat to determine cell susceptibility to the intrinsic apoptotic signaling pathway. The expression of Cx43 in BxPC-3 cells caused an increase in the Bax/Bcl-2 ratio (Fig. 4A and B). Of note, the main change in Bax or Bcl-2 was mitochondria-localized protein (Fig. 4A and B). As Cx43 translocated to the mitochondria during apoptosis, we speculated that potential interactions occurred between Cx43 and Bcl-2 family proteins. BxPC-3 cells were co-transfected with HA-Cx43/Flag-Bax or HA-Cx43/Flag-Bcl-2. Co-immunoprecipitation revealed that Cx43 co-precipitated with Bax, but not with Bcl-2 (Fig. 4C). Reciprocal co-immunoprecipitation showed consistent results. As the above experiments relied on exogenous proteins, we sought to examine whether an interaction occurs between endogenous Cx43 and Bax proteins. We further examined the interaction in BxPC-3 cells treated with gemcitabine (Fig. 4D). There was no Cx43/Bcl-2 interaction to be found regardless of the overexpression of these proteins or apoptotic stimuli. On the contrary, gemcitabine increased the interaction between Cx43 and Bax (Fig. 4D). These findings suggest that Cx43 directly interacts with Bax, but not Bcl-2, to regulate the apoptosis of pancreatic cancer cells.

*Cx43 (241-382aa) interacts with Bax to permeabilize mitochondrial membrane*. We then examined the importance of the Cx43/Bax interaction in regulating the apoptosis of pancreatic cancer cells. We examined the Cx43 protein to find the key region for its interaction with Bax. Cx43 includes 4 transmembrane regions and a connexin homolog domain (CNX). Different lengths of Cx43 plasmids were constructed as shown in Fig. 5A. Co-immunoprecipitation indicated that 241-382aa

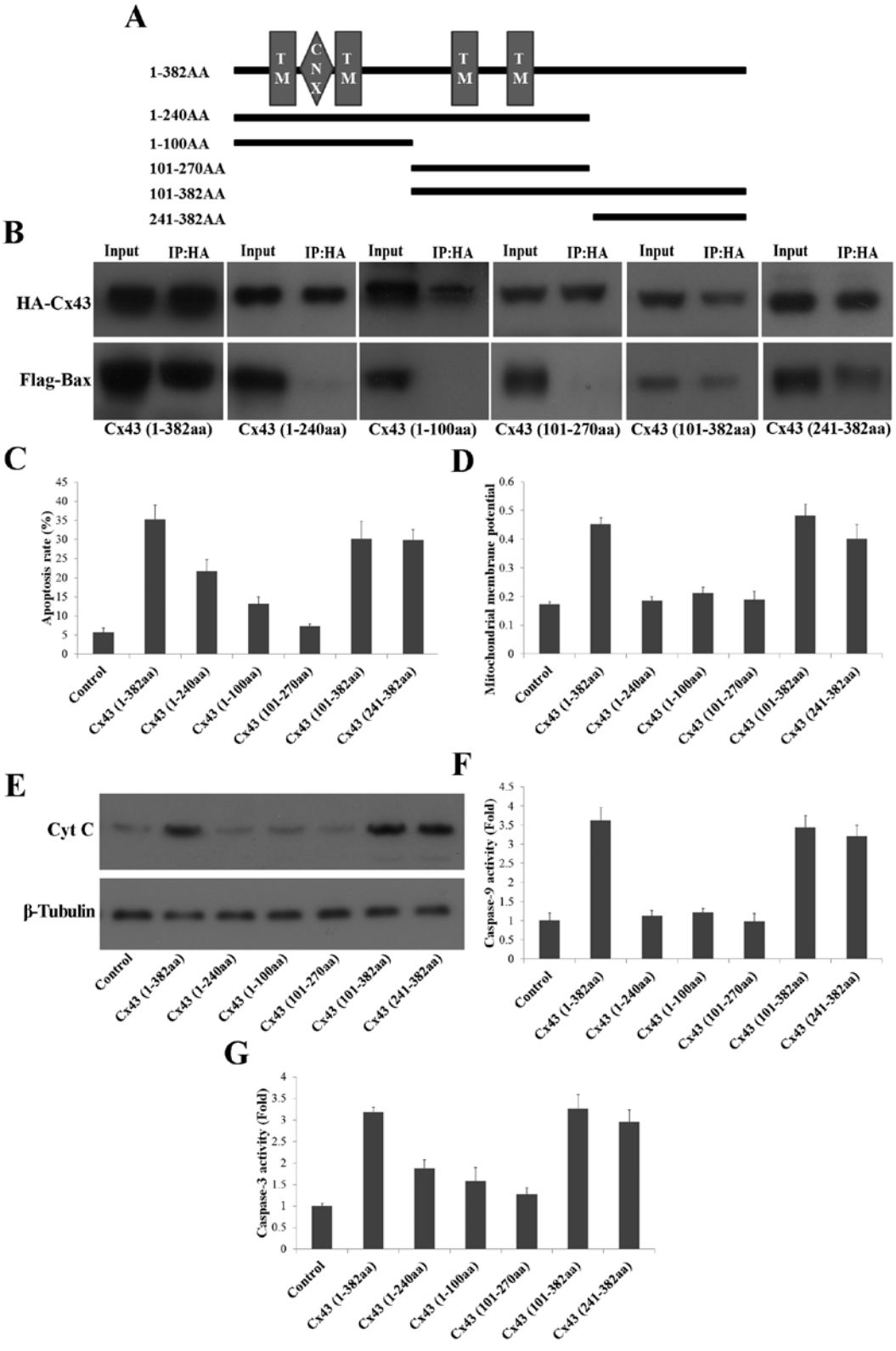


Figure 5. The 241-382aa fragment of Cx43 is required for initiating the mitochondrial apoptotic pathway. (A) As indicated, different lengths of the Cx43 plasmid were constructed with HA tag (TM, transmembrane domain; CNX, connexin homolog domain). (B) Cx43 was immunoprecipitated by HA antibody. Immunoblotting was performed with Flag antibody. IP, immunoprecipitation. After cells were transfected with different lengths of Cx43 plasmid or control plasmid, the apoptotic rate (C), mitochondrial membrane potential (D), caspase-9 activity (F) and caspase-3 activity (G) were analyzed. Data represent the means ± SEM values from at least 3 separate experiments. Release of Cyt c was assessed by western blot analysis of cytosolic fraction, and β-tubulin was used as the loading control (E).

was required for the interaction with Bax (Fig. 5B). The Bax interacting region (241-382aa) induced apparent apoptosis of pancreatic cancer cells. Even though certain Cx43 plasmid lengths did not interact with Bax, there was still some impact on pancreatic cell apoptosis (Fig. 5C). However, the Bax interacting region (241-382aa) was required for depolarizing the

mitochondrial membrane and releasing Cyt c (Fig. 5D and E). Caspase 9, as a mitochondria-mediated specific caspase, was activated by Cx43 fragments containing the 241-382aa region (Fig. 5F). Caspase-3, as a downstream effector caspase, was activated by either Bax interacting or non-interacting Cx43 fragments (Fig. 5G). These results indicate that the interaction between Cx43 and Bax is necessary for initiating the mitochondrial apoptotic pathway.

## Discussion

Cx43 has been reported to exhibit anti-tumor effects in various types of cancer. The downregulation of Cx43 promotes an aggressive breast cancer cell phenotype (9). Cx43 is related to the occurrence, development and metastatic potential of gastric cancers (10). The tumor suppressive role of Cx43 has several aspects, including cell proliferation, invasion and metastasis. The expression of Cx43 in pancreatic cancer cells caused apparent growth inhibition. The subsequent rescue experiment further demonstrated that growth inhibition was regulated by Cx43. Of note, pancreatic cancer cells showed an increase in the apoptotic rate with Cx43 expression. When the cells were treated with gemcitabine, which is the most widely used drug for pancreatic cancer, sensitivity to apoptosis correlated with the level of Cx43. Increased sensitivity to apoptotic stimuli was evident in the cells with higher levels of Cx43, such as CFPAC-1 cells. In the PANC-1 cells, the lower level of Cx43 rendered resistance to gemcitabine treatment. Cx43 has shown similar apoptotic regulation in other types of cancer. The overexpression of Bcl-2 in Cx43-transfected cells confers resistance to apoptosis induced under low-serum conditions in human glioblastoma cells (11). Cx43 increases the sensitivity of prostate cancer cells to TNFα-induced apoptosis (12). The protective effect of Cx43 has also been found in certain normal cells. In human epithelial cells, Cx43 has been shown to protect cells from oxidative stress (13). The different role of Cx43 is due to sophisticated mechanisms. Cx43 exerts its function through either GJ-dependent or -independent signaling pathways. Even in the case of GJs, it can transmit pro-apoptotic and anti-apoptotic signals to control the 'destiny' of cells.

We then tried to unravel the mechanism related to Cx43-regulated apoptosis. To differentiate between a GJ-dependent and -independent pathway, we used β-GA (a broad spectrum inhibitor of GJ) and a Cx43 mutant (a specific inhibitor of Cx43-mediated GJ). Even when GJs were significantly decreased, Cx43-regulated apoptosis was not compromised in pancreatic cancer cells. These data suggest that a GJ-dependent pathway is not required for Cx43-regulated apoptosis. GJ-independent pathway has attracted much attention in recent years (14). Cx43 controls $Ca^{2+}$ homeostasis to regulate cell death induced by a variety of insults, which has no correlation with Cx43-mediated GJs (15). Increasing evidence has indicated that Cxs, including Cx43, may control cell growth and inhibit tumorigenicity independent of GJs (14,16). The c-terminal tail of Cx43 is thought to be crucial for a GJ-independent pathway. Cx43 interacts with a large number of signaling proteins via intracellular carboxyl tail to regulate cellular functions (17,18). From the results of the present study, it can be concluded that GJ-independent pathways play major roles in regulating the apoptosis of pancreatic cancer cells.

Our study showed that Cx43 translocated to the mitochondria with gemcitabine treatment. Other studies have also demonstrated that Cx43 is located at the mitochondria of cardiomyocytes besides the cell membrane (19,20). In response to Wnt signaling, Cx43 interacts with β-catenin and translocates to the nucleus to regulate downstream gene expression (21). Further evidence has indicated that GJ-independent control of cell growth occurs through the aberrant localization of Cxs (14). The aberrant localization of Cxs leads to interactions with other signaling molecules or to the formation of hemichannels on organelles. Two major apoptotic pathways have been identified in mammalian cells: intrinsic and extrinsic pathways. The extrinsic apoptotic pathway is triggered by the engagement of so-called 'death receptors' on the cell surface. The intrinsic pathway is provoked by intercellular organelles, such as the mitochondria, endoplasmic reticulum and other organelles (22,23). Our data showed that the intrinsic apoptotic pathway was activated by mitochondrial Cx43 in pancreatic cancer cells. MMP depolarization, the Cyt c release and specific caspase activation, characteristics of the mitochondrial apoptotic pathway, were induced by Cx43. Consistent with our results, other reports have shown that Cx43 is a key regulator of mitochondrial physiology and myocyte apoptosis (19,24). Mitochondrial Cx43 is also a new player in the pathophysiology of myocardial ischemia-reperfusion injury-related apoptosis (19,25).

During stress, the Bcl-2 family is mainly responsible for the fate of cells (whether apoptosis will be induced or not) (26). Mitochondrial Bcl-2 family proteins are important to the intrinsic apoptotic pathway. The ratio of Bax/Bcl-2 was increased with Cx43 expression. These results are consistent with those from a previous study on human glioblastoma cells by microarray analysis (27). Changes in Bax or Bcl-2 proteins were mainly related to mitochondrial localization. Co-immunoprecipitation assay showed that Cx43 interacted with Bax, instead of Bcl-2 to regulate the apoptosis of pancreatic cancer cells. It is possible that Cx43 interacts with other Bcl-2 family members. Further studies are required to elucidate potential interactions between Cx43 and other Bcl-2 family proteins.

To understand the importance of the Cx43/Bax interaction in regulating cell apoptosis, different lengths of the Cx43 plasmid were constructed. Our data indicate that 241-382aa is required for the Cx43/Bax interaction. This region is heavily modified by post-transcriptional regulation. The ERK (28), casein kinase 1 (29), c-Src (30) and PKC (31) phosphorylation sites reside in the 241-382aa region. These modifications are involved in regulating GJ and protein interactions. The 241-382aa fragment induced apparent apoptosis. While some Cx43 fragments, such as 1-240aa, did not interact with Bax, apoptosis still occurred. This suggested that other pathways were also involved, such as a GJ-dependent pathway. However, the Bax interacting region (241-382aa) was required for inducing the Cyt c release, MMP change and caspase-9 activation. Therefore, the Cx43/Bax interaction was involved in prompting the mitochondrial apoptotic pathway. Therefore, subsequent mitochondrial membrane depolarization relies on Bax. Bax recruits Cx43 to the mitochondria, where Cx43 forms hemichannels to transduce apoptotic signals, including calcium signaling and Cyt c release.

Taken together, our data indicate that Cx43 regulates the growth and apoptosis of pancreatic cancer cells. Cx43 interacts with Bax to initiate the mitochondrial apoptotic pathway.

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