Cx32 inhibits TNFα-induced extrinsic apoptosis with and without EGFR suppression

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Abstract. Tumor necrosis factor α (TNFα) and TNF-related apoptosis-inducing ligand (TRAIL) can trigger the extrinsic apoptosis pathway. Our previous study indicated that connexin32 (Cx32) inhibited streptonigrin-induced intrinsic apoptosis via the epidermal growth factor receptor (EGFR) pathway. However, whether Cx32 can exert effects on the extrinsic apoptosis pathway through EGFR signaling remains unclear. In the present study, we investigated the role of Cx32 in extrinsic apoptosis induced by treatment with TNFα + cycloheximide (CHX) or afatinib in human cervical cancer (CaCx) cells. In stable inducible Cx32-transfected HeLa cells (HeLa-Cx32), Cx32 expression was induced by treatment with doxycycline (Dox). Furthermore, C-33A cells, which natively express high levels of Cx32, were used as a cell model for knockdown of Cx32 with siRNA. To determine the non-junctional function of Cx32 in apoptosis, 18α-glycyrrhetinic acid (18α-GA), a gap junction intracellular communication (GJIC) inhibitor, was used. Our results showed that Cx32 could inhibit apoptosis induced by TNFα + afatinib with or without the GJIC inhibitor. In clinical cervical tissue samples, we found that the expression of survivin was markedly higher in CaCx than in normal cervix tissue, which was in accordance with the expression of Cx32 in our previous study. In HeLa-Cx32 cells, we also found that Cx32 upregulated the expression of Cox-2. In addition, Cx32 upregulated EGFR expression in low-density culture (lacking GJ formation). Cx32 could also promote the expression of EGFR, phospho-STAT3 and phospho-ERK in HeLa-Cx32 cells following TNFα treatment. After knocking down Cx32 in C-33A cells, the expression levels of survivin and TNFα were downregulated. The present study verifies that Cx32 exerts an inhibitory effect on extrinsic apoptosis in CaCx cells, and suggests that Cx32 may regulate the progression and micro-environment of CaCx cells.

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Introduction

Gap junctions (GJs), principally composed of connexins (Cxs), are important for communication between tumor cells and stromal cells. Increased GJ coupling has been reported to hinder metastatic potential in a number of animal tumor models, including breast cancer and melanoma (1). Although many studies sustain the viewpoint that Cxs are tumor suppressors, recent evidence suggests that, in some tumor types, they may promote certain stages of tumor progression through junctional and non-junctional pathways (2). Cx26, but not Cx40 or Cx43, was shown to suppress tumorigenic features in cervical cancer (CaCx) HeLa cells, even though all three Cxs increased GJ intracellular communication (GJIC) (3). Downregulation in Cx32 results in the proliferation and metastasis of hepatocellular carcinoma (HCC), and the restoration of Cx32 expression may be a prospective strategy for the treatment of HCC (4). However, accumulation of cytoplasmic Cx32 can increase the self-renewal of cancer stem cells (CSCs) to expand the CSC population in HCC (5).

Tumor necrosis factor α (TNFα) activates caspase-8 in the extrinsic pathway of apoptosis. Aberrant secretion of TNFα facilitates a number of human diseases and has been implicated in tumor development and inflammation (6). In particular, TNFα polymorphisms have been associated with cervical cancer (7). Persistent high-risk human papillomavirus (HPV) infection gives rise to inflammation-associated CaCx

progression. HPV-negative head and neck cancers express epidermal growth factor receptor (EGFR) to a high level, and a monoclonal antibody for EGFR (cetuximab) is currently the only targeted therapy that has improved survival in patients with this disease (8). To prevent the retention of HPV within cells, TNFα-induced apoptosis is a key defense strategy (9); however, the function of Cx32 proteins in this mechanism of extrinsically triggered cell death remains unknown.

Our previous study demonstrated that Cx32 regulated EGFR expression and exerted a pro-tumor effect in CaCx (10). Cx32 can suppress endogenous apoptosis induced by streptonigrin in CaCx. However, the role of Cx32 in TNFα-induced extrinsic apoptosis is unclear. A high CaCx systemic inflammation score has been correlated with more advanced FIGO stages and poor tumor differentiation (11). In the present study, we investigated whether Cx32 was a key regulator of tumor growth associated with TNFα-related inflammation.

Materials and methods

Materials. Dimethyl sulfoxide (DMSO), 18α-glycyrrhetinic acid (18α-GA), 2-aminoethoxydiphenyl-borate (2-APB), anti-β-tubulin and anti-β-actin mouse IgG primary antibodies, and secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-Cx32 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against Cox2, EGFR, p-ERK1/2 (Thr202/Tyr204), STAT3, p-STAT3 (Tyr705), TNFα and survivin were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). TNFα was obtained from PeproTech, (Rocky Hill, NJ, USA). Hygromycin B, G418 and doxycycline (Dox) were obtained from Calbiochem (San Diego, CA, USA). An Annexin V-FITC apoptosis detection kit was purchased from BioTool, LLC (Houston, TX, USA). Cycloheximide (CHX) was purchased from Beijing Dingguo Changsheng Biotechnology, Co., Ltd. (Beijing, China). Lipofectamine™ 2000 was purchased from Gibco (Carlsbad, CA, USA). Calcein-AM (acetoxymethyl ester) was obtained from Invitrogen (Carlsbad, CA, USA). All other reagents were purchased from Sigma-Aldrich unless stated otherwise.

Clinical tissue specimens. The clinical cervical carcinoma and para-carcinoma tissue samples were obtained from the Affiliated Tumor Hospital of Xinjiang Medical University (Urumqi, China). Cervical tissue samples (n=15) were resected during surgery. The use of these clinical samples was approved by the ethics committee of Xinjiang Medical University Affiliated Tumour Hospital.

Cell lines and cell culture. The C-33A cell line was acquired from the American Type Culture Collection (ATCC; Manassas, VA, USA). C-33A cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum (FBS). Cx32 under the control of a bidirectional tetracycline-inducible promoter was stably transfected into HeLa cells (HeLa-Cx32) for subsequent induction of Cx32 expression via incubation with Dox (1 μg/ml) for ~48 h (12). Prior to treatment with Dox, 100 μg/ml G418 sulfate and 200 μg/ml hygromycin B were added to the medium [Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS] to select for stably transfected cells. Besides high-density culturing, we also use a low-density culture method, and the HeLa-Cx32 cells were seeded into 150-mm dishes. At this low-density, there was a lack of cell-cell contacts, which prevented the formation of GJs and enabled investigation into the non-junctional function of Cx32 in apoptosis.

GJ functional assay. GJIC function was evaluated using a 'parachute' dye coupling assay, as described by Goldberg *et al* (13) and Koreen *et al* (12). The experiment was devided into control group, Dox group, Dox+18α-GA group and Dox+2APB group. In this assay, donor and receiver HeLa-Cx32 cells were grown to confluence in 12-well plates. After the cells were cultured to confluence, donor cells were labeled with 5 μM calcein-AM for 30 min at 37˚C. The donor cells were then rinsed, trypsinized and seeded onto the receiver cells at a 1:150 donor/receiver ratio. The donor cells were incubated at 4 h at 37˚C to allow attachment to the monolayer of receiver cells and the formation of GJs. Cells were subsequently observed under a fluorescence microscope (Olympus IX71; Olympus Corp., Tokyo, Japan). Donor cells can be labeled and observed by strong calcein-AM staining. Calcein-AM from donor cells can be intracellularly transferred into receiver cells when GJIC is present. The level of GJIC was measured as the average number of receiver cells containing Calcein-AM per donor cell observed by fluorescence microscopy.

Cx32 siRNA and EGFR interference assay. After growing C-33A cells to 30-50% confluence, 50 nM of non-specific (NS) siRNA (negative control) or Cx32-siRNA (Guangzhou RiboBio, Co., Ltd., Guangzhou, China) and Lipofectamine™ 2000 were mixed and added to cells. Lipofectamine™ 2000 was used to transfect cells according to the manufacturer's protocol. After incubation with the siRNAs for 48 h, further experiments were conducted in the cells.

For the subsequent knockdown of EGFR, the following EGFR siRNAs were synthesized: siEGFR_1,5'-GGCTGGTTA TGTCCTCATT-3'; siEGFR_2, 5'-CCTTAGCAGTCTTATCT AA-3'; and siEGFR_3, 5'-GGAACTGG ATATTCTGAAA-3'. Among them, siEGFR_1 was determined to be the most effective by western blot analysis, and was selected for an EGFR targeting assay.

For the subsequent knockdown of Cx32, the following Cx32 siRNAs were synthesized: siCx32_1, 5'-CCGGCATTC TACTGCCATT-3'; siCx32_2, 5'-GGCTCACCAGCAACAC ATA-3'; and siCx32_3, 5'-GCAACAGCGTTTGCTATGA-3'. Among them, siCx32_3 was chosen for further experiments after confirmation by western blot analysis.

Apoptosis assay. Approximately 1-2x10^5 HeLa-Cx32 cells/well were seeded into 6-well plates. After adherence, the cells were incubated with Dox or DMSO for 48 h. TNFα (100 ng/ml) was then added to the HeLa-Cx32 cells for 24 h, and cells were incubated with CHX (0.1 or 1 μg/ml) or afatinib (1.25 μM) except EGFR siRNA group. The cells were washed twice with phosphate-buffered saline (PBS) and trypsinized, terminated by medium and harvested. After the cells were centrifuged at 1,000 rcf for 5 min at room temperature and resuspended with PBS twice, binding buffer and Annexin V-FITC with propidium iodide (PI) were used to stain cells for 15 min away

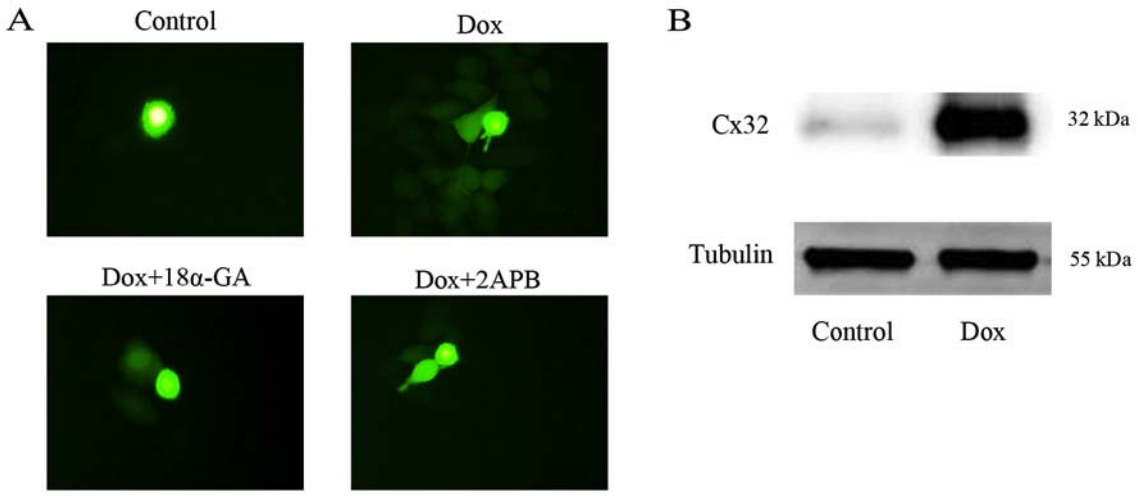

Figure 1. Inducible Cx32 expression model in HeLa cells (HeLa-Cx32) confirmed by western blot analysis. (A) Parachute assay of HeLa-Cx32 cells divided into the following 4 groups: Control, Dox, Dox+18α-GA and Dox+2APB. GJ function was detected after Cx32 induction with Dox, although was subsequently inhibited by 18α-GA or 2APB treatment. (B) Western blot analysis verified that Dox could induce the expression of Cx32 in HeLa-Cx32 cells. *P<0.05; n=3; n=1 represents an independent experiment.

from light at room temperature. Subsequently, the cells were immediately analyzed with a flow cytometer. Expo32 software was used to determine the rate of early apoptosis.

Western blotting. Homogenized tissue samples (50-100 mg) or cells were rinsed with PBS and treated with ice-cold lysis-buffer [1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM Na_3VO_4 and 1:1,000 protease inhibitors) for at least 30 min. After scraping, collection and ultrasonication, the lysate solutions were centrifuged at 12,000 rcf for 30 min at 4˚C, and the supernatant was retained. A BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure protein concentration. For western blotting, equal amounts (20 μg) of protein were prepared and separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% (w/v) skimmed dry milk in wash buffer [TBS and 0.05% Tween-20 (TBST)] for 1 h. The membranes were then incubated with monoclonal antibodies against Cx32 (1:1,000), EGFR (1:1,000), p-ERK (1:1,000), STAT3 (1:1,000), p-STAT3 (1:1,000), Cox-2 (1:1,000), survivin (1:1,000), TNFα (1:1,000), β-actin (1:10,000) and β-tubulin (1:10,000) overnight at 4˚C. HRP-conjugated secondary antibodies were applied to the membranes for 1-2 h at room temperature, and the membrane was then washed with TBST. Immunoreactive bands on the membrane were visualized using Western Lightning chemiluminescence reagents (Thermo Fisher Scientific). β-tubulin and β-actin were used as control markers. The control bands were set as '100' and the fold changes in each sample's ratio to the control bands were used as the finalized data.

Statistical analysis. All experiments were repeated at least three times. The data were presented as the mean ± standard error (SE) and analyzed using the SPSS 16.0 software. Statistical significance (P<0.05) was analyzed by one-way ANOVA (>2 groups) or a Student's t-test (2 groups). Western blot data was analyzed with the ImageJ software. Histograms or scatter diagrams were constructed with the Prism software. P<0.05 indicates a significant difference.

Results

Inducible Cx32 expression model in HeLa cells (HeLa-Cx32) confirmed by western blot analysis. The GJ function of the HeLa-Cx32 cell model was assessed with a parachute assay, as depicted in Fig. 1A. Results of the control group, Dox group, Dox+18α-GA group and Dox+2APB group are displayed in the figure. The results showed that after induction with Dox, GJs were formed, and also indicated that 18α-GA and 2APB could effectively suppress GJ function. The drug concentrations of 10 μM 18α-GA and 50 μM 2APB were selected according to our former study (10). As shown in Fig. 1B, western blotting indicated that Dox induced high-level expression of Cx32, and thus this cell model was used in subsequent experiments.

Cx32 expression and EGFR-related signal molecules in the different HeLa-Cx32 cell groups. As shown in Fig. 2A, a low-density culture was established to prevent GJ formation and the expression of EGFR was subsequently detected. We found that Cx32 could modulate EGFR expression without GJ formation. Furthermore, after incubation with TNFα for 24 h, the high expression levels of Cx32 in HeLa-Cx32 cells (induced by Dox) could promote the expression of EGFR, p-STAT3 and p-ERK, without changing the expression of total STAT3 (Fig. 2B). These results indicated that Cx32 could upregulate EGFR not only in the absence of GJ formation, but also after treatment with TNFα.

Cx32 inhibits apoptosis induced by TNFα plus CHX or afatinib in HeLa-Cx32 cells. According to the reported ability of TNFα to induce apoptosis (14), we used TNFα (50 ng/ml) + CHX (0.1 or 1 μg/ml) to induce apoptosis, as depicted in Fig. 3A. The results showed that apoptosis induced by TNFα and CHX co-treatment was inhibited by Cx32 upregulation following treatment with the GJ inhibitor 18α-GA. Afatinib

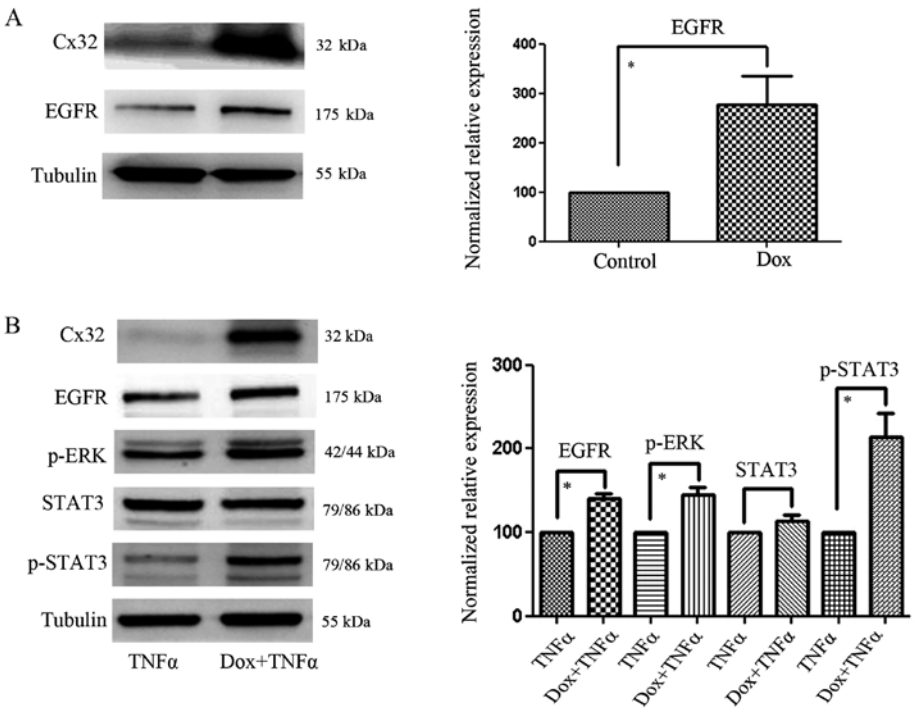

Figure 2. Cx32 expression and EGFR-related signal molecules in the different HeLa-Cx32 cell groups. (A) A low-density culture of HeLa-Cx32 cells was established to block GJ formation, and the expression of EGFR was detected. (B) After incubation with TNFα for 24 h, high-level Cx32 expression (induced by Dox) could promote the expression of EGFR, phospho-STAT3 and phospho-ERK, without changing the expression of STAT3. *P<0.05; n=3-5, n=1 represents an independent experiment.

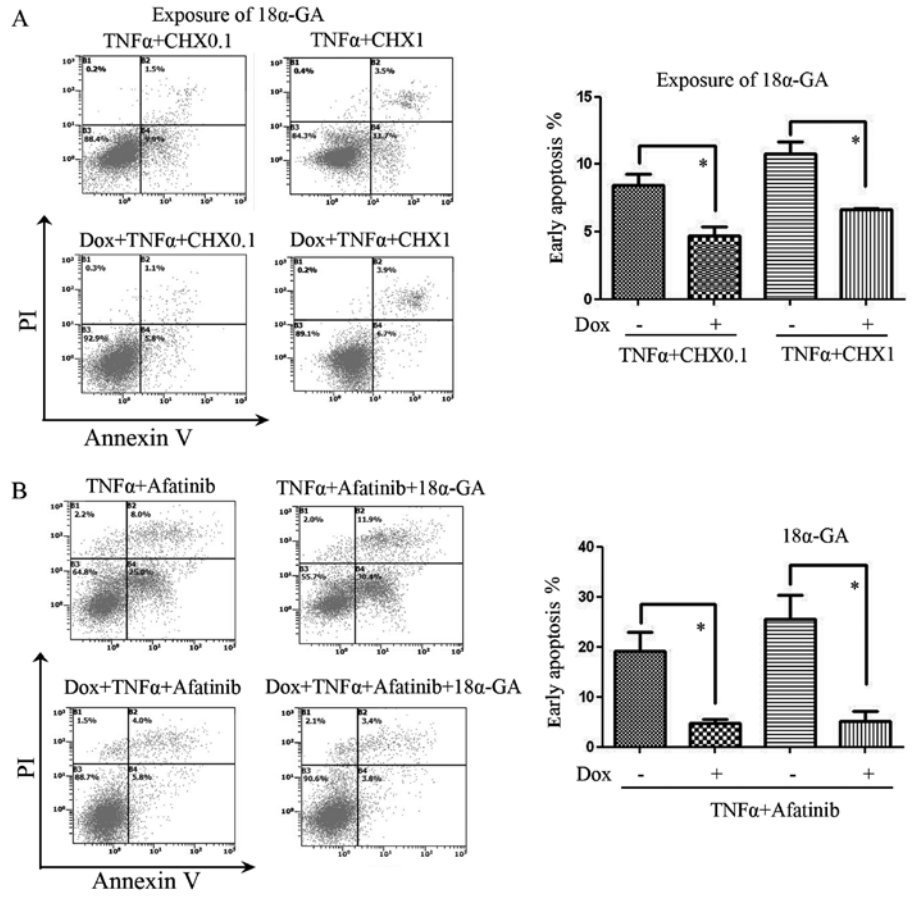

Figure 3. Cx32 inhibited apoptosis induces by TNFα plus CHX or afatinib in HeLa-Cx32 cells. (A) Cell apoptosis induced by TNFα+CHX co-treatment was inhibited by Cx32 expression following treatment with the GJ inhibitor 18α-GA. (B) Afatinib was used in co-treatment with TNFα to inhibit EGFR, and the results showed that, with and without GJ inhibition by 18α-GA, the anti-apoptotic effect of Cx32 against TNFα was present. *P<0.05; n=3-4; n=1 represents an independent experiment.

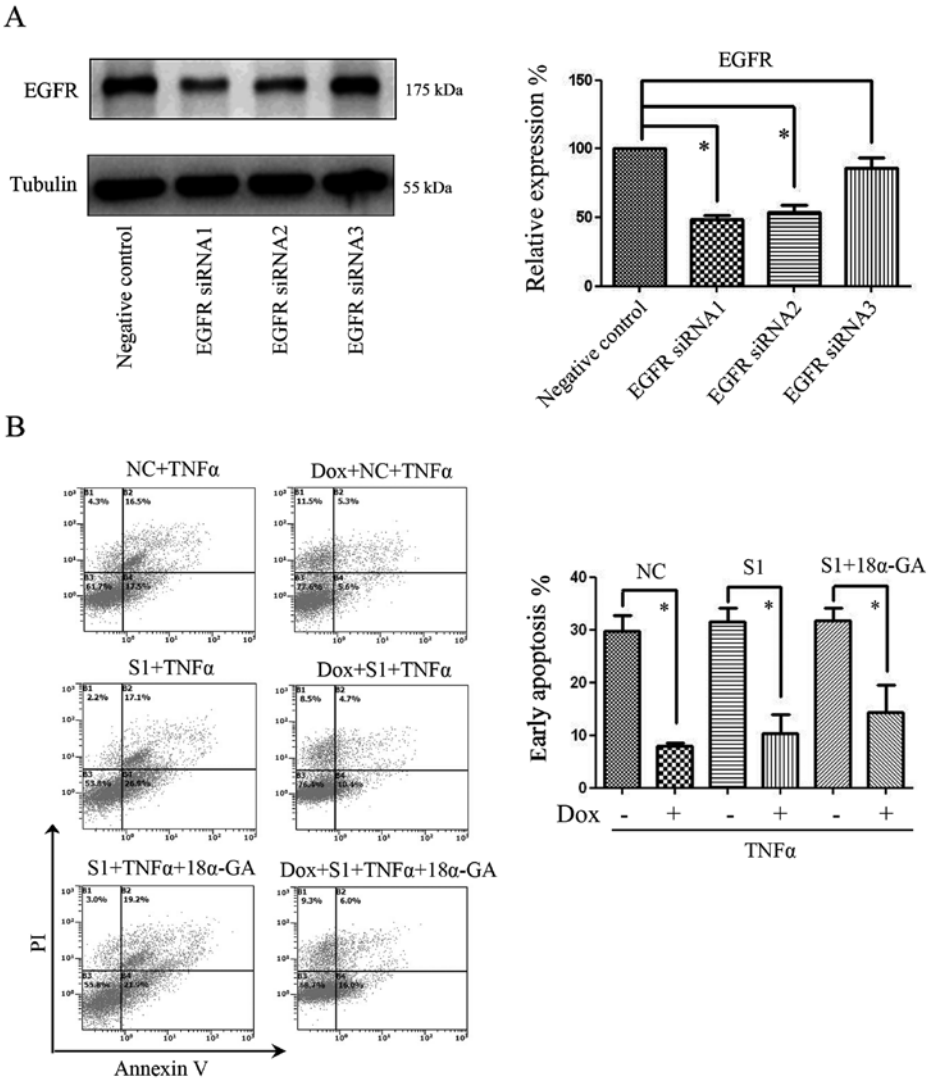

Figure 4. Effect of Cx32 on the apoptosis of HeLa-Cx32 cells after transfection with EGFR siRNA. (A) Using NS siRNA as a negative control (NC), three EGFR siRNAs (S1, S2 and S3) were used to knock down EGFR expression. EGFR siRNA1 (S1) was determined to be the most efficient fragment, and thus was used in further experiments. (B) The results showed that, with or without GJ inhibition by 18α-GA, EGFR siRNA did not reverse the anti-apoptotic effect of Cx32 against TNFα. *P<0.05; n=3-4; n=1 represents an independent experiment. S1, EGFR siRNA1.

(1.25 μM) was also used in co-treatment with TNFα to inhibit EGFR. The results showed that, with or without GJ inhibition by 18α-GA, the anti-apoptotic effect of Cx32 against TNFα + afatinib was present (Fig. 3B).

Effect of Cx32 on the apoptosis of HeLa-Cx32 cells after transfection with EGFR siRNA. Due to the multi-functionality of EGFR inhibitors, we used siRNA to specifically reduce EGFR expression in our further experiments. Using an NS siRNA as a negative control, three EGFR siRNAs (S1, S2 and S3) were used to knock down EGFR expression. Among these, EGFR siRNA1 (S1) was determined to be the most efficient fragment, and thus was used in our further experiments (Fig. 4A). The results showed that with or without GJ inhibition by 18α-GA, the anti-apoptotic effect of Cx32 against TNFα was present, even after transfection with EGFR siRNA (Fig. 4B).

Cx32 expression correlates with the expression of Cox-2, survivin and TNFα in cervical cancer cells. Cox-2 is an important factor for the prognosis of CaCx. After the upregulation of Cx32 was induced with Dox, we found that Cox-2 was also upregulated (Fig. 5A). Our previous study demonstrated that Cx32 expression was higher in CaCx than in normal cervical tissue. Compared with para-carcinoma tissue exhibiting low Cx32 expression, the expression of survivin in CaCx was markedly increased and coincided with Cx32 variation (Fig. 5B). Furthermore, in the CaCx cell line C-33A, after knockdown of Cx32 with siRNA, the expression levels of survivin and TNFα were found to be reduced (Fig. 5C). As the expression levels of TNFα, EGFR and survivin have been associated with anti-apoptotic processes and the tumor micro-environment, these results suggest that Cx32 may serve as a tumor enhancer in CaCx.

Discussion

Previous results have indicated that GJs may enhance the bystander effect in tumor cells (15). However, through the upregulation of death receptor 5 and downregulation of Cx43, carbenoxolone (an inhibitor of GJIC) has been found to enhance

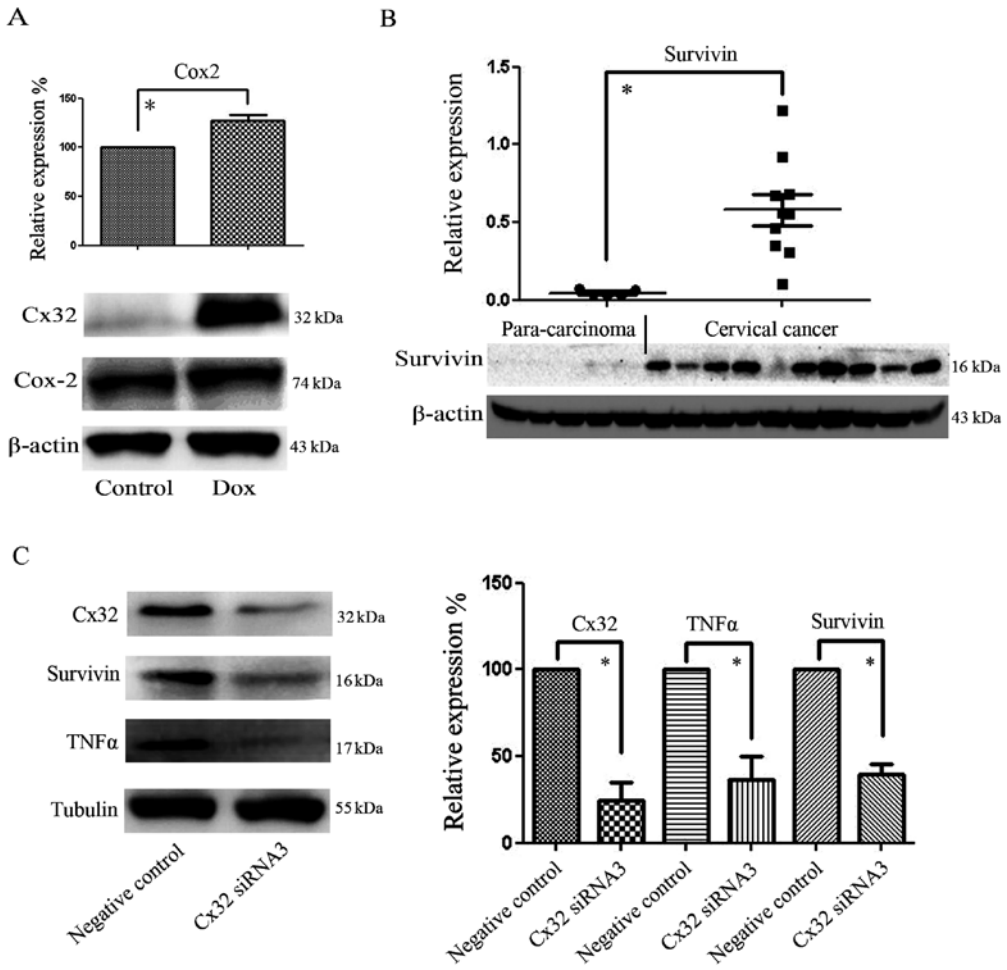

Figure 5. Cx32 expression correlates with the expressions of Cox-2, survivin and TNFα in cervical cancer cells. (A) After the expression of Cx32 was induced with Dox, Cox-2 was also upregulated in HeLa-Cx32 cells (n=3). (B) Western blot analysis of survivin expression in cervical cancer and para-carcinoma tissues expressing different levels of Cx32 (n=5-10). (C) After siRNA knockdown of Cx32 in C-33A cells, the expression levels of survivin and TNFα were reduced (n=3). *P<0.05; n=1 represents an independent experiment.

TRAIL-induced apoptosis (16). In contrast to the effects of Cx26, irradiated HeLa cells expressing Cx32 have previously showed enhanced survival capacity and greater metabolic activity relative to control cells (17,18). However, whether the promotion of GJIC involving Cx32 can increase or decrease the efficacy of antitumor drugs is not well defined (2). As the effect of GJs on apoptosis is complex, the present study focused on the non-junctional function of Cx32 in the apoptosis of CaCx cells.

Cx proteins may act as signaling effectors and activate the canonical mitochondrial apoptotic pathway, independently of their functional roles within GJs or hemichannels (19). Among the Cx proteins, Cx43 is the most widely studied. In a series of breast cancer samples, elevated levels of Cx43 were found to serve as positive prognostic markers, while elevated levels of Cx30 were shown to be negative prognostic markers (20). Thus, regarding the impacts of Cx proteins on tumors, Cx family members and the host tissues should be considered and assessed. Additionally, Cx mutations and aberrancies in their distribution require evaluation. For instance, aberrant trafficking of a Leu89Pro Cx32 mutant was found to be associated with X-linked dominant Charcot-Marie-Tooth disease (21). Strong Cx43 expression has been detected in the inner mitochondrial membrane within cardiomyocytes (22). According to our previous study, Cx32 is also expressed in the nucleus of CaCx tissue (10), and thus the present study further explored the function of Cx32 in CaCx cells.

Through activation of PKC-δ, EGF can protect CaCx ME180S cells from apoptosis induced by TNFα (23). EGFR and ErbB2 are important mediators of TNFα-regulated anti-apoptotic signals in intestinal epithelial cells (24). To determine whether the anti-apoptotic function of Cx32 was related to EGFR signaling, we used afatinib and siRNA to inhibit the function and expression of EGFR, respectively. However, the results showed that the effect of Cx32 on extrinsic apoptosis was not significantly altered after EGFR inhibition. In addition to afatinib, erlotinib can also be used to inhibit EGFR. The process of cell autophagy may serve as a protective mechanism against EGFR inhibitors, as inhibition of autophagy has been found to enhance the sensitivity of erlotinib in EGFR-mutated non-small cell lung cancer (25). Whether the resistive effects of Cx32 against afatinib and TNFα in the present study are related to autophagy is unclear and warrants further investigation.

A previous study documented that STAT3 inhibition markedly increased the sensitivity of HPV-related cancer to TRAIL-based therapy (26). Notably, it has been reported that EGFR inhibition may be enhanced by inhibition of the STAT3

pathway; compared with the inhibition of each pathway alone, combined blockade of both the EGFR and STAT3 pathways was more effective against human ovarian cancer *in vitro* and *in vivo* (27). Our results showed that in cell groups treated with TNFα, p-STAT3 expression was higher in the Cx32 high-expression group, which may explain the resistance of Cx32 to EGFR inhibition. A previous study reported that inhibition of the EGFR oncogene induced formation of an EGFR-TRAF2-RIP1-IKK complex, which stimulated an NF-κB-mediated transcriptional survival program (28). In addition, TNFα regulates NF-κB signaling, and thus whether NF-κB signaling is a key factor in the resistance of Cx32 to EGFR inhibition requires further investigation. Additionally, a previous study found that TNFα-induced NF-κB activation was not blocked by EGFR or Src inhibition, suggesting that TNFα may exert both EGFR-dependent and -independent effects (29).

Overexpression of survivin is involved in drug resistance in cancer cells, and reduces patient survival rate after chemotherapy and radiotherapy. Antagonism of survivin function renders cancer cells sensitive to the pro-apoptotic affects of TNFα, indicating that survivin blocks the extrinsic pathway of apoptosis (30). Our data showed that survivin expression was reduced in C-33A cells after knockdown of Cx32 with siRNA. This result demonstrates the potential relationship between Cx32 and survivin, which may account for the anti-apoptotic effect of Cx32 in CaCx cells.

Several studies have shown that TNFα, Fas and TRAIL serve as critical factors in the tumor environment (31). Abnormal secretion of TNFα contributes to a number of human diseases, and has been implicated in tumor development and inflammation (6). Notably, in endotoxemic mice, inhibition of EGFR activation decreased the production of TNFα in the myocardium (32). As our western blot results showed that Cx32 expression was correlated with both EGFR and TNFα expression, it is possible that the relationship between EGFR and TNFα is co-regulated by Cx32. One speculation is that Cx32 effects the tumor micro-environment by changing the expression of TNFα, EGFR, survivin and associated factors, although this requires further investigation.

In conclusion, high expression of Cx32 appeared to produce anti-apoptotic effects independently of GJs, and also modulated the expression levels of TNFα, Cox-2 and survivin, which may alter the tumor micro-environment in CaCx.

Acknowledgements

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