RESEARCH ARTICLE

ANXA2 Regulates the Behavior of SGC-7901 Cells

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Abstract

ANXA2, a member of the annexin family, is overexpressed and plays important roles in tumor development. However, the significance of ANXA2 expression in gastric carcinoma has not been clarified.To elucidate its roles in growth of gastric cancer, ANXA2 expression in SGC-7901 cells was inhibited with a designated siRNA, then cell proliferation, cell cycling, apoptosis and motility were determined by MTT assay, flow cytometry, Hoechst 33342 staining and wound healing assay, respectively. To further assess the behavior of ANXA2 deleted SGC-7901 cells, changes of microstructures were observed under fluorescence microscopy, laser scanning confocal microscopy and electron microscopy. We found that inhibition of ANXA2 expression caused cell proliferation to decrease significantly with G1 arrest, motility to be reduced with changes in pseudopodia/filopodia structure and F-actin and β-tubulin expression, and apoptosis to be enhanced albeit without significance. At the same time, ANXA2 deletion resulted in fewer pseudopodia/filopodia, non-stained areas were increased, contact inhibition among cells reappeared, and expression of F-actin and β-tubulin was decreased, with induction of polymerized disassembled forms. Taken together, these data suggest that ANXA2 overexpression is important to maintain the malignancy of cancer cells, and this member of the annexin family has potential to be considered as a target for the gene therapy of gastric carcinoma.

Keywords: ANXA2 - cell microstructure - gastric carcinoma - motility - proliferation

Asian Pac J Cancer Prev, **14 (10)**, 6007-6012

Introduction

Gastric cancer is the second most common cause of cancer types in the world and the incidence of gastric cancer is higher in Asians than in Western populations. Different etiologic factors, such as Helicobacter pylori infection (Kim et al., 2012), a diet high in salt, smoking, and environment nitrates, have been associated with the development of gastric cancer. Surgical resection is the most effective treatment for gastric cancer and the efficacy of chemotherapy remains limited. In recent years, molecularly gene therapy for treatment in gastric cancer has been given great concern. Therefore, identification of novel targets for gene therapy are major goals in this field.

Annexin A2 (ANXA2) is a member of the annexin family (Diaz et al., 2004), and plays important roles to regulate DNA synthesis (Yuan et al., 2007), cell proliferation (Wu et al., 2012), cytoskeleton reorganization (Singh et al., 2004), tumor invasion (Zhai et al., 2011), and metastasis (Zhou et al., 2012). The excessive expression of ANXA2 was reported to enhance the malignancy of numerous cancers in digestion system (Emoto et al., 2001; Esposito et al., 2006; Mohammad et al., 2008; Nedjadi et al., 2009).

To investigate how ANXA2 is involved with the cell malignancy of gastric cancer and evaluate its potential for gastric cancer gene therapy, we inhibited ANXA2 expression in SGC-7901 cells via siRNA method and analyzed the effects of ANXA2 on the cell proliferation, motility, apoptosis, and microstructures.

Materials and Methods

Cell culture

SGC-7901, a human gastric carcinoma cell line was purchased from Shanghai Institute of Cell Biology (Shanghai, China). The cells were cultured at 37°C and 5% CO_2 in an incubator. The medium, RPMI-1640, was supplemented with 10% (v/v) fetal bovine serum.

siRNA plasmid construction and transfection

The designated siRNA targeting to ANXA2 mRNA was named as siRNA2 (5'-GCACACTTGGAGAGCTG AGGTC-3'), and the negative control siRNA (5'-GCATC TAAGGTATCGTTGTGGCTC-3') was named as siRNASCR. The genes of the both siRNA were cloned into a dual promoter plasmid vector, pU6H1-GFP, and the constructed plasmids were respectively named as pU6H1-GFP-siANA2 and pU6H1-GFP-siRNASCR. The amplified and endotoxin removing constructed plasmids

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were induced into SGC-7901 cells using the optimized calcium phosphate transfection method as the follows: (1) One day prior to transfection, transfer the SGC-7901 cells into 6-well format with fresh RPMI 1640 + 10FBS medium so that they will be 80% confluent on the day of the experiment. (2) Four hours before transfection, replace RPMI 1640 + 10FBS medium with DMEM + 10 FBS medium (without antibiotics). (3) Gently mix 4 μg DNA, 85 μl 2 × HBS buffer (50 mM HEPES, 280 mM NaCl, 10 mM KCl, 12 mM Glucose, 1.5 mM Na2HPO4·2H_2O, pH 7.05) and 75 μl H_2O in a 2 ml tube. Slowly add 10.5 μl 2 M $CaCl_2$ and then pipette to mix the mixture for several seconds. (4) Incubate them for 25 minutes at room temperature. (5) Add the DNA + calcium phosphate mixture into cell culture in 6-well format gently and evenly. (6) Culture the transfected cells for 17 hours at 37°C and then replace the medium with RPMI-1640 + 10 FBS medium. (7) Measure the transfection efficiency by counting the cells expressing reporter gene under fluorescence microscope. The experiments were repeated three times independently.

Semi-quantitative RT-PCR assay

SGC-7901 cell samples were harvested at 72 and 96 h individually post transfection. Total RNA was isolated from cells using an RNeasy kit (Biozol reagent; BIOER, Hangzhou, China) following the manufacturer's instructions. Complementary deoxyribonucleic acids (cDNAs) were reverse-transcribed from 1 μg of total RNA. The primer sequences of ANXA2 were as follows: sense, 5'-GCGTCTAATCCGACAGCA-3'; reverse, 5'-GCCGACTTCCTTCACCAT-3'. The products of PCR were checked by 1% agarose gel electrophoresis, and the abundance of each mRNA was detected and normalized to that of the house-keeping gene GAPDH mRNA. The primer sequences were as follows: sense, 5'-AATCCCATCACCATCTTCCA-3'; reverse, 5'-CCTGCTTCACCACCTTCTTG-3'. All PCR experiments were done in triplicate.

Western blot assay

When the cell confluency was about 90 % at 72 and 96 h post transfection, the SGC-7901 cell lysate was collected to run a 10 % SDS-PAGE and nitrocellulose membrane (PALL, CA) transfer. The transferred membrane was incubated with polyclonal rabbit-anti human ANXA2 antibody (1:2000, Santa Cruz Biotech, CA) first, then with HRP labeled goat-anti rabbit antibody (1:4000, Biosynthesis Biotech, Beijing, China). The membrane was developed using an ECL Kit (Pierce, Shanghai, China) following the manufacture's instruction. Experiment was repeated for three times independently.

MTT assay

Cell proliferation inhibition was analyzed by the 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 20 μl MTT (5 mg/ml) was added into 96-well after transfected with siRNA2 or siRNAscr for 24 h, 48 h, 72 h, 96 h, 120 h. Later, 150 μl DMSO was added to each well to dissolve formazan crystals. Absorbance was checked at 562 nm using an ELISA reader (BIO-TEK, USA). Three separate experiments were operated and the data were determined by comparison to DMSO-treated control cells.

Apoptosis assay

SGC-7901 cells were transfected with pU6H1-GFP-siANA2 or pU6H1-GFP-siRNASCR for 72 h. Cells were fixed with 4% paraformaldehyde in PBS (15 min, room temperature) and washed with PBS. Coverslips were stained with Hoechest 33342 and inspected with a fluorescence microscope.

Cell cycle assay

Cell cycles were examined by flow cytometry. SGC-7901 cells were transfected with pU6H1-GFP-siANXA2 or pU6H1-GFP- siRNASCR for 72 h. 2×10^5 cells were collected and fixed in 70% ethanol overnight at 4°C. Then, cells were washed twice with phosphate buffer solution, digested by 10 μl (10 mg/ml) and stained with 10ul of propidium iodide (50 ug/ml) at room temperature for 30min. The DNA histograms were generated with a flow cytometer (Guava easyCyte 8HT, Millipore, USA).

Wound healing assay

Cells were plated at 5×10^5 cells/well in 6-well dishes and incubated overnight yielding confluent monolayers for wounding. Briefly, 24 h post transfection, a small hole was created in cell monolayer with a 1ml pipette tip linked to vacuum pump. Photographs were taken 24 h and 24 h after wounding. Wound areas were measured by Gene Tools software. Migration rate = (1-At/A0) ×100%. A0 is the wound area at 0h, and At is the wound area at 24h, respectively.

Observation for the microstructure changes

Observation under scanning electron microscope (SEM): The siRNA2 group, siRNASCR group and wild type group SGC-7901 cells were cultured on the slide cover-glasses in the 6-wells plate for 72 h after transfection. The cover-glass was rinsed using PBS and fixed for 1 hour at 4°C using 2.5% Glutaraldehyde (Sigma, St. Louis, NY). The fixed cells were dehydrated using gradient diluted ethanol solutions (30%, 50%, 70%, 80% × 2, 90% × 2, 95% × 2, 100%×2, 15 mins/each). The dehydrated cells were further treated following the instruction book of the environmental scanning electron microscope (Quanta2000, Philips-FEI, Netherlands) and observed under this microscope.

Observation under transmission electron microscope (TEM): The cell grouping, fixing and dehydrating are same as the above. The cell samples were treated and observed according to the instruction book of the transmission electron microscope (JEM-2100, Japan).

Observation for the microstructure associated proteins

F-actin: The siRNA2 group, siRNASCR group and wild type group SGC-7901 cells were cultured on the slide cover-glasses in the 6-wells plate for 72 h after transfection. The cover-glass was rinsed using PBS for three times, fixed using 3.7% paraformaldehyde for 10

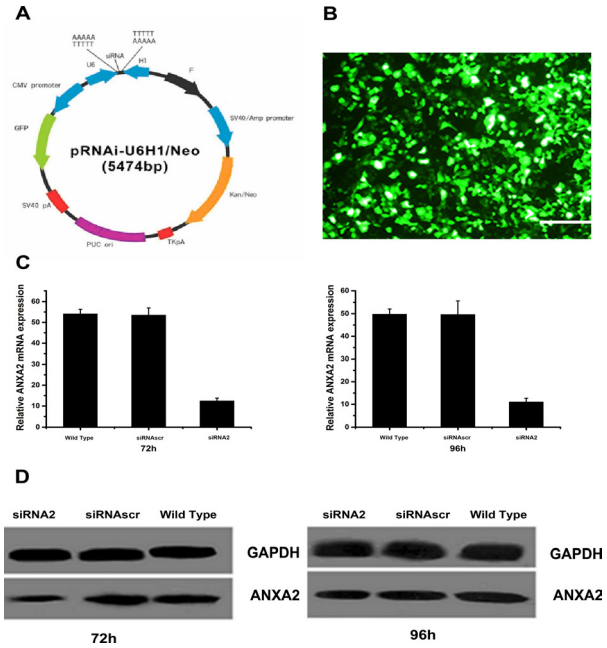

Figure 1. ANXA2 Expression was Knocked Down by SiRNA in SGC-7901 Cells. (A) The map of vector. (B) Transfection efficiency. pU6H1-GFP-siANXA2 transfected SCG-7901 cells for 72 h were observed under fluorescence microscope (100 ×). (C and D) 72 and 96 hrs post-transfection, the expression of ANXA2 was confirmed using western blot and semi-quantitative RT-PCR. The results indicated that the ANXA2 mRNA level in siRNA2 groups was significantly decreased (p<0.05) and the protein level was obviously blocked at least 63% in siRNA groups cells compared with siRNASCR group cells. Triplicate experiments were performed with essentially identical results

minutes at 37°C and treated with 0.1% TritonX-100 for 10 minutes at 37°C. The cover-glass was firstly incubated with 10% normal goat serum (Ding-Guo Biotech, Beijing, China) for 45 minutes at room temperature, and then with FITC-labeled phalloidine (2 μg/ml) (Ding-Guo Biotech, Beijing, China) for 60 minutes at room temperature. The cells were observed to check the F-actin changes under a laser scanning confocal microscope (TCS SP5, Leica, Germany).

β-tubulin: The steps before antibody incubation are same with the steps before FITC-labeled phalloidine incubation in F-actin staining. After the normal goat serum was removed, the cover-glass was incubated with rabbit-anti-human β-tubulin polyclonal antibody (1:50) (Ding-Guo Biotech, Beijing, China) at 4°C for overnight, and then with Cy3-labeled goat-anti-rabbit IgG (1:100) at 4°C for overnight. The cells were observed to check β-tubulin distribution under the fluorescence.

Statistical analysis

Experiments were performed independently at least three times. Results were expressed as the mean ± standard deviation (SD) and student's t-test was performed to compare results from different trails. Values were considered statistically significant when p<0.05.

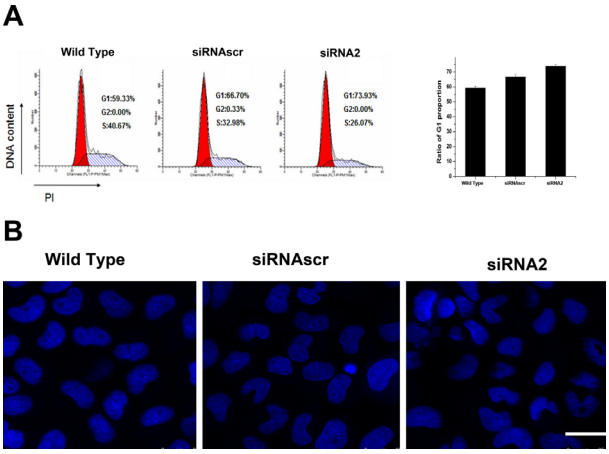

Figure 2. RNAi-mediated Depletion of ANXA2 Expression Reduced Cell Proliferation. (A) knockdown of ANXA 2 induced cell cycle arrest at G0/G1 phase. When compared with the siRNASCR group, the percentage of G0/G1 phase in the siRNA2 group was increased by 9.7% (P < 0.05) at 72 hrs after transfection. (B) Down-regulation of ANXA2 did not enhanced cell apoptosis. Hoechest 33342 staining were performed to analyze cell apoptosis at 72 hours post siRNA2 transfection. Scale bar, 100 μm

Results

ANXA2 expression was knocked down by siRNA in SGC-7901 cells

Over-expression of ANXA2, which has been reported in many kinds of digestive tract tumor cells, is supposed to be associated with cell proliferation, differentiation, invasion and metastasis during the pathogenesis of cancers. ANXA2 is also up-regulated in gastric carcinoma and it could contribute to the progression of gastric carcinoma (Zhang et al., 2012), however, the mechanisms of ANXA2 in gastric carcinoma have not been fully elucidated. In this study, we investigated the roles ANXA2 plays in the gastric cancer cell (SGC-7901 cell) progression including cell proliferation and motility. To study the role of ANXA2 in the malignant progression of gastric carcinoma, we designated siRNA to inhibit the expression of ANXA2 in SGC-7901 cells. Three visions of transfected cells were selected randomly and about 90% of cells emitted green fluorescence at 72 h after transfection under fluorescence microscope (Figure 1B). ANXA2 mRNA and protein expression was confirmed to be dramatically down-regulated in siRNA2 group compared to siRNASCR group and wild type group SCG-7901 cells (P<0.05). In addition, there was no obvious difference of ANXA2 expression between the wild type group and siRNASCR group SCG-7901 cells (P>0.05) (Figure 1C and D). These data indicate that the transfected ANXA2 siRNA significantly and specifically inhibits the ANXA2 expression in SGC-7901 cells.

RNAi-mediated depletion of ANXA2 expression reduced cell proliferation

The effect of down-regulation of ANXA2 on proliferation of SGC-7901 cells was assessed by cell cycle assay, MTT assay, and apoptosis. Firstly, to elucidate

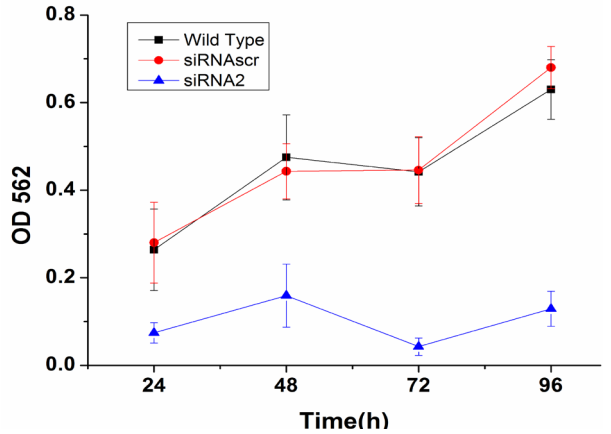

Figure 3. Down-regulation of ANXA2 Decreased Cell Growth. MTT assay was performed at 24 h, 48 h, 72 h and 96 h post siRNA transfection of SGC-7901 cells

the growth suppression effect of siRNA2 on SGC-7901 cells, we performed cell cycle distribution analysis by flow cytometry at 72 h after transduction. As shown in (Figure 2A), ANXA2 knockdown induced cell cycle arrest in G1. When compared with the siRNASCR group, the percentage of G0/G1 phase in the siRNA2 group was increased by 9.7% ($P < 0.05$) at 72 h after transfection.

The results demonstrate that ANXA2 silencing may induce cell cycle arrest at G1 phase

To further detect the effect of ANXA2 on the growth of SGC-7901 cells, viability curves for siRNA2, wild type and siRNASCR group SCG-7901 cells were determined by MTT assay. As shown in (Figure 3), the growth of siRNA2 group cells was inhibited notably when compared with other groups ($P<0.01$). However, there was no significant differences in cell growth between the wild type and siRNASCR groups SCG-7901 cells ($P>0.05$). These results indicate that down-regulation of ANXA2 expression by RNAi markedly inhibits the growth of SGC-7901 cells.

Moreover, cells stained with Hoechst 33342 were observed under fluorescence microscope to detect cell apoptosis. However, Figure 2B displayed no any different morphological changes after tansfection with pU6H1-GFP-siANXA2 or pU6H1-GFP-siRNASCR. The results indicated that down regulation of ANXA2 has no significant affect on the apoptosis of SGC-7901 cells.

RNAi-mediated depletion of ANXA2 expression impaired cell motility

To analyze the role of ANXA2 in gastric carcinoma cell motility, wound healing assay was performed. Migration of the cells to wounded areas was analyzed, and the percentage of wound closure was determined. As shown in (Figure 4A), wound closure was markedly reduced in siRNA2-transfected monolayers compared with controls. There was no significant difference of migration rates between siRNAscr group and NC group. Thus, siRNA-mediated down-regulation of ANXA2 expression inhibited SGC-7901 cells wound closure ability, suggesting that ANXA2 played an essential role in migration of SGC-7901 cells.

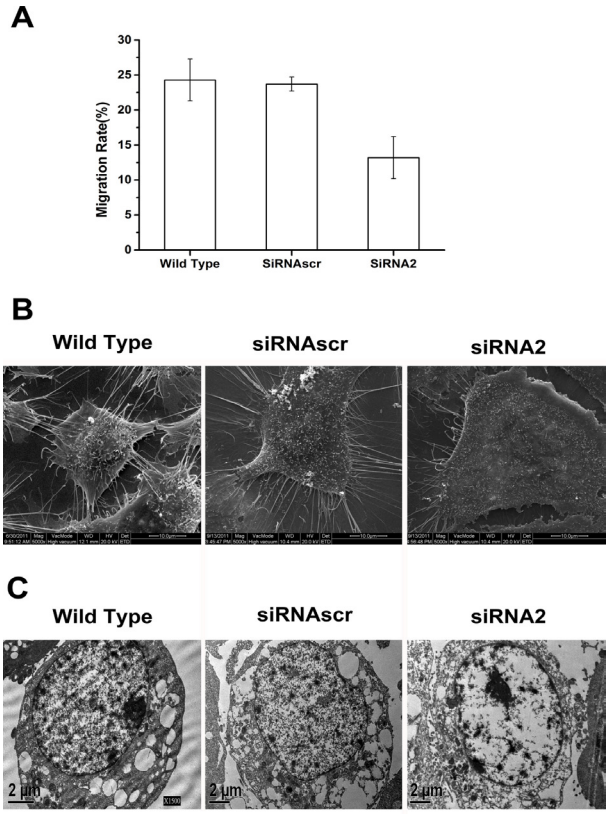

Figure 4. The Cell Microstructure Changes after RNAi-mediated Depletion of ANXA2 Expression. (A) Down-regulation of ANXA2 decreased SGC-7901 cell migration. Wound healing assay was performed at 24 h post siRNA transfection in SGC-7901 cells. (B) The cultured cells were observed under scanning electron microscope. The pseudopodia/filopodia of wild type and siRNASCR cells is much more vigorous than those of RNAi-mediated ANXA2 deleted SGC-7901 cells. The contact inhibition reappears in siRNA2 group. Scale bar, 10 μm. (C) The observation for cultured cells under transmission electron microscope. Much more cytosol transparent bubbles are formed in siRNA2 group cells than in wild type and siRNASCR SGC-7901 cells. Scale bar, 2 μm

The cell microstructure changes after RNAi-mediated depletion of ANXA2 expression

The enhanced motility is necessary for the cancer cells with powerful migration behavior, and pseudopodia/filopodia are essential structures for such a cell type. Pseudopodium is the thin, sheet-like protrusion filled with polymerized actin meshwork, and filopodia is the finger-like protrusion filled with tight bundles of filamentous actin. Filopodia promotes cell motility directly, so abundant filopodia bundles enhance cancer cells migration and metastasis.

To explore the correlation of ANXA2 with cancer cell motility, the microstructures of pseudopodia/filopodia in siRNA2 group and wild type group SGC-7901 cells were observed under scanning electron microscope and transmission electron microscope. As shown in Figure 4B, depletion of ANXA2 produced much more vigorous pseudopodia/filopodia than the other groups, and the contact inhibition among cells reappeared in siRNA2

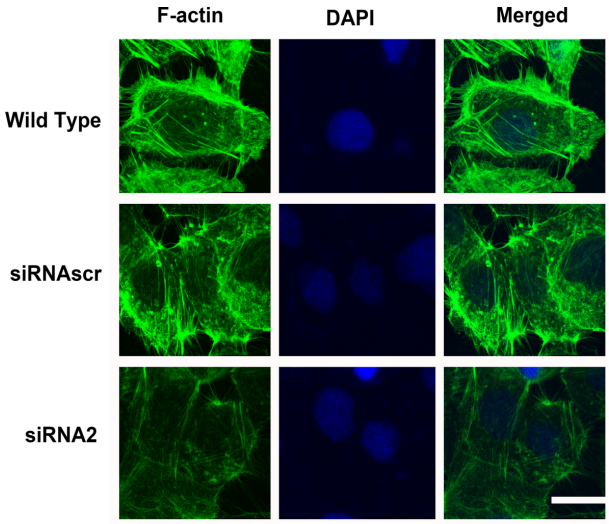

Figure 5. The F-actin Changes after RNAi-mediated Depletion of ANXA2 Expression. Under laser confocal microscope DAPI staining is for nuclei. F-actin expression is significantly enhanced and vigorously bundled in wild type and siRNASCR SGC-7901 cells, and distinctly inhibited and disassembled in siRNA2 group cells. Scale bar, 25 μm

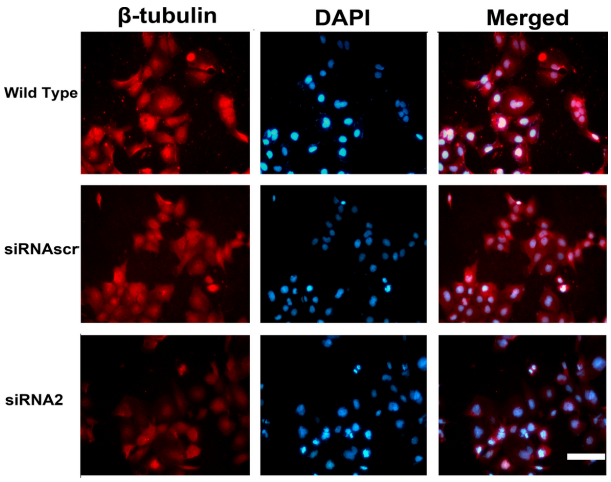

Figure 6. The β-tubulin Changes after RNAi-mediated Depletion of ANXA2 Expression. Immunofluorescence staining assay for β-tubulin, DAPI staining is for nuclei. β-tubulin expression is significantly enhanced and vigorously bundled in wild type and siRNASCR SGC-7901 cells, and distinctly inhibited and disassembled in siRNA2 group cells. Scale bar, 100 μm

group cells. When compared with the wild type group, much more cytosol transparent bubbles could be observed in siRNA2 group cells (Figure 4C). These findings demonstrate that ANXA2 expression may enhance cell motility for the invasion and metastasis of cancers and the unlimited proliferation of cancer cells.

The F-actin changes after RNAi-mediated depletion of ANXA2 expression

F-actin expression and its polymerization are essential for the development of pseudopodia/filopodia, we hypothesize that ANXA2 may increase the F-actin polymerization when the pseudopodia/filopodia sprouting and elongating is enhanced.

ANXA2 mediates the F-actin reorganization by its binding to F-actin, thus ANXA2 is important to stabilize the membrane-associated protein complexes in actin cytoskeleton (Garrido-Gomez et al., 2012). Any effect on F-actin bundles assembly or disassembly may be important to regulate the motility, invasion and growth of cancer cells. In order to explore the ANXA2 expression affect on F-actin changes in SGC-7901 cells, we display F-actin using FITC-labeled phalloidine staining. As shown in (Figure 5), F-actin expression was obviously enhanced and vigorously bundled along the inner face of membrane in wild type and siRNAscr groups SGC-7901 cells, but distinctly inhibited and disassembled in siRNA2 group cells. The results indicate a close correlation between ANXA2 expression and F-actin changes in SGC-7901 cells.

The β-tubulin changes after RNAi-mediated depletion of ANXA2 expression

Microtubules are the essential elements to form the active microtentacles that facilitate the moti-lity of cancer cells and enhance the malignancy of cancers consequently. Cancer cells are more deformable than normal cells depending on the enhanced stability of microtubules, and the increased deformability facilitates cancer metastasis. By the enhanced microtubule stability and assembly, cancer cells present an increased deformability to conquer the deforming force during their metastasis through microcapillary vessels system. Tubulin is important to increase cell deformability and motility. Using β-tubulin immunofluorescence staining assay, the significantly inhibited β-tubulin expression and polymerization was observed in siRNA2 group SGC-7901 cells comparing with in wild type and siRNAscr groups cells (Figure 6).

Discussion

The result shows that ANXA2 deletion induced β-tubulin expression decreased and microtubules disassembled. The enhanced microtubule disassembly may result in cell lamellipodia formation inhibited, cell motility decreased, and cell proliferation disrupted. Reported data explored that the microtubule disassembly destabilizes vascular vessels, and inhibits angiogenesis and VEGFR2 expression, so the disassembly suppresses the invasion and metastasis of cancer. Our results indicate that the expression and polymerization of β-tubulin were evidently disrupted in ANXA2 deleted SGC-7901 cells, and ANXA2 may play important roles for the expression and polymerization of β-tubulin to enhance the motility, angiogenesis, invasion, and metastasis of cancer cells. This result implies that SGC-7901 cells may utilize ANXA2 to sustain their microtubule assembly for microtentacles formation and enhance their metastatic potential.

In conclusion, transfection SGC-7901 cells with siRNA targeting human ANXA2 led to significant down-regulation of ANXA2 expression, which resulted in a marked impairment of cell migration ability, significant suppression of cell proliferation but not

enhancement of apoptosis in SGC-7901 cells. Moreover, we found that down regulation of ANXA2 induced the pseudopodia shorted and spared, non-stained areas increased, mitochondria decreased, and the expression and polymerization of F-actin and β-tubulin changed. These findings demonstrate that ANXA2 expression may enhance cell motility for the invasion and metastasis of cancers and the unlimited proliferation of cancer cells. Combing our previous data together, our results indicate that expression of ANXA2 is closely related to the proliferation of SGC-7901 cells and maintain the malignancy of cancer cell, therefore, ANXA2 might be a key potential target contributing to gastric cancer gene therapy or drug therapy.

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