Down-regulation of β-1,3-N-acetylglucosaminyltransferase-8 by siRNA inhibits the growth of human gastric cancer

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Received November 15, 2010; Accepted March 2, 2011

DOI: 10.3892/mmr.2011.448

Abstract. β-1,3-N-acetylglucosaminyltransferase-8 (β3Gn-T8) is the most recently identified enzyme in the β3Gn-T family, but its biological function is poorly understood. To elucidate the effects of β3Gn-T8 on gastric cancer behavior, β3Gn-T8 was down-regulated in AGS cells using small interfering RNA (siRNA). The mRNA and protein expression levels of β3Gn-T8 were detected using RT-PCR and Western blotting, respectively, and sequence-specific inhibition using siRNA was also measured using RT-PCR in human SPCA-1 and SGC-7901 cells. The cell proliferation rate was determined using MTT and the percentage of apoptotic cells was measured using flow cytometry. AGS cells transfected with β3Gn-T8 siRNA were subcutaneously transplanted into nude mice and tumorigenicity was assessed. The siRNA efficiently suppressed β3Gn-T8 expression in AGS cells, and the down-regulation of β3Gn-T8 caused significant inhibition of tumor cell growth *in vitro*. The apoptotic rate of AGS cells increased to 10.13% 48 h after siRNA transfection, which was five times that of the control cells. Furthermore, the knockdown of β3Gn-T8 expression reduced the tumorigenicity of gastric cancer cells in nude mice, suggesting that β3Gn-T8 has potential as a gastric cancer therapeutic target.

Introduction

Gastric cancer is the fourth most common malignancy and the second leading cause of cancer-related death in the world. Even with innovative therapeutic regimens, the mortality rate associated with gastric cancer is high, because gastric cancer is usually detected at an advanced stage. In China, approximately 20 per 100,000 individuals have been estimated to die from gastric cancer. In certain areas, the mortality rates reach as high as 60 deaths per 100,000 individuals (1). Despite treatment advances, chemotherapy is only marginally effective in most advanced cases. For patients who are refractory to or intolerant of the current chemotherapy regimens, treatment options are limited. Hence, more effective therapies with fewer side effects and early diagnostic methods are required. Tumor-associated antigens, such as CEA and CA, are commonly used as tumor markers for cancer detection. However, the ability to detect gastric cancer at an early curable stage is limited due to the low sensitivity and poor specificity of current diagnostic methods. MMP-10 has also been reported to be a good marker for the detection of gastric cancer, and MIA and MMP-10 may be good therapeutic targets for cancer treatment (2). However, these new testing methods remain in the early stages of development. Therefore, better early detection strategies for cancer and new targets for cancer therapy are clearly required.

Glycobiology research focuses on the potential use of glycans for the therapy and diagnosis of disease, and some tumor markers are glycoproteins that are usually detected using monoclonal antibodies. Each tumor marker has a variable profile of its usefulness in screening, determining diagnoses and prognoses, assessing the response to therapy and monitoring for cancer recurrence. These glycoproteins are produced by tumor cells in response to cancer or under certain benign conditions and indicate biological changes that signal the existence of malignancy. For example, carbohydrate epitopes, including T, Tn, Globo H, Lewis y, sialyl Lewis x, sialyl Lewis a and polysialic acid, are commonly found in various cancers (3). Glycosyltransferase mRNA expression has been found to be significantly altered in gastric carcinomas isolated from surgical specimens (4). More recently, the polypeptide N-acetyl-galactosaminyltransferase 3, which is involved in the initiation of O-glycosylation, has been identified as a possible new marker of early gastric cancer (5). Many recent studies have concluded that some aberrant sugar chains of cell surface glycoproteins are associated with cancer cell differentiation and malignant potential, such as altered cell adhesion, invasion, metastasis, recurrence and prognosis (6). Tumor formation may be due to increased or aberrant glycosylation of carbohydrate chains, changes in the density of carbohydrates on the cell surface, or the exposure of chains

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Key words: gastric cancer, β-1,3-N-acetylglucosaminyltransferase-8, RNAi, growth, cancer therapy

usually covered by other structures. These reports show that sugar chains on the cell surface may play an important role in tumor formation and development, but the mechanisms through which glycosyltransferases regulate cancer cell behavior remain to be identified.

The biosynthesis of complex glycoprotein sugar chains is tissue-specific and developmentally regulated by the activity of a series of glycosyltransferases. Since more glycosyltransferases have been identified and their biological roles revealed, increasing attention has been paid to enzymes participating in the regulation of cancer proliferation and development. We and another group have cloned and identified a new gene, UDP-GlcNAc: βGal β-1,3-N-acetylglucosaminyltransferase 8 (β3Gn-T8) (also known as β3 galactosyltransferase 7), and have evaluated the enzymatic activity of prokaryotic expression genes (7,8). The function of β3Gn-T8 *in vivo* is unclear due to its inherently low enzymatic activity; indeed, β3Gn-T8 was demonstrated to stimulate the activity of β3Gn-T2 (9,10), in the first report to show that mixed glycosyltransferases enhance enzymatic activity through the formation of a hetero-complex *in vitro*. In addition, β3Gn-T8 has been shown to transfer GlcNAc to the non-reducing terminus of the tetraantennary N-glycan Galβ1-4GlcNAc *in vitro*, as measured using an enzyme activity assay. Ishida *et al* showed that most of the cell lines established from colon cancer expressed higher levels of the β3Gn-T8 transcript than cell lines derived from gastric cancer (7). However, in the present study, β3Gn-T8 expression was also detected in SGC-7901 cells from human gastric cancer (7). We found that β3Gn-T8 was expressed in all four tumor cell lines tested (SPCA-1, SGC-7901, H460 and K562) using purified rabbit anti-β3Gn-T8 antiserum as the primary antibody and alkaline phosphatase-conjugated goat anti-rabbit IgG as the secondary antibody. In addition, β3Gn-T8 expression in stomach and cervical tumors was higher than in normal tissues, suggesting that this glycoprotein plays a role in tumorigenesis. We therefore chose gastric cancer as our model. The AGS cell line was derived from fragments of a tumor resected from a patient who received no prior therapy (11); the line was cured of a prior mycoplasma infection at the ATCC and is widely used in biomedical research. To elucidate the effects of β3Gn-T8 on gastric cancer behavior and the biological functions of this enzyme, we detected the expression of β3Gn-T8 in the MKN28 (well-differentiated), AGS (moderately differentiated) and SGC7901 (poorly differentiated) gastric adenocarcinoma cell lines, and found a higher expression of β3Gn-T8 in AGS cells (data not shown).

RNAi, a cellular mechanism in which double-stranded RNA triggers the degradation of the corresponding gene transcripts, is a system within living cells that helps to control which genes are active and how active they are (12-14). A major breakthrough in the application of RNAi technology in targeted gene silencing in mammalian cells was the advent of synthetic small interfering RNA (siRNA), and RNAi has become a routine tool for the knockdown of gene expression in basic research. The strong and specific suppression of gene expression using RNAi is currently being evaluated as a potentially useful method for developing gene silencing therapies for cancer. In this study, siRNAs that targeted β3Gn-T8 were used to inhibit gene expression at both the mRNA and protein levels in AGS cells. To examine the sensitivity and specificity of siRNA on β3Gn-T8 expression, the mRNA expression levels in SPCA-1 human lung adenocarcinoma cells and SGC-7901 gastric cancer cells were evaluated. We further investigated whether β3Gn-T8 siRNA inhibits tumor growth *in vitro* and *in vivo*, which may be used as a novel approach to cancer therapy.

Materials and methods

Cell culture. AGS human gastric cancer cells (ATCC; Manassas, VA, USA) were maintained in F12 nutrient mixture (Ham's; Gibco BRL, Grand Island, NE, USA) containing 10% fetal bovine serum (FBS; Gibco BRL) in a humidified tissue culture incubator at 37˚C in a 5% CO_2 atmosphere. SPCA-1 human lung adenocarcinoma cells and SGC-7901 gastric cancer cells (ATCC; Manassas) were cultured in RPMI-1640 (Gibco BRL) supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA).

siRNA design. The sequences used for β3Gn-T8 interference were determined from the complete ORF of the human β3Gn-T8 gene using the BLOCK-iT™ RNAi Designer web application (Invitrogen, Carlsbad, CA, USA). Since some regions of the mRNA may be either highly structured or bound by regulatory proteins, three siRNA target sites were selected at different positions along the length of the mRNA sequence. No correlation between the position of target sites on the mRNA and siRNA potency was observed. Next, the potential target sites were compared to the appropriate genome database and any target sequences with >16-17 contiguous base pairs of homology to other sequences were eliminated, to ensure that the selected gene was specifically targeted. After the siRNAs were designed, they were sent to Shanghai Genepharma Co. (China) to be synthesized. The following sequences were used: β3Gn-T8 si-1, sense 5'-CAUUCAACCAGACGCUCAAdTdT-3', anti-sense 5'-UUGAGCGUCUGGUUGAAUGGG-3'; β3Gn-T8 si-2, sense 5'-CAUUCGGCUCUGGAAACAAdTdT-3', anti-sense 5'-UUGUUUCCAGAGCCGAAUGCT-3'; β3Gn-T8 si-3, sense 5'-CCCAGAAGCUGGACAACUUdTdT-3', anti-sense 5'-AAGUUGUCCAGCUUCUGGGTT-3'; and negative siRNA, sense 5'-UUCUCCGAACGUGUCACGUdTdT-3', anti-sense 5'-ACGUGACACGUUCGGAGAATT-3'.

Cell transfection. One day prior to transfection, cells were plated in 2-ml growth medium without antibiotics to be 50-80% confluent at the time of transfection. To obtain the highest transfection rate with low non-specific effects, the transfection conditions were optimized by varying the siRNA and RNAi-Mate Reagent (Genepharma Co.) concentrations. A total of 0.5 ml siRNA/RNAi-Mate complex (0.3 μg siRNA and 0.9 μg RNAi-Mate Reagent) and 1.5 ml medium without serum or antibiotics were added to each well for a final volume of 2 ml. The siRNA had a dose-dependent inhibitory effect (10-40 nmol); however, too much siRNA (>80 nmol) led to AGS cell toxicity and death. The final concentration of 40 nmol siRNA used in this study was derived from a dose response assay. The cells were harvested at different time points.

RT-PCR analysis. Total RNA was extracted from the cultured cells using TRIzol Reagent (Invitrogen) according to the

manufacturer's instructions. For cDNA synthesis, 2 μg total RNA was used as the template in a 25-μl reverse transcription reaction. To detect β3Gn-T8 mRNA expression, PCR amplification was performed according to the following thermal cycling parameters: 1 cycle at 94˚C for 5 min; 30 cycles of 94˚C for 45 sec, 52˚C for 45 sec and 72˚C for 1 min; and a final extension cycle at 72˚C for 10 min. The PCR primer sequences used were as follows: β3Gn-T8 (362 bp), sense 5'-CCCTGACTTCGCCTCCTAC-3' and anti-sense 5'-GGTCTTTGAGCGTCTGGTTGA-3'; β-actin (250 bp), sense 5'-CCTCTATGCCAACCACAGTGC-3' and anti-sense 5'-GTACTCCTGCTTGCTGATCC-3'. The samples were analyzed in triplicate and the products were separated using electrophoresis on 10-g/l agarose gels.

Western blot analysis. After transfection, the cells were washed twice with cold PBS, lysed in a radioimmunoprecipitation assay buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% deoxycholic phenylmethyl-sulfonyl fluoride, 1 μg/ml aprotinin and 1 mM DTT] for 10 min, and then scraped. The extracts were centrifuged at 4,000 x g at 4˚C for 15 min and the proteins were separated using SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline and then incubated with primary antibodies for 1 h at room temperature. Signals were visualized with enhanced chemiluminescence reagents (Amersham Biosciences) and images were quantified using ImageQuant 5.1 (Amersham Biosciences). Rabbit anti-human β3Gn-T8 polyclonal antibody (pAb) was purified by our lab. The anti-β-actin rabbit monoclonal antibody (mAb) and the anti-rabbit secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

MTT analysis. Cells ($5x10^3$) were plated in a 96-well plate and 180 μl culture medium was added to each well. On the next day, the culture medium was replaced with fresh medium containing β3Gn-T8 siRNA or negative control siRNA. Six wells were used for each time point (24-h intervals for 4 days). Cell viability was then determined for each time point by adding 20 μl MTT (5 g/l; Sigma, St. Louis, MO, USA) to each well and incubating the cells for 4 h. The reaction was stopped by the addition of 150 μl DMSO (Sigma) and the absorbance of samples at 570 nm was then measured. A growth curve was plotted for each sample as the log cell number vs. time, and the growth rates were derived from the slope of each growth curve. Three independent experiments were performed and the results were used for plotting the relative growth rate with SD.

Flow cytometry. Apoptosis was identified and quantified using flow cytometry with PI staining. Both adherent and floating cells were collected after β3Gn-T8 siRNA treatment, washed with ice-cold PBS and fixed with 70% ice-cold ethanol overnight at 4˚C. The cells were then resuspended in 1X binding buffer at $1x10^6$ cells/ml. A total of 100 ml of the solution was transferred to a 5-ml culture tube, and then 5 μl Annexin V-FITC (20 μg/ml; Beyotime Biotechnology, China) and 5 μl PI (20 μg/ml; Beyotime Biotechnology) were added. The cells were gently vortexed and incubated for 15 min at room temperature in the dark. After adding 400 μl 1X binding buffer to each tube, the cells were analyzed using flow cytometry (Becton Dickinson, Mountain View, CA, USA) within 1 h.

Tumor growth in nude mice. Four-week-old female nude mice (SPF BALB/c, SCXK2007-0005) were obtained from the Laboratory Animal Center of Soochow University and kept in a room at constant temperature (23±2˚C) and humidity (50-70%) with a 12-h light-dark cycle. After being grown to subconfluency, transfected (β3Gn-T8 siRNA or negative control siRNA) and non-transfected cells were trypsinized and harvested, washed twice with PBS and resuspended in 0.2 ml PBS ($5x10^6$ cells/0.2 ml). Each group had 10 nude mice. Each week, the tumor diameter was measured, and the volume was calculated according to the formula V=0.4 x largest diameter x smallest diameter. The growth curve of each tumor was plotted and the tumor growth ratio was calculated. Four weeks after injection of the cells, the mice were sacrificed, the weights of the tumors were recorded and β3Gn-T8 protein levels in the samples from nude mice in the two groups were compared using Western blotting. The animal treatment protocol used in this study was approved by the Institutional Animal Care and Use Committee.

Statistical analysis. The results shown are the mean ± SD. A P-value <0.05 was considered statistically significant. Statistical analyses were calculated using SPSS 11.5. Each experiment was repeated three times.

Results

Changes in β3Gn-T8 mRNA and protein levels in AGS cells after siRNA transfection. The most appropriate method of siRNA preparation depends on the type of experiment being performed. The use of chemically synthesized siRNA is recommended for sensitive assays and should be combined with careful experimental setup to determine the best transfection conditions and the effect of the transfection conditions and the transfection on a downstream assay. Therefore, to determine whether β3Gn-T8 expression was altered after siRNA treatment of AGS cells, RT-PCR was performed, and the correct PCR products were confirmed using sequencing. RT-PCR results showed that siRNA led to a marked inhibition of β3Gn-T8 mRNA expression. In the negative control siRNA- and β3Gn-T8 siRNA (si-1, si-2 and si-3)-transfected cells, the mRNA expression ratios (β3Gn-T8/β-actin) were 0.982±0.006, 0.633±0.058, 0.22±0.046 and 0.447±0.017, respectively. The β3Gn-T8 mRNA expression was lower in the si-2 group than in the si-1 and si-3 groups (P<0.05), while no difference was found between the negative control siRNA group and untreated AGS cells (P>0.05; Fig. 1A and B).

The result of Western blotting showed that the expression of β3Gn-T8 protein in AGS cells transfected with β3Gn-T8 siRNA was strongly suppressed. Densitometry analysis showed that the expression of β3Gn-T8 protein in the negative control, si-1, si-2 and si-3 groups was 96.13, 64.52, 29.47 and 51.72% that of the untreated AGS cells, respectively. The expression level of β3Gn-T8 protein was lower in the si-2 group than in the si-1 and si-3 groups (P<0.05; Fig. 2A and B), while no difference was found between the negative control group and untreated cells (P>0.05). The results of RT-PCR and Western

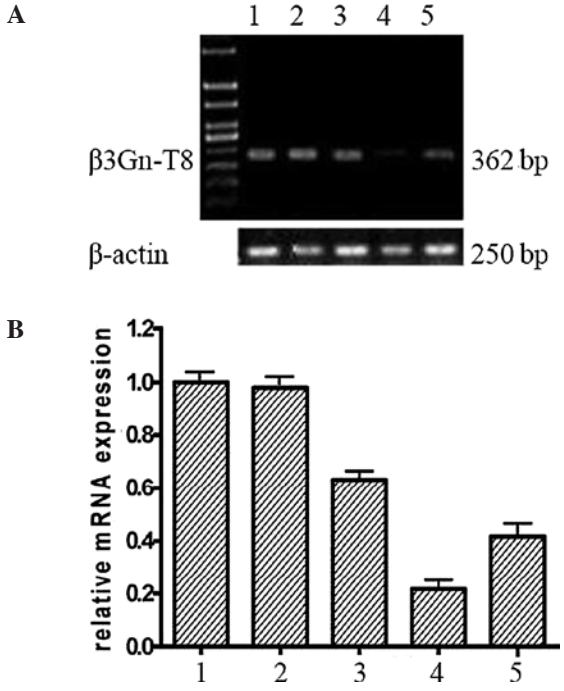

Figure 1. Expression of β3Gn-T8 mRNA in different groups of transfected cells. (A) Assay for β3Gn-T8 gene silencing efficiency using RT-PCR. Only the β3Gn-T8-specific siRNA inhibited the expression of the β3Gn-T8 gene. β3Gn-T8 knockdown was not observed with the negative control. (B) Densitometry measurements of the bands. β3Gn-T8 siRNA induced gene silencing. P<0.05 compared to non-transfected and negative control siRNA-transfected cells. There was no significant difference between the non-transfected and negative control siRNA-transfected cells. (1) Non-transfected cells, (2) negative control siRNA-transfected cells, (3) β3Gn-T8 si-1-transfected cells, (4) β3Gn-T8 si-2-transfected cells and (5) β3Gn-T8 si-3-transfected cells.

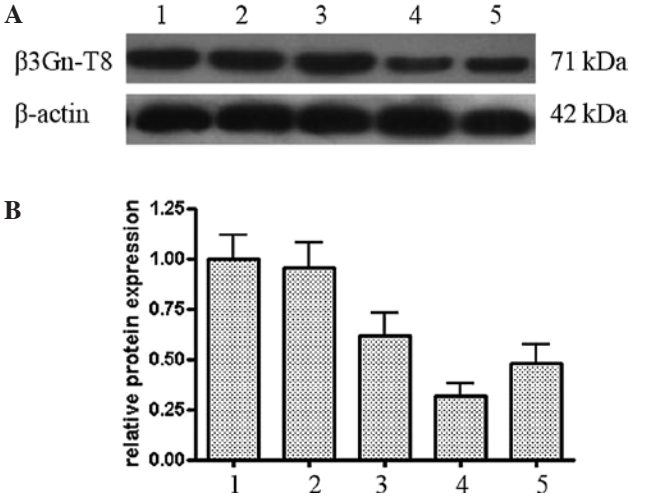

Figure 2. Expression of β3Gn-T8 protein in different groups of transfected cells. (A) Assay for β3Gn-T8 gene silencing efficiency using Western blotting. Only the β3Gn-T8-specific siRNA inhibited the expression of β3Gn-T8 protein. The silencing efficiency was not significantly different between the non-transfected and the negative control siRNA-transfected cells. (B) Densitometry measurements of the bands. (1) Non-transfected cells, (2) negative control siRNA-transfected cells, (3) β3Gn-T8 si-1-transfected cells, (4) β3Gn-T8 si-2-transfected cells and (5) β3Gn-T8 si-3-transfected cells.

blotting established the efficiency of siRNA transfection and identified si-2 as the most effective β3Gn-T8 siRNA.

Sensitivity and specificity of β3Gn-T8 si-2 siRNA in SPCA-1 and SGC-7901 cells. To confirm the specific knockdown of β3Gn-T8 using siRNA, the siRNA was transiently transfected into SPCA-1 and SGC-7901 cells. The inhibitory effect of the β3Gn-T8 siRNA was confirmed using RT-PCR; mRNA expression was significantly suppressed in SPCA-1 and SGC-7901 cells transfected with 40 nmol/l β3Gn-T8 si-2. Notably, β3Gn-T8 si-2 caused a 76.93% reduction in β3Gn-T8 mRNA in SPCA-1 cells (Fig. 3A and B) and a 79.14% reduction in SGC-7901 cells (Fig. 3C and D). The negative control siRNA had no effect on the β3Gn-T8 mRNA level in either cell line. The differences in the results between SPCA-1 and SGC-7901 cells were not significantly different, indicating that si-2 specifically targets β3Gn-T8 expression.

Knockdown of β3Gn-T8 in AGS cells inhibits tumor growth in vitro. Based on these data, a validated siRNA sequence to target β3Gn-T8 expression was identified. To determine whether the inhibition of β3Gn-T8 affected AGS cell proliferation, cell viability was determined using the MTT assay. Using this approach, cell viability was found to be significantly reduced after treatment with β3Gn-T8 si-2 (P<0.05) at 24, 48, 72 and 96 h post-transfection compared to non-transfected and negative control siRNA-transfected cells (Fig. 4A). The cell proliferation rates in si-2-transfected cells at these time points were 31.27, 40.19, 44.71 and 50.64%, respectively. Treatment of AGS cells with β3Gn-T8 si-2 was associated with a time-dependent inhibition of cell growth, whereas no significant inhibitory effect was observed in the non-transfected and negative control siRNA-transfected cells (P>0.05).

Knockdown of β3Gn-T8 in AGS cells induces apoptosis in vitro. We further studied the effect of β3Gn-T8 si-2 on the induction of apoptosis in AGS cells. The apoptotic rate was 10.13% at 48 h post-β3Gn-T8 si-2 transfection, and there was no significant difference in the percentage of apoptotic cells between non-transfected (2.57%) and negative control siRNA-transfected (2.97%) cells (P>0.05; Fig. 4B).

Knockdown of β3Gn-T8 in AGS cells reduces tumor growth in nude mice. Since the suppression of β3Gn-T8 inhibited AGS cell proliferation *in vitro*, we next examined whether these *in vitro* data were correlated with the *in vivo* effects. AGS cells transfected with β3Gn-T8 si-2 were grown in nude mice to form tumors which were measured at regular time points, as described in the Materials and methods. The average tumor size was smaller in mice injected with β3Gn-T8 si-2-transfected cells (741±19 mm^3) than non-transfected (1.379±124 mm^3; P<0.05) and negative control siRNA-transfected (1.131±117 mm^3; P<0.05) cells (Fig. 5A and B). The tumor growth ratio of mice injected with β3Gn-T8 si-2-transfected cells (45.62%) was also lower than that of the non-transfected (86.71%; P<0.05) and negative control siRNA-transfected (80.31%; P<0.05) cells (Fig. 5C). Tumor growth in mice injected with β3Gn-T8 si-2-transfected cells was inhibited, although no difference was observed using Western blotting in β3Gn-T8 protein levels in the tumor samples from nude mice of different groups.

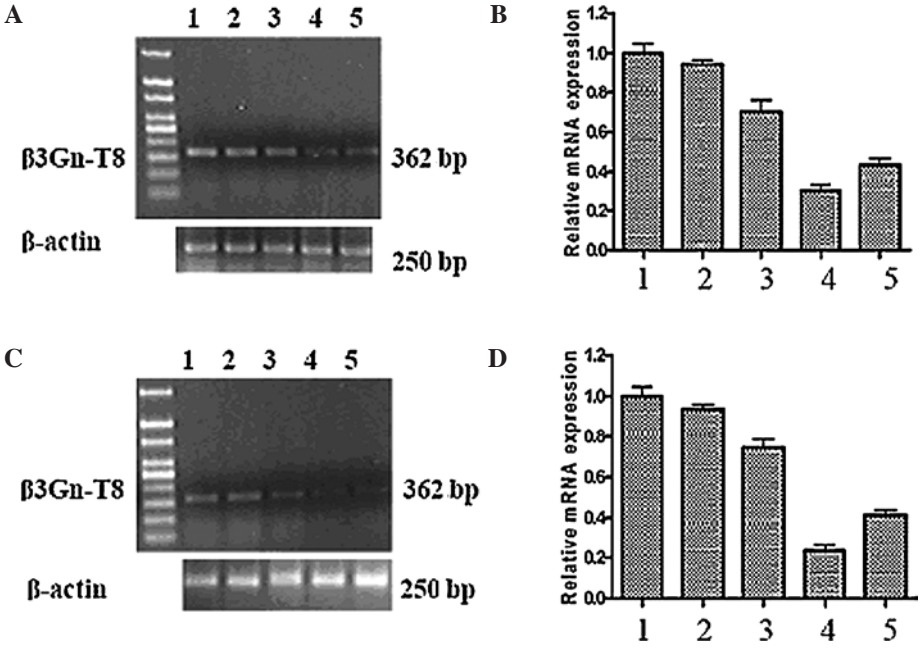

Figure 3. Effects of β3Gn-T8 siRNA knockdown in SPCA-1 and SGC-7901 cells. (A and C) β3Gn-T8 mRNA expression levels quantified using RT-PCR in SPCA-1 (A) and SGC-7901 (C) cells. (B and D) Densitometry measurements of the bands in SPCA-1 (B) and SGC-7901 (D) cells. β-actin was used as the internal loading control. The negative control siRNA had no effect on the β3Gn-T8 mRNA level in either cell line. β3Gn-T8 si-2 had the most suppressive effect on the β3Gn-T8 mRNA level in both cell lines. (1) Non-transfected cells, (2) negative control siRNA-transfected cells, (3) β3Gn-T8 si-1-transfected cells, (4) β3Gn-T8 si-2-transfected cells and (5) β3Gn-T8 si-3-transfected cells.

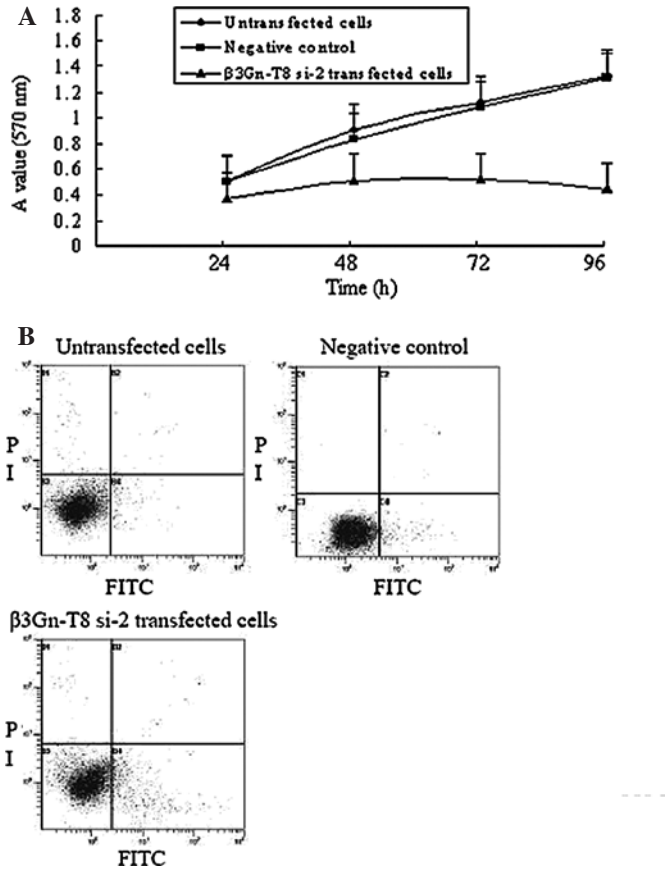

Figure 4. (A) Effects of negative control siRNA and β3Gn-T8 si-2 treatment on cell viability. β3Gn-T8 si-2 group vs. negative control group ($P<0.05$); β3Gn-T8 si-2 group vs. non-transfected group ($P<0.05$). (B) The percentage of apoptotic cells at 48 h post-transfection was analyzed using flow cytometry. The apoptotic rates were 2.57, 2.97 and 10.13%, respectively. β3Gn-T8 si-2 group vs. negative control group ($P<0.05$); β3Gn-T8 si-2 group vs. non-transfected group ($P<0.05$).

Discussion

The results of RT-PCR show that β3Gn-T8 transcripts are constitutively expressed at high levels in AGS gastric cancer cells, as shown in Fig. 1A. Furthermore, Western blot analysis using human β3Gn-T8 antibody revealed the presence of β3Gn-T8 in AGS cells, as shown in Fig. 2A. The effectiveness of siRNA inhibition is highly sequence-dependent; we identified one siRNA sequence that showed a robust effect using RT-PCR, as shown in Fig. 3A and C. To evaluate the potential functional significance of altered β3Gn-T8 in gastric cancer cells, we examined the effect of the β3Gn-T8 siRNA in AGS cells, and found that the down-regulation of β3Gn-T8 decreased malignant phenotypes. We observed that AGS cells transfected with β3Gn-T8 siRNA proliferated slowly, compared to proliferation in the control groups, indicating that β3Gn-T8 siRNA has an anti-proliferative effect. We also noted that AGS cells treated with β3Gn-T8 siRNA displayed a significantly increased rate of apoptosis compared to that of the control groups ($P<0.05$). In addition, RNAi-mediated β3Gn-T8 knockdown inhibited gastric cancer growth in nude mice. A siRNA targeting β3Gn-T8 was efficiently delivered into cancer cells and resulted in the inhibition of tumor growth *in vitro* and *in vivo*.

Gastric cancer is a complex disease with numerous factors contributing to transformation and progression. Gastric cancer cells are often characterized by increased resistance to apoptosis, which enables their survival under abnormal growth stimulation and mediates their increased resistance to various forms of cellular stress (15,16). Hence, apoptosis deficiency has been shown to contribute to tumorigenesis. The balance between apoptotic and anti-apoptotic signal pathways plays a role in the pathogenesis of various types of cancer.

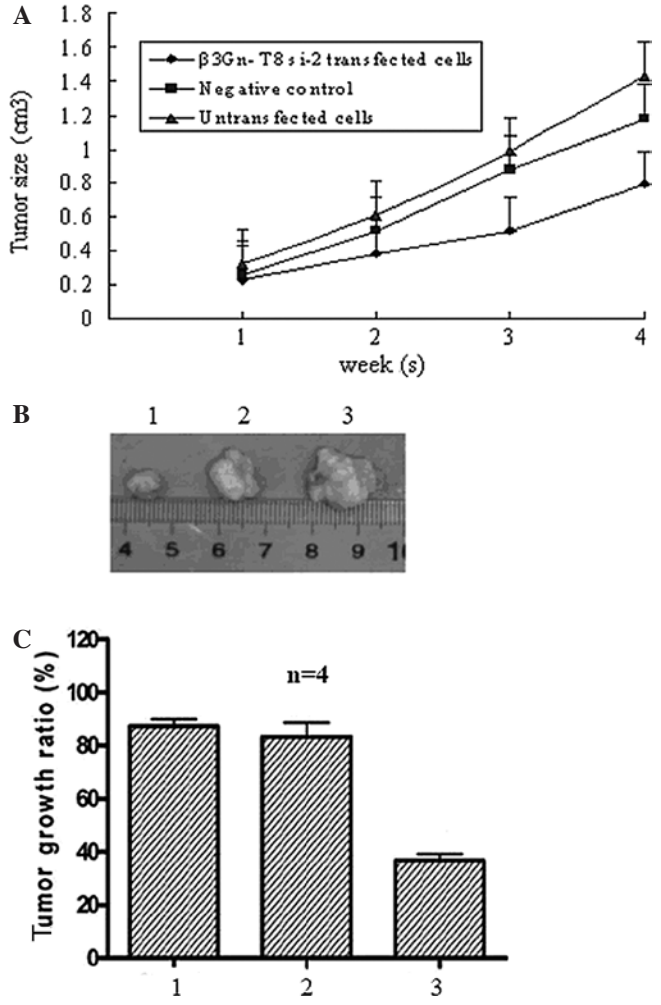

Figure 5. Tumor growth in the nude mice injected with β3Gn-T8 si-2 within 4 weeks post-injection. (A) The tumor growth curve showed a significant growth tendency in mice injected with β3Gn-T8 si-2 ($P<0.05$). (B) Representative image of a tumor. (1) β3Gn-T8 si-2-transfected cells, (2) negative control siRNA-transfected cells and (3) non-transfected cells. (C) The tumor growth ratio in mice injected with β3Gn-T8 si-2-transfected cells was lower than in those injected with non-transfected cells ($P<0.05$) or negative control siRNA-transfected cells ($P<0.05$). (1) Non-transfected cells, (2) negative control siRNA-transfected cells and (3) β3Gn-T8 si-2-transfected cells.

Cell proliferation, which is regulated by telomerase activity, and cell loss, which is regulated by apoptosis, determine tumor growth. There are many factors involved in the mechanisms of cell proliferation and apoptosis, and many recent studies have indicated that aberrant glycosylation promotes key events involved in proliferation and apoptosis. Numerous reports have demonstrated that glycosyltransferases directly modify carbohydrates on cell surface receptors and cell adhesion molecules, which then promotes or inhibits tumor cell growth. For example, α-4GT1, which encodes α-1,4-glycosyltransferase, appears to be an inhibitor of apoptosis (17). C2GnT-I is an enzyme that acts in the Golgi to modify glycoproteins destined for export to the cell surface, which then interact with the extracellular milieu. It has also been reported that C2GnT may act as a tumor-suppressor gene, since loss of function resulted in resistance to apoptosis (18). Apoptosis has previously been found to be associated with Ley expressed in various types of cancer (19) and Lex in colonic adenocarcinoma HT29 cells caused by enhanced α3→3FT (20). Several endogenous apoptotic triggers are expressed in the skin, including galectin-1 and galectin-7, which are members of the galectin family of immunoregulatory lectins. Galectins bind oligosaccharide ligands on cell surface glycoproteins and glycolipids to regulate cell growth, adhesion and death. Therefore, we conclude that β3Gn-T8 is an enzyme that acts in the Golgi to modify glycoproteins destined for export to the cell surface, which then interact with the extracellular milieu. Protein glycosylation is a complex process that is affected by glycosyltransferase abundance and activity, localization within the Golgi and the rate of glycoprotein transport through the Golgi. Moreover, a single glycosyltransferase modifies many different acceptor glycoproteins. Thus, modest changes in enzymatic activity or localization profoundly disrupt normal cellular glycosylation, thereby controlling the biological behavior of cells. We do not believe that cell proliferation and apoptosis is regulated by β3Gn-T8 alone. However, we demonstrated that AGS cells transfected with β3Gn-T8 siRNA showed a significant reduction in proliferative ability, suggesting that the down-regulation of β3Gn-T8 in gastric cancer cells is an important factor in determining cancer cell proliferation.

Synthetic siRNA is a powerful tool for silencing mammalian gene expression for gene function studies. However, the down-regulation of β3Gn-T8 expression using RNAi in AGS gastric cancer cells had not previously been performed. In addition, the effect of RNAi has been reported to differ greatly in the targeting of different sequences of the gene. Therefore, it is important to determine the most effective sequence. To better understand the molecular mechanisms associated with β3Gn-T8 regulation of cell proliferation and apoptosis in gastric cancer, several pairs of siRNA that targeted β3Gn-T8 were designed and transfected into AGS cells. We showed that siRNA technology represented an extremely powerful tool for inhibiting cancer cell growth. In siRNA design, the 19-nt mRNA target sequence should preferentially contain approximately 50% GC content and be flanked by 5'-AA and 3'-UU residues (21,22). Both siRNA sequences identified in this study were found to substantially inhibit gene expression. However, to better investigate the siRNA effect, β3Gn-T8 siRNA-2 was used in our study. These inhibitory effects were due to post-transcriptional silencing mechanisms in which the synthetic double-stranded siRNA effectively silenced β3Gn-T8 expression in AGS gastric cancer cells. This observation is consistent with recent studies, which have shown that the introduction of a 21-nt double-stranded RNA (dsRNA) molecule into cancer cells strongly suppressed the expression of specific mRNAs (21,22). Although β3Gn-T8 siRNA retarded the growth of tumors in nude mice, the β3Gn-T8 protein levels in the tumor samples from nude mice of different groups did not vary at 1 month post-injection, which was most likely due to the time-dependent effect of synthetic siRNA. Some reports have indicated that the inhibitory effect of synthetic siRNA may last 6-7 days. Therefore, after tumorigenesis, β3Gn-T8 expression may return after the loss of RNAi in tumor tissues. We also detected a relationship between the gene silencing and cell viability; apoptosis was increased by β3Gn-T8 siRNA, by at most approximately 10%. The nude mouse assay was used since a previous study had shown it to be appropriate for *ex vivo* testing using a cell line pre-transfected with siRNA (21). Based

on its specific anti-apoptotic function, β3Gn-T8 represents a suitable target for anti-tumor approaches; the inhibition of β3Gn-T8 blocks anti-apoptotic and mitotic progression in tumor cells and, as a result, tumor development is suppressed.

To elucidate whether β3Gn-T8 knockdown differentially affected cell growth, 40 nM siRNA was used for transfection in this study. At this concentration, the reduction in mRNA and protein levels was not complete, nor was it expected to be. Higher siRNA concentrations do not always result in absolute protein or mRNA reduction. For example, the siRNA reduction of Akt-1 was reported to be 51 and 73% effective for 20 and 80 nM siRNA, respectively. In our study, siRNA concentrations above 40 nM were neither necessary nor possible as there was a nearly complete elimination of AGS cells and a significant reduction in the proliferation of other cancer types. Therefore, we conclude that the amount of β3Gn-T8 mRNA and protein knockdown may be correlated with the amount of cell growth inhibition.

Our findings confirm that chemically synthesized siRNA specifically blocks β3Gn-T8 gene expression, induces cell apoptosis and inhibits the growth of carcinoma cells. Understanding the regulation of glycosyltransferase expression in AGS cells and developing novel strategies to modify cellular glycosylation may provide new therapeutic approaches for overcoming tumor cell resistance to apoptosis in this disease. Moreover, since altered glycosylation and resistance to apoptosis are hallmarks of many types of tumors, these findings may be relevant for understanding how subtle changes in glycosylation are capable of profoundly altering tumor cell biology.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China to Q.C. (no. 30670462).

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