

RNAi-mediated gene silencing of ST6GalNAc I suppresses the metastatic potential in gastric cancer cells

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Abstract

Background ST6GalNAc I is a sialyltransferase controlling the expression of sialyl-Tn antigen (STn), which is overexpressed in several epithelial cancers, including gastric cancer, and is highly correlated with cancer metastasis. However, the functional contribution of ST6GalNAc I to development or progression of gastric cancer remains unclear. In this study, we investigated the effects of suppression of ST6GalNAc I on gastric cancer in vitro and in vivo.

Methods Gastric cancer cell lines were transfected with ST6GalNAc I siRNA and were examined by cell proliferation, migration, and invasion assays. We also evaluated the effect of ST6GalNAc I siRNA treatment in a peritoneal dissemination mouse model. The differences in mRNA levels of selected signaling molecules were analyzed by polymerase chain reaction (PCR) arrays associated with tumor metastasis in MKN45 cells. The signal transducer and activator of transcription 5b (STAT5b) signaling pathways that reportedly regulate the insulin-like growth factor-1 (IGF-1) were analyzed by Western blot.

Results ST6GalNAc I siRNA inhibited gastric cancer cell growth, migration, and invasion in vitro. Furthermore,

intraperitoneal administration of ST6GalNAc I siRNA-liposome significantly inhibited peritoneal dissemination and prolonged the survival of xenograft model mice with peritoneal dissemination of gastric cancer. PCR array confirmed that suppression of ST6GalNAc I caused a significant reduction in expression of IGF-1 mRNA. Decreased IGF-1 expression in MKN45 cells treated with ST6GalNAc I siRNA was accompanied by reduced phosphorylation of STAT5b.

Conclusion ST6GalNAc I may regulate the gene expression of IGF-1 through STAT5b activation in gastric cancer cells and may be a potential target for treatment of metastasizing gastric cancer.

Keywords STn · ST6GalNAc I · Gastric cancer · Peritoneal dissemination · STAT5b

Introduction

Gastric cancer remains one of the leading causes of cancer death worldwide [1, 2]. Many patients are diagnosed at stages unsuitable for curative surgery and have extremely poor prognoses, with 5-year survival ranging from 2 % to 15 % [3]. Approximately one half of all cases of unresectable gastric cancer exhibit peritoneal metastasis, which is the most life-threatening form of metastasis; it is associated with acute clinical symptoms such as malignant ascites, intestinal obstruction, and rapid progression of the disease [4–6]. Although peritoneal metastasis remains the major problem in the treatment of gastric cancer, there is currently no effective treatment strategy available to improve survival.

Altered glycosylation is a common phenotypic change observed in cancer cells, and overexpression of sialylated

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antigens at the surface of cancer cells has been widely reported [7, 8]. The sialyl-Tn antigen (Neu5Ac α 2-6GalNAc α 1-O-Ser/Thr), also known as STn and CD175s, is a simple mucin-type carbohydrate antigen. STn is rarely expressed in normal tissue but is aberrantly expressed in a variety of carcinomas, including gastric [9–11], colorectal [12], ovarian [13], breast [14, 15], and pancreatic [16] carcinomas, in which it is highly correlated with cancer invasion and metastasis [17–19].

STn is formed by the sialylation of the Tn antigen: GalNAc linked to serine or threonine. GalNAc is the first sugar added during O-linked glycan synthesis, and this basic unit can be extended to form multiple glycan structures. The sialylation of GalNAc prevents further sugar additions and effectively truncates the O-linked glycan extension [20, 21]. Given that antibodies against STn are cancer specific [17, 22], serum STn levels are used as a prognostic indicator for cancer aggressiveness and metastatic potential [23–25] in colorectal [26] and ovarian cancer [13].

The generation of STn is primarily associated with a single sialyltransferase, ST6GalNAc I, which adds sialic acid in an α -2-6 linkage to a serine or threonine residue (Tn antigen) [27–29]. Because the expression of STn is correlated with, or induced by, the expression of ST6GalNAc I in some colorectal, gastric, and breast cancer cell lines [28–30], the emergence of STn is thought to be partly caused by aberrant expression of ST6GalNAc I, with or without concomitant decrease or loss of other glycosyltransferases that compete with ST6GalNAc I for their substrate [31].

The relationship between STn and cancer progression has been demonstrated experimentally by ST6GalNAc I-transfected, STn-positive gastric cancer cells. Pinho et al. [32], reported that forced expression of STn leads to major morphological and cell behavior alterations in gastric carcinoma cells that were reversible by specific antibody blockade. STn antigen is able to modulate a malignant phenotype and induce a more aggressive cell behavior, such as decreased cell–cell aggregation and increased extracellular matrix (ECM) adhesion, migration, and invasion. Ozaki et al. [33], reported that forcing STn expression in gastric cancer lines resulted in increased metastasis and decreased survival in nude mice after intraperitoneal injection of tumor cells. This enhanced metastatic capability of STn-expressing cells was abrogated by pretreatment with anti-STn antibodies.

Given a strong association between STn expression and cancer progression, STn might be a favorable target for treating peritoneal metastasis of gastric cancer. Nevertheless, STn may only play a partial role in the metastatic process, and unknown functions of ST6GalNAc I may be involved through mechanisms other than STn synthesis.

In this study, we found that suppression of ST6GalNAc I strongly inhibited gastric cancer cell growth, migration,

and invasion, and simultaneously downregulated the insulin-like growth factor-1 (IGF-1)-dependent axis induced by the STAT5b signaling pathway. We also show that intraperitoneal (i.p.) administration of ST6GalNAc I siRNA-liposome to a peritoneal dissemination mouse model significantly inhibited tumor metastasis and prolonged survival, suggesting ST6GalNAc I as a possible target for treatment of peritoneal dissemination in gastric cancer.

Materials and methods

Cell lines and culture conditions

The gastric cancer cell lines MKN45 and MKN74 were purchased from the Japanese Cancer Research Resource Bank (Osaka, Japan), JRST and HSC-39 were purchased from Immuno-biological Laboratories (Gunma, Japan), and KATO III and NUGC4 were purchased from RIKEN Cell Bank (Tsukuba, Japan). We also used luciferase-expressing transfectants (MKN45-luc) purchased from the Japanese Cancer Research Resource Bank (Osaka, Japan). All cell lines were cultured in RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA) containing 10 % heat-inactivated fetal bovine serum (FBS; Life Technologies), 2 mM L-glutamine, and 1 % penicillin/streptomycin G1146 (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C in a humidified atmosphere containing 5 % CO₂.

Reagents and antibodies

Two monoclonal antibodies against STn (Sialyl-Tn) were used in this study. The fluorescein isothiocyanate (FITC)-conjugated STn antibody (STn219) and the TAG72 antibody (B72.3) were purchased from Abcam (Cambridge, UK). Mouse IgG1 and mouse FITC-conjugated IgG1 (Dako, Glostrup, Denmark) were used as control-irrelevant antibodies.

Immunofluorescence analysis

Cells seeded on counting chamber slides were blocked with 5 % bovine serum albumin (BSA) for 1 h and then incubated with the FITC-conjugated STn antibody (STn219; Abcam) and FITC-conjugated anti-mouse whole IgG (Dako) for 30 min on ice. Immunofluorescent images were acquired by fluorescence microscopy (Biozero BZ-8000, Keyence, USA).

Flow cytometry analysis

Cells (1×10^6) were incubated with the FITC-conjugated STn antibody (STn219; Abcam) in $1 \times$ phosphate-buffered

saline (PBS), 1 % BSA, for 30 min on ice, washed twice with PBS containing 1 % BSA, and then incubated for another 30 min on ice with FITC-conjugated anti-mouse whole IgG (Dako) in PBS containing 1 % BSA. Cells were then analyzed by flow cytometry using a FACS Calibur instrument (Becton-Dickinson, Franklin Lakes, NJ, USA).

Quantitative RT-PCR

Total RNA was isolated from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. Reverse transcription was carried out with 1 μ g purified RNA using the SuperScript VILO cDNA synthesis kit (Invitrogen). All TaqMan primers mixed with probes were purchased from Invitrogen. Primers for the experiment were as follows: ST6GalNAc I, forward 5'-ACAGTCCCTGGCAAAGCC TA-3', reverse 5'-TGTGTGTTGAGGGCATTGTTC-3'; β -actin, forward 5'-GCCATCCTCACCTGAAGTA-3', reverse 5'-GAAGGTGTGGTGCCAGATTT-3'. β -Actin was used as a control for RNA integrity.

The TaqMan reactions were performed using an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). The results were expressed as the ratio of the number of copies of the product gene to the number of copies of the housekeeping gene (β -actin) from the same RNA (respective cDNA) sample and PCR run.

Preparation of siRNAs

Three formulations of siRNA directed against ST6GalNAc I (Gene ID: 55808) were purchased from Invitrogen. We used ST6GalNAc I stealth siRNA (Invitrogen). ST6GalNAc I HSS125080 (SiRNA 1); (5'-CCUGGAACACUUUG CACCACCCUUU-3') and ST6GalNAc I HSS125081 (SiRNA 2); (5'-GCUCUGUGACCAGGUGAGUGCUU AU-3'), and stealth RNAi negative-control high GC (12935-300; Invitrogen).

Transfection of siRNAs

Gastric cancer cell lines were transfected with siRNAs by use of Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Cell lines were plated at a density of 5×10^5 cells per 6-well dish. Cells were then transfected with 90 pmol Stealth siRNA and 9 μ l Lipofectamine RNAiMAX reagent per dish and incubated 72 h.

Western blot and immunoprecipitation

Protein extracts of cells were resolved over 4–20 % or 15–25 % gradient SDS-polyacrylamide gels (Cosmo Bio, Tokyo, Japan), then transferred onto polyvinylidene

fluoride membranes (Millipore, Bedford, MA, USA). Membranes were incubated with 5 % non-fat dry milk in Tris-buffered saline (TBS) and probed with antibodies against ST6GalNAc I (Atlas Antibodies, Stockholm, Sweden), IGF-1 (Abcam), and β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBST (0.1 % Tween 20 in TBS), then with peroxidase-coupled antibodies as the secondary antibody (GE Healthcare, Little Chalfont, UK). The proteins were visualized with enhanced chemiluminescence (ECL; GE Healthcare). The blots were quantified using LAS-4000UV mini and MultiGauge software (Fujifilm, Tokyo, Japan). For immunoprecipitation study, proteins from cell extracts were immunoprecipitated using anti-STAT5b antibody (Santa Cruz Biotechnology) bound to protein A Sepharose (Sigma). Immunoprecipitates were analyzed by Western blot with anti-phospho STAT5 (Tyr699) antibodies (Cell Signaling Technology, Denver, CO, USA). Immunoblotting study for the indicated antibodies were performed three times to confirm the results.

Cell proliferation assay

Cells (5×10^4) were plated in 24-well culture microtiter plates (Thermo Scientific Nunc, Roskilde, Denmark) and incubated at 37 °C in 5 % CO₂. Every 24 h, one plate was quantified for cell viability by adding 50 μ l/well of Premix WST-1 (Takara, Ootu, Japan) and incubating for 2 h at 37 °C and 5 % CO₂. The absorbance of the samples was measured against a background control as a blank using a microtiter plate reader (Infinite M1000 Pro; Tecan, Durham, NC, USA). The wavelength for measuring the absorbance is 450 nm and the reference wavelength is 620 nm.

Cell migration and invasion assay

Cell migration and invasion assay were carried out according to the manufacturer's protocol (Cell Biolabs, San Diego, CA, USA). Cells (3×10^5) in RPMI were placed on 8.0- μ m-pore-size membrane inserts in 24-well plates, and RPMI with 10 % FBS was placed at the bottom of the wells. After 24 or 48 h, the cells remaining in the top chamber were carefully removed from the upper surface of the filters using a cotton swab. The cells from the underside of the membrane were fixed with methanol and stained with methylene blue by 400 μ l Cell Stain Solution (Cell Biolabs). The migration and invasion cells were counted under a light microscope with a low-magnification objective with at least five individual fields per insert. Data are presented as the mean number of cells per high-power field based on triplicate measurements from two independent experiments.

Therapeutic studies with ST6GalNAc I siRNA

To evaluate the effect of ST6GalNAc I siRNA treatment on tumor dissemination, we conducted two sets of experiments. In vitro experiments demonstrated that ST6GalNAc I-siRNA 1 was a more powerful suppressor of ST6GalNAc I mRNA compared to ST6GalNAc I-siRNA 2. We therefore chose to use ST6GalNAc I siRNA 1 for the in vivo experiments. In the first set of experiments, three groups of mice ($n = 6$ for each group) were used for tumor formation and histological evaluations: 1×10^7 MKN45-luc cells stably expressing luciferase were injected peritoneally into 7- or 8-week-old female BALB/c nude mice purchased from Charles River Japan (Yokohama, Japan). Then, each group was injected i.p. with PBS, random siRNA, or ST6GalNAc I siRNA (70 μ g siRNA) twice a week for 4 weeks (total, seven times), starting 2 days after injection of MKN45-luc cells. Intraperitoneal growing tumors in mice were visualized using an IVIS Lumina Imaging System (Xenogen, Alameda, CA, USA). Tumor dimensions and weights of the mice were measured twice a week. Mice were killed at 26 days after injection of cells and the tumors were surgically isolated. The peritoneal nodules were excised, fixed in 10 % neutral buffered formalin, and snap-frozen in dry-iced acetone for immunohistochemical examination. In the second set of experiments, three groups of mice ($n = 6$ for each group) were used to determine the effect of i.p. administration of ST6GalNAc I siRNA on survival using the same dosage schedule as already described. Body weights were measured twice a week. For this in vivo experiment, we use stealth RNAi negative-control high GC siRNA/InvivoFectamine 2.0 Reagent (Invitrogen) complex and ST6GalNAc I stealth siRNA/InvivoFectamine 2.0 Reagent complex. The siRNA/InvivoFectamine reagent complexes were prepared as follows: an equal volume of InvivoFectamine reagent and an siRNA solution were mixed by rotation at 50 °C for 30 min. The final mixture of 200 μ l containing 70 μ g siRNA was used in each i.p. injection. All animal procedures were approved by the Sapporo Medical University Institutional Animal Care and Use Committee.

Immunohistochemistry

For immunohistochemistry, paraffin-embedded blocks prepared from mouse peritoneal nodules were fixed. Slides were deparaffinized in xylene, followed by treatment with a graded series of alcohol washes, rehydration in PBS (pH 7.5), and blocked against endogenous peroxidase with H_2O_2 . Slides were incubated with anti-TAG72 antibody (B72.3) (1:50 dilution; Abcam) at 4 °C overnight. After washing with PBS, biotinylated antibody (1:50 dilution; Vectastain Universal Elite ABC Kit, Vector Laboratories,

Fig. 1 Expression of sialyl-Tn antigen (STn) and ST6GalNAc I in gastric cancer cell lines. **a** Expression of STn in MKN45, JRST, HSC-39, MKN74, NUGC4, and KATOIII cell lines that were examined by immunofluorescence. **b** Flow cytometry analysis. MKN45, JRST, and HSC-39 showed 37.9 %, 24.4 %, and 20.7 % STn-positive cells, respectively; in contrast, MKN74, NUGC4, and KATOIII showed 5.3 %, 4.2 %, and 1.1 % STn-positive cells, respectively. **c** ST6GalNAc I expression evaluated by qPCR in gastric cancer cell lines. ST6GalNAc I expression levels were calculated as ratios in relationship to the expression in MKN74. **d** Suppressive effect of ST6GalNAc I siRNA on STn expression evaluated by qPCR in MKN45 cells. MKN45 cells were treated with random siRNA, ST6GalNAc I siRNA 1, or ST6GalNAc I siRNA 2. ST6GalNAc I expression levels were calculated as ratios relative to the expression in cells treated with random siRNA. **e** Immunoblot analysis of ST6GalNAc I expression levels in MKN45 cells treated with random siRNA, ST6GalNAc I siRNA 1, or ST6GalNAc I siRNA 2 for 72 h. β -Actin was used as a loading control. Representative results from two independent experiments are shown. **f** STn expression shown by immunofluorescence analysis and flow cytometry analysis. MKN45 transfected with random siRNA showed 34.2 % STn-positive cells. In contrast, ST6GalNAc I siRNA 1- and ST6GalNAc I siRNA 2-transfected cells showed 2.9 % and 3.0 % STn-positive cells, respectively. One representative image of four experiments is shown. * $P < 0.05$. $\times 100$

Burlingame, CA, USA) was added to tissue sections. STn was visualized by Vectastain Elite ABC reagent (Vector Laboratories), followed by incubation with 3,3'-diaminobenzidine (DAB). Nuclei were counterstained with hematoxylin. Negative controls were performed by omitting the primary antibody.

Statistical analysis

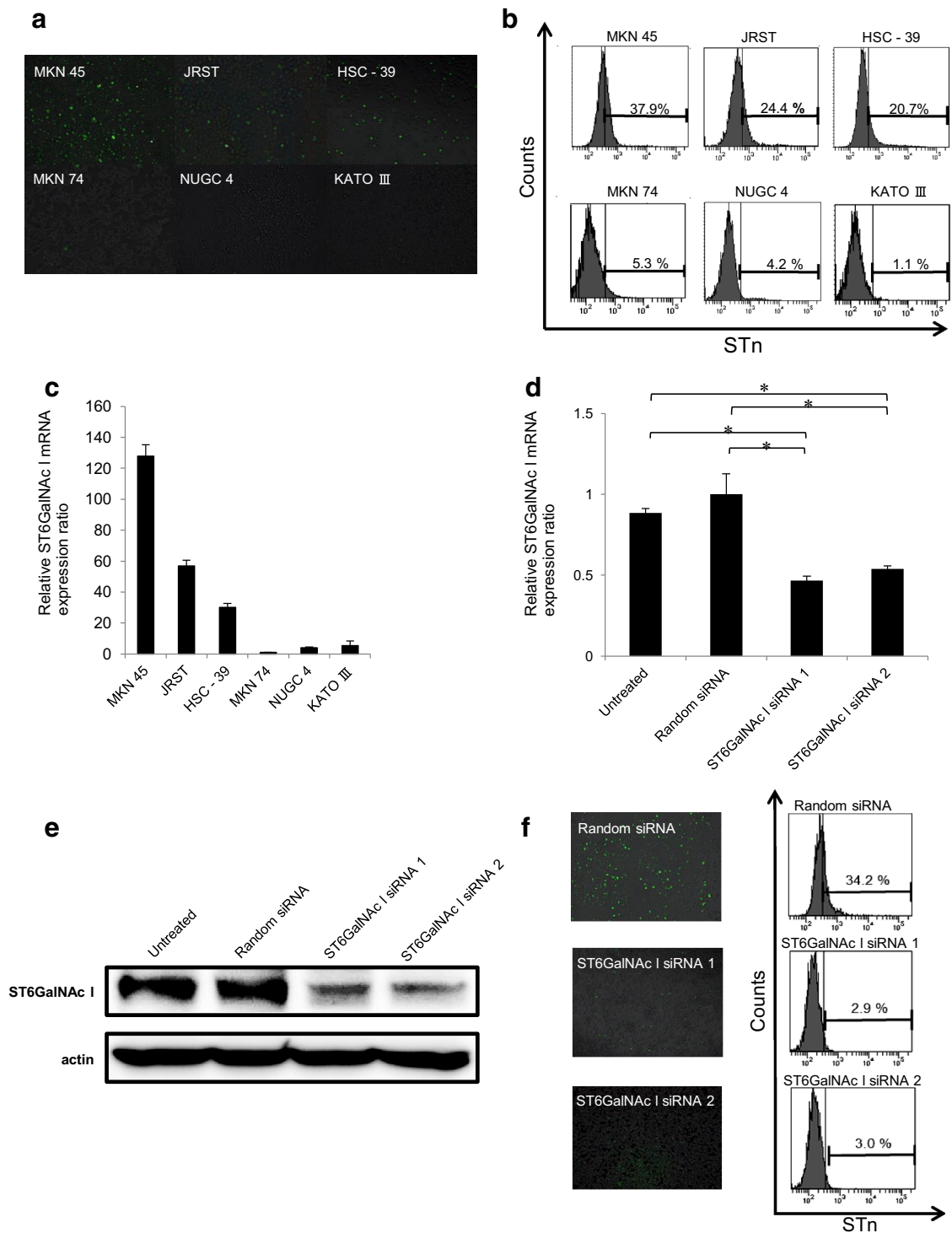
All data were expressed as the mean \pm SE and analyzed using the unpaired *t* test. Survival curves were calculated according to the Kaplan–Meier method. Differences between survival curves were examined with the log-rank test. The accepted level of significance was $P < 0.05$. PRISM (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses.

Results

Expression of STn and ST6GalNAc I in gastric cancer cell lines

We evaluated the expression level of STn in various human gastric cancer cell lines by using immunofluorescence staining and flow cytometry (Fig. 1a, b) and found that STn expression was at low to moderate levels in MKN45, JRST, and HSC-39 cells. In contrast, the expression of STn was almost negative in MKN74, NUGC4, and KATOIII cells.

The expression of ST6GalNAc I was evaluated by quantitative RT-PCR. A relatively high expression of ST6GalNAc I mRNA was detected in MKN45, JRST, and



HSC-39 cells with relative ST6GalNAc I expression values ranging from 40 to 130; a low expression was observed in NUGC4 and KATOIII cells, with relative ST6GalNAc I expression values ranging from 5 to 10 when compared to the lowest mRNA expression of MKN74 (Fig. 1c), thus resembling a cell-surface expression pattern of STn.

In human gastric cancer tissues, the ST6GalNAc I expression was observed as widespread granular perinuclear and diffuse cytoplasmic staining regardless of the histological subtype (Supplemental Fig. 1).

To assess the suppressive effect of siRNA on STn expression in MKN45 cells, we first examined expression

of mRNA in ST6GalNAc I siRNA-transfected MKN45 cells by quantitative RT-PCR. In this particular experiment, to eliminate potential off-target effects, we used two types of siRNAs, ST6GalNAc I siRNA 1 and ST6GalNAc I siRNA 2, which target different parts of the molecule. The results showed that ST6GalNAc I mRNA expression in MKN45 cells transduced with both ST6GalNAc I siRNA 1 and ST6GalNAc I siRNA 2 was significantly reduced as compared with the expression in untreated cells and cells transfected with random siRNA (Fig. 1d). As determined by Western blotting, STn expression in MKN45 transduced with ST6GalNAc I siRNA 1 or ST6GalNAc I siRNA 2 was substantially reduced as compared with the expression in untreated and random siRNA-treated cells (Fig. 1e). In immunofluorescence staining and flow cytometry, STn expression in MKN45 transduced with ST6GalNAc I siRNA 1 or ST6GalNAc I siRNA 2 were apparently reduced as compared with random siRNA transfectants (Fig. 1f).

Effect of ST6GalNAc I suppression on cell growth

Next, we investigated the effects of ST6GalNAc I siRNA on the proliferation of gastric cancer cell lines by WST-1 assays (Fig. 2). In STn-expressing cells (MKN45, JRST, and HSC-39), the proliferation of ST6GalNAc I siRNA 1- or ST6GalNAc I siRNA 2-transfected cells was significantly inhibited as compared with untreated and random siRNA-treated cells. In contrast, there was no obvious inhibition over the entire experimental period of proliferation in ST6GalNAc I siRNA 1- or ST6GalNAc I siRNA 2-transfected MKN74 cells without expression of STn. In contrast, in the STn-overexpressing MKN74 cells that were transfected with ST6GalNAc I plasmid, the proliferation was significantly increased compared to that of STn-negative mock-transfected cells (Supplemental Fig. 2a).

Suppression of ST6GalNAc I reduces gastric cancer cell migration and invasion

To evaluate the effects of siRNA-mediated downregulation of ST6GalNAc I on cell migration and invasion capacity, we examined cell migration in a non-Matrigel-coated Boyden chamber and cell invasion in a Matrigel-coated invasion chamber. In STn-expressing cells (MKN45, JRST, and HSC-39), the migration rates of the ST6GalNAc I siRNA 1- or ST6GalNAc I siRNA 2-transfected cells were significantly decreased compared to that of untreated or random siRNA-transfected cells ($P < 0.05$) (Fig. 3a). As shown in Fig. 3b, in STn-expressing cells, siRNA-mediated

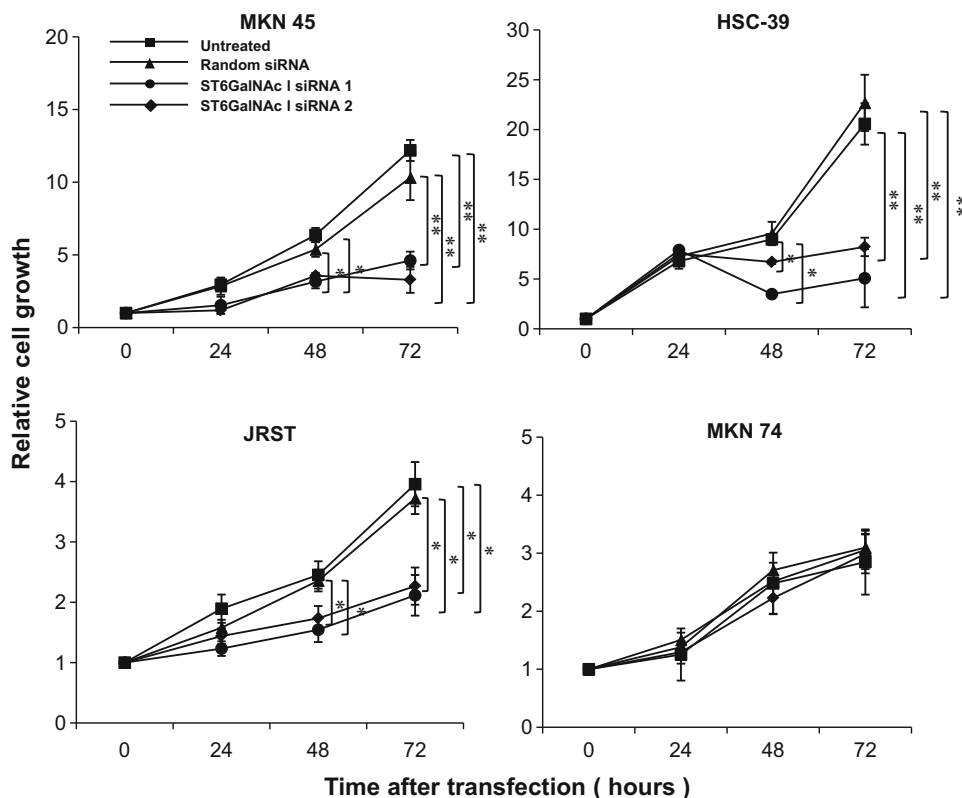
downregulation of STn also significantly reduced cell invasion capacity when compared to that of untreated or random siRNA-transfected cells.

In contrast, there was no obvious reduction of tumor cell migration and invasion in ST6GalNAc I siRNA 1- or ST6GalNAc I siRNA 2-transfected MKN74 cells without expression of STn. On the other hand, in the STn-overexpressing MKN74 cells, which were transfected with ST6GalNAc I plasmid, tumor cell migration and invasion were significantly increased compared to those of mock-transfected cells (Supplemental Fig. 2b, c).

In vivo inhibition of peritoneal dissemination in the mouse xenograft model by i.p. administration of ST6GalNAc I siRNA

To confirm that downregulation of ST6GalNAc I inhibits peritoneal dissemination of MKN45, we tried to suppress the peritoneal dissemination by i.p. administration of ST6GalNAc I siRNA. MKN45-luc cells were injected into the abdominal cavity of three separate groups of nude mice (1×10^7 cells per mouse). Starting 2 days after injection of MKN45-luc, 200 μ l ST6GalNAc I siRNA-liposome (containing 70 μ g siRNA), random siRNA-liposome, or PBS, respectively, was injected into the peritoneal cavity of the recipient mice twice a week for 4 weeks. After seven injections (day 26), the mice were killed to examine peritoneal dissemination, and the tumor masses and serum STn levels were measured. In vivo growth of MKN45-luc cells was significantly inhibited by treatment with ST6GalNAc I siRNA-liposome compared with the growth in mice treated with random siRNA-liposome or PBS, as visualized by optical imaging of luciferase activity in the IVIS system (Fig. 4a). The time course (at 7, 14, 21, and 26 days) of tumor growth is shown by the quantification of photon counts (Fig. 4b). As shown in Fig. 4c, the number and sizes of peritoneal metastatic foci tended to be smaller in ST6GalNAc I siRNA-liposome-injected mice than in random siRNA-liposome-injected or PBS-treated mice. Serum STn levels in ST6GalNAc I siRNA-treated mice (120 ± 38 U/ml) were significantly lower than those in random siRNA-liposome-treated mice (45 ± 4 U/ml) ($P < 0.01$). Tumor weights were significantly higher in random siRNA- or PBS-treated mice compared with those in ST6GalNAc I siRNA-treated mice ($1,979 \pm 167$ versus 565 ± 89 mg, respectively; Fig. 4d). To investigate whether the inhibition of tumor dissemination was associated with the downregulation of STn expression, the disseminated nodules were excised and subjected to STn immunohistochemical staining. STn was markedly downregulated in the mice treated with ST6GalNAc I siRNA compared with those treated with PBS or random siRNA (Fig. 4e).

Fig. 2 Suppression of ST6GalNac I by siRNA inhibits cell proliferation in gastric cancer cell lines. Cell proliferation of untreated, random siRNA-, or ST6GalNac I siRNA 1- or ST6GalNac I siRNA 2-transfected gastric cancer cells was measured by the WST-1 assay. Results are from triplicate measurements from two independent experiments. * $P < 0.05$, ** $P < 0.01$



Effect of ST6GalNac I siRNA i.p. administration on survival in a gastric cancer peritoneal dissemination mouse model

We also observed the effects of ST6GalNac I siRNA injection on survival in a peritoneal dissemination model using MKN45 cells. MKN45 cells were injected into the abdominal cavity of three separate groups of nude mice (1×10^7 cells per mouse). Starting 2 days after injection of MKN45, 200 μ l ST6GalNac I siRNA-liposome (containing 70 μ g siRNA), random siRNA-liposome, or PBS, respectively, was injected into the peritoneal cavity of the recipient mice twice a week for 4 weeks. As shown in Fig. 5a, the survival of mice treated with ST6GalNac I siRNA was significantly prolonged relative to that of random siRNA- or PBS-treated mice. The weight of mice treated with ST6GalNac I siRNA was significantly greater than that of mice treated with random siRNA or PBS (Fig. 5b).

PCR array of tumor metastasis-associated mRNAs in ST6GalNac I siRNA-transfected MKN45

To clarify the mechanisms whereby ST6GalNac I modulates cell proliferation, migration, and invasion, we analyzed the differences in mRNA levels of selected signaling molecules by PCR arrays that were designed to assess the expression levels of mRNAs associated with tumor metastasis (Supplemental

Fig. 3). We chose MKN45 because its expression level of ST6GalNac I mRNA was the highest in six tested gastric cancer cell lines (Fig. 1c). Genes showing expression that changed at least 1.5 fold in ST6GalNac I siRNA-transfected cells compared to expression in random siRNA-transfected cells are shown in Supplemental Fig. 3b. Alterations in the expression levels were observed in cell growth and proliferation molecules such as insulin-like growth factor-1 (IGF-1), cell adhesion molecules such as integrin beta 4 (ITGA4), and intermediate filament proteins such as vimentin (VIM). Among all these mRNA moieties, the decrease in IGF-1 expression was the most pronounced in ST6GalNac I siRNA-transfected MKN45 cells (2.126-fold decrease). In fact, as shown in Fig. 6a, expression of IGF-1 was substantially suppressed by ST6GalNac I siRNA transfection, whereas it was overexpressed by transient transfection with ST6GalNac I plasmid when analyzed by immunoblot assay. These results indicate that ST6GalNac I directly or indirectly regulates the expression of IGF-1 upstream target genes involved in gastric cancer cells.

Mechanism of ST6GalNac I siRNA-induced downregulation of IGF-1

To explore the molecular mechanisms by which ST6GalNac I siRNA downregulates IGF-1 mRNA expression in MKN45 cells, we examined the STAT5b signaling pathways that reportedly regulate IGF-1 synthesis [34–39]. As

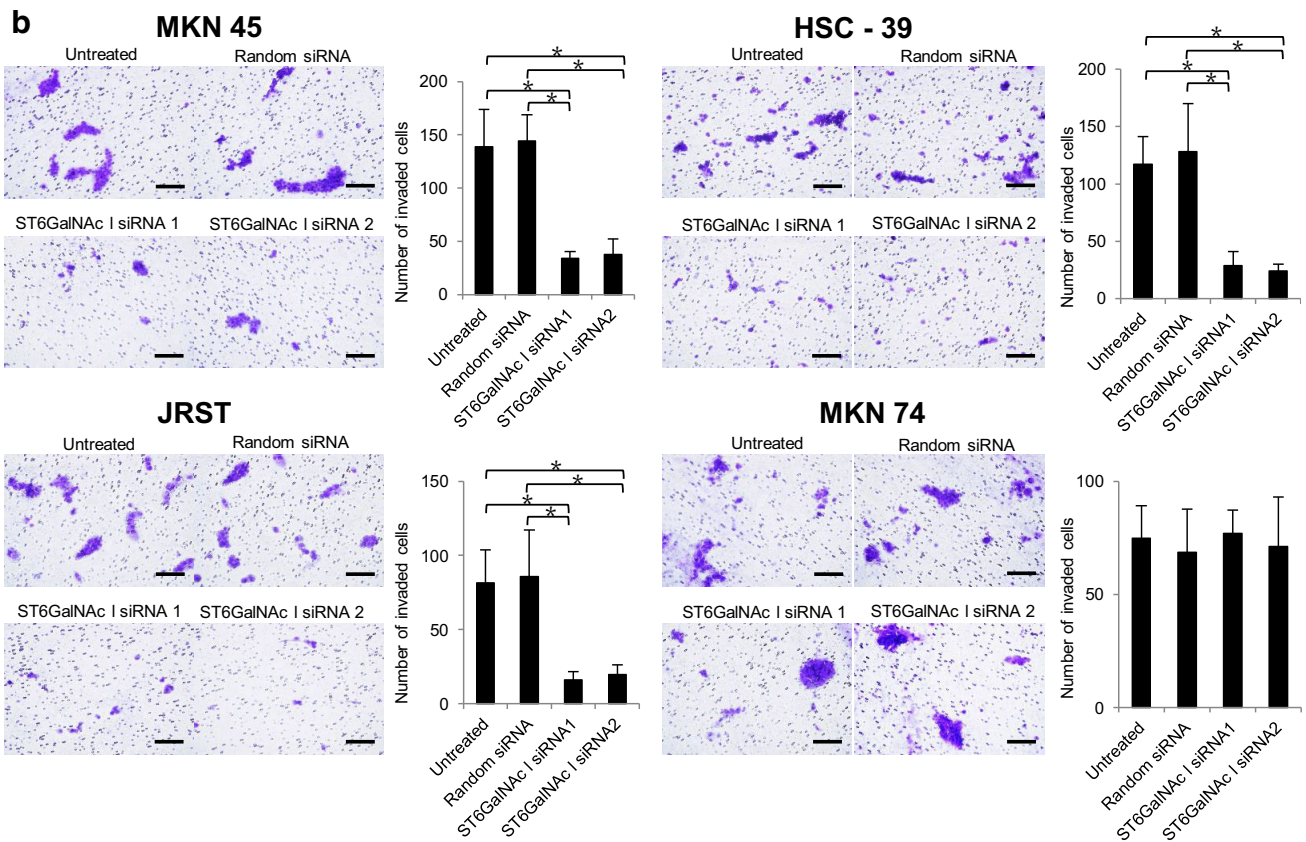
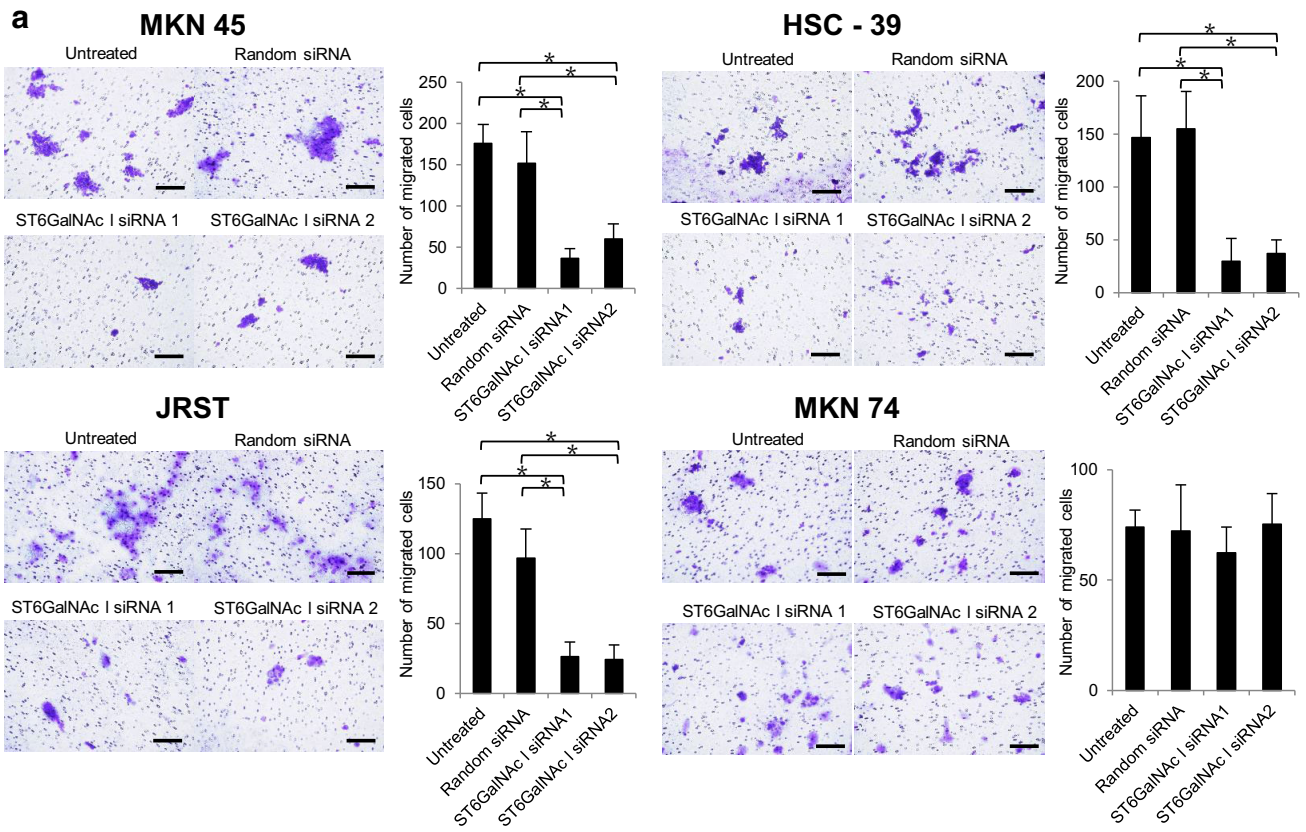
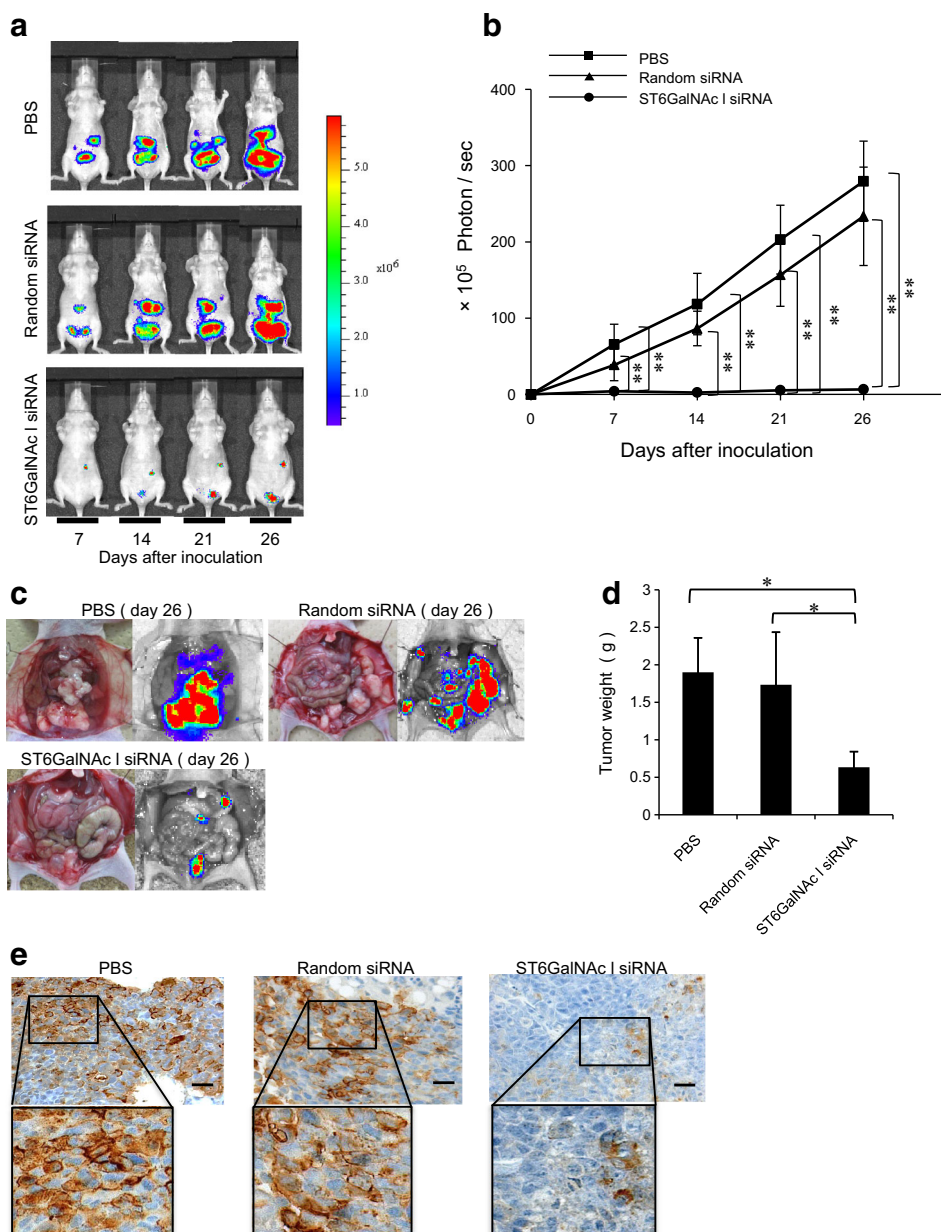


Fig. 3 Suppression of ST6GalNac I by siRNA inhibits cell invasion and migration in gastric cancer cell lines. **a** In vitro migration assays utilized a modified Boyden chamber system. Equal numbers (3×10^5) of transfected cells were placed in the top chamber and allowed to migrate for 24 h. Representative photographs are shown at *left*. *Right panel* shows number of migrated cells that were transfected with the indicated siRNA. Results from triplicate measurements from two independent experiments. $*P < 0.05$. **b** Invasion assays of ST6GalNac I siRNA-transfected gastric cancer cell lines using a modified Boyden chamber system. After 48 h, invading cells were stained with methylene blue and counted. *Right panel* shows number of invaded cells that were transfected with the indicated siRNA. $*P < 0.05$. Bars 100 μm

shown in Fig. 1e, expression of ST6GalNac I in MKN45 cells was substantially suppressed by ST6GalNac I siRNA transfection, whereas it was enhanced by transient transfection with ST6GalNac I plasmid (Supplemental Fig. 4). Phosphorylation of STAT5b was inhibited in MKN45 cells treated with ST6GalNac I siRNA compared with the phosphorylation in cells transfected with random siRNA. In contrast, phosphorylation of STAT5b was more enhanced by transient transfection with ST6GalNac I plasmid as compared with controls (Fig. 6b). These results

Fig. 4 siRNA-mediated downregulation of ST6GalNac I inhibits peritoneal dissemination of gastric cancer cells in a mouse model. **a** Inhibition of tumor cell growth in the peritoneal cavity by ST6GalNac I siRNA-liposome administration. Representative images of mice treated with ST6GalNac I siRNA, random siRNA, or phosphate-buffered saline (PBS) alone are visualized by the IVIS system at the indicated days after inoculation of MKN45-luc. Color bar $\times 10^6$ photon/s. **b** Results of quantitative photon-counting analysis of mice treated as depicted in (a). Each point represents mean \pm SD ($n = 6$). Similar results were obtained in two independent experiments. **c** Mice treated as depicted in a were killed and subjected to laparotomy (*left*) to visualize disseminated cells by the IVIS system (*right*) 26 days after intraperitoneal inoculation of MKN45-luc. **d** Peritoneal tumor masses as indicated in c were resected and weighed. **e** STn expression in tumor tissue assessed by immunohistochemistry as indicated in c. $*P < 0.05$; $**P < 0.01$. Bars 100 μm



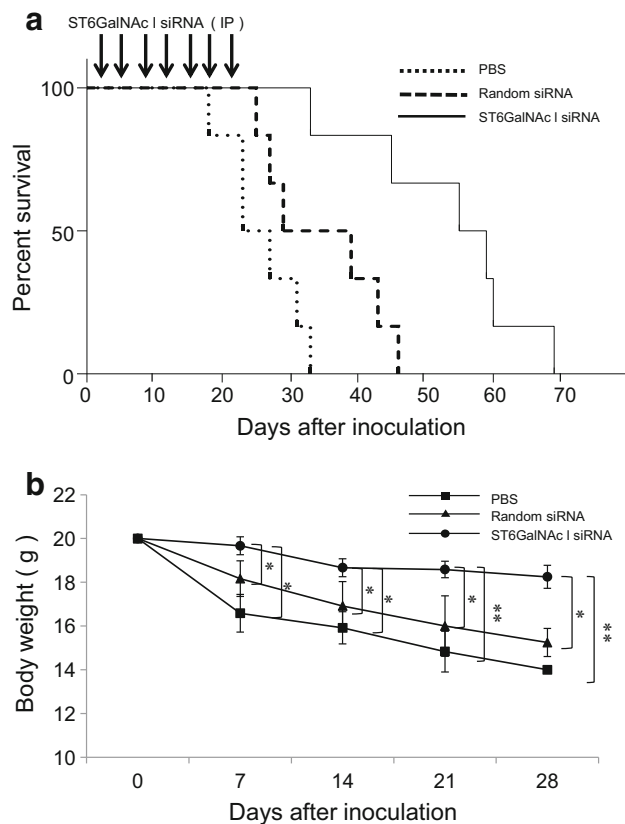


Fig. 5 Effect of i.p. administration of ST6GalNAc I siRNA on survival in a peritoneal dissemination mouse model. **a** ST6GalNAc I siRNA-liposome was administered i.p. 2 days after inoculation of MKN45 cells as indicated. Kaplan–Meier curve reveals that i.p. administration of ST6GalNAc I siRNA-liposome significantly prolonged survival of the mice (PBS vs. random siRNA, $p = 0.072$; random siRNA vs. ST6GalNAc I siRNA, $p = 0.0017$; PBS vs. ST6GalNAc I siRNA, $p = 0.0011$). **b** Weight of ST6GalNAc I siRNA-treated mice was significantly higher than that of control mice

suggest that ST6GalNAc I regulates the STAT5b signaling pathways, leading to regulation of IGF-1 expression.

Discussion

Because of its high specificity, STn has been suggested as a promising target in the treatment of cancer. However, it remains unclear how STn antigen-expressing tumors acquire a growth advantage in vivo, and currently there are no approved therapies that target STn. In this study, we found that ST6GalNAc I regulates the gene expression of IGF-1 through activation of the STAT5b pathway in gastric cancer cells. Therefore, ST6GalNAc I appears to be important in the migration and invasive potential of gastric cancer cells in association with STn expression. These results provide a basis for identifying potential molecular targets for cancer treatment via ST6GalNAc I-mediated IGF-1 regulation in gastric cancer cells.

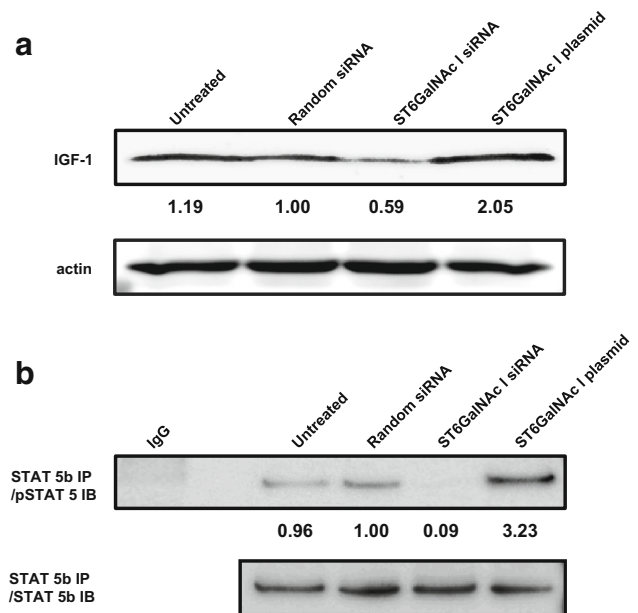


Fig. 6 ST6GalNAc I siRNA-regulated IGF-1 expression and STAT5b signaling in MKN45 cells. **a** Immunoblot analysis of IGF-1 expression levels in MKN45 cells treated for 72 h with random siRNA, ST6GalNAc I siRNA, or ST6GalNAc I expression plasmid. Relative mean density presented as ratio to random siRNA as indicated. β -Actin was used as a loading control. Representative results from two independent experiments. **b** STAT5b phosphorylation in MKN45 cells treated as in **a** was determined by immunoprecipitation (IP) with anti-STAT5b antibody, followed by immunoblotting (IB) with antiphospho-STAT5 (Tyr699) antibody. Relative mean density presented as ratio to random siRNA as indicated. Representative results from two independent experiments

In the present study, we explored the potential of anti-dissemination therapy involving suppression of STn expression by ST6GalNAc I siRNA treatment. We observed that all six gastric cancer cell lines showed ST6GalNAc I mRNA expression by quantitative RT-PCR, and among them, MKN45 showed the highest expression, which was 130 times as much as that in MKN74 cells, which showed the lowest expression (Fig. 1c). Moreover, we confirmed that gastric cancer specimens obtained from gastric cancer patients expressed ST6GalNAc I in both intestinal and diffuse types of the cancer as determined by immunohistochemistry (Supplemental Fig. 1).

Inhibition of ST6GalNAc I expression in vitro in MKN45 cells almost completely suppressed STn expression (Fig. 1f). Moreover, growth, invasion, and migratory capacity were significantly suppressed in ST6GalNAc I siRNA-transfected cells compared with mock transfectants (Figs. 2, 3). It should be noted that off-target effects were considered to be well controlled by using either of these siRNAs because we used two independent siRNAs against the same target (ST6GalNAc I) mRNA and found comparable gene silencing efficacy in an in vitro experiment (Figs. 1, 2, 3).

Moreover, we showed that overexpression of ST6GalNAc I is associated with increase of growth, invasion, and migratory capacity of MKN 74 cells. These observations clearly indicated that regulation of ST6GalNAc I expression has a direct impact on tumor development of gastric cancer cells including proliferation, migration, and invasion ability.

As an *in vivo* therapeutic model, we focused on peritoneal dissemination because the peritoneum is the most frequent site of gastric cancer recurrence. Prevention or treatment of peritoneal dissemination may be an important therapeutic strategy for improving the quality and quantity of life for gastric cancer patients. In this respect, it is important to consider that the metastatic cancer grows in an enclosed cavity. In this environment, local administration of the siRNA-liposome complex could produce a high concentration of the agent for attachment to the target cancer cells during a limited time period [40]. In fact, our *in vivo* studies showed that STn expression was effectively downregulated in the tumor nodules in mice intraperitoneally injected with the ST6GalNAc I siRNA. In addition, both tumor weight and serum STn levels were significantly reduced (Fig. 4). Moreover, we showed that mice treated with ST6GalNAc I siRNA survived significantly longer than mice treated with PBS alone or random siRNA (Fig. 5a), even without any apparent side effects (data not shown), indicating that ST6GalNAc I siRNA reduced dissemination ability and tumor growth of cancer cells *in vivo* as well as *in vitro*.

Nevertheless, it may be difficult to fully explain such profound antitumor effects solely by decreased STn expression. Therefore, we conducted focused quantitative RT-PCR arrays of metastasis-associated genes in MKN45 cells treated with ST6GalNAc I siRNA to explore the potential role of ST6GalNAc I. The array showed a significant decrease in IGF-1 mRNA expression in MKN45 cells treated with ST6GalNAc I siRNA compared with that of random siRNA-treated cells (Supplemental Fig. 3). Among the different growth factors influencing the initiation, progression, and metastasis of gastric cancer, IGF plays a critical role in the stimulation of gastric cancer cell proliferation, survival, angiogenesis, and resistance to apoptosis [41]. Recent studies have shown that elevation of serum IGF-1 increases the risk for developing several types of cancer [41–43]. In gastric cancer, aberrant activation of IGF-1 receptor by autocrine/paracrine loops has been suggested because expression of both IGF and its receptor is high in tumor tissue. Our findings suggest that reduced expression of IGF-1 by ST6GalNAc I siRNA transfection may be involved in suppression of dissemination, probably by inhibiting autocrine/paracrine growth stimulation. The JAK2-STAT pathway is one of the major intracellular pathways in the IGF system that is frequently activated in

various types of cancer cells [44, 45]. Among a variety of STAT proteins, STAT5b directly regulates transcription of IGF-1 [34–37]. Moreover, expression of STAT5b was associated with tumor progression in human solid cancer [46–48].

In the present study, ST6GalNAc I inhibition suppressed STAT5b phosphorylation (Fig. 6b), and as a result decreased IGF-1 expression was observed (Fig. 6a). Conversely, overexpression of ST6GalNAc I increased STAT5b phosphorylation and IGF-1 expression (Fig. 6a, b). These results provide further clues about the role of ST6GalNAc I in activation of STAT5b and other signaling molecules linked to IGF-1 during gastric cancer development and dissemination. Interestingly, Kim et al. reported that the sialic acid residues on gangliosides may be essential for the activation of JAK-STAT signaling [49], which suggests the possibility that sialylation by ST6GalNAc I may affect this pathway. However, a detailed investigation is warranted to elucidate the crosstalk between ST6GalNAc I and JAK/STAT signaling pathway-associated molecules.

In conclusion, we have demonstrated that *in vitro* and *in vivo* transfection with ST6GalNAc I siRNA in human gastric cancer cells inhibited the expression of STn, resulting in several antitumor activities such as inhibition of cell growth, migration, and invasion as well as blocking of the IGF-1-dependent axis and suppression of STAT5b signaling pathways. These findings suggest that ST6GalNAc I could be an effective therapeutic target in peritoneal dissemination of gastric cancer; thus, *i.p.* administration of ST6GalNAc I siRNA to disseminated tumor cells in the peritoneal cavity prolonged the survival of model mice. The present report provides a basis for developing new targets in the treatment of dissemination through studies in additional animal models and clinical trials. As STn apparently is essential in causing metastasis associated with pancreatic [16], colorectal [12], ovarian [13], and breast cancers [14, 15], our approach may also be useful in treating metastatic conditions in other organs in addition to the stomach.

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Conflict of interest The authors have no conflict of interest.

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