Morphological Changes, Cadherin Switching, and Growth Suppression in Pancreatic Cancer by GALNT6 Knockdown

Abstract
Pancreatic cancer reveals the worst prognosis among human cancers with little improvement in its clinical outcome in the last three decades. We previously suggested that polypeptide N-acetylgalactosaminyltransferase 6 (GALNT6), which catalyzes O-type glycosylation of Mucin 1, might be a promising molecular target for drug development for breast cancer. In this study, we report upregulation of GALNT6 in pancreatic cancer cells where Mucin proteins are highly O-glycosylated. We found that knockdown of GALNT6 with small interfering RNA in pancreatic cancer cells decreased the amount of Mucin 4 protein as well as that of its transcript, reduced the levels of human epidermal growth factor receptor 2 and extracellular signal–regulated kinase, and significantly reduced pancreatic cancer cell viability. Interestingly, knockdown of GALNT6 caused drastic morphological changes of pancreatic cells, accompanied with the cadherin switching from P-cadherin to E-cadherin. Considering important roles of Mucin 4 in growth and invasion, our findings imply that targeting GALNT6 is a very promising therapeutic strategy for treatment of pancreatic cancer patients who still have very limited treatment modalities.

Introduction
Pancreatic cancer is the fourth leading cause of cancer-related death in the United States and has kept the lowest 5-year survival rate among common cancers due to the difficulty of diagnosis at an early stage, a progressive phenotype, and limited options of treatment with relatively low efficacy [1]. Current treatment options of pancreatic cancer are based on either gemcitabine or a combination regimen called FOLFIRINOX, which is composed of folinic acid (FOL), 5-fluorouracil (F), irinotecan (IRIN), and oxaliplatin (OX) [2]. However, because these treatment modalities are not highly effective, more effective and less toxic treatment modalities should be developed for pancreatic cancer patients.

Accumulating evidences have suggested that mucins are involved in development, invasiveness, metastasis, and drug resistance of pancreatic cancer [3]. Mucins, which are present as a membrane-bound form or as a secreted form, enhance several oncogenic signal pathways and suppress cell adhesion [4]. Particularly, in pancreatic cancer, Mucin 4 protein expression is known to be elevated and extensively modified with O-linked oligosaccharides (O-glycosylation) [3]. Increase of O-glycosylated Mucin 4 was correlated with poor prognosis in patients with pancreatic cancer [5,6].

The O-glycosylation is one of common posttranslational protein modifications in cancer cells, and aberrant O-glycosylation is generally considered to enhance functions of oncogenic proteins [7]. For example, O-glycosylation is indicated to play important roles in protein processing, stability, secretion, and thus activation of signaling pathways [8]. Such abnormal O-glycosylation in cancer cells is attributed to upregulated polypeptide N-acetylgalactosaminyltransferase 6 (GALNT6), which catalyzes O-type glycosylation of Mucin 1. Therefore, targeting GALNT6 might be a promising molecular target for drug development for breast cancer.
expression of polypeptide N-acetylgalactosaminyl transferase (GALNT) family proteins that are involved in the initiation of the O-glycosylation by transferring N-acetyl-alpha-D-galactosamine (GalNAc) to target substrates [9]. We previously reported that GALNT6 was highly transactivated and stabilized Mucin1 protein through the O-glycosylation in breast cancer cells [10]. However, the underlying mechanism to cause aberrant O-glycosylation of Mucins in pancreatic cancer has not been fully investigated.

In the present study, we demonstrate that GALNT6 is overexpressed in a subset of pancreatic cancer cell lines examined and plays imperative roles in growth and invasion of pancreatic cancer cells. We also reveal evidences supporting that GALNT6 is essential for O-glycosylation and stabilization of Mucin 4 protein, and that knockdown of GALNT6 causes drastic morphological changes of pancreatic cells accompanied with the cadherin switching from P-cadherin to E-cadherin. Our results imply that GALNT6 is a promising molecular target for drug development for pancreatic cancer.

Material and Methods

Cell Lines

Human pancreatic cancer cell lines ASPC-1, Capan-2, HPAF-II, Panc02.03, Panc08.13, PANC-1, and PL45 were purchased from the American Type Culture Collection (Rockville, MD), KP-1N, MiaPaCa-2, and SUIT-2 cells were purchased from the Japanese Collection of Research BioResources Cell Bank (Saitama, Japan). KLM-1, PK-45P, and PK-59 cells were provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. KP4 cell was provided by RIKEN BioResource Center (Tsukuba, Japan). The stocks of cell lines that had been deposited in liquid nitrogen were used in this study. All cells were cultured at 37°C in atmosphere of humidified air with 5% of CO₂ and in appropriate culture media (Life Technologies, Grand Island, NY), such as RPMI1640 for ASPC-1, KLM-1, KP-1N, Panc02.03, Panc08.13, PK-45P, and PK-59; DMEM for KP4, PANC-1, and PL45; EMEM for HPAF-II, MiaPaCa-2, and SUIT-2; and McCoy for Capan-2. Each medium was supplemented with 15% (for Panc02.03 and Panc08.13) or 10% (for other cell lines) of fetal bovine serum (Fisher Scientific, Pittsburgh, PA) and 1% of antibiotic-antimycotic solution (Fisher Scientific).

Gene Silencing by RNA Interference

To knock down endogenous GALNT6 expression in pancreatic cancer cells, we used small interfering RNAs (siRNAs) synthesized from Sigma-Aldrich (St. Louis, MO). KLM-1 and Panc02.03 cells were transfected with 200 pmol of oligo siRNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. The target sequences of siRNAs are 5′-GAGAUAACCUCCUGUGACA-3′ for si-GALNT6 and 5′-GCACUGUUUCAAUGCUCUU-3′ for si-GALNT6-2, as previously described [10]. The siRNA Universal Negative Control (Sigma-Aldrich) was used for si-control.

Cell Viability Assay

For methyl thiazolyl tetrazolium (MTT) assay, pancreatic cancer cells were seeded into 24-well plates at 5 × 10⁴ cells per well and transfected with oligo siRNA. Then, cancer cells were cultured at 37°C under 5% CO₂ for an additional 72 hours. The Cell Counting Kit–8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used for MTT reaction and examined the cell viability. After reaction for 1 to 3 hours, 100 μl of supernatant was transferred into a 96-well plate and read in the iMark microplate absorbance reader (Bio-Rad, Hercules, CA) at 450 nm of wavelength. All of these experiments were done in triplicate.

Cell Invasion Assay

To examine cell invasiveness, 1 × 10⁶ cells were seeded on Transwell plates (Costar, Cambridge, MA) of 5-μm pore filters. After adding 500 μl of 10% serum-containing medium to the lower chamber of the well, pancreatic cancer cells were allowed to migrate for 72 hours. The migrated cells on the lower side of the membrane were photographed and counted with the ImageJ software [11].

Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from KLM-1 and Panc02.03 cells using RNeasy Mini Kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions. Total RNA (1 μg) was reversely transcribed using SuperScript III First-Strand Synthesis System (Invitrogen) to generate cDNA. Aliquots of cDNA samples were quantified by the real-time RT-PCR method. The RT-PCR was performed using primers listed below using the ViiA 7 system (Life Technologies). The expression level of GALNT6 or MUC4 was normalized with that of GAPDH. The PCR primer sequences were 5′-GGATGAAACCTACCCCATCA-3′ and 5′-ACCCATGGTGCTCAAAGTAGGA-3′ for GALNT6, 5′-GAGGAAATGAC-CAGCTGCTT-3′ and 5′-AGGGCCAGGGGTTGATAGAT-3′ for MUC4, and 5′-CGACCACCTTTTGCAGAATC-3′ and 5′-GGTTGACAGACAGGGTACTTTAT-3′ for GAPDH.

Western Blot Analysis

To detect the expression of endogenous proteins in pancreatic cancer cells, we performed Western blots with normalization of protein amount by β-actin. Cells were lysed with the IP lysis buffer (Thermo Scientific, Waltham, MA) and proteins were separated by electrophoresis using the any KD SDS-PAGE gel (Bio-Rad) and transferred onto nitrocellulose membrane (GE Healthcare, Pittsburgh, PA). After blocking with the Block ACE solution (AbD Serotec, Raleigh, NC), the membranes were incubated with the first antibodies: anti-GALNT6 (Atlas Antibodies, Stockholm, Sweden), anti–Mucin 4 (Santa Cruz Biotechnology, Santa Cruz, CA), anti–E-cadherin (Abcam Biotech, Cambridge UK), anti–P-cadherin (Abcam Biotech), anti–extracellular signal-regulated kinase (ERK) (Abcam Biotech), anti–human epidermal growth factor receptor 2 (HER2) (Abcam Biotech), anti–β-actin (Sigma-Aldrich), anti–Histone H3 (Abcam Biotech), or anti-Tubulin antibody (Millipore). Finally, the membrane was incubated with horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology), and protein bands were visualized by the enhanced chemiluminescence detection reagents (GE Healthcare). For the nuclear/cytoplasm fractionation, KLM-1 and Panc02.03 cells were lysed using NE-PER Nuclear and Cytoplasmic Extraction buffers (Thermo Scientific) for 10 minutes on ice and then quick-spin for 15 seconds to collect cytosolic lysate. Pellets were washed two times with cytoplasmic lysis buffer and then lysed with the nuclear lysis buffer for 45 minutes on ice. The final lysates were spun for 20 minutes at 14,000 rpm at 4°C to collect nuclear lysates.
**Immunocytochemical Staining**

Pancreatic cancer cells were seeded on Lab-Tek II chamber slide system (Nalge Nunc International, Penfield, NY) before transfection with oligo siRNAs. The cells were fixed with 4% paraformaldehyde in PBS for 15 minutes and rendered permeable with 0.1% Triton X-100 in PBS at 4°C for 2.5 minutes. Subsequently, the cells were covered with 3% BSA in PBS at 4°C for 3 hours to block nonspecific hybridization followed by incubation with antibodies of anti-GALNT6 (1:100), anti-E-cadherin (1:100), or anti-β-catenin (1:100). After washing with PBS for three times, cells were stained by Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (Molecular Probe, Eugene, OR) diluted at 1:1000. Finally, nuclei were counterstained with 4′,6-diamidino-2-phenylindole, and cells were examined by SP5 Tandem Scanner Confocal Microscope (Leica Microsystems, Buffalo Grove, IL) at the University of Chicago Microscopy Core Facility. Cytoskeleton structure was visualized by staining with Alexa Fluor 488-conjugated phalloidin (Molecular Probes).

**In vitro O-Glycosylation**

Recombinant GALNT6 protein was purchased from Abnova (Walnut, CA), and MUC4 peptide (TSSASTGHATPLPVTD) was synthesized from GenScript (Piscataway, NJ). Then 5 μM of the MUC4 peptide was incubated with 0.5 μg of recombinant GALNT6 protein in 25 mM of Tris-HCl (pH 7.4), 10 mM of MnCl2, and w/wo 50 μM of UDP-GalNAc at 37°C for 12 hours. The resultant peptides were desalted by Zip-tip C18 (Merck Millipore, Billerica, MA), mixed with 4 mg/ml α-cyano-4-hydroxycinnamic acid, and analyzed with 4800 Plus MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) mass spectrometer (Sciex, Framingham, MA).

**Statistical Analysis**

Statistical significance was calculated by Student’s t test using Prism 6 software (GraphPad, San Diego, CA). A difference of P < .05 was considered to be significant.

**Results**

**GALNT6 and Mucin 4 Expression in Pancreatic Cancer**

We first examined expression levels of GALNT6 in pancreatic cancers through publically available gene expression datasets. The Oncomine database revealed that GALNT6 is highly expressed in pancreatic cancer among 18 human cancer types listed (Supplementary Figure 1). Then, we confirmed by Western blot analyses high levels of GALNT6 expression in 8 of 14 pancreatic cancer cell lines (Figure 1A). We also examined expression levels of Mucin 4, a dominantly expressed Mucin protein in pancreatic cancer [3], and found that Mucin 4 protein levels are relatively higher in the pancreatic cancer cell lines with higher GALNT6 expression (Figure 1A).

**Knockdown Effects of Endogenous GALNT6**

To investigate the biological function of GALNT6 in pancreatic cancer cells, we used siRNA to knock down GALNT6 expression using two pancreatic cancer cell lines, KLM-1 and Panc02.03, in which GALNT6 was highly expressed (Figure 1A). Knockdown of...
GALNT6 by siRNA successfully decreased GALNT6 expression in these two pancreatic cancer cells compared with those transfected with si-control (Figure 1B). Concordantly, Western blot analyses showed significant reduction of GALNT6 protein in KLM-1 and Panc02.03 cells (Figure 1C). Interestingly, we found that both protein level and transcriptional expression level of Mucin 4 were significantly decreased by GALNT6 knockdown (Figure 1C and D). Furthermore, knockdown of GALNT6 significantly reduced cell viability (Figure 1E), which was verified by transfection of an additional siRNA targeting GALNT6 (Supplementary Figure 2). These results indicated that GALNT6 is likely to be essential for proliferation and/or survival of pancreatic cancer cells.

**O-Glycosylation of Mucin 4 by GALNT6**

Mucin 4 protein contains tandemly repeated peptides that are extensively O-glycosylated in pancreatic cancer cells [3]. Furthermore, previous reports showed that the GALNT family is directly involved in the O-glycosylation of Mucin 4 [12]. To examine whether GALNT6 can O-glycosylate Mucin 4, we performed in vitro O-glycosylation assay using recombinant GALNT6 protein and a Mucin 4 peptide (MUC4) corresponding to the core amino acids of tandemly repeated sequences (TSSASTGHATPLPVTD). As a result of MALDI-TOF mass spectrometry (MS) analysis, a molecule, which was 203.07 Da larger ($m/z = 1744.79$) than unmodified MUC4 peptide ($m/z = 1541.72$), was detected specifically in a UDP-GalNAc (+) sample (Figure 2A), indicating O-glycosylation of the MUC4 peptide by GALNT6. Furthermore, subsequent MS/MS analysis identified the shift of b10 fragment from $m/z$ of 901.45 corresponding to an unmodified MUC4 peptide to b10' position with $m/z$ of 1104.46 corresponding to an O-glycosylated MUC4 peptide (Figure 2B). This result illustrated that the 10th threonine was predominantly O-glycosylated by GALNT6.

**Suppression of Mucin 4 Signal Pathways by Knockdown of GALNT6**

Because Mucin 4 protein was significantly reduced by knockdown of GALNT6, we further examined HER2 and ERK proteins, which were reported to interact with Mucin 4 and play important roles in pancreatic cancer cell proliferation and survival [13]. We observed that GALNT6 knockdown significantly affected protein levels of HER2 and ERK in KLM-1 pancreatic cancer cells (Figure 3A). We also observed drastic morphological changes in KLM-1 cell by GALNT6 knockdown compared with the cells transfected with si-control as shown in Figure 3B. Hence, we examined protein levels of the cadherin molecules and found that knockdown of GALNT6 caused the cadherin switching, decrease of P-cadherin, and increase of E-cadherin (Figure 3A). These morphologic alterations were further assessed by immunostaining with a fluorescence-labeled phalloidin.
which interacts with F-actin and clarified changes in the cytoskeleton structure. The staining of F-actin was restricted to cellular membrane in the si-control cells, but that of F-actin was markedly increased and dispersed in the cytoplasm of the GALNT6-knockdown cells (Figure 3C).

Cell Morphological Changes by GALNT6 Knockdown

We have demonstrated that knockdown of GALNT6 induced drastic changes in the cytoskeleton structure. To identify the mechanism underlying these alterations, we examined representative cell adhesion molecules, an E-cadherin/β-catenin complex by immunocytochemical staining. The knockdown of GALNT6 significantly increased staining of E-cadherin as well as β-catenin in the cell-to-cell adhesion area in KLM-1 (Figure 4A) and in Panc02.03 cells (Figure 4B). These findings were in concordant with our Western blot results from cellular fractions of cytoplasm and nucleus. Knockdown of GALNT6 increased amount of β-catenin protein, and most of the β-catenin protein was retained in the cytoplasm of KLM-1 cells (Supplementary Figure S3).

Decreased Invasion and Survival of Pancreatic Cancer Cells by GALNT6 Knockdown

Because upregulated Mucin 4 in pancreatic cancer cells was also indicated to enhance the invasiveness [13], we further examined knockdown effects of GALNT6 in the cell invasion using Transwell plates. Our results revealed that the invasive capacities of KLM-1 and Panc02.03 cells were significantly decreased by GALNT6 knockdown (Figure 5A). Because a critical role of Mucin 4 in resistance to gemcitabine through activation of HER2 and ERK pathways was indicated [14], we examined the effect of GALNT6 knockdown on the gemcitabine sensitivity of pancreatic cancer cells. We first measured the half-maximum inhibitory concentration (IC50) value for gemcitabine to be 41.5 nM for KLM-1 cells and 28.4 nM for Panc02.03 cells. Then, we treated the IC50 value of gemcitabine in the KLM-1 and Panc02.03 cell lines transfected with si-control, si-GALNT6, or si-MUC4. As expected, knockdown of either GALNT6 or MUC4 sensitized these two pancreatic cancer cell lines to gemcitabine (Figure 5, B and C). Because si-GALNT6 sensitized pancreatic cancer cells to gemcitabine more than si-MUC4, GALNT6 might have additional O-glycan substrates involved in the survival of pancreatic cancer cells.

Discussion

Despite all the efforts and attempts in the last few decades, the outcomes of patients with pancreatic cancer have been improved little. The overall 5-year survival rate for pancreatic cancer patients is still less than 10%, and this disease is estimated to become the second leading cause of cancer-related death in the United States by 2030 [15]. Although molecular targeted therapies have been widely developed and applied for many types of cancer in the last two
Figure 4. Knockdown of GALNT6 induced morphological changes with increased cell adhesion complex. Immunocytochemistry of E-cadherin (green) and β-catenin (red) was conducted 72 hours after transfection with si-control (top) or si-GALNT6 (bottom) in KLM-1 cells (A) and Panc02.03 cells (B). 4′,6-Diamidino-2-phenylindole (blue) was costained to identify nucleus. Scale bars indicate 10 μm.
showed that GALNT6-induced O-glycosylation might be critically important to stabilize Mucin 4 protein. We also performed experiments suggested that GALNT6-induced O-glycosylation coexpressed in nearly half of the cell lines. Our knockdown studies suggested that O-glycosylation is one of the important mechanisms leading to abundant Mucin 4 protein in pancreatic tumor cells [6]. Mucin 4 can associate with the HER2 receptor and affect tumorigenic processes including proliferation, apoptosis, and epithelial-mesenchymal transition [17]. High expression of Mucin 4 also enhances a Src kinase pathway leading to lysosomal degradation of E-cadherin. It was reported that knockdown of Mucin 4 enhanced formation of an E-cadherin/β-catenin complex and the membrane translocation of β-catenin, resulting in the downregulation of the Wnt/β-catenin signaling pathway in pancreatic cancer cells [18]. One of the possible mechanisms leading to abundant Mucin 4 protein in pancreatic cancer is the increase of protein stability caused by extensive O-glycosylation in the backbone tandem-repeat region. Recent studies suggested that O-glycosylation is one of the important modifications in cancer cells conducted by GALNT family enzymes that are involved in several cellular functions by catalyzing O-glycan substrates. Indeed, we previously demonstrated that GALNT6 was overexpressed in breast cancer cells and stabilized Mucin1 protein through O-glycosylation [10].

In this study, we first analyzed GALNT6 and Mucin 4 expression levels in pancreatic cancer cells and found that these proteins were coexpressed in nearly half of the cell lines. Our knockdown experiments suggested that GALNT6-induced O-glycosylation might be critically important to stabilize Mucin 4 protein. We also showed that MUC4 transcription level was decreased by knockdown of GALNT6. Although the mechanism of this transcriptional downregulation of Mucin 4 is unclear, we suspect some regulatory mechanism between Mucin 4 protein and MUC4 gene expression. In fact, it was suggested that the cytosolic domain of Mucin 4 could be translocated into the nucleus and interact with some transcription factors leading to increased expression of the MUC4 gene [19]. In addition, we assessed critical roles of GALNT6 in pancreatic cancer cells in the Mucin 4 signaling pathways by investigating protein levels of HER2 and ERK and found that protein levels of these two proteins were abrogated by knockdown of GALNT6. More interestingly, knockdown of GALNT6 resulted in drastic cell morphologic changes, round shape, and enlarged cell size, like the mesenchymal to epithelial transition, accompanied by the increase in the cell adhesion molecules E-cadherin and β-catenin.

Both E-cadherin and P-cadherin interact with β-catenin and form the cell adhesion complex linked to the actin cytoskeleton structure. Normal epithelial tissues show high expression of E-cadherin, but epithelial tumor cells often lose or reduce E-cadherin expression [20]. Transition of E-cadherin to P-cadherin, also called "cadherin switching," is often related with tissue disorder, cellular dedifferentiation, and enhanced invasiveness of cancer cells [21]. Concordantly, we previously reported that P-cadherin was significantly upregulated in majority of human pancreatic cancers and its function was involved in the increased motility of pancreatic cancer cells [22]. Our results implied that knockdown of GALNT6 decreased P-cadherin expression and accelerated the cell adhesion complex including E-cadherin and β-catenin, resulting in reduction of the invasiveness of pancreatic cancer cells.

Gemcitabine is currently used as one of the major chemotherapeutic options to treat pancreatic cancer, but its clinical effect is very limited because of rapid acquisition of drug resistance. Mucin 4 is known to be involved in the chemoresistance and prolonged survival of cancer cells, and knockdown of Mucin 4 was shown to sensitize pancreatic cancer cells to gemcitabine through suppression of mitogen-activated protein kinase, c-Jun N-terminal kinase, and nuclear factor-κB pathways [23]. In concordance with these previous

**Figure 5.** Knockdown effects of GALNT6 in invasion and survival of pancreatic cancer cells. (A) Invasive ability of pancreatic cancer cells was assessed by the Transwell plate chamber assay. (B, C) Seventy-two hours after treatment with IC50 of gemcitabine, MTT assay was performed to examine cell viability of KLM-1 (B) and Panc02.03 (C) cells that were transfected with si-control, si-GALNT6, or si-MUC4. Asterisks indicated P value of <.001 (**), <.01 (**), or <.05 (*).
findings, our GALNT6 knockout experiments with gemcitabine treatment revealed enhancement of pancreatic cancer cell death, compared with gemcitabine alone, probably through the reduction of Mucin 4 protein.

In summary, our results implicate that GALNT6 is essential in O-glycosylation and stabilization of Mucin 4 protein. Because O-glycosylation of Mucin 4 has critical roles in pancreatic carcinogenesis, its modifying enzyme GALNT6 is an attractive molecular target for the development of novel treatment for pancreatic cancer.

**Authorship contributions**

Y. N. planned and supervised the entire project; J. P. made the study design and interpreted results; Y. E. T. designed all experiments and performed data analysis; T. K. supported real-time PCR experiments; M. J. supported cell culture and sample preparation; Y. H. and K. U. performed MS analysis. Y. E. T., J. P., and Y. N. wrote this article.

**Disclosure of Potential Conflicts of Interest**

Y. N. is a stockholder and a scientific advisor of OncoTherapy Science, Inc. J. P. is a scientific advisor of OncoTherapy Science, Inc.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2016.03.005.

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