Critical Roles of Mucin 1 Glycosylation by Transactivated Polypeptide N-Acetylgalactosaminyltransferase 6 in Mammary Carcinogenesis

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Abstract

The structure of O-glycosylated proteins is altered in breast cancer cells, but the mechanisms of such an aberrant modification have been largely unknown. We here report critical roles of a novel druggable target, polypeptide N-acetylgalactosaminyltransferase 6 (GALNT6), which is upregulated in a great majority of breast cancers and encodes a glycosyltransferase responsible for initiating mucin-type O-glycosylation. Knockdown of GALNT6 by small interfering RNA significantly enhanced cell adhesion function and suppressed the growth of breast cancer cells. Western blot and immunostaining analyses indicated that wild-type GALNT6 protein could glycosylate and stabilize an oncoprotein mucin 1 (MUC1), which was upregulated with GALNT6 in breast cancer specimens. Furthermore, knockdown of GALNT6 or MUC1 led to similar morphologic changes of cancer cells accompanied by the increase of cell adhesion molecules β-catenin and E-cadherin. Our findings implied that overexpression of GALNT6 might contribute to mammary carcinogenesis through aberrant glycosylation and stabilization of MUC1 and that screening of GALNT6 inhibitors would be valuable for the development of novel therapeutic modalities against breast cancer. Cancer Res; 70(7); 2759–69. ©2010 AACR.

Introduction

Breast cancer is the most common cancer among women worldwide, and more than a million women are diagnosed with breast cancer every year (1). Molecular-targeted drugs such as tamoxifen, aromatase inhibitors, and trastuzumab (Herceptin) developed recently have contributed to reduction of its mortality rate and provided a better quality of life to patients with breast tumors expressing estrogen receptor (ER) or human epidermal growth factor receptor (EGFR) 2 (HER2). However, a significant portion of patients has no clinical benefit from these treatments. Furthermore, increases in the risk of endometrial cancer with long-term tamoxifen administration as well as cardiac toxicity with trastuzumab treatment have been recognized as severe adverse events (2). Hence, development of novel molecular-targeted drugs for breast cancer with higher efficacy and low risk of adverse reactions is essentially important to improve clinical management. Toward development of such therapeutic agents, we had analyzed the genome-wide gene expression profile of 81 breast cancers as well as 29 normal human organs using cDNA microarray and have reported several novel target candidates for breast cancer therapy (3–11). In this article, we describe the identification and characterization of UDP-N-acetyl-α-D-galactosamine (GalNAc):polypeptide N-acetylgalactosaminyltransferase 6 (GALNT6), which was upregulated in a great majority of breast cancer cases.

The mucin-type O-glycosylation is initiated by GALNT family members that transfer GalNAc to serine or threonine residues on the target protein (reviewed in ref. 12). This modification occurs in the Golgi complex and is presumably controlled by the expressions and distributions of GALNT proteins (13). Interestingly, the structure of glycan chains that covalently attached to glycoproteins was altered in breast cancer cells. For instance, the O-glycans were often truncated (core 1–based type) in breast carcinoma cells, whereas their chains were extended (core 2–based type) in normal breast cells (reviewed in ref. 14). Mucin 1 (MUC1), a type I transmembrane protein, contributes to mammary carcinogenesis through interaction with EGFRs, ERα, and β-catenin (15). These aberrant O-type glycosylations were suggested to regulate the protein stability and subcellular distribution of MUC1 (16). However, the mechanisms of such
aberrant \(O\)-glycosylation of proteins in breast cancer cells have been largely unknown.

Here, we report that GALNT6 transfers GalNAc to MUC1 protein in vitro and in vivo, stabilizes MUC1 oncoprotein, and plays critical roles in proliferation and cytoskeletal regulation of breast cancer cells.

Materials and Methods

Cell lines and clinical samples. Human breast cancer cell lines (BT-20, HCC1937, MCF7, MDA-MB-231, MDA-MB-435S, SKBR3, T47D, YMB-1, BT-474, BT-549, HCC1143, HCC1500, HCC1599, MDA-MB-157, MDA-MB-453, OUCB-F, andZR-75-1), HBL-100, COS-7, HEK293T, and HeLa cell lines were purchased from the American Type Culture Collection (ATCC) in 2001 to 2003 and cultured under their respective depositors’ recommendation. No abnormalities were observed on the cellular morphology of these cell lines both at low and high densities of cultures by microscopy according to the guideline from ATCC (17). HBC-4 and HBC-5 cell lines were kindly provided by Dr. Takao Yamori (Division of Molecular Pharmacology, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan) with material transfer agreement in 2001. Human normal breast epithelial cell lines (HMEC and MCF10A) were purchased from Cambrex Bioscience, Inc. in 2007. The stocks of cell lines that had been deposited in liquid nitrogen were used in this study. We monitored the cell morphology of these cell lines by microscopy and confirmed to maintain their morphologic images in comparison with the original morphologic images from the above affiliation. During our recent test in 2009, no Mycoplasma contamination was detected in cultures of all of these cell lines using a Mycoplasma Detection kit (Roche). Tissue samples from surgically resected breast cancers and their corresponding clinical information were obtained from the First Department of Surgery, Sapporo Medical University (Hokkaido, Japan) and Department of Breast Surgery, The Cancer Institute Hospital of Japanese Foundation for Cancer Research (Tokyo, Japan) after obtaining written informed consents. This study, as well as the use of all clinical materials described above, was approved by individual institutional Ethical Committees.

Semiquantitative reverse transcription-PCR and Northern blot analyses. Reverse transcription-PCR (RT-PCR) and Northern blots analyses were performed as described previously (5, 18). The PCR primer sequences were

\[
\begin{align*}
\text{GALNT6} & : 5'-\text{GACATTTCCACGGTTCT} & 3' \\
\text{GALNT6} & : 5'-\text{GAGTCCAGGTAAGTGAATCTGTCC} & 3'
\end{align*}
\]

and

\[
\begin{align*}
\text{MUC1} & : 5'-\text{CGGCTCACGTTCTGGAGGACCC} & 3' \\
\text{MUC1} & : 5'-\text{CGAGTTGGACAAGCACTGAGTATG} & 3'
\end{align*}
\]

for GAPDH and \(\beta\)-actin, respectively. Western blot analyses were performed using polyclonal antibodies against GALNT6 and MUC1 proteins in breast cancer cells, we employed mouse anti-GALNT6 monoclonal antibodies (3G7, diluted at 1:300), and nuclei were counterstained with anti-GALNT6 monoclonal antibody, we also generated mouse anti-GALNT6 monoclonal antibodies, as described previously (20). The hybridomas were subcloned to assess the ability to recognize GALNT6 protein. After limiting dilution, the clones of 3G7 and 4H11 were selected for immunostaining and Western blot analyses, respectively.

Recombinant GALNT6 protein. The partial coding sequence of GALNT6 without a signal peptide (codons 35–179) was purified and inoculated into rabbits, as described previously (9). In addition, because of the limited amount of above polyclonal antibody, we also generated mouse anti-GALNT6 monoclonal antibodies, as described previously (20). The hybridomas were subcloned to assess the ability to recognize GALNT6 protein. After limiting dilution, the clones of 3G7 and 4H11 were selected for immunostaining and Western blot analyses, respectively.

Immunocytochemical staining. The immunocytochemical staining was performed as described previously (5, 6). Briefly, the cells were incubated with anti-GALNT6 polyclonal (diluted at 1:100) or anti-GALNT6 monoclonal antibodies (3G7, diluted at 1:300), and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). The Golgi apparatus and cytoskeleton structure were visualized by staining with anti–Golgi-58k monoclonal antibody (Sigma-Aldrich) and Alexa Fluor 594 phalloidin (Molecular Probes), respectively.

Western blot analysis. To detect the expression of endogenous GALNT6 and MUC1 proteins in breast cancer cells, we performed Western blot as described previously (5, 6). After SDS-PAGE, membranes blotted with proteins were incubated with an anti-GALNT6 polyclonal antibody or anti-GALNT6 antibody, respectively, as described previously (9). In addition, because of the limited amount of above polyclonal antibody, we also generated mouse anti-GALNT6 monoclonal antibodies, as described previously (20). The hybridomas were subcloned to assess the ability to recognize GALNT6 protein. After limiting dilution, the clones of 3G7 and 4H11 were selected for immunostaining and Western blot analyses, respectively.

Generation of anti–GALNT6-specific antibodies. To generate anti-GALNT6 polyclonal antibodies, partial recombinant GALNT6 protein (codons 35–179) was purified and inoculated into rabbits, as described previously (9). In addition, because of the limited amount of above polyclonal antibody, we also generated mouse anti-GALNT6 monoclonal antibodies, as described previously (20). The hybridomas were subcloned to assess the ability to recognize GALNT6 protein. After limiting dilution, the clones of 3G7 and 4H11 were selected for immunostaining and Western blot analyses, respectively.
Biotechnology), anti-E-cadherin (BD Biosciences), and anti-β-actin (Sigma-Aldrich) monoclonal antibodies. Particularly, the anti-MUC1 monoclonal antibody (VU4H5) can recognize the endogenous MUC1 proteins at various molecular weights, which are explainable by its structural features of the gene containing variable numbers of tandem repeat and the protein forming homodimers or heterodimers (21, 22).

**VVA lectin blot and pull down.** To detect the GalNAc-conjugated proteins, we did lectin Western blot as described previously (23). Briefly, whole-cell lysates, including glycoproteins of interest, were transferred onto a nitrocellulose membrane (GE Healthcare) after SDS-PAGE. After blocking with 5% bovine serum albumin, the membrane was incubated with 0.5 μg/mL of biotin-conjugated VVA lectin (EY Laboratories) and streptavidin–horseradish peroxidase (BD Biosciences). Similarly, the proteins bound to the biotin-conjugated VVA lectin were pulled down by streptavidin-agarose (Invitrogen) as described previously (24).

**Gene silencing by RNA interference.** To knock down endogenous GALNT6 expression in breast cancer cells, we used psil6BX3.0 vector for expression of short hairpin RNA (shRNA) against a target gene as described previously (25). Target sequences of the synthetic oligonucleotides for shRNA against GALNT6 were shown in Supplementary Table S1. Ten days after transfection with each of shRNA expression, we evaluated the knockdown effect on cell viability by MTT and colony formation assays as described previously (5). To examine the early-stage effects in cells in which GALNT6 or MUC1 was knocked down, we also used the synthesized duplex small interfering RNAs (siRNA; Sigma-Aldrich Japan) si-EGFP (5′-GCGACGACGACUUUUCAAG-3′) and si-GALNT6 (5′-GGAGAAUCCUUCCGGUGACA-3′) corresponding to the target sequence of sh-G6-2. The si-MUC1 (5′-GUUCAGUGGCCCCUUCUUCAAG-3′) was synthesized according to a previous report (26). The RNA interference (RNAi) rescue assay was conducted as described previously (27).

**Cell detachment assay.** The strength of cell to culture dish attachment was quantified by the "cell detachment assay" (28, 29). Total number of viable cells was evaluated by Cell Counting Kit-8 (Dojindo) before and after incubation with a dissociation solution containing 5 mmol/L EDTA in PBS (−) for 10 min.

**Establishment of GALNT6 stably expressed transformants.** Mock (no insert) or pCAGGS-GALNT6 (WT and H271D) HA expression vectors were transfected into HeLa cells using FuGENE6 (Roche). Then, the positive clones were selected under incubation with culture medium containing 0.8 mg/mL of neomycin (geneticin, Invitrogen). Two weeks later, the stable transformants were selected by the limiting dilution and screened for clones stably expressing HA-tagged GALNT6 protein (WT and H271D). Finally, we isolated individual clones of mock (001, 003, and 006), WT (101, 110, and 304), and H271D (102, 212, and 114).

**In vitro GalNAc transferase assay.** In vitro GalNAc transferase assay was performed as described previously (30, 31). As substrates, MUC1-a (AHGVTSAPDTR) and MUC1-b (RPAPGSTAPPA) peptides derived form the tandem repeat of MUC1 protein were synthesized by Sigma-Aldrich Japan and fluorescence labeled [dansylation (DNS)]. Briefly, the reaction was performed in 50 μL of reaction mixtures containing 25 mmol/L Tris-HCl (pH 7.4), 10 mmol/L MnCl2, 50 μmol/L UDP-GalNAc, 4 μmol/L DNS-MUC1 peptides, and 0.5 μg of recombinant GALNT6 (codons 35–622) protein. The reaction mixture was incubated at 37°C for 16 h and analyzed by reversed-phase high-performance liquid chromatography (HPLC) as described previously (30). For the confirmation of GalNAc conjugation, the reacted samples were further incubated with Acremonium sp. α-N-acetylgalactosaminidase (GalNAcase; Seikagaku Biobusiness) to remove the conjugated GalNAc from the MUC1-a peptide.

**Statistical analysis.** Statistical significance was calculated by Student’s t test using StatView 5.0 software (SAS Institute). A difference of P < 0.05 was considered to be statistically significant.

**Results**

**Identification of GALNT6 upregulated in breast cancer.** Through the previous studies of genome-wide gene expression profiles (3, 4), we first selected the genes that encode proteins having enzymatic activity, according to the reported information or the computer-assisted prediction by SMART program (32), and decided to perform further analysis for the GALNT6 gene that encodes an O-glycosyltransferase as a possible druggable target for breast cancer. We confirmed by semiquantitative RT-PCR analysis its upregulation in 7 of 12 clinical breast cancer specimens and in 12 of 19 breast cancer cell lines examined (Supplementary Fig. S1A). Subsequent Northern blot analysis revealed overexpression of its ∼5-kb transcript in breast cancer cell lines, whereas its expression was hardly detectable in normal human organs (Supplementary Fig. S1B) as concordant to the results of cDNA microarray analysis. We subsequently generated rabbit polyclonal antibodies and two kinds of mouse monoclonal antibodies (3G7 and 4H11), all of which could recognize the endogenous GALNT6 protein (∼75 kDa) in breast cancer cells without producing any nonspecific bands or background signals in SDS-PAGE and immunocytochemical staining (Supplementary Fig. S2A–D). The immunohistochemical staining analysis revealed its strong staining in breast cancer tissues but no positive staining in the normal human tissues, including normal mammary ductal cells, lung, heart, liver, and kidney (Fig. 1A), in concordance with the results of Northern blot analysis.

To further characterize the GALNT6 protein in breast cancer cells, we investigated the subcellular localization of endogenous GALNT6 in T47D breast cancer cells by immunocytochemical staining using anti-GALNT6 polyclonal antibody. The results showed that GALNT6 protein was clearly observed in the Golgi complex of T47D cells, as evaluated by co-staining with the Golgi marker Golgi-58k (Fig. 1B). Similarly, we observed strong staining of GALNT6 in the Golgi complex in breast cancer tissue section (Fig. 1C).

**Knockdown of GALNT6.** To investigate the biological significance of GALNT6 overexpression in breast cancer cells,
we generated shRNA expression vectors to knock down the endogenous expression of GALNT6 (sh-G6-1 and sh-G6-2). We found that introduction of both of sh-G6-1 and sh-G6-2 into T47D cells resulted in significant reduction of GALNT6 expression that was accompanied by suppression of cell proliferation, whereas no change was observed in cells transfected with a control shRNA vector (Fig. 2A, left). Moreover, we confirmed the results of sh-G6-2 specificity to GALNT6 by using two mismatched shRNAs (sh-mis-1 and sh-mis-2; Fig. 2A, right). To further examine the effects of GALNT6 knockdown, we introduced the synthesized oligoduplex siRNA against GALNT6 (si-GALNT6) into T47D cells. Interestingly, 4 days after transfection of siRNA, the GALNT6-depleted (si-GALNT6) cells showed round shape and enlarged cell size compared with the cells transfected with a control si-EGFP (Fig. 2B). These morphologic alterations caused by si-GALNT6 were further assessed by immunostaining with fluorescence-labeled phalloidin (Fig. 2C) and subsequently restored by exogenously introduced GALNT6 (Supplementary Fig. S3).

Stabilization of MUC1 by GALNT6. We noticed that the appearances of GALNT6-depleted cells were very similar to those of the cells in which MUC1 was knocked down (33, 34). Because MUC1 was reported to be one of candidate substrates of the GALNT family (31), we compared the knockdown effects of GALNT6 with that of MUC1 by siRNA using breast cancer cell lines T47D, MCF7, and SKBR3. First, as shown in Supplementary Fig. S4A, we confirmed the knockdown of GALNT6 and MUC1 expressions by Western blot analysis using an anti-GALNT6 monoclonal antibody (3G7) and an anti-MUC1 monoclonal antibody (clone VU4H5) that could specifically recognize endogenous MUC1 in breast cancer cells. As expected, either GALNT6 or MUC1 depletion caused very similar morphologic changes (round shape and enlarged cell size) and the attenuated cell proliferation in all the three cell lines examined (Supplementary Fig. S4B and C). These findings have indicated that GALNT6 is likely to be indispensable for the proliferation of breast cancer cells through the regulation of cytoskeleton structure possibly by modification of MUC1.
To investigate the interaction of GALNT6 and MUC1 in more detail, we knocked down GALNT6 expression by siRNA and examined its effect on the MUC1 protein in T47D cells. We found that knockdown of GALNT6 protein induced the reduction of cytoplasmic MUC1 protein (4 days after transfection; Fig. 3A and B), although the transcriptional level of MUC1 was unchanged (Fig. 3A). When we used another cell line, MCF7, we observed similar results (Supplementary Fig. S5A and B), suggesting that GALNT6 may influence the posttranslational modification and stabilization of MUC1 protein in breast cancer cells. We subsequently introduced the plasmid designed to express GALNT6 protein into MCF10A cells, in which the GALNT6 expression level was very low (Supplementary Fig. S2B), and found by immunocytochemical staining the remarkable enhancement of the signal intensity of cytoplasmic MUC1 protein by introduction of GALNT6 (Fig. 3C, yellow arrow), further supporting our hypothesis that GALNT6 plays a critical role for the stabilization of MUC1 protein. In addition, we examined the expression levels of these two molecules in breast cancer cells by Western blot analysis with anti-GALNT6 and anti-MUC1 monoclonal antibodies and found that GALNT6 and MUC1 proteins were co-overexpressed in breast cancer cell lines and clinical cancer tissue sections examined, but neither of the proteins was expressed in HMEC or normal breast ductal cells (Fig. 3D; Supplementary Fig. S6). Taken
together, our findings imply that upregulation of GALNT6 protein might contribute to mammary carcinogenesis through stabilization of MUC1 oncoprotein.

**GALNT6 O-glycosylates MUC1 in vitro and in vivo.** To investigate whether GALNT6 O-glycosylates MUC1 as a substrate, we generated the recombinant WT GALNT6 as well as the inactive GALNT6 mutant proteins (H271D and E382Q) and performed in vitro GalNAc transferase assay using MUC1 peptides (MUC1-a and MUC1-b) corresponding to the tandem repeat fragment of MUC1 protein. WT GALNT6 O-glycosylated MUC1 peptides as indicated by the left-shifted band in Fig. 4A. On the other hand, mutant GALNT6 proteins (H271D and E382Q) could not transfer GalNAc to MUC1 peptides even by 16-hour incubation (no left-shifted band appeared). In addition, we confirmed that treatment of GalNAcase removed GalNAc, which was transferred by the WT GALNT6, and restored the left-shifted peak of MUC1-a peptide (Fig. 4B).

To further investigate whether the exogenous introduction of GALNT6 protein can glycosylate the endogenous MUC1 protein in vivo, we performed Western blot analysis with anti-MUC1 monoclonal antibody using HeLa derivative cells in which stable GALNT6 expression was established; those in which mock or H271D expression vectors were introduced were used as controls (mock, WT, and H271D; see Materials and Methods). In cells with WT GALNT6 (clones 101, 110, and 304), we observed the highest molecular weight of MUC1 protein (>250 kDa), whereas in those with mock (clones 001, 003, and 006) or H271D (clones 102, 212, and 114), we did not observe the shifted band (Fig. 4C). Moreover, we confirmed that the shifted MUC1 protein corresponded to the O-glycosylated (GalNAc) MUC1 protein by immunoprecipitation with anti-MUC1 monoclonal antibodies followed by VVA lectin blotting and vice versa (Fig. 4D). To further examine whether GALNT6 stabilizes the MUC1 protein in vivo, we transfected WT or H271D constructs into MCF10A

Figure 3. GALNT6 is critical for MUC1 stabilization. A, T47D cells were transfected with si-EGFP or si-GALNT6 and collected at days 1, 2, and 4, followed by Western blot (top) and semiquantitative RT-PCR (bottom). B, 4 d after transfection with si-EGFP (top) or si-GALNT6 (bottom), T47D cells were costained with anti-GALNT6 polyclonal antibody (green) and anti-MUC1 monoclonal antibody (red). C, 2 d after transfection with a GALNT6 construct (pCAGGS-GALNT6-HA), MCF10A cells were costained with anti-HA rat (green) and anti-MUC1 monoclonal antibodies (red). Yellow and white arrows indicate MCF10A cells with and without expression of HA-GALNT6, respectively. D, Western blot analysis revealed co-overexpression of GALNT6 and MUC1 in breast cancer cell lines. Asterisk indicates a human normal breast epithelial cell line, HMEC.
normal epithelial cells and then performed immunocytochemical staining with anti-HA and anti-MUC1 antibodies. The WT GALNT6 (Fig. 5A) transformants augmented the signal intensity of MUC1 proteins (yellow arrows), whereas the H271D (Fig. 5B) did not affect that of MUC1 (white arrows), suggesting that GALNT6-mediated glycosylation of MUC1 protein is critical for stability of MUC1 protein.

**GALNT6 and MUC1 are involved in cytoskeletal regulation.** Because MUC1 was reported to disrupt cell adhesion (34, 35), we examined the participation of two cell adhesion molecules, β-catenin and E-cadherin, in which their involvement in carcinogenesis was reported, in the GALNT6-MUC1 pathway. We knocked down GALNT6 or MUC1 expressions by siRNA in T47D breast cancer cells and found by semi-quantitative RT-PCR and Western blot analyses that knockdown of either GALNT6 or MUC1 remarkably augmented the proteins of cell adhesion molecules (Fig. 6A, top) but did not alter their transcriptional levels (Fig. 6A, bottom). We also immunostained T47D cells with or without GALNT6 knockdown using anti–β-catenin or anti–E-cadherin monoclonal antibodies (Fig. 6B and C) and identified cell morphologic changes (round shape and enlarged size) accompanied by stronger staining of β-catenin (Fig. 6B) and E-cadherin (Fig. 6C) proteins. The results of MUC1-depleted T47D cells were quite similar to those of GALNT6-depleted cells (Supplementary Fig. S7). Because the increase of the cell adhesion complex might enhance cell-to-plate dish attachment, we performed the cell detachment assay and found the inverse correlation between MUC1 expression level and strength of the cell attachment (Supplementary Fig. S8).

**Discussion**

Among all human genes, approximately 2,000 to 3,000 genes are estimated to encode druggable proteins, which include membrane or nuclear receptors, ion channels, protein kinases, and other enzymes (36). The comparison of whole-genome expression profiles between a large set of normal and cancer cells has been considered to be an effective approach to identify potential targets for development of anticancer drugs (37). Because the reduction of adverse reactions caused by drugs, particularly by anticancer agents, is one of the very serious issues to be solved in clinical management, we have focused on isolation of cancer-specific molecules that were upregulated commonly in cancer cells but were not or undetectably expressed in normal human organs.
We have identified several cancer-specific molecules and characterized them for possible application to development of cancer therapy (5, 6, 20, 38, 39). In the present study, we characterized a novel breast cancer-specific molecule, **GALNT6**, encoding an O-glycosyltransferase and showed its potential as a druggable target by showing its critical roles in the growth of breast cancer cells.

O-type glycosylation is one of common modifications that have multiple functions related to the folding, stability, and targeting of various glycoproteins, and is initiated by members belonging to the GALNT family in the Golgi complex (15). Accumulating lines of evidence have suggested that the GALNT family members are involved in several cellular functions by catalyzing substrates specific to each member. For instance, glycosylation by GALNT3 prevents proteolytic processing of fibroblast growth factor 23 and that by GALNT14 promotes ligand-stimulated clustering of death receptors (40, 41). However, our results uncovered characteristics of GALNT6 distinct from these two GALNT members.

Abnormalities of the glycan structure of proteins are frequently observed in breast cancer cells (42). Immunostaining analysis in the present study revealed very intense staining of GALNT6 in the Golgi apparatus of breast cancer cells (Fig. 1), but no staining in adjacent normal cells, suggesting its potential roles in mammary carcinogenesis through protein glycosylation. Subsequent functional analyses of GALNT6 revealed that GALNT6 is tightly linked to regulation of cytoskeleton structure and also proliferation of breast cancer cells throughout stabilization of MUC1 protein (Figs. 2 and 3). Accordingly, we assume that GALNT6 plays a fundamental role in aberrant glycosylation of MUC1 in breast cancer cells because our results have shown that GALNT6 is coexpressed with MUC1 protein (Fig. 3D) and indispensable to stabilize it (Fig. 3; Supplementary Fig. S5). The critical roles of GALNT6-MUC1 pathway were validated by knockdown of either GALNT6 or MUC1 that resulted in similar effects on cell shape and proliferation (Supplementary Fig. S4). In particular, the effects of GALNT6 depletion seem greater than those of MUC1 depletion (Supplementary Fig. S4C), which suggests that GALNT6 may have additional functions through glycosylation of an unidentified substrate. Therefore, further identification of novel substrates of GALNT6 is needed.
warranted to clarify unveiled pathophysiologic roles of GALNT6 in breast cancer.

We then investigated enzyme activity of GALNT6 and showed that WT GALNT6 initiated O-glycosylation of MUC1 in vitro and in vivo (Fig. 4). Our findings implicated that the activity of GALNT6 is essential to stabilize MUC1 protein (Fig. 5) and that screening of GALNT6 inhibitors would be valuable to develop a novel therapeutic agent for breast cancer. Moreover, because GALNT6 is exclusively up-regulated in breast cancer cells, but not expressed in normal human vital organs (Fig. 1A), GALNT6 is supposed to be one of ideal therapeutic targets and may enable us to develop an anticancer agent with minimum risk of adverse effects.

To further characterize biological significance of the GALNT6 and MUC1 interaction, we examined the status of β-catenin and E-cadherin because these two molecules are involved in carcinogenesis and also important in the regulation of cell morphology. According to previous reports, it is likely that MUC1 captures β-catenin through interaction with its cytoplasmic tail and thereby inhibits complex formation of cell adhesion molecules (34, 35). We found that GALNT6-MUC1 pathway has a very significant role in stabilization and localization of these two molecules and formation of the cell adhesion complex (Fig. 6). To quantify the GALNT6/MUC1-mediated disruption of cell adhesion, we performed the cell detachment assay (Supplementary Fig. S8) and showed that the detachment (cell to dish) was clearly increased by reduced MUC1 protein in concordance with previous findings (33).

In summary, our findings suggest a model as described in Fig. 6D. In breast cancer cells, upregulation of GALNT6 could cause stabilization of MUC1 protein throughout its glycosylation activity. Subsequently, the accumulation of glycosylated MUC1 protein may induce the abnormalities of cell adhesion molecules such as β-catenin and E-cadherin, resulting in the antiadhesive effect. Moreover, the elevated MUC1 protein promotes cancer cell proliferation partly by interactions with EGFR, c-Src, Grb2, and ERα (43, 44), although further in-depth analysis will be required to elucidate the precise mechanism of the GALNT6-MUC1 pathway in breast cancer cells. Our results reported in this study should contribute to shed light on the unique roles of abnormal glycosylation in mammary carcinogenesis, and also indicate a possibility to develop the inhibitors for enzymatic activity of GALNT6 as therapeutic modalities against breast cancer.

**Figure 6.** GALNT6 and MUC1 are involved in cytoskeletal regulation. A, Western blot (1st to 5th panels) and semiquantitative RT-PCR (6th to 10th panels) analyses for β-catenin (CTNNB1) and E-cadherin (CDH1) in si-GALNT6–transfected or si-MUC1–transfected cells. β-Actin and ACTB, quantity controls at protein and transcriptional levels, respectively. B and C, immunocytochemistry of β-catenin and E-cadherin in si-EGFP–transfected or si-GALNT6–transfected T47D cells. Green, anti-GALNT6 polyclonal antibody; red, monoclonal antibodies against β-catenin (B) or E-cadherin (C). D, schematic representation of GALNT6-MUC1 pathway in mammary carcinogenesis. Overexpression of GALNT6 attributes to aberrant glycosylation and stabilization of MUC1, induces the elevated interaction with several signal transducers, and thereby results in proliferation and anti–cell adhesion of breast cancer cells.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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