

## Critical Role of Estrogen Receptor Alpha O-Glycosylation by N-Acetylgalactosaminyltransferase 6 (GALNT6) in Its Nuclear Localization in Breast Cancer Cells<sup>1,2</sup>



Boya Deng<sup>\*</sup>, Yunus Emre Tarhan<sup>\*</sup>, Koji Ueda<sup>†</sup>, Lili Ren<sup>\*</sup>, Toyomasa Katagiri<sup>‡</sup>, Jae-Hyun Park<sup>\*</sup> and Yusuke Nakamura<sup>\*</sup>

<sup>\*</sup>Department of Medicine, The University of Chicago, Chicago, IL 60637, USA; <sup>†</sup>Cancer Proteomics Group, Cancer Precision Medicine Research Center, Japanese Foundation for Cancer Research, Tokyo, 135-8550, Japan; <sup>‡</sup>Division of Genome Medicine, Institute for Genome Research, Tokushima University, Tokushima, Japan

### Abstract

Alteration of protein O-glycosylation in various human cancers including breast cancer is well known, but molecular roles of their aberrant glycosylations on cancer have not been fully understood. We previously reported critical roles of polypeptide N-acetylgalactosaminyltransferase 6 (GALNT6 or GalNAc-T6) that was upregulated in a great majority of breast cancer tissues. Here we further report O-glycosylation of estrogen receptor alpha (ER- $\alpha$ ) by GALNT6 and the significant role of its nuclear localization in breast cancer cells. Knockdown of *GALNT6* expression in two breast cancer cell lines, T47D and MCF7, in which both ER- $\alpha$  and GALNT6 were highly expressed, by small interfering RNA could significantly attenuate expression of ER- $\alpha$ . Immunocytochemical analysis clearly demonstrated the drastic decrease of ER- $\alpha$  protein in the nucleus of these cancer cells. Accordingly, the downstream genes of the ER- $\alpha$  pathway such as *MYC*, *CCND1*, and *CTSD* were significantly downregulated. We confirmed GALNT6-dependent ER- $\alpha$  O-glycosylation and identified O-glycosylation of S573 in an F domain of ER- $\alpha$  by GALNT6 through LC-MS/MS analysis. We also obtained evidences showing that the glycosylation of ER- $\alpha$  at S573 by GALNT6 is essential for protein stability and nuclear localization of ER- $\alpha$  in breast cancer cells. Furthermore, we designed cell membrane-permeable peptides including the O-glycosylation site and found a significant decrease of the cell viability of breast cancer cells by treatment of these peptides in a GALNT6 expression-dependent manner. Our study suggests that targeting the GALNT6 enzymatic activity as well as the GALNT6/ER- $\alpha$  interaction could be a promising therapeutic approach to ER- $\alpha$ -positive breast cancer patients.

*Neoplasia* (2018) 20, 1038–1044

### Introduction

Breast cancer is one of the major malignancies affecting women across the world. A total of 266,120 women are estimated to be diagnosed breast cancer and 40,920 women would die of breast cancer in the United States in 2018 [1]. Approximately 70% of breast cancers express/overexpress or have somatic mutations in an estrogen receptor-alpha (ER- $\alpha$ ) gene, which plays critical roles in development and progression of breast cancer. Inhibitors of an estrogen/ER- $\alpha$  signaling pathway such as selective ER- $\alpha$  modulators (e.g., tamoxifen and raloxifene), ER- $\alpha$  downregulators (e.g., fulvestrant), and aromatase inhibitors (AIs) have been used for hormone receptor-positive breast cancer and significantly improved the prognosis breast cancer patients [2–4]. However, these treatment modalities often become ineffective because of the intrinsic and acquired endocrine

Abbreviations: GALNT6, N-acetylgalactosaminyltransferase 6; ER- $\alpha$ , estrogen receptor-alpha; GalNAz, tetraacetylated N-azidoacetyl-galactosamine; *CCND*, Cyclin D1; *CTSD*, Cathepsin D; VVA, *Vicia villosa* agglutinin; GAPDH, glyceraldehyde phosphate dehydrogenase.

Address all correspondence to: Yusuke Nakamura, MD, PhD, Department of Medicine, 900 E. 57th Street, KCBD 6130, Chicago, IL 60637, USA.

E-mail: [ynakamura@bsd.uchicago.edu](mailto:ynakamura@bsd.uchicago.edu)

<sup>1</sup>Statement of significance: Findings uncover the O-glycosylation of ER- $\alpha$  by GALNT6 and demonstrate effective strategy to ER- $\alpha$ -positive breast cancer treatment.

<sup>2</sup>Conflict of interest statement: Y. N. is a stock holder and a scientific advisor of OncoTherapy Science, Inc. J. P. is a scientific advisor of Cancer Precision Medicine Inc. Received 4 May 2018; Revised 12 August 2018; Accepted 20 August 2018

© 2018 The Authors. Published by Elsevier Inc. on behalf of Neoplasia Press, Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1476-5586

<https://doi.org/10.1016/j.neo.2018.08.006>

resistance [5,6]. Hence, development of novel molecular-targeted drugs for breast cancer to overcome endocrine resistance with higher efficacy and low risk of adverse reactions is crucial to further improve clinical outcome of breast cancer patients.

Polypeptide N-acetylgalactosaminyltransferase 6 (GALNT6) is an enzyme which mediates the mucin-type O-glycosylation and has been reported to be aberrantly expressed in many types of human cancer [7–9]. GALNT6 expression level was much higher in breast cancers compared to other cancer types [10], especially in the estrogen receptor (ER)-positive breast cancer tissues [11]. We previously reported upregulation of GALNT6 in a great majority of breast cancers and demonstrated its critical roles in breast cancer through decrease of cellular adhesion ability and disruption of mammary acinar morphogenesis [12,13]. We also found its upregulation in pancreatic cancer cells, in which GALNT6 could cause a cadherin switch (from E-cadherin to P-cadherin) affecting cellular adhesion to the underlying matrix [14]. High GALNT6 expression was also reported to be correlated with an increased risk of recurrence, lymph node metastasis, and chemoresistance in ovarian cancer [15]. It was also shown that GALNT6 was highly upregulated in colon adenocarcinomas compared with adjacent colon tissues, implying its important role in colon carcinogenesis [8]. GALNT6 was identified as an independent prognostic factor for the poor prognosis of gastric cancer patients; high GALNT6 was significantly associated with the low expression levels of E-cadherin as well as the high expression levels of MMP9 in gastric cancer tissues [16].

Here we demonstrate a possibility of ER- $\alpha$  as a novel substrate of GALNT6 and an essential role of GALNT6-mediated O-glycosylation for the nuclear localization of ER- $\alpha$  in breast cancer cells. We also show that cell membrane-permeable peptides including the O-glycosylation site of ER- $\alpha$  inhibit the interaction of ER- $\alpha$ /GALNT6, alter cellular phenotypes, and cause the cell death. Our data suggest that targeting the GALNT6 enzymatic activity as well as the GALNT6/ER- $\alpha$  interaction could be a promising therapeutic approach to ER-positive breast cancers.

## Materials and Methods

### Cell Culture

Human cancer cell lines T47D, MCF7, SKBR3, HCC1937, and HeLa were purchased from American Type Culture Collection and cultured according to provider's protocols. Three HeLa cell-derived cell lines stably expressing HA-tagged wild-type GALNT6 protein (HeLa-GALNT6-WT) or HA-tagged enzyme-dead H271D-substituted GALNT6 protein (HeLa-GALNT6-H271D) and the cells transfected with an empty vector (HeLa-Mock) were established as previously described [12], and these cells were cultured in the medium containing 0.8 mg/ml of G418 (Geneticin). Transfection of plasmids in the study was performed by using FuGENE 6 reagents (Roche) according to the manufacturer's protocols. Estradiol was purchased from Sigma-Aldrich, USA.

### Screening of Novel O-Glycosylation Substrates Induced by GALNT6

For screening a candidate O-glycosylation substrate(s) of GALNT6, we performed *in vivo* metabolic labeling of O-glycosylated proteins using GalNAz (tetraacetylated N-azidoacetylgalactosamine), which is an azide-labeled sugar for O-glycans [17]. After 72 hours of treatment with 50  $\mu$ M of GalNAz, total proteins were extracted from HeLa cells stably expressing mock, GALNT6-WT, or GALNT6-H271D by using CellLytic M

reagent (Sigma-Aldrich) with 1% of Protease Inhibitor Cocktail Set III (Calbiochem). The O-glycosylated proteins containing GalNAz were co-conjugated with biotin by Click-iT reaction buffer kit (Invitrogen) and then were immunoprecipitated with NeutrAvidin beads (Thermo Fisher). After on-beads Lys-C/Trypsin digestion, eluted O-glycosylated proteins were analyzed by tandem mass spectrometry (MS/MS) analysis which performed beta-elimination reaction to remove O-glycans and simultaneously substitute originally-glycosylated Ser/Thr to [Ser-1Da] / [Thr-1Da] (-OH > -NH<sub>2</sub>).

### GALNT6 Knockdown by Small Interfering RNA (siRNA)

We used siRNA (Sigma-Aldrich) for GALNT6 knockdown and SIC001 Mission siRNA Universal Negative Control (Sigma-Aldrich) as control. Briefly, GALNT6-siRNA or control siRNA was transfected into cells by using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's protocols. Seventy-two hours later, cells were collected for further analysis. The target sequences of siRNA are 5'-GAGAAUCCUUCGUGACA-3' for si-GALNT6 as previously described [12,14].

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

Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA (1  $\mu$ g) 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 (qPCR). The qPCR was performed using primers listed below using the ViiA 7 system (Life Technologies). The expression levels of target genes were normalized with that of GAPDH. The PCR primers were as shown: *GALNT6* (Hs00926629\_m1), *ESR1* (Hs00174860\_m1), *MYC* (Hs00153408\_m1), *CCND1* (Hs00765553\_m1), *CTSD* (Hs00157205\_m1), and *GAPDH* (Hs02758991\_g1) TaqMan Gene Expression Assays (Thermo Fisher Scientific).

### Western Blot

Western blot was performed as described previously [14]. Protein bands were visualized by ECL detection reagents (GE Healthcare). The primary antibodies used in this study were as follows: anti-human ER- $\alpha$  monoclonal antibody (1:400, Santa Cruz), anti-human GALNT6 polyclonal antibody (1:500, Sigma-Aldrich), anti-Flag M2 monoclonal antibody (1:1000, Sigma-Aldrich), anti-HA monoclonal antibody (1:1000, Roche), and anti- $\beta$ -actin monoclonal antibody (1:10,000, Sigma-Aldrich). The secondary antibodies were goat anti-rabbit or anti-mouse IgG-HRP antibodies (1:10,000-1:30,000, Santa Cruz). For the detection of O-glycosylated proteins, we performed lectin blotting using biotinylated *Vicia villosa* agglutinin (VVA) lectin (1:1000, Vector Laboratories) and Streptavidin-HRP (1:10,000, Thermo Scientific).

### Identification of an O-Glycosylation Site(s) of ER- $\alpha$ by MS Analysis

For identification of an O-glycosylation site(s) on ER- $\alpha$  protein, HeLa-GALNT6 stable cells (WT) were transfected with pCAGGSn3FC-ER- $\alpha$  expression vector [18,19] and collected after 48 hours of incubation. Cells were lysed with lysis buffer, and Flag-tagged ER- $\alpha$  protein was immunoprecipitated with anti-Flag monoclonal antibody and Protein A/G agarose (Invitrogen). After washing with the lysis buffer five times, immunocomplexes were

loaded to an SDS-PAGE gel, and protein bands were visualized by Coomassie stains (Bio-Rad) according to the manufacturer's instruction. The protein band located at the 66 kDa (where ER- $\alpha$  located) was excised with a clean, sharp scalpel. The excised ER- $\alpha$  band was reduced in 10 mM Tris (2-carboxyethyl) phosphine (Sigma-Aldrich) with 50 mM ammonium bicarbonate (Sigma-Aldrich) for 30 minutes at 37°C and alkylated in 50 mM iodoacetamide (Sigma-Aldrich) with 50 mM ammonium bicarbonate for 45 minutes in the dark at 25°C. Trypsin/Lys-C (Promega) solution was added and incubated at 37°C for 12 hours. The resulting peptides were extracted from gel fragments and further incubated with 20% ammonia aqueous solution at 45°C for 12 hours in order for  $\beta$ -elimination of O-glycans, resulting in substitution of -OH residues on originally glycosylated Ser/Thr to -NH<sub>2</sub>. Following acidification of samples (pH = 3.5), peptides were desalted by Oasis HLB cartridge (Waters). Then peptides were analyzed with Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific) combined with UltiMate 3000 RSLC nano-flow HPLC system (Thermo Scientific) with HCD MS/MS mode. The MS/MS spectra were searched against *Homo sapiens* protein sequence database in SwissProt using Mascot search engine in Proteome Discoverer 2.2 software (Thermo Scientific), in which peptide identification filters were set at "false discovery rate < 1%" and "Mascot expectation value < 0.05." Carbamidomethylation of Cys was set for a fixed modification. Oxidation of Met and  $\beta$ -elimination of Ser/Thr (-0.984016 Da) were set for variable modifications.

### Immunocytochemistry

Immunocytochemistry was performed as previously described [14]. For siRNA knockdown, at 72 hours after adding siRNA, the cells were fixed. For cell-permeable peptide treatment, at 24 hours after seeding of cells, peptides were added and then incubated for 1 or 2 hours (1 hour for T47D cells and 2 hours for MCF7 cells), and then the cells were fixed. The primary antibodies used in immunocytochemical analysis included anti-human GALNT6 polyclonal antibody (1:500, Sigma-Aldrich), anti-ER- $\alpha$  antibody (1:400, Santa Cruz), and Alexa Fluor Phalloidin 594 (1:40, ThermoFisher Scientific). The secondary antibodies were Alexa Fluor 488 anti-Mouse IgG antibodies (1:1000, Life Technologies) and Alexa Fluor 594 anti-Rabbit IgG antibodies (1:1000, Life Technologies). Finally, cells were stained with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and examined by TCS SP5 Confocal Laser Scanning Microscope (Leica Microsystems).

### Peptide Design and Synthesis

We used two peptides synthesized by Genscript, USA. The amino acid sequences corresponded to a part of ER- $\alpha$  including the O-glycosylation site; wild-type peptide, 11R-GGG-LATAGSTSSHS, and a substituted peptide at the glycosylation site: 11R-GGG-LATAGPTSSHS. The peptides had a purity of >95%.

### Cell Viability Analyses

For methyl thiazolyl tetrazolium (MTT) assay, breast cancer cells were seeded into 24-well plates (BD Falcon) at a density of  $2.5 \times 10^4$  cells per well at day 0, and 24 hours after seeding of cells, we added 1 mM each of the peptide and then examined the cell viability 4 hours after the peptide addition. We used the Cell Counting Kit-8

(Dojindo Molecular Technologies, Inc.) for MTT assay and examined the cell viability at 4 hours after the peptide addition.

### Time-Lapse Microscopy

To examine cellular morphological changes caused by the peptide treatment, we added either PBS or 1 mM each of either wild-type peptide or substituted peptide to the culture medium when breast cancer cells reached 70% confluency and then cultured at 37°C in humidified air with 5% CO<sub>2</sub>. The cell phenotypes were photographed with 20 $\times$  magnification at 5-minute intervals over 24 hours by using an automated imaging system with high resolution (EVOS FL Auto 2 Imaging System, Thermo Fisher Scientific).

### Statistical Analysis

Student's *t* test, chi-square test, and Fisher's exact test were applied for comparisons. Results were considered significantly when *P* values were < .05.

## Result

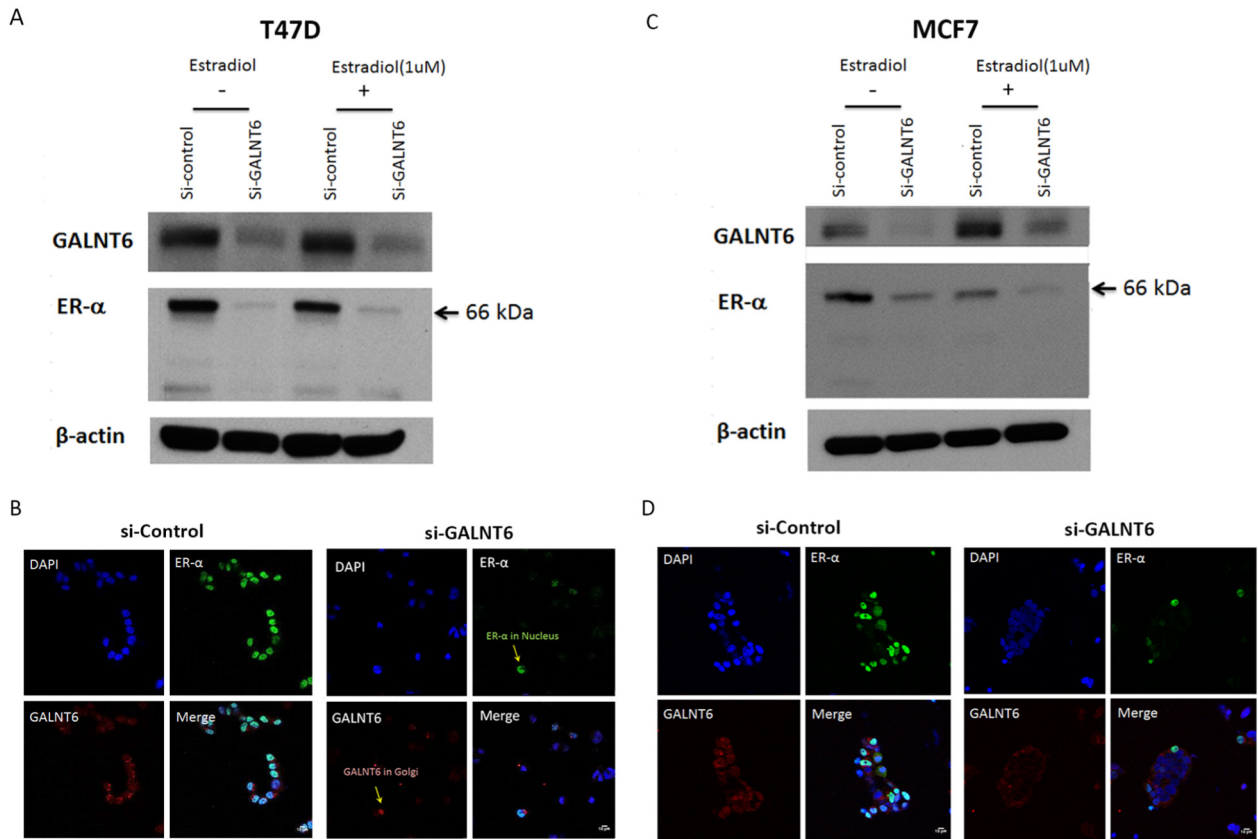
### Identification of a Novel GALNT6 Substrate(s)

We screened a GALNT6 substrate(s) using the three stable cell lines (HeLa-GALNT6-WT, HeLa-GALNT6-H271D, and HeLa-Mock) and identified 182 possible O-glycosylated proteins in HeLa-GALNT6-WT cells, 190 in HeLa-GALNT6-H271D cells, and 117 in HeLa-Mock cells (Supplementary Figure 1 and Supplementary Table 1). Among them, ER- $\alpha$  glycosylation was uniquely observed in the GALNT6-WT-expressing cells but not in two other cell lines. Since ER- $\alpha$  is a well-known and important protein in mammary carcinogenesis, we focused on this protein for further analysis and attempted to examine the biological significance of O-glycosylation of ER- $\alpha$  by GALNT6 in breast cancer cells.

### Knockdown Effects of Endogenous GALNT6

We chose four cell lines for further analysis: two cell lines, T47D and MCF7 cells, expressed both ER- $\alpha$  and GALNT6; SKBR3 cells expressed ER- $\alpha$  but not GALNT6; HCC1937 cells expressed neither ER- $\alpha$  nor GALNT6 (Supplementary Figure 2). We firstly used siRNAs to knock down endogenous GALNT6 expression in two breast cancer cell lines, T47D and MCF7, in which GALNT6 was highly expressed [12] and the ER- $\alpha$  expression was detected (Supplementary Figure 2). Knockdown of GALNT6 by siRNA could significantly decrease GALNT6 expression in these two cancer cells compared with those transfected with si-Control in a condition both with and without estradiol treatment (Figure 1, A and C). Concordantly, immunocytochemistry analysis revealed significant decrease of ER- $\alpha$  in the nucleus in GALNT6 knockdown cells by siRNAs (Figure 1, B and D). After knockdown of GALNT6 by siRNAs, we evaluated expression levels of ER- $\alpha$  downstream genes such as *CCND1*, *MYC*, and *CTSD* (Figure 2). The qPCR results showed that at 72 hours after the knockdown of GALNT6, expression levels of these downstream genes correspondingly decreased in the conditions either with or without addition of estradiol. In T47D cells, *CCND1* and *MYC* were significantly downregulated regardless of addition of estradiol (*P* < .05), and *CTSD* expression level was downregulated only in a condition without estradiol. In MCF7 cells, all of the three downstream genes were significantly downregulated in conditions regardless of addition of estradiol (*P* < .05).



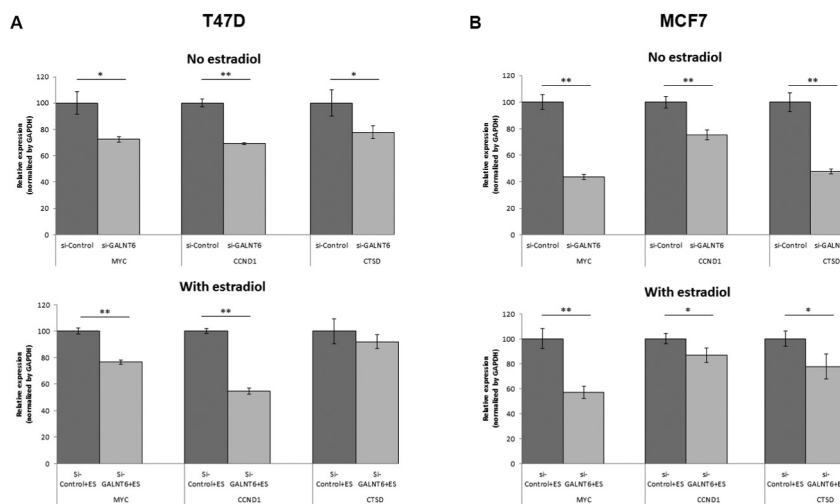


**Figure 1.** Knockdown effect of GALNT6 in T47D and MCF7 cell lines. (A, C) Western blotting showed the GALNT6 and ER- $\alpha$  protein levels 72 hours after transfection of siGALNT6 compared with si-Control, with or without estradiol treatment (1 nM, 24 hours). (B, D) Immunocytochemistry showed the ER- $\alpha$  (green) expressions in nuclear of T47D and MCF7 72 hours after transfection of si-GALNT6. GALNT6 was investigated by immunostaining with a fluorescence-labeled GALNT6 (red), and DAPI was co-stained to identify nucleus (blue). Scale bar indicates 10  $\mu$ m.

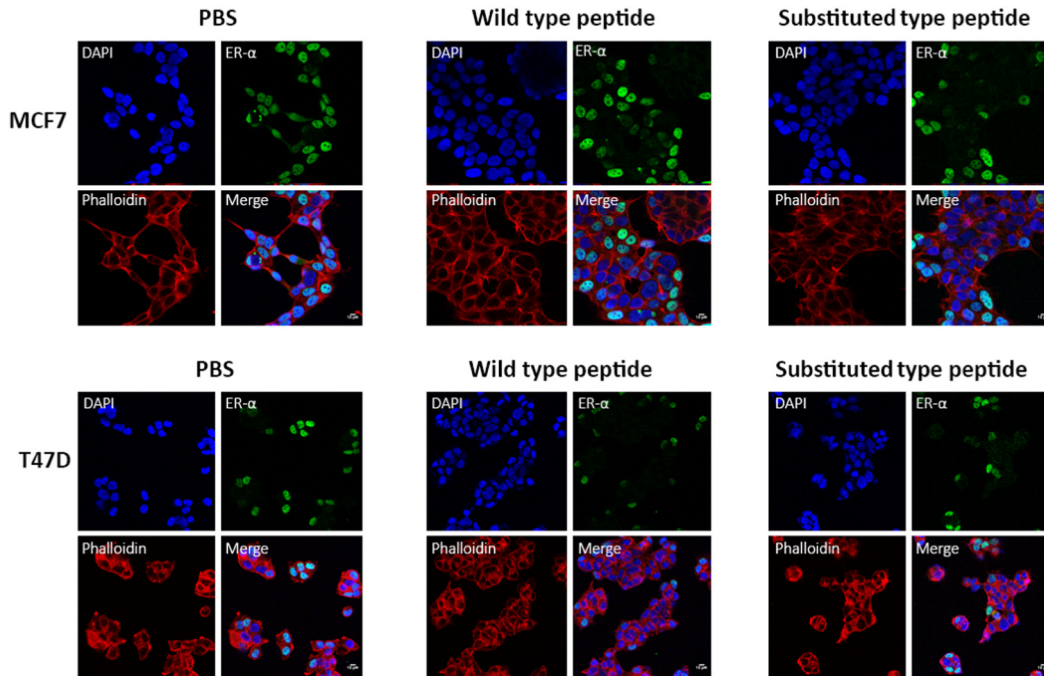
**GALNT6 Induced In Vivo O-Glycosylation of ER- $\alpha$**

To further investigate an O-glycosylation site(s) of ER- $\alpha$  by GALNT6, we introduced the Flag-tagged full-length ER- $\alpha$  expressing vector into three HeLa-derivative cells in which WT-GALNT6 or

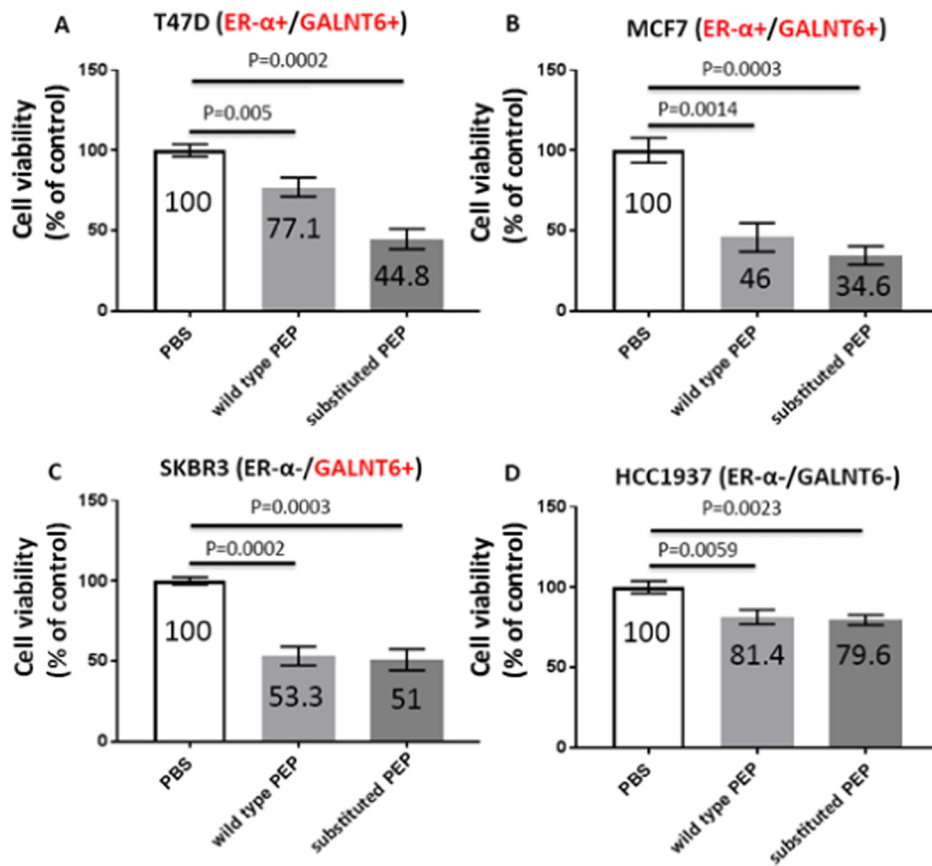
H271D-enzyme-dead-GALNT6 expression was introduced, or HeLa cells transfected with the mock vector. By immunoprecipitation with anti-Flag antibody followed by VVA lectin blotting (specific to GalNAc-Ser/Thr), we observed a glycosylated protein band of



**Figure 2.** Knockdown effect of GALNT6 to ER- $\alpha$  downstream genes in T47D and MCF7 cell lines. (A, B) Transcriptional levels of *CCND1*, *MYC*, and *CTSD* after GALNT6 siRNA knockdown (72 hours) in T47D cell line (A) and MCF cell line (B), with or without estradiol treatment (1 nM, 24 hours), were examined by qPCR (\* $P < .05$ , \*\* $P < .01$ ).



**Figure 3.** Cell-permeable peptides treatment on breast cancer cell lines. Immunocytochemistry of ER- $\alpha$  (green) was conducted 1 hour (MCF7) or 2 hours (T47D) after treatment of permeable peptides. Cell morphology was further investigated by immunostaining with a fluorescence-labeled phalloidin (red), and DAPI was co-stained to identify nucleus (blue). Scale bar indicates 10  $\mu$ m.



**Figure 4.** (A, B, C, D) The proliferation assay to analyze the antitumor effect of the cell-permeable peptides (1 mM) on breast cancer cell lines. Four hours after the peptide treatment in four cell lines, T47D, MCF7, SKBR3, and HCC1937, we performed the cell viability with both wild-type and substituted peptides.

approximately 66 kDa in WT-GALNT6 cells (Supplementary Figure 3A, *black arrow*) but not in GALNT6-H271D-expressing cells or mock cells, suggesting that GALNT6 could mediate O-glycosylation of ER- $\alpha$ . To identify an O-glycosylation site(s) of ER- $\alpha$  by GALNT6, we visualized protein bands with Coomassie stains, cut the protein band which was specifically detected in the HeLa-GALNT6-WT cells, and conducted LC-MS/MS analysis (Figure 3B). We then identified an O-glycosylation site at Ser573 in an F domain of ER- $\alpha$  protein.

### *The Growth Suppression of Breast Cancer Cell Lines with Cell Membrane-Permeable Peptides*

To further examine the significance of ER- $\alpha$  glycosylation by GALNT6, we synthesized two cell membrane-permeable peptides corresponding to a part of ER- $\alpha$  including the S573 (Supplementary Figure 4); one corresponds to the wild-type peptide sequence, and the other has a substitution of this serine to proline. As shown in immunocytochemical staining (Figure 3), ER- $\alpha$  expression levels in T47D and MCF7 cells were significantly reduced in 1-2 hours (1 hour for T47D cells and 2 hours for MCF7 cells) after addition of 1 mM of each peptide. Four hours after the peptide treatment, three cell lines, T47D, MCF7, and SKBR3, in which GALNT6 was expressed, showed significant decrease of cell viability with both wild-type and substituted peptides (Figure 4, A-C and Movies S1-S3). We did not observe significant differences in the cell viability in ER- $\alpha$ -positive cells (T47D and MCF7 cells) and ER- $\alpha$ -negative SKBR3 cells with peptide treatment. We suspect that since these peptides bound to GALNT6 protein, glycosylation of other GALNT6 substrates that were critical for cell survival was also suppressed and the cell death was induced. However, the growth-inhibitory effect with the peptide treatment was very modestly observed in HCC1937 cells that expressed neither ER- $\alpha$  nor GALNT6 compared with the three GALNT6-positive cell lines (Figure 4D and Movie S4), supporting that the peptides worked in a GALNT6-dependent manner.

### **Discussion**

ER- $\alpha$  belongs to members of the nuclear hormone-receptor superfamily, which acts as a ligand-inducible transcriptional factor [20]. The human ER- $\alpha$  gene (*ESR1*) encodes a 66-kDa protein with 595 amino acids [21] which consists of four major domains: an N-terminal domain (NTD), a ligand-binding domain (LBD), a DNA-binding domain (DBD), and an F domain. The NTD is able to transactivate multiple downstream genes through binding to the TATA box-binding protein (TBP) [22]. The LBD can bind ligands and coregulatory proteins. The DBD binds to estrogen response elements in DNA [20], and the F domain modulates the transcriptional activity, coactivator interactions, dimerization, and stability of the protein [23]. Through the activation by binding to estrogen, ER- $\alpha$  is translocated into the nucleus, binds to DNA, and modulates transcription of its downstream genes [24].

O-type glycosylation is one of the important posttranslational protein modifications and takes place on serine or threonine residues [25,26]. GALNT6, which is one of the GALNT family members functioning in the Golgi complex, mediates O-type glycosylation and plays a critical role in folding of multiple glycoproteins and maintenance of the protein stability [27]. In our previous studies, we demonstrated that GALNT6 could O-glycosylate and stabilize Mucin 1 (MUC1) and plays critical roles in proliferation and cytoskeletal

regulation of breast cancer cells [12]. In addition, through a signaling pathway involving fibronectin, one of the GALNT6 O-glycosylation substrates, GALNT6 would enhance transformational potentials of mammary epithelial cells and *in vivo* invasiveness of breast cancer cells [13]. Furthermore, we reported that GALNT6 knockdown in pancreatic cancer cells decreased mRNA and protein levels of Mucin 4 (MUC4), in which the 10th threonine residue of the tandem repeat was predominantly O-glycosylated by GALNT6, and reduced the levels of human epidermal growth factor receptor 2 [14]. We also found that GALNT6 could bind to the ATPase domain of GRP78 and stabilizes the GRP78 protein through O-glycosylations, which also contributed to proper subcellular localization and antiapoptotic function of GRP78 protein in cancer cells [28]. EGFR was also shown to be a substrate of GALNT6 in ovarian cancer cells [15].

In this study, we found O-glycosylation of ER- $\alpha$  by GALNT6 and demonstrated the biological significance of ER- $\alpha$  O-glycosylation in cancer cell lines by two approaches: reduced expression by siRNA and inhibition of the interaction between ER- $\alpha$  and GALNT6 with cell membrane-permeable peptides. We demonstrated that these two approaches could significantly decrease the ER- $\alpha$  expression in the nucleus and reduce the cell viability on GALNT6-positive breast cancer cells. We identified S573 in the F domain of ER- $\alpha$  was O-glycosylated in GALNT6-positive cells but not in GALNT6-negative or GALNT6-enzyme-dead cells, which strongly suggested that this O-glycosylation is mediated by GALNT6. The F domain of ER- $\alpha$  is known to be the key covalent modification target in the function of ER- $\alpha$ . All previous studies for ER- $\alpha$  were involved in mice or insects; one report demonstrated that one major *in vivo* glycosylation site in mER- $\alpha$  is located at Thr575 near the carboxyl terminus [29]. We compared the amino acid sequences between two species and found that S573 in human is not compatible to Thr575 of mER- $\alpha$ . Hence, it is difficult to discuss the biological significance of glycosylation of hER- $\alpha$  and mER- $\alpha$  in mammary carcinogenesis at this point.

The membrane-permeable arginine-rich peptide could facilitate efficiently the nonspecific uptake of peptides into cells and is considered to be a good peptide delivery system [30,31]. To investigate the biological significance of GALNT6-mediated ER- $\alpha$  O-glycosylation, we designed two cell membrane-permeable peptides corresponding to the S573 O-glycosylation site in the F domain: one with the wild-type sequence and the other with the substitution of this serine residue to proline. We found that both peptides could significantly decrease the ER- $\alpha$  expression levels, particularly its nuclear localization, and cause the significant decrease of the cell viability in the GALNT6-positive cancer cells. Although one peptide included the amino acid substitution at the glycosylation site, it is likely that this substituted peptide still kept the binding affinity to GALNT6 as similar to the wild-type peptide. As expected, neither of peptides revealed the significant growth inhibitory effect in the ER- $\alpha$ /GALNT6 double-negative cancer cells. Our data indicate that (1) in the GALNT6-positive breast cancer cells, the wild-type and substituted peptides bind to GALNT6, block GALNT6 activity, and lead to cell death, and (2) in the GALNT6/ER- $\alpha$  double-positive breast cancer cells, the stronger effect on the cell viability was observed in MCF7 cells where ER- $\alpha$  levels were much higher than another cell line. The modest effect was observed in the ER- $\alpha$ /GALNT6 double-negative cells probably due to the toxic effect of the peptide exposure or due to the cross-reactivity of these peptides to other GALNT family members that also play significant roles in cancer cell growth.



In conclusion, our data for the first time, which confirmed the O-glycosylation of ER- $\alpha$  by GALNT6, indicate that targeting GALNT6 as well as the GALNT6/ER- $\alpha$  interaction may be an effective strategy for development of novel drugs to treat hormone receptor-positive but hormone therapy-resistant breast cancer.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neo.2018.08.006>.

### Authors' Contributions

Y. N. designed and supervised the project, provided funding, and edited the manuscript; B. D. performed Western blotting; ICC, IP-VVA, cell viability assay, and time-lapse movie; analyzed and interpreted data; and wrote the manuscript; Y. T. (contributed equally to work) performed cell culture and RNA extraction; L. R. performed qPCR; K. U. performed the MS/MS analysis; T. K. and J. P. assisted experiments.

### References

- [1] Siegel RL, Miller KD, and Jemal A (2017). Cancer statistics, 2017. *CA Cancer J Clin* **67**(1), 7–30.
- [2] Johnston SR (2010). New strategies in estrogen receptor-positive breast cancer. *Clin Cancer Res* **16**(7), 1979–1987.
- [3] Derks MGM, Blok EJ, Seynaeve C, Nortier JWR, Kranenbarg EM, Liefers GJ, Putter H, Kroep JR, Rea D, and Hasenburg A, et al (2017). Adjuvant tamoxifen and exemestane in women with postmenopausal early breast cancer (TEAM): 10-year follow-up of a multicentre, open-label, randomised, phase 3 trial. *Lancet Oncol* **18**(9), 1211–1220.
- [4] *Tamoxifen for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group* *Lancet* **351**(9114), 1451–1467.
- [5] Clarke R, Leonessa F, Welch JN, and Skaar TC (2001). Cellular and molecular pharmacology of antiestrogen action and resistance. *Pharmacol Rev* **53**(1), 25–71.
- [6] Ali S, Rasool M, Chaoudhry H, Pushparaj PN, Jha P, Hafiz A, Mahfooz M, Abdus Sami G, Azhar Kamal M, and Bashir S, et al (2016). Molecular mechanisms and mode of tamoxifen resistance in breast cancer. *Bioinformatics* **12**(3), 135–139.
- [7] Potapenko IO, Lüders T, Russnes HG, Helland Å, Sørli E, Kristensen VN, Nord S, Lingjærde OC, Børresen-Dale AL, and Haakensen VD (2015). Glycan-related gene expression signatures in breast cancer subtypes; relation to survival. *Mol Oncol* **9**(4), 861–876.
- [8] Lavrsen K, Dabelsteen S, Vakhrushev SY, Levann AMR, Haue AD, Dylander A, Mandel U, Hansen L, Frödin M, and Bennett EP, et al (2018). De novo expression of human polypeptide N-acetylgalactosaminyltransferase 6 (GalNAcT6) in colon adenocarcinoma inhibits the differentiation of colonic epithelium. *J Biol Chem* **293**(4), 1298–1314.
- [9] Li Z, Yamada S, Inenaga S, Imamura T, Wu Y, Wang KY, Shimajiri S, Nakano R, Izumi H, and Kohno K, et al (2011). Polypeptide N-acetylgalactosaminyltransferase 6 expression in pancreatic cancer is an independent prognostic factor indicating better overall survival. *Br J Cancer* **104**(12), 1882–1889.
- [10] Ramaswamy S, Tamayo P, Rifkin R, Mukherjee S, Yeang CH, Angelo M, Ladd C, Reich M, Latulippe E, and Mesirov JP, et al (2001). Multiclass cancer diagnosis using tumor gene expression signatures. *Proc Natl Acad Sci U S A* **98**(26), 15149–15154.
- [11] Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, Speed D, Lynch AG, Samarajiwa S, and Yuan Y, et al (2012). The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* **486**(7403), 346–352.
- [12] Park JH, Nishidate T, Kijima K, Ohashi T, Takegawa K, Fujikane T, Hirata K, Nakamura Y, and Katagiri T (2010). Critical roles of mucin 1 glycosylation by transactivated polypeptide N-acetylgalactosaminyltransferase 6 in mammary carcinogenesis. *Cancer Res* **70**(7), 2759–2769.
- [13] Park JH, Katagiri T, Chung S, Kijima K, and Nakamura Y (2011). Polypeptide N-acetylgalactosaminyltransferase 6 disrupts mammary acinar morphogenesis through O-glycosylation of fibronectin. *Neoplasia* **13**(4), 320–326.
- [14] Tarhan YE, Kato T, Jang M, Haga Y, Ueda K, Nakamura Y, and Park JH (2016). Morphological changes, cadherin switching, and growth suppression in pancreatic cancer by GALNT6 knockdown. *Neoplasia* **18**(5), 265–272.
- [15] Lin TC, Chen ST, Huang MC, Huang J, Hsu CL, Juan HF, Lin HH, and Chen CH (2017). GALNT6 expression enhances aggressive phenotypes of ovarian cancer cells by regulating EGFR activity. *Oncotarget* **8**(26), 42588–42601.
- [16] Guo Y, Shi J, Zhang J, Li H, Liu B, and Guo H (2017). Polypeptide N-acetylgalactosaminyltransferase-6 expression in gastric cancer. *Oncotargets Ther* **10**, 3337–3344.
- [17] Hang HC, Yu C, Kato DL, and Bertozzi CR (2003). A metabolic labeling approach toward proteomic analysis of mucin-type O-linked glycosylation. *Proc Natl Acad Sci U S A* **100**(25), 14846–14851.
- [18] Kim NH, Yoshimaru T, Chen YA, Matsuo T, Komatsu M, Miyoshi Y, Tanaka E, Sasa M, Mizuguchi K, and Katagiri T (2015). BIG3 Inhibits the estrogen-dependent nuclear translocation of PHB2 via multiple karyopherin-alpha proteins in breast cancer cells. *PLoS One* **10**(6), e0127707.
- [19] Kim JW, Akiyama M, Park JH, Lin ML, Shimo A, Ueki T, Daigo Y, Tsunoda T, Nishidate T, and Nakamura Y, et al (2009). Activation of an estrogen/estrogen receptor signaling by BIG3 through its inhibitory effect on nuclear transport of PHB2/REA in breast cancer. *Cancer Sci* **100**(8), 1468–1478.
- [20] Kumar V, Green S, Stack G, Berry M, Jin JR, and Chambon P (1987). Functional domains of the human estrogen receptor. *Cell* **51**(6), 941–951.
- [21] Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, and Shine J (1986). Sequence and expression of human estrogen receptor complementary DNA. *Science* **231**(4742), 1150–1154.
- [22] Wärnmark A, Wikström A, Wright AP, Gustafsson JA, and Härd T (2001). The N-terminal regions of estrogen receptor alpha and beta are unstructured in vitro and show different TBP binding properties. *J Biol Chem* **276**(49), 45939–45944.
- [23] Koide A, Zhao C, Naganuma M, Abrams J, Deighton-Collins S, Skafar DF, and Koide S (2007). Identification of regions within the F domain of the human estrogen receptor alpha that are important for modulating transactivation and protein-protein interactions. *Mol Endocrinol* **21**(4), 829–842.
- [24] Levin ER (2005). Integration of the extranuclear and nuclear actions of estrogen. *Mol Endocrinol* **19**(8), 1951–1959.
- [25] Holt GD, Snow CM, Senior A, Haltiwanger RS, Gerace L, and Hart GW (1987). Nuclear pore complex glycoproteins contain cytoplasmically disposed O-linked N-acetylglucosamine. *J Cell Biol* **104**(5), 1157–1164.
- [26] Davis LI and Blobel G (1986). Identification and characterization of a nuclear pore complex protein. *Cell* **45**(5), 699–709.
- [27] Chia J, Goh G, and Bard F (2016). Short O-GalNAc glycans: regulation and role in tumor development and clinical perspectives. *Biochim Biophys Acta* **1860**(8), 1623–1639.
- [28] Lin J, Chung S, Ueda K, Matsuda K, Nakamura Y, and Park JH (2017). GALNT6 Stabilizes GRP78 protein by O-glycosylation and enhances its activity to suppress apoptosis under stress condition. *Neoplasia* **19**(1), 43–53.
- [29] Jiang MS and Hart GW (1997). A subpopulation of estrogen receptors are modified by O-linked N-acetylglucosamine. *J Biol Chem* **272**(4), 2421–2428.
- [30] Noguchi H, Matsushita M, Okitsu T, Moriwaki A, Tomizawa K, Kang S, Li ST, Kobayashi N, Matsumoto S, and Tanaka K, et al (2004). A new cell-permeable peptide allows successful allogeneic islet transplantation in mice. *Nat Med* **10**(3), 305–309.
- [31] Sato S, Nakamura T, Katagiri T, Tsuchikawa T, Kushibiki T, Hontani K, Takahashi M, Inoko K, Takano H, and Abe H, et al (2017). Molecular targeting of cell-permeable peptide inhibits pancreatic ductal adenocarcinoma cell proliferation. *Oncotarget* **8**(69), 113662–113672.