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Polypeptide *N*-acetylgalactosaminyltransferase 6 Disrupts Mammary Acinar Morphogenesis through *O*-glycosylation of Fibronectin[1,2]

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Abstract

A high expression of short and immature *O*-glycans is one of the prominent features of breast cancer cells, which would be attributed to the upregulated expression of glycosyltransferases. Therefore, a detailed elucidation of glycosyltransferases and their substrate(s) may improve our understandings for their roles in mammary carcinogenesis. Here we report that overexpression of polypeptide *N*-acetylgalactosaminyltransferase 6 (GALNT6), a glycosyltransferase involved in the initial step of *O*-glycosylation, has transformational potentials through disruptive acinar morphogenesis and cellular changes similar to epithelial-to-mesenchymal transition in normal mammary epithelial cell, MCF10A. As one of the critical *O*-glycan substrates, we identified fibronectin that was *O*-glycosylated *in vivo* and thereby stabilized by GALNT6. Because knockdown of fibronectin abrogated the disruptive proliferation caused by introduction of GALNT6 into epithelial cells, our findings suggest that GALNT6-fibronectin pathway should be a critical component for breast cancer development and progression.

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Introduction

Alterations in *O*-type glycosylation of breast cancer cells induce diverse biologic and pathologic consequences influencing growth and survival of the cells and their ability for invasion and metastasis [1]. Accumulating evidences have shown that aberrant expression of glycosyltransferases confers the altered *O*-glycan structures in breast cancer cells [2,3]. In this regard, we previously characterized oncogenic roles of GALNT6 that was upregulated exclusively in breast cancer cells and regulated cell proliferation and cytoskeleton structure [4]. In accordance to biologic relevance, GALNT6 expression was related with poor prognosis of breast cancer patients, indicating its application as a molecular marker for risk of cancer metastasis [5]. However, the molecular mechanism of how GALNT6 contributes to breast malignancy by enhancing the *O*-glycosylation remains unclear.

The three-dimensional culture of MCF10A mammary epithelial cells was developed to assess oncogenic ability by monitoring the disruption of well-ordered architecture of mammary gland, which is regarded as an early aspect of mammary carcinogenesis [6]. Under the three-dimensional culture conditions, MCF10A cells form the acinar structure composed of a monolayer of polarized cells, but the well-organized architecture was disrupted by the introduction of certain cancer-related genes [7–9]. In addition to investigating cell morphogenesis, the three-dimensional culture-based monitoring system provides more accurate physiological conditions to assess oncogenic functions related to invasive behavior and epithelial-to-mesenchymal transition (EMT) [7].

In this study, we generated GALNT6-expressing MCF10A stable transfectants and clarified transformational potentials of GALNT6 by the three-dimensional culture method in the aspects of disruption of acinar structure formation as well as EMT-like cellular alterations.

Abbreviations: EMT, epithelial-to-mesenchymal transition; GALNT6, polypeptide *N*-acetylgalactosaminyltransferase 6; VVA, *Vicia villosa*; WT, wild-type

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Materials and Methods

Cell Lines

Human breast cancer cell lines (T47D and HCC1500) and an immortalized breast epithelial cell line (MCF10A) were purchased from American Type Culture Collection (Rockville, MD) and Cambrex Bioscience (Walkersville, MD), respectively. Detailed cell culture and test methods were previously described [4]. The cell viability was assessed by Cell Counting Kit 8 (Dojindo, Kumamoto, Japan).

Three-dimensional Cell Culture with MCF10A-GALNT6 Stable Transfectants

Each of the pCAGGS-HAc expression vectors of mock (no insert), GALNT6 wild-type (WT), and inactivated GALNT6 mutant (H271D) was transfected into MCF10A cells using FuGENE6 transfection reagent (Roche, Basel, Switzerland), as previously described [4]. Then, the positive clones were selected under incubation with culture medium containing 0.4 mg/ml of Geneticin (Invitrogen, Carlsbad, CA) and validated by Western blot analysis and immunocytochemical staining. The cells were seeded on the growth factor–reduced Matrigel (BD Biosciences, San Jose, CA) and maintained in mammary epithelial cell medium media (Cambrex Bioscience) with 2% of the Matrigel [6]. Each size of cell colonies was quantified by ImageJ software by National Institutes of Health (Bethesda, MD) [10].

Western Blots and Immunocytochemical Staining

SDS-PAGE, immunoprecipitation, and immunocytochemical staining were performed as described previously [4], with anti–E- or N-cadherins (BD Biosciences), anti-fibronectin (Santa Cruz Biotechnology, Santa Cruz, CA), anti–β-actin (Sigma-Aldrich, St Louis, MO), anti–integrin α_6 (Chemicon, Billerica, MA), and anti–active caspase-3 (Abcam, Cambridge, UK) antibodies. The cytoskeleton structure was visualized by staining with Alexa Fluor-488/594 phalloidins (Molecular Probe, Eugene, OR). The GalNAc (*N*-acetyl-α-D-galactosamine)–conjugated glycoproteins were detected by *Vicia villosa* (VVA) lectin (Vector, Burlingame, CA) with or without incubation with recombinant GALNT6 proteins *in vitro* [4].

Knockdown Experiments

To deplete endogenous expression of fibronectin, we introduced a synthesized oligo-duplex small interfering RNA (siRNA) against the *FN1* gene (5′-AAGTGGTCCTGTCGAAGTATT-3′) into MCF10A-GALNT6 stable transfectants once a week [11]. We used si-GALNT6 (5′-GAGAAAUCCUUCGGUGACA-3′) to deplete endogenous expression of GALNT6 and used si-EGFP (5′-GAGAAAUCCUUCGGUGACA-3′) as a control [4]. Semiquantitative reverse transcription–polymerase chain reaction was performed as described previously [12]. The polymerase chain reaction primer sequences were 5′-CTGCAGTATATCCGCTTAGCC-3′ and 5′-TAAGTCCATGCAAAGGAGACTAGC-3′ for *ACTB* and 5′GGAGTTGATTATACCATCACTG-3′ and 5′-TTTCTGTTTGATCTGGACCT-3′ for *FN1* (GenBank no. NM_002026).

Statistical Analysis

Statistical significance was examined by Student's *t* test using R statistical package [13]. A difference of $P < .05$ was considered to be statistically significant.

Results

Breast Cancer Cells Express Multiple O*-glycan Substrates of GALNT6*

MUC1 was one of important substrates of GALNT6 in breast cancer cells (T47D, MCF7, and SKBR3). However, our previous findings indicated the presence of additional *O*-glycan substrates that would be *O*-glycosylated by GALNT6 [4]. Indeed, VVA-lectin Western blot analysis indicated the presence of multiple substrates whose *O*-glycosylations were diminished by knockdown of GALNT6 (Figure 1*A*, *lane 1 vs 4*). The glycosylations of these candidate bands were restored by *in vitro* incubation with recombinant WT GALNT6 protein (Figure 1*A*, *lane 4 vs 5*). This result was in concordance with our previous speculation that GALNT6 might have additional functions through *O*-glycosylation of unidentified substrates [4].

Overexpression of GALNT6 Induces EMT-like Morphologic Alterations

To investigate unidentified substrates of GALNT6, we generated transfectants stably expressing WT- or enzyme-dead- (H271D) GALNT6 in MCF10A cell that expressed a very low level of GALNT6 and MUC1 proteins [4]. In contrast to T47D cells (Figure 1*A*), VVA-lectin analysis recognized only a few bands that showed intensity enhancement by overexpression of GALNT6 (Figure 1*B*, *left*) and we observed no effect on the proliferation of MCF10A cells (Figure 1*B*, *right*). It might be due to the lower expression of some GALNT6 substrates in MCF10A cells. However, we observed significant morphologic changes from epithelial-like cells to mesenchymal-like cells by introduction of WT-GALNT6 (Figure 1*C*). Because such EMT-like changes have been strongly implicated in relation to cancer progression with abolished cell adhesion [14], we further performed Western blot analysis using the antibody to E-cadherin that is the representative cell adhesion molecule and an epithelial cell maker and found that E-cadherin expression was reduced by the overexpression of WT-GALNT6. However, E-cadherin expression level was elevated by H271D-GALNT6 induction than transfection of mock control, indicating a dominant negative effect of H271D-GALNT6 to the weakly expressed endogenous GALNT6 protein (Figure 1*D*, *left*). Interestingly, a mesenchymal cell marker, N-cadherin was inversely correlated with the expression levels of E-cadherin (Figure 1*D*, *right*) as known as "cadherin switching" that was supposed to be regulated in transcriptional levels [15]. Hence, it is likely that substrates of GALNT6 and their downstream targets might affect such a competitive transcription of two cadherins. In addition, the reduction of E-cadherin was also observed in HCC1500 breast cancer cells, which showed a low level of GALNT6, by the overexpression of GALNT6 (Figure W1).

Overexpression of GALNT6 Disrupts Acinar Formation of MCF10A Cells in Three-dimensional Culture

The three-dimensional cell culture enabled us to monitor cell morphogenesis in a relatively physiological condition compared with the conventional two-dimensional cell culture. Hence, we performed three-dimensional cell culture of MCF10A-GALNT6 stable cells and observed irregular and invasive proliferation of these cells into Matrigel matrix after 10 days of incubation (Figure 2*A*). Subsequently, we quantified each acinar size and found that the disruptive proliferation of MCF10A cells was induced by the overexpression of WT-GALNT6, although it was not observed in MCF10A-H271D-GALNT6 stable cells (Figure 2*B*). In addition, we performed immunocytochemical