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Mucin Glycosylating Enzyme GALNT2 Regulates the Malignant Character of Hepatocellular Carcinoma by Modifying the EGF Receptor

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Abstract

Extracellular glycosylation is a critical determinant of malignant character. Here, we report that N-acetylgalactosaminyltransferase 2 (GALNT2), the enzyme that mediates the initial step of mucin type-O glycosylation, is a critical mediator of malignant character in hepatocellular carcinoma (HCC) that acts by modifying the activity of the epidermal growth factor receptor (EGFR). GALNT2 mRNA and protein were downregulated frequently in HCC tumors where these events were associated with vascular invasion and recurrence. Restoring GALNT2 expression in HCC cells suppressed EGF-induced cell growth, migration, and invasion *in vitro* and *in vivo*. Mechanistic investigations revealed that the status of the O-glycans attached to the EGFR was altered by GALNT2, changing EGFR responses after EGF binding. Inhibiting EGFR activity with erlotinib decreased the malignant characters caused by siRNA-mediated knockdown of GALNT2 in HCC cells, establishing the critical role of EGFR in mediating the effects of GALNT2 expression. Taken together, our results suggest that GALNT2 dysregulation contributes to the malignant behavior of HCC cells, and they provide novel insights into the significance of O-glycosylation in EGFR activity and HCC pathogenesis. *Cancer Res; 71(23); 1–10. ©2011 AACR.*

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third leading cause of cancer-related death worldwide (1). The primary curative treatment for HCC is hepatic resection. Although clinical treatment of HCC is continuously evolving, the prognosis of HCC patients remains poor. To improve the survival of HCC patients, further understanding of HCC pathogenesis and novel treatment agents are needed.

Glycosylation is the most common posttranslational modification of proteins. Aberrant glycosylation is a hallmark of most human cancers and affects many cellular properties, including cell proliferation, apoptosis, differentiation, transformation, migration, invasion, and immune responses (2). Tumor-associated carbohydrate antigens have drawn global attention to develop diagnostic reagents and vaccines for cancer therapy (3). However, the glycogenes responsible for the expression of these antigens and their pathophysiologic roles in human cancers are still largely unknown.

Two major types of protein glycosylation in mammalian cells exist: N-linked and O-linked. The most frequently occurring O-glycosylation is the mucin type, initiated by the transfer of UDP-N-acetylgalactosamine (UDP-GalNAc) to the hydroxyl group of serine (S) or threonine (T) residue forming Tn antigen (GalNAcα-S/T; ref. 4). This reaction is catalyzed by a large family of polypeptide GalNAc transferases (GALNT), consisting of at least 20 members in humans, namely GALNT1 to 14 and GALNTL1 to L6 (5, 6).

Studies have shown that O-glycans and *GALNT* genes play critical roles in a variety of biological functions and human disease development. For instance, loss of GALNT1 activity in mice results in bleeding disorder (7). Risk of epithelial ovarian cancer (8) and coronary artery disease (9) have been associated with single nucleotide polymorphisms of *GALNT1* and *GALNT2*, respectively. GALNT3 expression is a potential diagnostic marker for lung (10) and pancreatic (11) cancers. GALNT6 modifies mucin 1 glycosylation and regulates proliferation of breast cancer cells (12).

The epidermal growth factor receptor (EGFR) is a promising therapeutic target as its overexpression is associated with various cancers and plays a crucial role in tumor malignancy (13). Overexpression of EGFR in HCC (14) and upregulation of EGF in advanced HCC compared with the control liver tissue

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and early HCC (15) suggest the potential role of EGFR-ligand interaction in HCC progression. Phase 2 clinical trials of erlotinib, an EGFR inhibitor, showed 9% partial response and 25% partial response combined with bevacizumab (antibody for VEGF) for advanced HCC (16). The phase 3 clinical trial of erlotinib for advanced HCC is still ongoing. However, the clinical efficacy is still unsatisfactory. Thus, to improve the effect of EGFR-targeted therapies, molecular mechanisms by which EGFR regulates HCC properties should be further investigated.

In HCC, the expression pattern and function of GALNT family have never been reported, although O-glycosylation can regulate multiple cellular properties. Here, we report that GALNT2 is frequently downregulated in HCC. Moreover, GALNT2 modifies EGFR O-glycosylation and activity, and plays a critical role in the malignant phenotype of HCC cells *in vitro* and *in vivo*.

Materials and Methods

Tissue samples

Postsurgery fresh tissue samples were collected from patients receiving treatment at the National Taiwan University Hospital (Supplementary Table S1). The tumor samples were taken from the central part of the resected tumor and the paired nontumor samples were taken 2 cm away from the tumor. For immunohistochemistry, specimens were fixed in 4% (w/v) paraformaldehyde/PBS. For RNA extraction, specimens were soaked in RNA*later* (Qiagen Corp.) at 4°C overnight and then stored at −20°C. Samples used for Western blotting were stored at −80°C. Ethics approval was obtained from the local hospital ethic committees and a written consent was obtained from each patient before sample collection.

Cell line and cell culture

Human liver cancer cell lines Huh7, PLC5, and HepG2 were purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan) in 2008. HA22T, SUN387, and HCC36 cells were kindly provided by Shiou-Hwei Yeh (National Taiwan University, Taiwan) in 2010. All cell lines were authenticated by the provider based on morphology, antigen expression, growth, DNA profile, and cytogenetics. Cells were maintained with Dulbecco's modified Eagle's medium (DMEM; Biowest) containing 10% FBS (PAA Laboratories), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Biowest) in tissue culture incubator at 37°C, 5% CO_2. All cell culture experiments were conducted with cells at less than 30 passages after receipt. Cells were tested to be *Mycoplasma* free prior to experiments.

cDNA synthesis and real-time reverse transcriptase PCR

The total RNA was isolated using Trizol reagent (Invitrogen, Life Technologies) according to the manufacturer's protocol. In real-time PCRs, quantitative PCR System Mx3000P (Stratagene) was used. Primers were designed by Primer 3 (v.0.4.0; Supplementary Table S2). Relative quantity of gene expression normalized to *GAPDH* was analyzed with MxPro Software (Stratagene).

Immunohistochemistry

Paraffin-embedded tissue sections were incubated with anti-GALNT2 polyclonal antibody (1:75, Sigma) diluted with 5% nonfat milk/PBS for 16 hours at 4°C. After rinsing twice with PBS, Super Sensitive Link-Label immunohistochemistry Detection System (BioGenex) was used and the specific immunostaining was visualized with 3,3-diaminobenzidine liquid substrate system (Sigma). All sections were counterstained with hematoxylin. Negative controls were done by replacing primary antibody with control IgG. Tumor cell proliferation was assessed by Ki67 immunoreactivity. Anti-Ki67 rabbit polyclonal antibody (Vector Laboratories) was applied to the slides at 1:500 dilution. Cells with positively stained nuclei were counted in 5 random fields.

Plasmid construction

Reverse transcriptase PCR (RT-PCR) was done for cloning of full-length human *GALNT2* (Accession No. NM_004481) from nontumorous liver total RNA (BD Biosciences). The sense primer was 5′-ATGCGGCGGCGCTCGCGGAT-3′, and the antisense primer was 5′-CTGCTGCAGGTTGAGCGTGA-3′. The RT-PCR products were cloned into pcDNA3.1/myc-His (Invitrogen Life Technologies) to generate the *GALNT2*/myc-His fusion gene. The insert was confirmed by DNA sequencing.

Transfection

Overexpression of *GALNT2* gene was achieved by transfecting cells with pcDNA3.1/*GALNT2*/mycHis plasmids using Lipofectamine 2000 (Invitrogen, Life Technologies) according to the manufacturer's protocol. The transfected cells were selected with 600 μg/mL of G418 for 14 days and then pooled for further studies.

siRNA knockdown of *GALNT2* expression

SMARTpool siRNA oligonucleotides against *GALNT2* and siCONTROL nontargeting siRNA were synthesized by Dharmacon Research (Thermoscientific). For knockdown of *GALNT2*, cells were transfected with siRNA using DharmaFECT 4 (Thermoscientific) with a final concentration of 100 nmol siRNA for 48 hours.

Western blot analysis

GALNT2 proteins were detected with rabbit anti-GALNT2 polyclonal antibody (Sigma). For detection of EGFR and its downstream signaling molecules, anti-phospho-tyrosine antibody 4G10 (Upstate Biotechnology) and antibodies against total EGFR, EGFR pY 845, EGFR pY1068, p-Src, Src, p-Shc, Shc, p-AKT, AKT, p-ERK1/2, and ERK1/2 (Cell Signaling Technology, Inc.) were used. Detection of glycoproteins decorated with Tn antigens was achieved by using biotinylated *Vicia villosa* agglutinin (VVA, Vector Laboratories) with or without neuraminidase (Sigma) treatment. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected with anti-GAPDH monoclonal antibody (BD Pharmingen).

Lectin pull down and immunoprecipitation

To detect the Tn, T, and T/sialyl T on glycoproteins, VVA, peanut agglutinin (PNA), and Jacalin agarose beads (Vector

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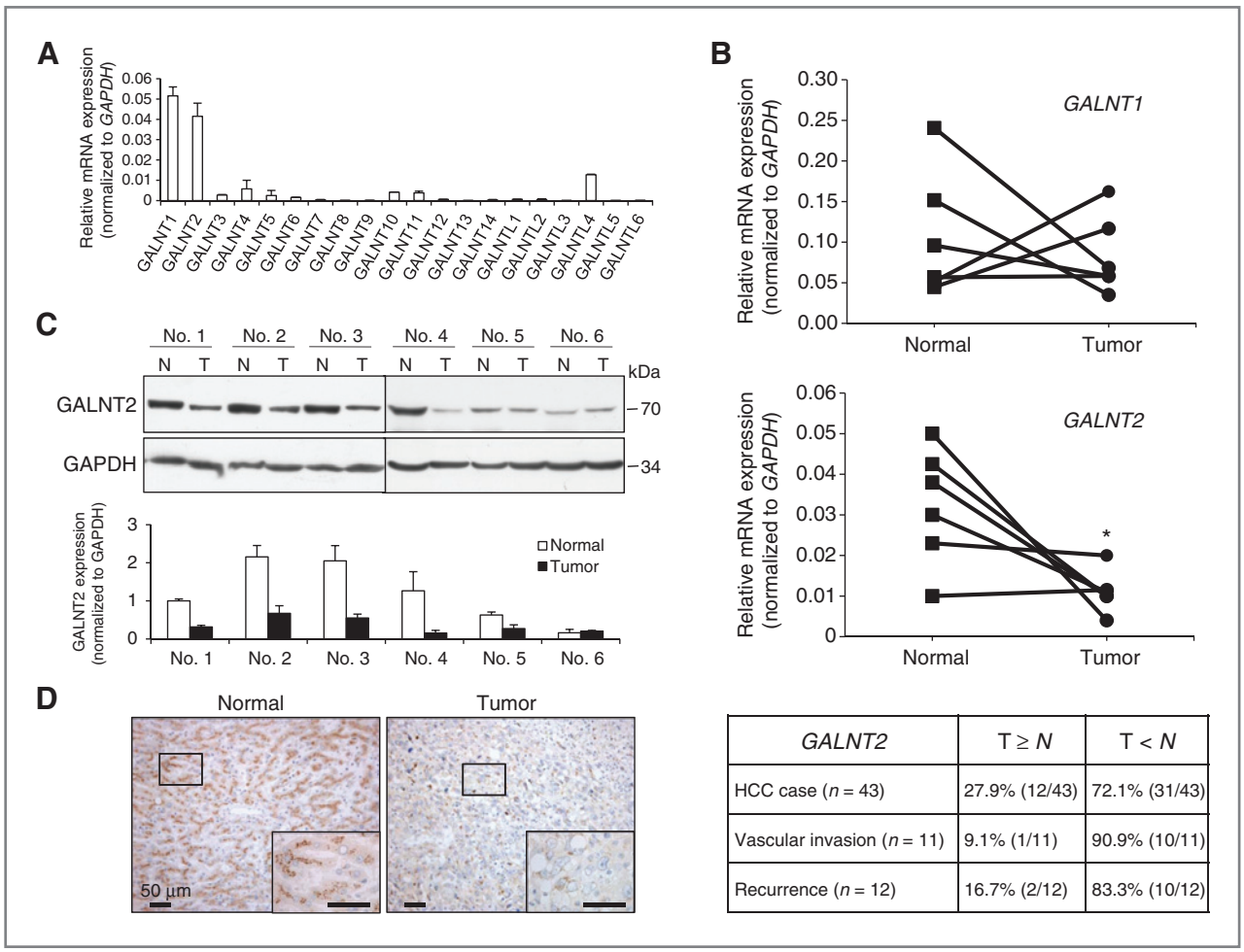
Laboratories) were used, respectively. Briefly, cell lysates (0.5 mg) were incubated with VVA, PNA, or Jacalin agarose beads for 4 hours. Peptide: N-Glycosidase F (PNGaseF, Sigma) was used to remove N-glycans. Protein G sepharose beads (Amersham Pharmacia) conjugated with 1 μg of anti-EGFR antibody were used in immunoprecipitation. The precipitated proteins were then subjected to Western blotting.

Matrigel invasion assay

Cell invasion assays were done in BioCoat Matrigel Invasion Chambers (Becton Dickinson) according to the manufacturer's protocol, as previously described (17). Briefly, 500 μL DMEM with or without chemoattractants was loaded in the lower part of the chamber and 3×10^4 of transfected cells in 500 μL serum-free DMEM were seeded onto the upper part. Chemoattractants were 10% FBS (PAA Laboratories), 100 ng/mL EGF (Sigma), or 50 ng HGF (Sigma). Cells were allowed to invade the matrigel for 24 hours. In some experiments, 70 μmol/L erlotinib (Santa Cruz Biotechnology) or dimethyl sulfoxide (DMSO) was included in the upper-chamber medium. The invading cells were fixed and stained with 0.5% (wt/vol) crystal violet (Sigma). The numbers in each well were counted and values are presented as mean ± SD.

Transwell migration assay

The transfected cells (3×10^4) were resuspended in serum-free DMEM and added to the top well of each migration chamber with an 8-μm pore size membrane (Corning). Cell migration was induced by 10% FBS (PAA Laboratories), 100 ng/mL EGF (Sigma), or 50 ng HGF (Sigma) in the bottom chambers



GALNT2	T ≥ *N*	T < *N*
HCC case (*n* = 43)	27.9% (12/43)	72.1% (31/43)
Vascular invasion (*n* = 11)	9.1% (1/11)	90.9% (10/11)
Recurrence (*n* = 12)	16.7% (2/12)	83.3% (10/12)

Figure 1. Expression of GALNT2 in human HCC. A, expression of the *GALNT* family genes in primary liver tissues. The expression of *GALNT1-14* and *GALNTL1-L6* mRNA, as indicated, in pooled nontumorous parts of HCC patients ($n = 8$) was analyzed by real-time RT-PCR. The relative level of mRNA was normalized to *GAPDH* and obtained from 3 separate experiments. Error bars, ±SD. B, *GALNT1* and *GALNT2* mRNA expression in HCC tissues. *GALNT1* (top) and *GALNT2* (middle) expression in 6 paired HCC tissues (from No. 1–6) and *GALNT2* expression in 43 paired HCC tissues ($n = 43$) were analyzed (bottom). T, tumor tissues; *N*, paired nontumorous liver tissues. Paired *t* tests were done. *, $P < 0.05$, T/*N* ratio < 0.5 is considered as T < *N*. C, expression of GALNT2 in paired HCC tissues ($n = 6$) by Western blotting. Representative images are shown. The signals were quantified from 3 separate experiments. Paired *t* tests also showed significant decrease of GALNT2 expression in HCC tumors (not shown). D, immunohistochemistry of GALNT2 in paired HCC tissues. Representative images (patient No. 3) are shown. The subcellular localization of GALNT2 was shown in the Golgi apparatus of normal hepatocytes. Amplified images are shown in the lower right. The negative control did not show any specific signals (data not shown). Scale bars, 50 μm.

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and analyzed after 24 hours. To assess the effect of erlotinib on cell migration, erlotinib (70 μmol/L) or DMSO was included in the upper-chamber medium.

Cell growth analysis

Cells (4×10^4) were seeded in 6-well plates with serum-free DMEM or DMEM containing 10% FBS (PAA Laboratories), 100 ng/mL EGF (Sigma), or 50 ng HGF (Sigma). Viable cells were determined at 24-hour intervals for 72 hours using hemocytometer with trypan blue exclusion. Erlotinib (70 μmol/L) and DMSO control were used to assess the effect of EGFR inhibitor.

Bromodeoxyuridine incorporation and immunofluorescence microscopy

Cells were plated in chamber slides and subjected to serum starvation for 16 hours, and treated with 10% FBS (PAA Laboratories), 100 ng/mL EGF (Sigma), or 50 ng HGF (Sigma) for 2 hours and then bromodeoxyuridine (BrdU; 10 μmol/L) for 0.5 hours. The cells were fixed and incubated with anti-BrdU antibody (Sigma). BrdU staining was completed with Cy3-conjugated goat anti-mouse IgG (Jackson Immunoresearch), and counterstained by 4′,6-diamidino-2-phenylindole (DAPI). For GALNT2 staining, cells cultured in complete DMEM were stained with anti-GALNT2 antibody (Sigma) and Cy3-conjugated goat anti-rabbit IgG (Jackson Immunoresearch).

Internalization of EGFR

Cells were starved for 4 hours and then treated with EGF (100 ng/mL; Sigma) for 10 minutes at 37°C. The cells were washed with ice cold PBS, fixed, and then immunostained by anti-EGFR (Cell Signaling) and anti-EEA1 (early endosome antigen 1) antibody (Santa Cruz) and counterstained by DAPI (Sigma).

Tumor growth in nude mice

For tumor growth analysis, 6-week-old female BALB/c nude mice (National Laboratory Animal Center, Taiwan) were injected subcutaneously with 5×10^6 of Mock cells ($n = 6$) or *GALNT2* transfectants ($n = 6$). At day 28 after injection, tumors in each group were excised for analyses. Animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of National Taiwan University College of Medicine.

Statistical analysis

Student t test was used for statistical analyses. Data are presented as means ± SDs. We conducted paired t tests for the analysis of paired HCC tissues. Where appropriate, a 2-way ANOVA followed by a Bonferroni *post hoc* test for significance was applied, as indicated in figure legends. $P < 0.05$ was considered statistically significant.

Results

Expression of GALNT2 is frequently downregulated in human HCC

To investigate the potential role of *GALNT* gene family in HCC, we first analyzed *GALNT1-14* and *GALNTL1-L6*

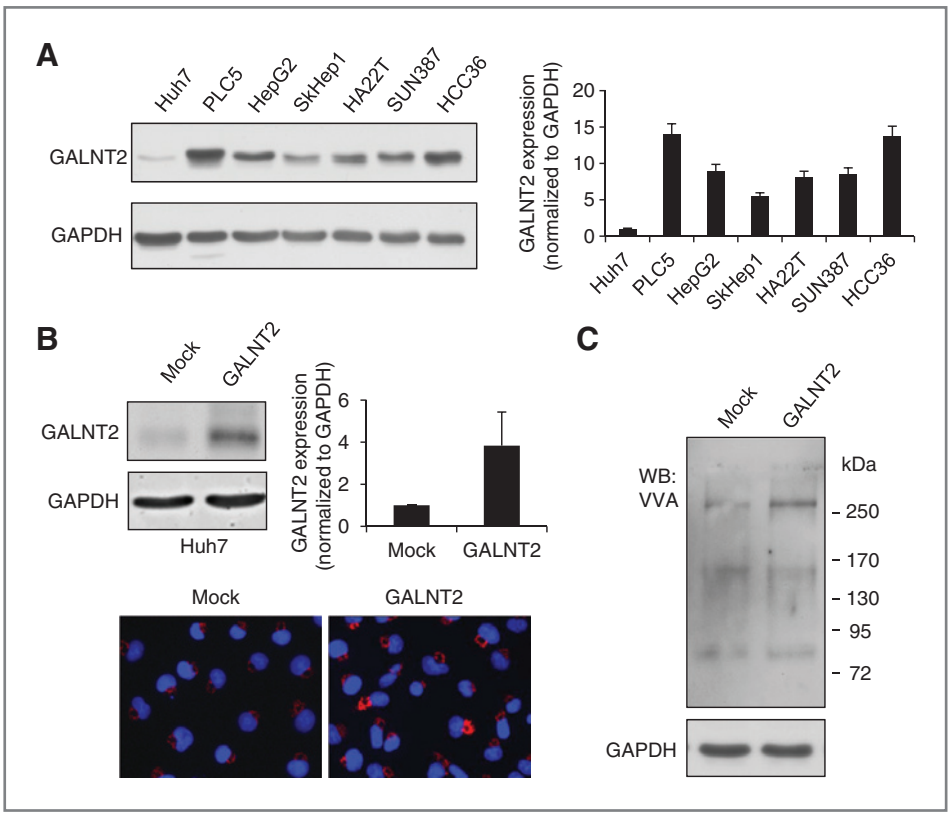

Figure 2. Stable transfection of Huh7 cells with GALNT2. A, expression of GALNT2 in HCC cell lines. The GALNT2 protein expression was analyzed by Western blotting, and the relative intensity of signals is presented as the mean ± SD. $n = 3$. B, reexpression of GALNT2 in Huh7 cells. GALNT2 overexpression was confirmed by Western blotting. Immunofluorescence microscopy showed overexpression of GALNT2 (red) in > 90% of GALNT2 stable transfectants. Nuclei were counterstained with DAPI (blue). The changes in carbohydrates on cellular proteins were detected by *Vicia villosa* agglutinin (VVA), specific for GalNAc-O-Ser/Thr.

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expression in primary liver tissues by real-time RT-PCR. Among them, only *GALNT1* and *GALNT2* were found to be highly expressed in the nontumorous liver tissues ($n = 8$) (Fig. 1A). We therefore further analyzed *GALNT1* and *GALNT2* expression in paired HCC tissues ($n = 6$). Paired t tests showed that *GALNT2*, but not *GALNT1*, exhibited significant downregulation in HCC tissues compared with their noncancerous parts (Fig. 1B, upper and middle panel). Moreover, real-time RT-PCR revealed downregulation of *GALNT2* expression in 72.1% (31/43) of HCC tissues (Fig. 1B, lower panel). Interestingly, *GALNT2* downregulation was found in 90.9% (10/11) and 83.3% (10/12) of HCC patients with vascular invasion and recurrence, respectively. Consistent findings of lower expression levels of GALNT2 protein in HCC tissues were observed by Western blotting and immunohistochemistry (Fig. 1C and D). These results suggest that GALNT2 expression is frequently downregulated and associated with invasive properties of HCC.

Stable transfection of Huh7 cells with GALNT2

To investigate the role of GALNT2 in HCC, we first analyzed GALNT2 expression in 6 HCC cell lines by Western blotting. We found that Huh7 expressed lower levels of GALNT2, whereas PLC5 expressed higher levels of GALNT2 in these cell lines (Fig. 2A). We therefore chose Huh7 cells and PLC5 cells to overexpress and knockdown the GALNT2 expression, respectively. For establishing stable transfectants, G418-resistant clones of transfected Huh7 cells were pooled. Mock and GALNT2 stable transfectants were obtained from the pooled colonies of Huh7 cells transfected with pcDNA3.1 and GALNT2/pcDNA3.1 plasmids, respectively. The overexpression of GALNT2 was confirmed by Western blot analysis and immunofluorescence microscopy (Fig. 2B). An increased binding of VVA lectin to glycoproteins in GALNT2 overexpressing lysates was observed, reflecting enhanced O-glycosylation (Fig. 2C).

GALNT2 suppresses malignant phenotypes in Huh7 cells

To investigate effects of GALNT2 on malignant phenotypes in Huh7 cells, cell growth, migration, and invasion were analyzed. We found that GALNT2 suppressed FBS- and EGF-induced cell growth (Fig. 3A). Our data further showed that GALNT2 was able to inhibit FBS- and EGF-induced cell proliferation (Fig. 3B), but has no significant effect on apoptosis and cell cycle (data not shown). Furthermore, reexpression of GALNT2 significantly inhibited FBS- and EGF-induced migration and invasion revealed by transwell migration and matrigel invasion assays, respectively (Fig. 3C and D). In contrast, we did not observe significant changes in cell growth, proliferation, migration, and invasion when cells treated with HGF or under serum-free conditions. To further confirm the effects of GALNT2 overexpression on Huh7 cells, GALNT2 overexpression was knocked down by siRNA. Our data showed that the suppressive effects of GALNT2 overexpression were significantly blocked by GALNT2 siRNA but not control siRNA (Supplementary Fig. S1). To know whether EGF plays a role in FBS-induced malignant phenotypes, EGFR inhibitor erlotinib was used to treat mock and GALNT2 stable transfectants. Our data showed that cell growth, migration, and invasion were

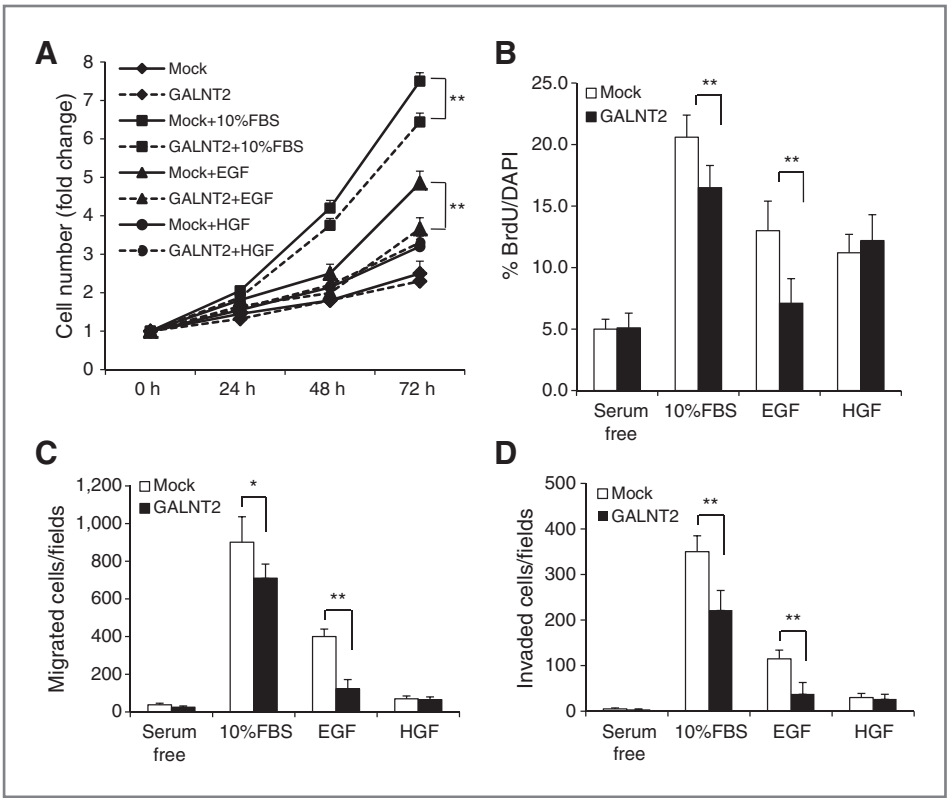

Figure 3. Effects of GALNT2 on malignant phenotypes in Huh7 cells. A, effects of GALNT2 on cell growth analyzed by trypan blue exclusion assays. Cells were grown in serum-free DMEM or DMEM containing 10% FBS, 100 ng/mL EGF, or 50 ng/mL HGF, and the number of live cells at different time points were counted. The results were graphed after standardization by Mock (0 h) to 1.0. Results were analyzed by 2-way ANOVA and are represented as the mean ± SD from 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$. B, effects of GALNT2 on cell proliferation. Proliferative cells were analyzed by BrdU incorporation assays. **, $P < 0.01$; $n = 3$; error bars, mean ± SD. C, effects of GALNT2 on cell migration by transwell migration assays. Data are represented as mean ± SD from 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$. D, effects of GALNT2 on invasion by matrigel invasion assays. Data are represented as mean ± SD from 3 independent experiments. **, $P < 0.01$.

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significantly suppressed by erlotinib (Supplementary Fig. S2). These results suggest that GALNT2 can suppress the malignant behavior of Huh7 cells and the EGF-mediated pathway may be involved in this process.

GALNT2 inhibits tumor growth in nude mice

To investigate the effect of GALNT2 on tumor growth *in vivo*, Mock and GALNT2 transfected Huh7 cells were subcutaneously xenografted in nude mice. We observed that GALNT2 significantly suppressed tumor volume (Fig. 4A) and tumor weight (Fig. 4B) after 28 days. Immunohistochemical analysis showed a significant decrease in the percentage of Ki67-positive cells in GALNT2 tumors compared with Mock tumors (Fig. 4C and D). These results suggest that GALNT2 inhibits HCC tumor cell growth and proliferation *in vivo*.

GALNT2 modifies glycosylation and activity of EGFR in Huh7 cells

Because we found that GALNT2 can suppress EGF-induced malignant phenotypes, we analyzed whether EGFR glycosylation and activity were modulated by GALNT2 expression. We observed that endogenous EGFRs in HCC cells could not be pulled down by VVA (data not shown). Interestingly, EGFR could be easily pulled down by VVA after neuraminidase treatment (Fig. 5A, upper panel). In addition, more EGFR molecules were pulled down after the removal of N-glycans by PNGaseF than those without treatment. We also showed that EGFRs could be precipitated by PNA after neuraminidase treatment and pulled down by jacalin without neuraminidase treatment. To further confirm the presence of O-glycans, EGFR was immunoprecipitated, followed by neuraminidase treatment, and then immunoblotted with VVA. The results obtained consistently show the expression of sialyl Tn structure on EGFRs (Fig. 5A, lower panel), suggesting that short O-glycans, preferentially sialyl Tn, were decorated on EGFRs. Notably, forced expression of GALNT2 enhanced the expression of sialyl Tn on EGFR (Fig. 5B). To know the effects of glycosylation on EGFR molecules, we analyzed the cell surface expression of EGFR, EGF-induced endocytosis, and EGF-induced dimerization of EGFR. Our data showed that neither the surface expression (Fig. 5C) nor EGF-induced dimerization (data not shown) of EGFR was affected by GALNT2. Interestingly, fluorescence microscopy showed that GALNT2 inhibited colocalization of EGFR with EEA1 when cells were treated with EGF (Fig. 5C). In addition, endocytosis triggered by Alexa488-EGF was suppressed by GALNT2 (Supplementary Fig. S3A). Overexpression of GALNT2 significantly inhibited the elimination of biotinylated EGFR from the cell surface (Supplementary Fig. S3B). These results suggest that GALNT2 overexpression inhibits EGF-triggered endocytosis of EGFR.

We next examined the effect of GALNT2 on tyrosine phosphorylation of cellular proteins. We found that GALNT2 mainly suppressed tyrosine phosphorylation of proteins located at 175 and 140 kDa (Fig. 5D). We then investigated whether phosphorylation and activity of EGFR (175 kDa) were modulated by GALNT2. Our data showed that GALNT2 expression inhibited the EGF-induced phosphorylation of EGFR at Y845 and Y1068, as well as the total tyrosine phosphorylation levels revealed by 4G10 mAb (Fig. 5E). Furthermore, phosphorylation levels of EGFR downstream signaling molecules, including p-Src, p-Shc,

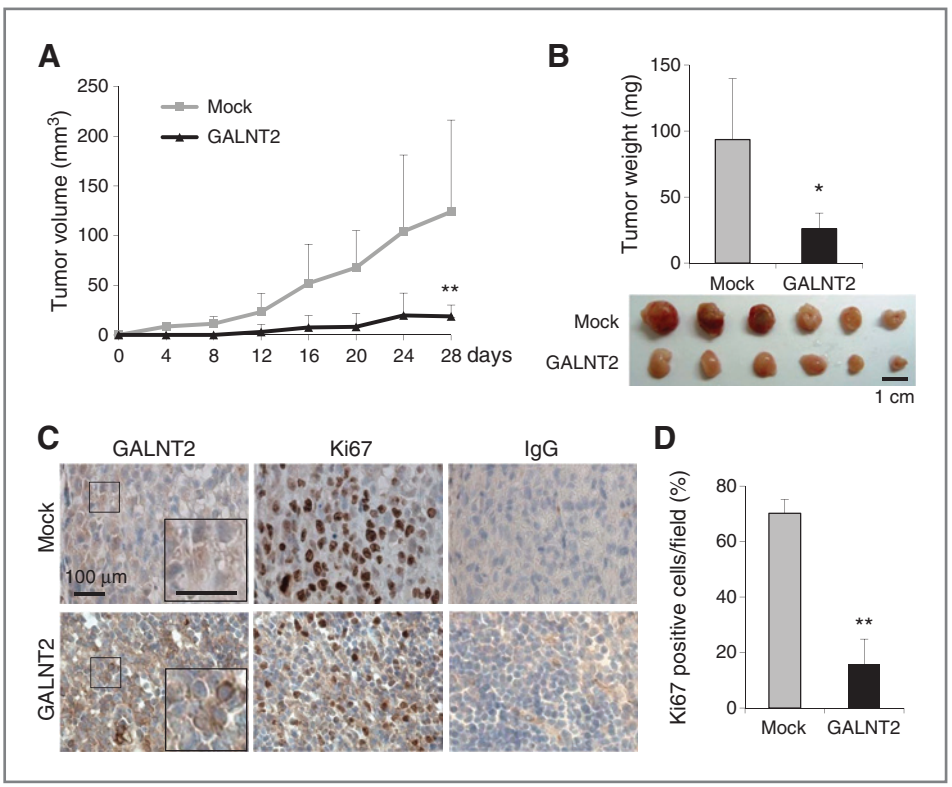

Figure 4. GALNT2 inhibits tumor growth in nude mice. A, the effect of GALNT2 on tumor volumes observed for 28 days. Data are shown as mean ± SD. **, $P < 0.01$, $n = 6$ for each group. B, GALNT2 inhibited tumor weights. After implantation for 28 days, tumors were excised and their weights were shown as mean ± SD. *, $P < 0.05$, $n = 6$. C, immunohistochemistry of xenografts. Paraffin-embedded sections were immunostained with GALNT2, Ki67, or control IgG. Scale bars, 100 μm. D, GALNT2 inhibited tumor cell proliferation *in vivo*. The number of Ki67-positive cells was counted from randomly selected microscopic fields. Data are shown as mean ± SD from 6 tumors and 5 fields for each tumor were counted. **, $P < 0.01$.

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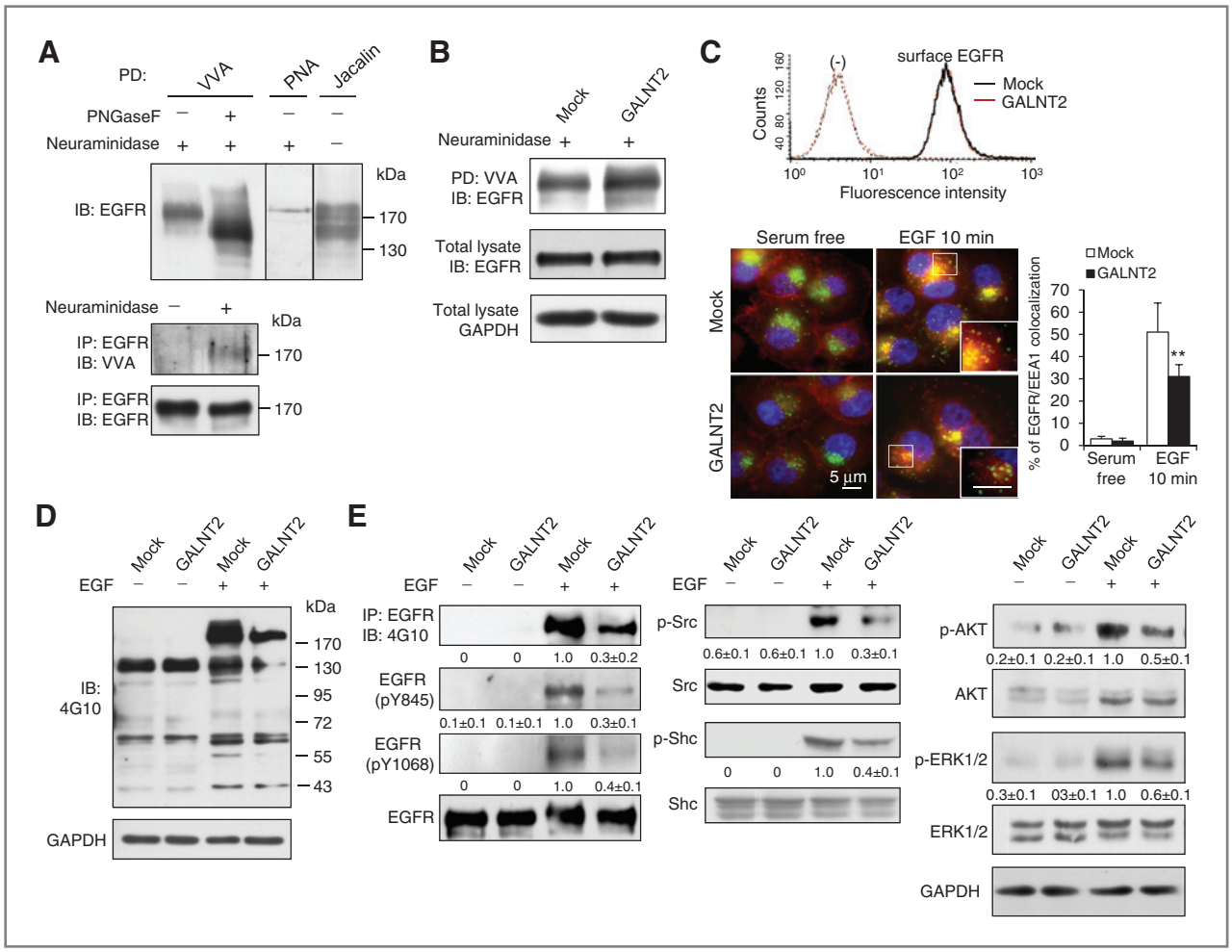

Figure 5. GALNT2 modifies glycosylation and activity of EGFR in Huh7 cells. A, EGFR was decorated with short O-glycans. In the upper panel, cell lysates of Huh7 cells was treated with/without neuraminidase, and then pulled down (PD) by lectins. PNGaseF was used to remove N-glycans in cell lysates before lectin pulldown. The pulled down molecules were immunoblotted (IB) with anti-EGFR antibody. In the lower panel, cell lysates were immunoprecipitated with anti-EGFR antibody, and then immunoblotted with VVA or anti-EGFR antibody. B, GALNT2 increased VVA binding to the neuraminidase-treated EGFR. The cell lysates treated with neuraminidase were pulled down with VVA and then immunoblotted with anti-EGFR antibody. C, GALNT2 suppressed EGF-induced endocytosis. Top panel, GALNT2 did not change the surface expression of EGFR by flow cytometry. Bottom panel, representative images of EGF-induced endocytosis. Endocytosis was triggered by 100 ng/mL EGF for 10 minutes. EGFR (red) and early endosome antigen 1 (EEA1; green) were immunostained with anti-EGFR and anti-EEA1 antibody, respectively. Nuclei were stained with DAPI (blue). Scale bars indicate 5 μm. A significant decrease in the percentage of colocalization between EGFR and EEA1 was observed in GALNT2 stable transfectants compared with mock transfectants. Data are represented as mean ± SD from 3 independent experiments. Five fields for each image were quantified. D, GALNT2 inhibited EGF-induced tyrosine phosphorylation. Phosphorylated tyrosines in cell lysates were immunoblotted with 4G10 antibody. E, GALNT2 suppressed EGF-induced phosphorylation of EGFR and its downstream signaling molecules. Mock or GALNT2 stable transfectants were starved for 4 hours and then treated with (+) or without (−) EGF (100 ng/mL) for 10 minutes. Cell lysates were immunoprecipitated with anti-EGFR polyclonal antibody and then immunoblotted with antibodies mentioned. Alternatively, cell lysates were directly immunoblotted with various antibodies, as indicated. Signals of Western blotting were quantified by ImageQuant5.1 (Amersham Biosciences) and presented as means ± SD from 3 independent experiments.

pAkt, and p-ERK1/2, also diminished (Fig. 5E). These results suggest that GALNT2 expression downregulates EGF-induced phosphorylation of EGFR and, which in turn, suppresses the downstream signaling pathways of EGFR.

Effects of GALNT2 knockdown on HCC cells

To verify the effect of GALNT2 on HCC cells, endogenous GALNT2 expression was knocked down with siRNA. Our data showed that GALNT2 pooled siRNA efficiently inhibited GALNT2 protein expression in PLC5 cells and suppressed the expression of short O-glycans recognized by VVA lectins compared with the control siRNA (Fig. 6A). In addition, the knockdown of GALNT2 diminished binding of VVA to neuraminidase-treated EGFR, indicating that less sialyl Tn were present on the EGFR in the GALNT2-knockdowned cells (Fig. 6B). In these cells, the EGF-induced phosphorylation of EGFR at Y1068 and Y845 was increased (Fig. 6B), suggesting that EGFRs decorated with less sialyl Tn exhibit higher activity. Moreover, our results showed that knockdown of GALNT2 enhanced EGF-induced cell growth (Fig. 6C), migration (Fig.

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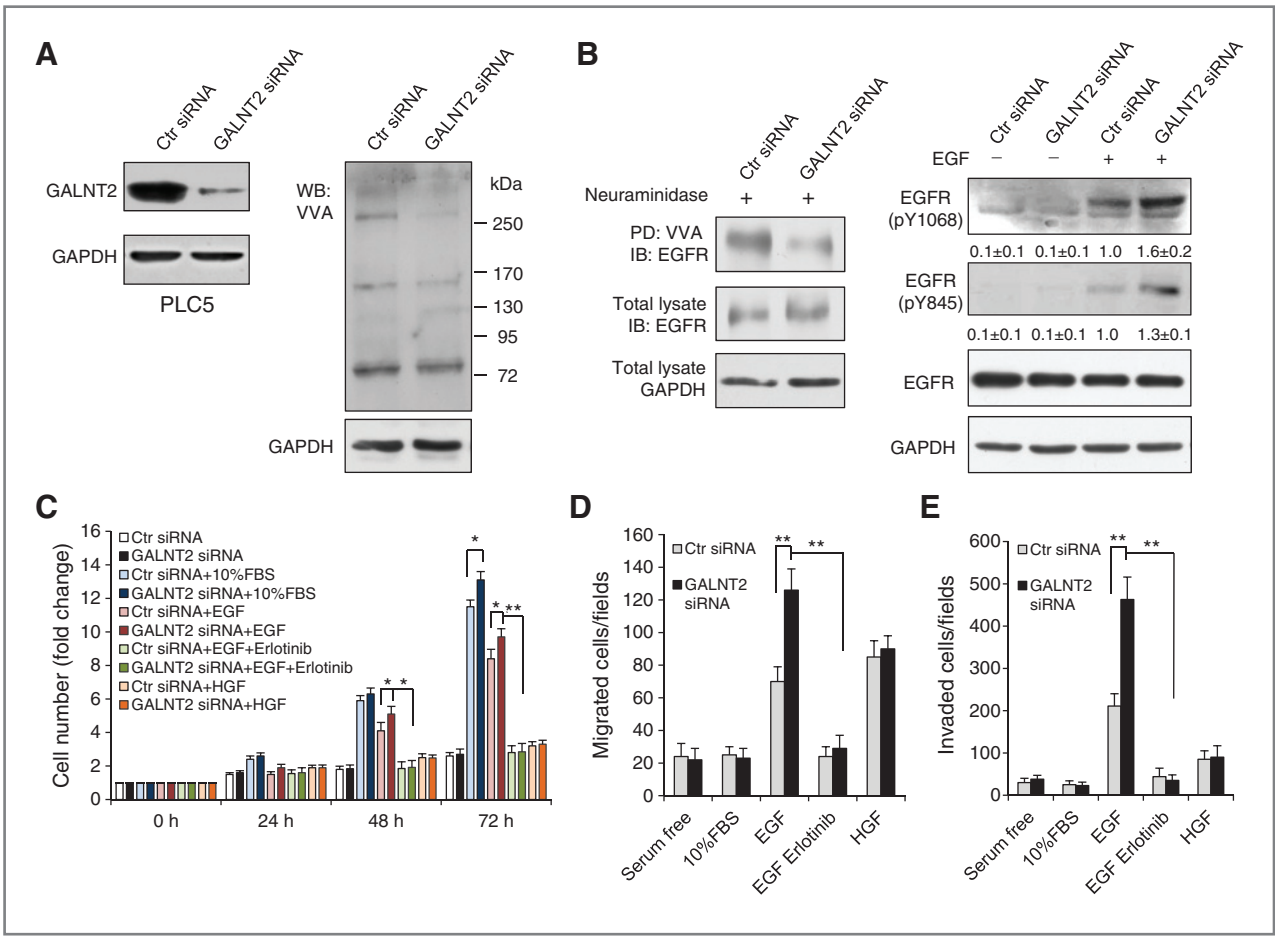

Figure 6. Effects of GALNT2 knockdown on PLC5 cells. A, knockdown of GALNT2 with siRNA in PLC5 cells. GALNT2 expression was knocked down by *GALNT2* siRNA compared with the control (Ctr) siRNA by Western blotting. Binding of VVA to cellular proteins was decreased in GALNT2-knockdowned cells. B, knockdown of GALNT2 inhibited O-glycosylation but increased EGF-induced phosphorylation of EGFR. The neuraminidase-treated cell lysate of PLC5 cells knockdowned with the control (Ctr) siRNA or *GALNT2* siRNA was pulled down (PD) by VVA and then immunoblotted (IB) with anti-EGFR polyclonal antibody. The cells transfected with the control (Ctr) siRNA or *GALNT2* siRNA were starved for 4 hours and then treated with (+) or without (−) EGF (100 ng/mL) for 10 minutes. The phosphorylation of EGFR was analyzed. C–E, effects of GALNT2 knockdown and EGFR inhibitor erlotinib on EGF-induced malignant phenotypes. Effects of GALNT2 knockdown on malignant properties including cell growth (C), cell migration (D), and cell invasion (E) were analyzed. Cells were treated with 10% FBS, 100 ng/mL EGF, or 50 ng/mL HGF. Some were treated with DMSO control (−) or 70 μmol/L of erlotinib, as indicated. Data are shown as mean ± SD from 3 independent experiments. A 2-way ANOVA followed by a Bonferroni *post hoc* test for significance was applied, *, $P < 0.05$; **, $P < 0.01$.

6D), and invasion (Fig. 6E) in PLC5 cells, whereas no significant changes were observed for cells treated with HGF or under serum-free conditions. Surprisingly, PLC5 cells invaded in higher numbers than migrated. In addition, we observed that FBS-induced cell growth, but not migration and invasion, was enhanced by GALNT2 siRNA. Notably, the increase in the EGF-triggered malignant properties induced by GALNT2 knockdown was significantly suppressed by erlotinib, suggesting that EGFR plays a critical role in the phenotypic changes mediated by GALNT2.

To further confirm the effect of GALNT2 on HCC cells, we knocked down GALNT2 expression in another HCC cell line HA22T. Our data showed that knockdown of GALNT2 with siRNA increased EGFR activation but decreased VVA binding to EGFR (Supplementary Fig. S4A). In addition, EGF-induced cell growth, migration, and invasion were significantly increased by GALNT2 siRNA (Supplementary Fig. S4B–D). These results further show a role of GALNT2 in regulating the malignant behavior of HCC cells.

Discussion

We showed that GALNT1 and GALNT2 are the major GALNT enzymes in human liver. Downregulation of GALNT2 was frequently found in primary HCC tissues and associated with vascular invasion and recurrence. Interestingly, GALNT2 modulates the structure of short O-glycans on EGFR. Moreover, GALNT2 could regulate the malignant phenotype and phosphorylation levels of EGFR and its downstream signaling molecules. Here, we show for the first time that GALNT2 can modulate the malignant behavior of HCC cells, and that O-glycosyltransferase is a novel regulator of EGFR function.

Expression of short O-glycans, such as Tn, sialyl Tn, T, and sialyl T, are found in many types of cancer and exploited to

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develop cancer vaccines (18). Changes in these structures often alter the function of the cell and its antigenic property, as well as its potential to invade and metastasize (18). The T-antigen expression is associated with lower survival probability and is an independent prognostic factor in colorectal cancer (19). Sialyl Tn expression is associated with poor clinical outcome in endometrial and colorectal cancer patients (20, 21). In contrast, the presence of sialyl Tn in keratoacanthoma is associated with tumor regression (22). Several short O-glycans have also been detected in human HCC, but not in the normal liver, by monoclonal antibodies or lectins (23, 24). However, the role of short O-glycans in HCC progression and prognosis remains unknown. This study reveals that GALNT1 and GALNT2 are the major GalNAc transferases in liver tissues, and that GALNT2 can modulate the sialyl Tn expression in HCC cells and suppress their malignant properties. Thus, it is of great interest to further investigate the significance of the short O-glycans and the GALNT family in HCC malignancy.

Because there is no consensus sequence for GalNAc addition by GALNTs and O-glycans have never been reported on EGFR, it has long been thought that EGFR carries only N-glycans. This study also showed that neuraminidase-treated EGFR can be pulled down by VVA and PNA. Binding of VVA to the neuraminidase-treated EGFR was enhanced after removal of N-glycans, and GALNT2 enhanced the VVA binding to neuraminidase-treated EGFR. Although the exact sites of O-glycosylation on EGFR require further investigation, our data strongly suggest that EGFR carries short O-glycans. Interestingly, we found that EGF-induced endocytosis of EGFR was suppressed by GALNT2 overexpression, suggesting that changes in O-glycosylation on EGFR could modulate EGFR internalization and thereby regulate its downstream signaling. Indeed, it has been reported that clathrin-mediated internalization is essential for sustained EGFR signaling (25). To our knowledge, this study is for the first time to show that EGFR may express short O-glycans.

We found that GALNT2 can modulate EGF-induced phenotypes in all tested HCC cell lines. In contrast, there is no significant effect for GALNT2 on HGF-triggered phenotypes. These findings suggest that the effect of GALNT2 exhibits selectivity to EGF-, but not HGF-induced phenotypes. We also observed that GALNT2 can significantly modulate 10% FBS-triggered cell growth in HCC cells. Because many substrates for GALNT2 are present in HCC cells, it remains possible that GALNT2 mediates its effects through other receptors in addition to EGFR. EGFR inhibitors prevent the development of HCC in animal models (26) and erlotinib has shown some activity in the treatment of human HCC (16, 27). Sorafenib, a multikinase inhibitor, has been shown to provide a significant survival benefit for patients with advanced HCC (28). However, the efficacy of these agents still remains to be improved. So far, no single target is identified to play the major role in HCC progression, suggesting that multiple pathways should be targeted for HCC treatment. There are currently many clinical trials evaluating TKIs for HCC, including those tested in combination with erlotinib as a first-line therapy (29). A better understanding of the molecular mechanism that regulates the activity and signaling of RTKs is important for developing novel-targeted treatments. This study has identified a novel mechanism by which the activity and downstream signaling of RTKs can be modified by O-glycosyltransferase, which may offer novel insights into the development of new therapeutic agents for HCC.

In conclusion, the results obtained in this study suggest that GALNT2 could modify EGFR glycosylation and activity, and thereby regulate the malignant behavior of HCC cells. This study not only shows a pathophysiologic role of GALNT2 in HCC cells but also contributes to shed light on the significance of abnormal O-glycosylation in HCC tumor progression. Understanding effects and mechanisms of O-glycosylation on the activity of EGFR or other receptor tyrosine kinases by *GALNT* family genes may offer a novel strategy for the development of HCC therapeutic agents. These include anti-microRNAs, siRNAs, carbohydrate mimetics, or small molecule compounds that can modulate GALNT gene expression or enzyme activity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Mucin Glycosylating Enzyme GALNT2 Regulates the Malignant Character of Hepatocellular Carcinoma by Modifying the EGF Receptor

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