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Roles of *N*-Acetylglucosaminyltransferase III in Epithelial-to-Mesenchymal Transition Induced by Transforming Growth Factor β1 (TGF-β1) in Epithelial Cell Lines*

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Background: The inhibitory effects of GnT-III on cancer metastasis remain unclear.
Results: GnT-III influenced EMT-like changes through not only prolongation of E-cadherin turnover but also suppression of β-catenin·p-Smad complex formation.
Conclusion: GnT-III plays important roles in TGF-β-induced EMT-like changes.
Significance: The expression of E-cadherin is regulated not only by transcriptional factors but also by post-transcriptional modifications.

The epithelial-to-mesenchymal transition (EMT) plays crucial roles in embryonic development, wound healing, tissue repair, and cancer progression. Results of this study show how transforming growth factor β1 (TGF-β1) down-regulates expression of *N*-acetylglucosaminyltransferase III (GnT-III) during EMT-like changes. Treatment with TGF-β1 resulted in a decrease in E-cadherin expression and *GnT-III* expression, as well as its product, the bisected *N*-glycans, which was confirmed by erythro-agglutinating phytohemagglutinin lectin blot and HPLC analysis in human MCF-10A and mouse GE11 cells. In contrast with GnT-III, the expression of *N*-acetylglucosaminyltransferase V was slightly enhanced by TGF-β1 treatment. Changes in the *N*-glycan patterns on α3β1 integrin, one of the target proteins for GnT-III, were also confirmed by lectin blot analysis. To understand the roles of GnT-III expression in EMT-like changes, the MCF-10A cell was stably transfected with *GnT-III*. It is of particular interest that overexpression of *GnT-III* influenced EMT-like changes induced by TGF-β1, which was confirmed by cell morphological changes of phase contrast, immunochemical staining patterns of E-cadherin, and actin. In addition, GnT-III modified E-cadherin, which served to prolong E-cadherin turnover on the cell surface examined by biotinylation and pulse-chase experiments. GnT-III expression consistently inhibited β-catenin translocation from cell-cell contact into the cytoplasm and nucleus. Furthermore, the transwell assay showed that GnT-III expression suppressed TGF-β1-induced cell motility. Taken together, these observations are the first to clearly demonstrate that GnT-III affects cell properties, which in turn influence EMT-like changes, and to explain a molecular mechanism for the inhibitory effects of GnT-III on cancer metastasis.

The epithelial-to-mesenchymal transition (EMT)[3] is a process whereby epithelial cells form an organized, tightly connected sheet and trans-differentiate into disorganized motile mesenchymal cells. EMT is therefore envisioned as a differentiation or morphogenetic process in which new tissue types are generated during embryogenesis, which contributes to the pathogenesis of diseases such as metastatic cancer and tissue fibrosis (1–3). Although the significance of EMT during cancer progression and even its relevance in human cancer tissues remains a matter for debate, EMT occurs at the invasive front and produces single migratory cells that lose E-cadherin expression in colorectal carcinoma and other cancers (4, 5). Transforming growth factor β (TGF-β) is a critical multifunctional cytokine and founding member of the TGF-β superfamily. TGF-β functions by binding to its receptor and activating signaling cascades that result in the modulation of gene transcription involved in EMT, including Smad-interacting protein 1 (*SIP1*), *Snail*, and *Slug*, which are zinc finger-containing transcription factors that repress E-cadherin expression and induce EMT (6). The loss of E-cadherin expression is considered a crucial step in the progression of a tumor to invasive carcinoma, and it is also a fundamental event in EMT (7).

Changes in glycan structure are associated with many physiological and pathological events, including cell growth, migration, differentiation, and tumor invasion (8, 9). *N*-Acetylgluco-

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[3] The abbreviations used are: EMT, epithelial-to-mesenchymal transition; E4-PHA, erythro-agglutinating phytohemagglutinin; FN, fibronectin; GnT-III, *N*-acetylglucosaminyltransferase III; GnT-V, *N*-acetylglucosaminyltransferase V; L4-PHA, leuko-agglutinating phytohemagglutinin; PA-*N*-glycan, pyridylaminated *N*-glycan.

saminyltransferase III (GnT-III) catalyzes the attachment of a GlcNAc to a core mannose of *N*-glycan via a β1,4-linkage to form the bisecting GlcNAc structure, and it has been found to play an important role in the suppression of cancer metastasis. Introduction of the bisecting GlcNAc suppresses further processing and elongation of *N*-glycans catalyzed by other branching enzymes such as *N*-acetylglucosaminyltransferase IV (GnT-IV) or *N*-acetylglucosaminyltransferase V (GnT-V), which are strongly associated with cancer metastasis, because neither of them can utilize the bisected oligosaccharide as a substrate (10–12). GnT-V activity and β1,6-branched *N*-glycans levels are reportedly also increased in highly metastatic tumor cell lines (13, 14). Cancer metastasis is consistently and effectively suppressed in *GnT-V* knock-out mice (15). GnT-III has therefore been proposed as an antagonist of GnT-V, thereby contributing to the suppression of cancer metastasis (16, 17). GnT-III is generally regarded as a key glycosyltransferase in *N*-glycan biosynthetic pathways. In fact, lung metastasis of highly metastatic mouse melanoma B16 cells, which express relatively higher levels of GnT-V, is significantly suppressed by overexpression of *GnT-III* (18). It is intriguing that enhancement of cell-cell adhesion was reported in these *GnT-III* transfectants (19). These results strongly suggest that remodeling of glycosyltransferase-modified *N*-glycan structures could modulate cell adhesion and thereby cancer metastasis as well. It is therefore very important to clarify the molecular mechanisms for the regulation of glycosyltransferase expression under physiological and pathological conditions.

However, GnT-III expression was significantly up-regulated by cell-cell adhesion in an E-cadherin-dependent manner (20). GnT-III activity was greatly increased when cells were cultured under dense conditions, compared with sparse culture conditions. Up-regulation of *GnT-III* was observed only in epithelial cells that expressed E-cadherin and not in MDA-MB231 cells, which is an E-cadherin-deficient cell line. The expression levels of *GnT-III* were up-regulated by cell-cell interaction via the E-cadherin·catenin·actin complex, because disruption of actin polymerization or lack of *α-catenin* expression interfered with the regulation of GnT-III. The reintroduction of *α-catenin* into *α-catenin*-deficient cells rescued GnT-III expression that had been enhanced under cell-cell adhesion (21), clearly indicating that the E-cadherin·catenin complex is essential for cell-cell adhesion-regulated *GnT-III* expression. Unexpectedly, a recent study has shown that *GnT-III* expression is strongly up-regulated by knockdown of *β-catenin* or inhibition of Wnt/β-catenin signaling (22). Considering that β-catenin is an essential molecule in both cadherin-mediated cell adhesion and canonical Wnt signaling, it is logical to propose that *GnT-III* expression may be closely regulated by at least two pathways, *i.e.* the positive effect of E-cadherin-catenin-mediated cell adhesion signaling and the negative effect of Wnt/β-catenin signaling.

In this study, we used an EMT model to investigate whether EMT affects GnT-III and *N*-glycan expression, which in turn affects EMT. We found that TGF-β1-induced EMT dramatically down-regulated GnT-III expression, and overexpression of *GnT-III* inversely influenced EMT through prolonged E-cadherin turnover on the cell surface. These results clearly suggest that *N*-glycan expression is precisely regulated by physiological and pathological conditions and plays important roles in intracellular signaling and cell behavior.

EXPERIMENTAL PROCEDURES

Cell Line and Cell Culture—Epithelial GE11 cells, derived from β1 integrin knock-out embryonic stem cells, kindly provided by Dr. Arnoud Sonnenberg (Division of Cell Biology, Netherlands Cancer Institute, Netherlands) (23), were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G, and 0.1 mg/ml of streptomycin, under a humidified atmosphere containing 5% CO_2. MCF-10A, a human nontumorigenic immortalized breast epithelial cell line, was cultured in DMEM/F-12 medium, supplemented with 5% horse serum, 20 ng/ml EGF, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, and 100 ng/ml cholera toxin, under a humidified atmosphere containing 5% CO_2 at 37 °C. Cells at 5×10^5 were plated on 10-cm dishes, followed by incubation with TGF-β1 (PeproTech) at 5 ng/ml for 4 days.

Western Blot, Immunoprecipitation, and Lectin Blot Analyses—Cells cultured under different conditions, as indicated, were washed with PBS and then lysed with lysis buffer (10 mM Tris-HCl, 1% Triton X-100, 150 mM NaCl, aprotinin, leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Insoluble materials were removed by centrifugation at 15,000 rpm for 10 min at 4 °C. Equal amounts of protein were separated using 7.5% SDS-PAGE, transferred to nitrocellulose, and probed with the appropriate antibodies, as indicated, or with biotinylated erythro-agglutinating phytohemagglutinin (E4-PHA), biotinylated leuko-agglutinating phytohemagglutinin (L4-PHA), biotinylated *Datura stramonium*, *Aleuria aurantia,* and concanavalin A lectins (Seikagaku Kogyo Inc., Japan). Immunoreactive bands were visualized using a Vectastain ABC kit (Vector Laboratories) and an ECL kit (Amersham Biosciences). Monoclonal antibodies against E-cadherin, N-cadherin, fibronectin, α-catenin, and β-catenin were purchased from BD Biosciences, and the anti-α-tubulin antibody was from Sigma. Antibody against GnT-III (33A8) was obtained from Fujirebio Inc. (Tokyo, Japan). Monoclonal antibodies against Smad2-, p-Smad2-, and HRP-labeled anti-mouse IgG were obtained from Cell Signaling (Danvers, MA). Anti-pY654-β-catenin antibody was purchased from Invitrogen. For immunoprecipitation, the supernatant (2 mg of protein) was incubated for 1 h at 4 °C with anti-E-cadherin monoclonal antibody (3 μg/ml) (BD Biosciences), and anti-β1 integrin (P5D2) was obtained from the Developmental Studies Hybridoma Bank, University of Iowa. Protein G beads (30 μl in 50% slurry) were then added, followed by incubation overnight at 4 °C with a rotator. After washing three times with lysis buffer, the immunoprecipitates were subjected to 7.5% SDS-PAGE, and the separated proteins were transferred to a nitrocellulose membrane. The membrane was incubated with a lectin for lectin blot analysis or with an antibody for immunoblot analysis.

Microscopy and Cell Image—Cells were seeded on glass bottom dishes for 48 h before fixation. After washing two times with PBS, the cells were fixed for 30 min in 3.7% paraformaldehyde solution at 37 °C. For permeabilization, the cells were

TABLE 1
Primers used for RT-PCR experiments

Gene		Primer sequence (5′–3′)
E-cadherin	Forward	ACGCATTGCCACATACA
	Reverse	CGTTAGCCTCGTTCTCA
N-cadherin	Forward	GAAAGACCCATCCACG
	Reverse	CCTGCTCACCACCACTA
Vimentin	Forward	ATGGCTCGTCACCTTCG
	Reverse	AGTTTCGTTGATAACCTGTCC
Snail	Forward	AATCGGAAGCCTAACTACAGCG
	Reverse	GTCCCAGATGAGCATTGGCA
Slug	Forward	AGCAGTTGCACTGTGATGCC
	Reverse	ACACAGCAGCCAGATTCCTC
GnT-III	Forward	GCCGCGTCATCAACGCCATCAA
	Reverse	CAGGTAGTCGTCGGCGATCCA
GnT-V	Forward	GACCTGCAGTTCCTTCTTCG
	Reverse	CCATGGCAGAAGTCCTGTTT
GAPDH	Forward	AGCCACATCGCTCAGACA
	Reverse	TGGACTCCACGACGTACT

treated with 0.2% (v/v) Triton X-100 in PBS. The fixed cells were blocked with 2% BSA in PBS for 1 h and were then incubated with monoclonal antibodies against E-cadherin, N-cadherin, β-catenin, and TO-PRO3 (Invitrogen) in blocking buffer for 1 h at room temperature. Following three washes in PBS, the cells were incubated with a 1:500 dilution of Alexa Fluor® secondary antibody or with a 1:300 dilution of Alexa Fluor® 488 phalloidin for F-actin (Invitrogen) for 1 h at room temperature. After washing three times with PBS, the cells were analyzed using an Olympus fluorescence microscope (FV1000 system).

GnT-III Activity—After washing with PBS, the cultured cells were lysed by sonication. The cell lysate protein concentration was determined using a BCA protein assay kit (Pierce). Equal amounts of protein were used in GnT-III and GnT-V activity assays, as described previously (24). The specific activities of GnT-III and GnT-V were determined using a substrate, 4-(2-pyridylamino)-butylamine-labeled GlcNAcβ1–2Manα1–6(GlcNAcβ1–2 Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc-Asn (25). Each assay used 5 mM substrate (in 10 μl of total reaction solution). The activities of endogenous GnT-III and GnT-V were measured by high performance liquid chromatography (HPLC), expressed as the picomoles of GlcNAc transferred/h/mg of proteins (20).

RT-PCR for mRNA Expression Analysis—Total RNA was prepared with TRIzol (Invitrogen), and 2.0 μg of total RNA was reverse-transcribed using a Superscript III RNase H reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. The sequences of the primers used for the PCR amplification are shown in Table 1. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a control in PCR runs, and the reaction products obtained were submitted to electrophoresis in 1.6% agarose gels containing ethidium bromide. Real time PCR was performed with a StepOnePlus real time PCR system (Applied Biosystems, Inc., Foster City, CA) using SYBR® Premix Ex Taq™ II PCR master mixes (Takara, Japan). PCR primers were as follows: *GnT-III* forward sequence, TCAACGCCATCAACATCAAC, and reverse sequence, GTGGCGGATGTACTCGAAGG; and *GAPDH* forward sequence, AAATGGTGAAGGTCGGTGTG, and reverse sequence, TGAAGGGGTCGTTGATGG.

Preparation of Pyridylaminated (PA) N-Linked Oligosaccharide and Analysis of N-Glycans by the Reversed Phase HPLC—*N*-Glycan analysis was performed with minor modification as reported previously (20). The cells were lysed by homogenization, and the *N*-glycans were then released with peptide:*N*-glycosidase F (New England Biolabs Japan, Inc., Tokyo, Japan) from glycoprotein by incubation for 16 h. Pyridylamination of each sample, pyridylaminated *N*-glycan (PA-*N*-glycan), was performed according to the manufacturer's recommended procedure (pyridylamination manual kit, Takara). Excess 2-aminopyridine was removed with a cellulose cartridge. The PA-*N*-glycans prepared from cells were analyzed using a reversed-phase HPLC system (Shimazu Co., Japan) with an ODS80-TM column (4.6 × 150 mm; Tosoh). Elution was performed at a flow rate of 1.0 ml/min at 55 °C using 20 mM ammonium acetate buffer (pH 4.0) as solvent A and the same buffer containing 1% 1-butanol as solvent B. The column was pre-equilibrated with 4% solvent B, and after injection of a sample, the PA-*N*-glycans were separated by 4% of solvent B for 10 min and then a linear gradient of 4–30% of solvent B for 60 min. PA-*N*-glycans were detected using a fluorescence detector (Shimazu) at excitation and emission wavelengths of 320 and 400 nm, respectively.

GnT-III Expression and Retroviral Infection—The cDNA encoding human *GnT-III* was amplified for cloning into pENTR-D-Topo for the Gateway Conversion System (Invitrogen), according to the manufacturer's protocol. The cloned genes were inserted into the virus expression vector, pBABE-puro (Addgene, Inc. Cambridge, MA), and introduced into the Gateway Conversion System using an LR clonase reaction. The *GnT-III* construct was transfected into Phoenix-Ampho cells with Lipofectamine 2000 (Invitrogen) for the production of viral supernatants, and after virus infection the infected cells were selected with 5 μg/ml puromycin. For mock transfection, the same protocol was performed using only the empty virus expression vector.

Cell Surface Biotinylation and Immunoprecipitation—Cell surface biotinylation was performed as described previously (24). Briefly, cells were rinsed twice with ice-cold PBS and were then incubated with ice-cold PBS containing 0.2 mg/ml EZ-Link® Sulfo-NHS-Biotin (Pierce) for 2 h at 4 °C. After incubation, 50 mM Tris-HCl (pH 8.0) was used for the initial wash to quench any unreacted biotinylation reagent. The cells were then washed three times with ice-cold PBS and solubilized in lysis buffer. Insoluble material was removed by centrifugation at 15,000 rpm for 10 min at 4 °C. The supernatant (2 mg of protein) was incubated with streptavidin-agarose (15 ml in 50% slurry) (Upstate Biotechnology) for another 3 h at 4 °C with rotation. After washing three times with lysis buffer, the immunoprecipitates were subjected to 7.5% SDS-PAGE, and the separated proteins were transferred to a nitrocellulose membrane. The membrane was incubated using antibodies against E-cadherin, β-catenin, and α-catenin for immunoblot analysis.

Metabolic Labeling—The pulse-chase experiments were performed as described previously, with minor modifications (26). The TGF-β-pretreated cells grown at 90% confluence on poly-L-lysine-coated 6-well dishes were washed three times with FCS-free medium and then starved for 30 min in DMEM by excluding methionine and cysteine (Sigma). After starvation, the cells were pulse-labeled in 500 μl of DMEM containing 200 μCi of [^{35}S]methionine and -cysteine (PerkinElmer Life Sciences) for 60 min, and then changed with complete DMEM/

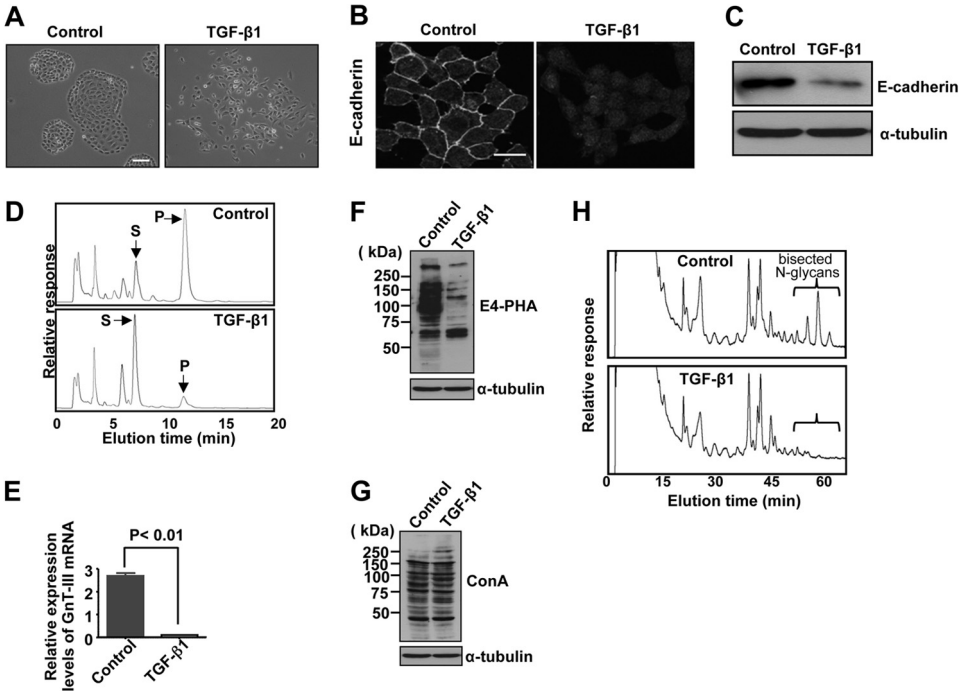

FIGURE 1. **Effects of TGF-β1 on cell morphology, E-cadherin expression, and changes in *N*-glycans in GE11 cells.** GE11 cells were grown in 6-well (2×10^5) or bottom dishes (2×10^4) for 24 h and then replaced with fresh complete medium with or without TGF-β1 (5 ng/ml) for another 4 days of incubation. *A,* cell morphology of the indicated cells was photographed. Photographs were taken of living cells using a ×10 objective. *Scale bar,* 100 μm. *B,* E-cadherin expressed on the cell surface was stained with anti-E-cadherin primary antibody, followed by incubation with Alexa Fluor-conjugated secondary antibody. *Scale bar,* 25 μm. *C,* total expression levels of E-cadherin were analyzed using Western blotting. Cell lysates from those cells were immunoblotted with anti-E-cadherin antibody. *D,* equal amounts of cell lysate proteins (20 μg) were used as the enzymatic source for the examination of GnT-III activities. *S,* substrate; *P,* product. *E,* mRNA expression of *GnT-III*. Quantitative RT-PCR was performed by monitoring in real time the increase in fluorescence of the SYBR Green dye on an ABI StepOnePlus. The mean number of cycles to the threshold (*CT*) of fluorescence detection was calculated for each sample, and the results were normalized to the mean *CT* of *GAPDH* for each sample tested. The changes in *N*-glycans were detected by E4-PHA (*F*) and concanavalin A (*ConA*) (*G*) lectin blot. α-Tubulin was used as a load control. *H, N*-glycans of GE11 cells cultured under normal conditions and treated with (*bottom*) or without (*top*) TGF-β for 4 days were released with peptide:*N*-glycosidase F (*PNGaseF*), as described under "Experimental Procedures," digested with sialidase, and subjected to reversed-phase HPLC. The elution times for those PA-bisected *N*-glycans were compared with standard PA-*N*-glycans.

F-12 containing 5% horse serum and 5 ng/ml TGF-β1 at the indicated times. The cells were lysed, and the cell lysates were immunoprecipitated with the anti-E-cadherin antibody and Dynabeads®. The immunoprecipitates were separated on 4–12% precast gels (Bio-Rad). After drying the gels, radioactive bands were visualized with a Fuji BAS 2500 BioImage analyzer.

Extraction of Nuclear β-Catenin—The nuclear proteins were prepared using the Thermo Scientific NE-PER nuclear and cytoplasmic extraction kit. Briefly, 5×10^6 cells were harvested with trypsin/EDTA and then centrifuged at $500 \times g$ for 5 min. After washing with PBS, the cells were transferred to 1.5-μl tubes, leaving the cell pellets as dry as possible, after which reagents were added to the nuclear proteins, according to the manufacturer's instructions. The expression levels of β-catenin were examined by Western blot analysis. The staining of α-tubulin was used as a loading control.

Cell Migration—Transwell (BD BioCoat™ control inserts, 8.0-mm inserts; BD Biosciences) were coated only on the bottom side with 10 mg/ml FN at 37 °C for 1 h. Cells pretreated with TGF-β1 for 4 days were starved in serum-free medium for 4 h, trypsinized, and suspended with 0.5 mg/ml trypsin inhibitor (Nacalai Tesque) in DMEM. The suspended cells were centrifuged, and the supernatants were removed. The cell pellets were resuspended with assay medium (0.1% BSA in DMEM containing 1% FBS) containing TGF-β1 and diluted to 4×10^5 cells/ml. To each FN-coated transwell were added 100-μl aliquots of the cell suspension; the cells were then incubated at 37 °C for 3 h. After incubation, cells on the upper side were removed by scraping with a cotton swab. The membranes in the transwells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet for 30 min. Cells that had migrated to the lower side were counted using a phase-contrast microscope.

RESULTS

Effects of TGF-β1 Treatment on GnT-III Expression in GE11 Cells—We have previously observed that GnT-III expression was markedly induced in cells cultured under dense conditions in an E-cadherin-α-catenin-actin-dependent manner, in several cell lines including epithelial GE11 cells (20, 21). Disruption or loss of E-cadherin expression diminishes this induction. We used an EMT model to understand the biological functions of the E-cadherin-dependent induction of GnT-III. It is well known that TGF-β promotes EMT by a combination of Smad-dependent transcriptional events and Smad-independent effects on cell·junction complexes (6). E-cadherin is one of the key targets for repression during EMT. TGF-β-induced EMT often coincides with a loss of E-cadherin expression. As shown in Fig. 1*A*, upon treatment with TGF-β1 for 4 days, the flat-with-epithelial shape of cell populations in the islands (aggregate) were converted to a diffused fibroblast-like morphology. As expected, the E-cadherin expression at cell-cell contacts

(Fig. 1*B*) or the total levels (Fig. 1*C*) were greatly down-regulated by TGF-β1. It is interesting that GnT-III activity (Fig. 1*D*) and the expression levels of *GnT-III* mRNA confirmed by quantitative RT-PCR were suppressed ~25-fold in TGF-β-treated cells, compared with that in control cells without TGF-β treatment (Fig. 1*E*). The products detected by E4-PHA (Fig. 1*F*), which preferentially recognizes bisecting GlcNAc, were dramatically suppressed by TGF-β1 treatment, whereas the total *N*-glycans confirmed by concanavalin A lectin seemed to be unchanged (Fig. 1*G*). Consistently, the pyridylaminated *N*-glycans analyzed by HPLC showed that the bisected *N*-glycans, which were compared with standard biantennary PA-bisected *N*-glycans, were dramatically decreased in the cells treated with TGF-β (Fig. 1*H*).

Effects of TGF-β1 Treatment on Expression of Epithelial Marker and Mesenchymal Marker Molecules in MCF10A Cells—To confirm whether the phenomena described above would also be observed in another cell line, human mammary epithelial MCF10A cells were examined. In full confluent cultures, MCF10A cells formed a compact epithelial morphology, although cells treated with TGF-β1 for 4 days showed a fibroblast-like spindle morphology, suggesting that MCF10A cells are also susceptible to EMT (Fig. 2*A*). In fact, upon treatment, the cells showed significant loss of both E-cadherin protein and mRNA expression, and up-regulation of mesenchymal markers such as N-cadherin and fibronectin (Fig. 2, *B–D* (*right* and *left panels*)). Furthermore, we investigated whether the expression of some of the transcription factors identified as EMT regulators correlated with these phenotypic changes. As shown in Fig. 2*C*, the expression levels of Snail and Slug mRNA were greatly increased in the presence of TGF-β1. The immunostaining data showed that E-cadherin expression in cell-cell junctions became jagged and faint in the cells treated with TGF-β (Fig. 2*B*, *upper right panel*) when compared with control cells (*upper left panel*). In contrast to E-cadherin, N-cadherin proteins were induced in the cells treated with TGF-β (Fig. 2*B*, *middle right panel*). F-actin distribution also dramatically changed from cell-cell borders to the stress fiber after TGF-β1 treatment. The downstream signaling of TGF-β, the phosphorylation of Smad2, was greatly increased after treatment (Fig. 2*D*).

Down-regulation of GnT-III Expression and Up-regulation of GnT-V Expression—As observed in GE11 cells, *GnT-III* expression and its activities were significantly suppressed by TGF-β1 treatment (Fig. 2, *E* and *F*). Consistent with a previous report (27), GnT-V expression and its activities were up-regulated in the presence of TGF-β1 (Fig. 2, *E* and *G*). To examine whether the increased expression levels of those glycosyltransferases were correlated with structural changes in *N*-glycans, we further investigated *N*-glycans on the integrin β1 subunit, which is a good target of those glycosyltransferases. As shown in Fig. 2*H*, the reactivity with E4-PHA lectin decreased, whereas the staining for L4-PHA or *D. stramonium* lectin, which recognizes branched GlcNAc, increased in TGF-β1-treated cells compared with control cells. The reactivity with *A. aurantia* lectin, which preferentially recognizes α1,6-fucose (core fucose) structure, was at a similar level. These results suggest that TGF-β-induced EMT specifically down-regulates GnT-III expression.

Overexpression of GnT-III-neutralized and TGF-β1-induced EMT—To understand the role of decreased *GnT-III* expression during the EMT process, we established a *GnT-III*-expressing MCF10A cell line. The levels of expression of GnT-III were verified by immunoblot analysis (Fig. 3*A*), E4-PHA lectin blot (Fig. 3*B*), and the specific activities (5,500 pmol/h/mg), which were comparable with activity (4,050 pmol/h/mg) of GE11 cells cultured under dense conditions. The two major bands that migrated at 62 and 52 kDa (Fig. 3*A*) are assumed to be derived from GnT-III, because these bands were also observed in the purified GnT-III from rat kidney (28). All MCF10A control cells had a bipolar, fibroblastic appearance after treatment with TGF-β1, although some of the *GnT-III*-expressing MCF10A cells still had cell aggregates with epithelial shapes (Fig. 3*C*). Furthermore, immunostaining for E-cadherin supported these morphology changes. E-cadherin expression at cell-cell contacts almost disappeared after treatment with TGF-β1 in MCF10A control cells but was still retained in *GnT-III*-expressing MCF10A cells (Fig. 3*D*). To confirm the expression of GnT-III-inhibited TGF-β-induced EMT, we compared the expression levels of E-cadherin and N-cadherin between control and GnT-III-overexpressing cells treated with TGF-β, and we found that the expression of N-cadherin, a marker for EMT, was significantly suppressed in the GnT-III-overexpressing cells (Fig. 3*E*). Furthermore, the expression levels of *N-cadherin* and *Snail* mRNA were significantly suppressed in the *GnT-III*-expressing cells, compared with those in control cells in the presence of TGF-β1 (Fig. 3*G*). However, GnT-III did not significantly affect the expression of E-cadherin mRNA (Fig. 3*G*). Taken together, these responses to *GnT-III* expression clearly suggest that that GnT-III has an inhibitory function for TGF-β-induced EMT through an indirect action.

TGF-β binds to type II and type I receptors (TβRII and TβRI). TβRII transphosphorylates TβRI, and the latter activates receptor-regulated Smad2 and Smad3. Previously, our group reported that α1,6-fucosylation of TβRII plays an important role in downstream signal transduction (29). To determine whether overexpression of *GnT-III* affected TGF-β-mediated signaling, phosphorylation levels of Smad2 were examined. As shown in Fig. 3*F*, there were no significant differences in the phosphorylation levels of Smad2, suggesting that a glycosyltransferase might have specificity for the modulation of certain target glycoproteins.

GnT-III Expression Prolonged E-cadherin Turnover on Cell Surface in TGF-β-induced EMT—E-cadherin plays crucial roles in EMT. It has four potential *N*-glycosylation sites in its extracellular domain (30). Several studies have shown that alterations in *N*-glycosylation could modulate cadherin-dependent cell-cell adhesion (19, 31–33). Taniguchi and co-workers (19) reported that overexpression of *GnT-III* enhanced cell-cell adhesion in B16 melanoma cells, because of a delay in the turnover of E-cadherin on the cell surface. Here, to understand the underlying mechanism for the neutralization of TGF-β-induced EMT observed in GnT-III-expressing MCF10A cells, E-cadherin turnover was examined and verified by total expression, expression on the cell surface by biotinylation, and by pulse-chase experiments. Treatment with TGF-β1 decreased E-cadherin expression in all three cell types, but the rate of

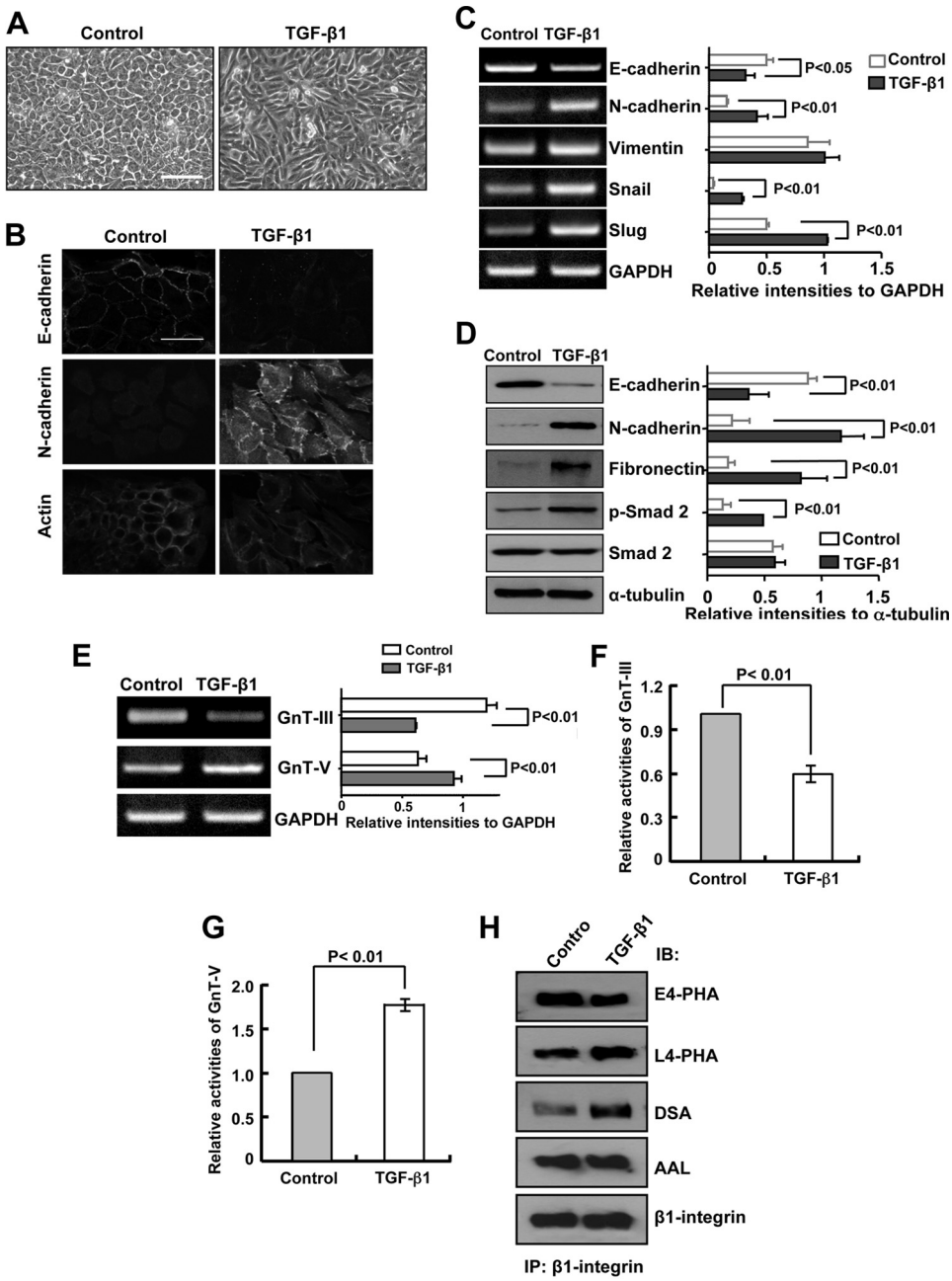

FIGURE 2. **TGF-β1-induced EMT in MCF10A cells.** *A,* MCF10A cells underwent morphological changes in response to TGF-β1 treatment as described in Fig. 1. Photographs were taken of living cells using a $\times$10 objective. *Scale bar,* 100 μm. *B,* cells treated with or without TGF-β1 were stained with anti-E-cadherin and N-cadherin primary antibodies, followed by incubation with Alexa Fluor-conjugated secondary antibody. Localization of F-actin was examined by staining with Alexa Fluor 488 phalloidin. *Scale bar,* 50 μm. *C,* RT-PCR using total RNA extracted from untreated (*control*) and TGF-β1-treated cells was carried out to examine the expression levels of *E-cadherin*, *N-cadherin*, *vimentin*, *Snail*, and *Slug*. The expression level of *GAPDH* was used as a load control. The relative ratios (each gene *versus GAPDH*) are shown in the *right panel*. The *p* values were calculated using Student's two-tailed *t* test. *Error bars* indicate the standard deviation. *D,* cell lysates from those cells were immunoblotted (*IB*) with anti-E-cadherin, N-cadherin, fibronectin, p-Smad2, and Smad2 antibodies. α-Tubulin was used as a load control. The relative ratios (each protein *versus* α-tubulin) are shown in the *right panel*. The *p* values were calculated using Student's two-tailed *t* test. *Error bars* indicate S.D. *E,* MCF 10A cells were cultured with or without TGF-β1 (5 ng/ml) for 4 days. The effects of TGF-β1 on expression of *GnT-III* and *GnT-V* were examined using RT-PCR, and the relative ratios are shown in the *right panel*. The quantitative data were obtained from three independent experiments. The *p* values were calculated using Student's two-tailed *t* test. *Error bars* indicate S.D. Equal amounts of cell lysates (20 μg) were used as the enzymatic source for assaying GnT-III and GnT-V activities. The relative activities of GnT-III (*F*) and GnT-V (*G*) are shown as folds, in which the activity for control cells without TGF-β1 treatment was set as 1. All of these quantitative data were obtained from three independent experiments. The *p* values were calculated using Student's two-tailed *t* test. *Error bars* indicate S.D. *H,* cell lysates from cells treated with or without TGF-β1 were immunoprecipitated (*IP*) using anti-β1 integrin antibody. Immunoprecipitates were run on a 7.5% SDS-polyacrylamide gel and probed with the biotinylated E_4-PHA, L4-PHA, *D. stramonium* (*DTA*), and *A. aurantia* (*AAL*) lectins. The same amounts of protein loaded were verified by probing with anti-β1 integrin antibody.

decrease in *GnT-III*-expressing cells was significantly lower than that in control or mock cells (Fig. 4, *A* and *B*). Consistently, biotinylation data showed that the down-regulation of E-cadherin expression induced by TGF-β1 on the cell surface was greatly neutralized by *GnT-III* expression (Fig. 4*C*), which was also confirmed by biotinylation data with a time course induced

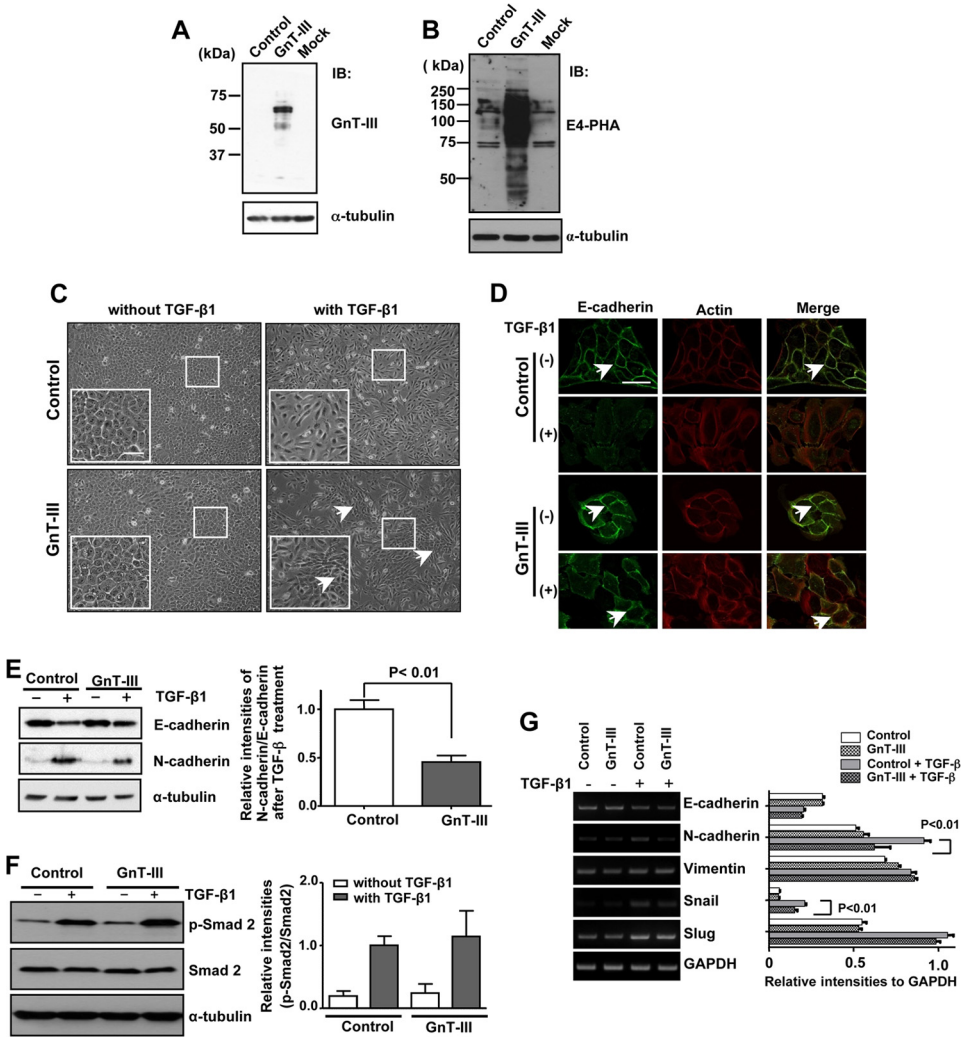

FIGURE 3. **Effects of GnT-III expression on cell morphological changes and cellular signaling in TGF-β-induced EMT.** *GnT-III* expression vector and vector only were transfected into MCF10A cells using the Phoenix retrovirus system, and stable expression cells were established as described under "Experimental Procedures." Cells were harvested and lysed for immunoblotting (*IB*). Equal amounts of protein (5 μg) were separated on 7.5% SDS-PAGE under reducing conditions, and the membranes were probed with antibody against GnT-III (*A*) and with the E4-PHA lectin (*B*). α-Tubulin was used as a loading control. *C,* wild-type MCF10A and *GnT-III*-expressing MCF10A cells underwent morphological changes in response to TGF-β 1 treatment as described in Fig. 1. Photographs were taken of living cells using a ×10 objective. *Insets*, representative cell morphology. *Arrows* indicate aggregated cells. *Scale bar,* 100 μm. *D,* indicated cells treated with or without TGF-β1 were stained with anti-E-cadherin antibody, followed by incubation with Alexa Fluor-conjugated secondary antibody. Localization of F-actin was examined by staining with Alexa Fluor 488 phalloidin. The *arrows* indicate E-cadherin expressed in the cell-cell contact. *Scale bar,* 50 μm. *E,* cell lysates from those cells were immunoblotted with anti-E-cadherin, N-cadherin, and α-tubulin antibodies (*left panel*). The relative intensity was a ratio of N-cadherin *versus* E-cadherin after TGF-β1 treatment (*right panel*). The *p* value was calculated using Student's two-tailed *t* test. *Error bars* indicate S.D. *F,* cell lysates were immunoblotted anti-pSmad2, Smad2, and α-tubulin antibodies (*left panel*). The relative intensity was a ratio of p-Smad2 *versus* Smad2 (*right panel*). The quantitative data were obtained from three independent experiments. α-Tubulin was used as a load control. *G,* RT-PCR using total RNA extracted from untreated and TGF-β1-treated control or *GnT-III*-expressing cells was carried out to examine the expression levels of *E-cadherin*, *N-cadherin*, *vimentin*, *Snail*, and *Slug*. The expression level of *GAPDH* was used as a load control. The relative ratios (each gene *versus GAPDH*) are shown in the *right panel*. The *p* value was calculated using Student's two-tailed *t* test. *Error bars* indicate S.D. *Control*, wild-type MCF 10A cells; *GnT-III*, cells transfected with *GnT-III*; *Mock*, cells transfected with vector only.

by TGF-β at 0, 2, 4, and 6 days (Fig. 4*D*). Furthermore, the pulse-chase experiment showed that the turnover rate of E-cadherin in *GnT-III*-expressing cells appeared to be lower than that in control cells (Fig. 4, *E* and *F*). The E-cadherin expression level chased at 0 h in *GnT-III*-expression cells was similar to that in control cells, suggesting that *GnT-III* expression does not affect *E-cadherin* expression at the transcription level. In fact, RT-PCR analysis confirmed that GnT-III did not significantly affect the expression of *E-cadherin* mRNA (Fig. 3*G*). However, the expression levels of E-cadherin chased at each chase point were higher in *GnT-III*-expressing cells than those in control cells at each matched point (Fig. 4*E*). The decay curve also showed the tendency (Fig. 4*F*). The lower band of the double bands (Fig. 4*E*) was β-catenin, which was confirmed by Western blot in this study (data not shown) and by analysis with mass spectrometry as reported by Pinho *et al.* (32). Concomitantly, the amounts of β-catenin associated with E-cadherin were similar to the changes in E-cadherin.

As shown in Fig. 4*G*, overexpression of *GnT-III* changed the pattern of *N*-glycans on E-cadherin, producing an increase in E4-PHA staining and a decrease in L4-PHA staining, which further supports the notion that GnT-III has an antagonistic effect on GlcNAc branching formation. Taken together, these results indicate that GnT-III expression neutralizes the down-

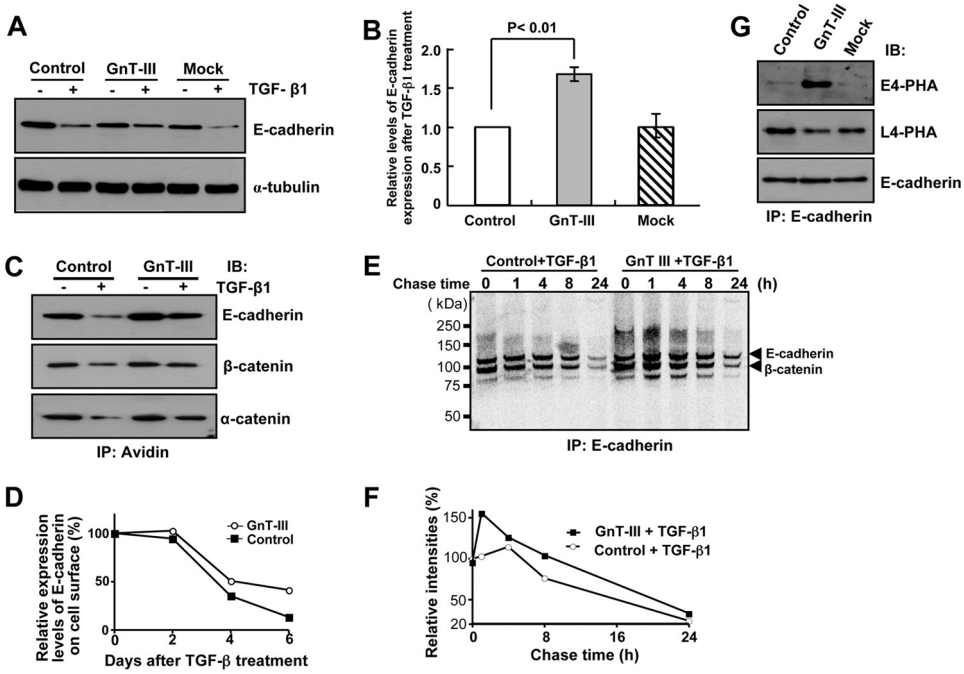

FIGURE 4. **Effects of overexpression of *GnT-III* on E-cadherin retained and E-cadherin·catenin complex formation.** *A,* indicated cells treated with or without TGF-β1 for 4 days were harvested and lysed for immunoblotting (*IB*). Equal amounts of protein (20 μg) were separated on 7.5% SDS-PAGE under reducing conditions, and the membranes were probed with antibody against E-cadherin and reprobed with anti-α-tubulin, which was used as loading control. *B,* ratios of E-cadherin retained after treatment with TGF-β1. The ratio of intensity of E-cadherin treated with TGF-β *versus* intensity of E-cadherin treated without TGF-β1 of control cells, as shown in *A* was set as 1. The quantitative data were obtained from three independent experiments. The *p* value was calculated using Student's two-tailed *t* test. *Error bars* indicate S.D. *C,* cell surface biotinylation was performed as described under "Experimental Procedures." Equal amounts of the cell lysates were immunoprecipitated (*IP*) with avidin-agarose. The immunoprecipitates were subjected to 7.5% SDS-PAGE. The membranes were probed with anti-E-cadherin, β-catenin, and α-catenin antibodies for immunoblot analysis. *D,* time course for cell surface biotinylation in cells treated with TGF-β for 0, 2, 4, and 6 days. The relative intensity was a ratio of E-cadherin *versus* α-tubulin. The quantitative data were obtained from three independent experiments. *E,* after metabolic labeling with [^{35}S]methionine and -cysteine for 30 min, cells were then chased at the indicated times. The cells were lysed, and the same amounts of cell lysate were immunoprecipitated with anti-E-cadherin antibody at the indicated times. *F,* relative intensity was a ratio of band intensity at each chasing point *versus* the band intensity at 0 time, which was 100%. The data were obtained form two independent experiments. *G,* cell lysates from indicated cells were immunoprecipitated using anti-E-cadherin antibody. Immunoprecipitates were run on a 7.5% SDS-polyacrylamide gel and probed with the biotinylated E4-PHA and L4-PHA lectins and anti-E-cadherin antibody.

regulation of E-cadherin expression induced by TGF-β through a post-transcriptional mechanism, *i.e.* prolongation of E-cadherin turnover on the cell surface.

Overexpression of GnT-III Inhibited TGF-β-induced Nuclear Localization of β-Catenin—β-Catenin is known to be a central player not only in E-cadherin-mediated cell-cell adhesion but also in the canonical Wnt signaling pathway. Loss of E-cadherin results in an increase in nuclear localization of β-catenin. Experiments were thus carried out to examine the effect of GnT-III expression on localization of β-catenin in cell-cell contact and the nuclei. Consistent with the observation with E-cadherin, after treatment with TGF-β1, β-catenin staining was lost in cell-cell contacts of control cells but still existed in cell-cell contacts of *GnT-III*-expressing cells (Fig. 5*A*). Conversely, β-catenin accumulated significantly in the nuclei and cytosol of control cells, but not *GnT-III*-expressing cells, after treatment with TGF-β1 (Fig. 5*A*). To further confirm this phenomenon, the nuclei were isolated, and the expression levels of β-catenin were compared in the membrane plus cytoplasmic fraction and the nuclear fraction using Western blot analysis. The expression levels of β-catenin in the membrane plus cytoplasmic fractions were greatly decreased in TGF-β1-treated control cells, compared with control cells without TGF-β1 or *GnT-III*-expressing cells treated with TGF-β1 (Fig. 5*B*). In contrast, the expression level of β-catenin in the nuclei of TGF-β1-treated control cells was higher than that in control cells without TGF-β1- or GnT-III-expressing cells treated with TGF-β1 (Fig. 5*B*). Furthermore, these results suggest that the expression of GnT-III plays an inhibitory role in TGF-β-induced EMT.

It has been reported that β-catenin·p-Smad2 complexes operate to promote mesenchymal gene responses to TGF-β1, and there is a strong correlation between the formation of these complexes and the initiation of EMT (34, 35), although the underlying mechanisms for the complex are unclear. Kim *et al.* (35) also reported that $\alpha 3\beta 1$ integrin was required for β-catenin phosphorylation at tyrosine 654, formation of pY654-β-catenin·p-Smad2 complex, and initiation of EMT in alveolar epithelia cells. We previously reported that GnT-III expression inhibited biological functions of $\alpha 3\beta 1$ integrin (36), so the levels of pY654-β-catenin were examined. Treatment with TGF-β1 did not affect the total expression levels of β-catenin but significantly suppressed the expression levels of pY-β-catenin in GnT-III-expressing cells, compared with that in control cells (Fig. 5*C*). However, as shown in Fig. 2*D*, the phosphorylation level of Smad2 in control cells treated with TGF-β1 was similar to that in *GnT-III*-expressing cells (Fig. 5*B*). The extent of complex formation induced by TGF-β1 was clearly suppressed in *GnT-III*-expressing cells compared with that in control cells (Fig. 5*D*). Thus, although the level of p-Smad2 was unchanged, the down-regulation of pY-β-catenin·p-Smad2

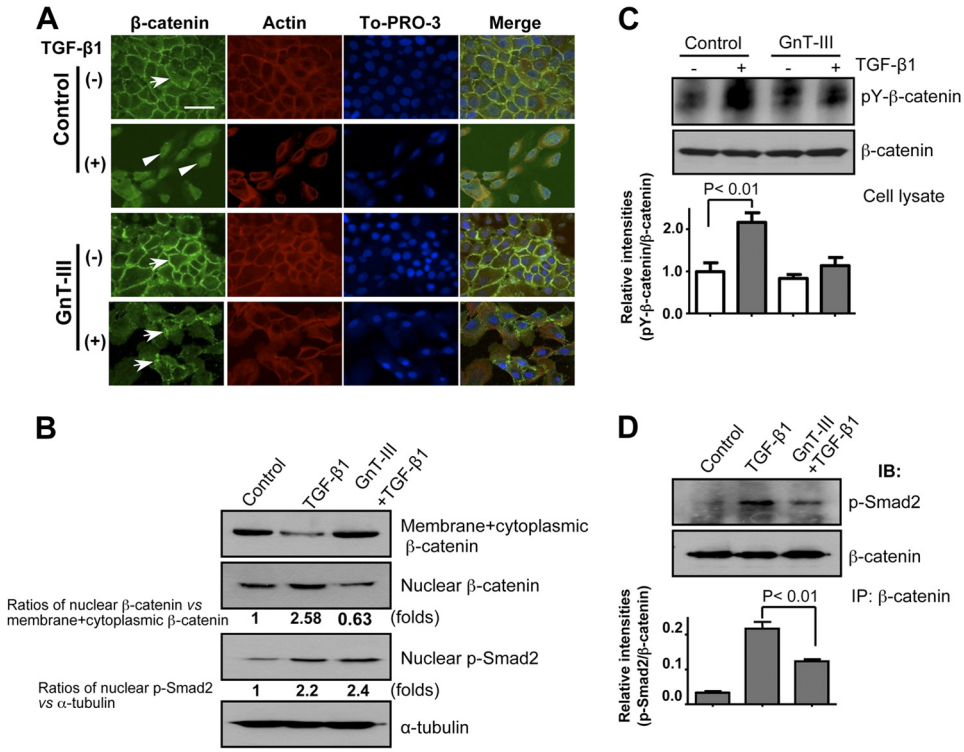

FIGURE 5. **Localization of β-catenin in *GnT-III*-expressing cells after treatment with TGF-β1.** *A,* to visualize the effects of overexpression of GnT-III on the localization of β-catenin, control and *GnT-III*-expressing cells were cultured with or without TGF-β as described above and then stained with anti-β-catenin primary antibody, phalloidin, or TO-PRO-3 and fluorescent secondary antibodies. *Arrows* indicate β-catenin expressed on cell-cell adhesion junctions. *Arrowheads* indicate localization of β-catenin in nuclei. *Scale bar,* 50 μm. *B,* nuclear proteins were prepared as described under "Experimental Procedures." The membrane plus cytoplasm or nuclear fractions of these indicated cells were probed with anti-β-catenin or anti-phospho-Smad2 antibody. α-Tubulin was used as a load control. The ratios of nuclear β-catenin *versus* membrane and cytoplasmic β-catenin or nuclear p-Smad2 *versus* α-tubulin are shown as folds, in which the ratio for control cells without TGF-β1 treatment was set as 1. *C,* cell lysates from indicated cells were immunoblotted (*IB*) with anti-pY-β-catenin (*upper panel*) and anti-β-catenin antibody (*middle panel*). The relative intensities showed ratios of pY-β-catenin *versus* β-catenin (*lower panel*). The quantitative data were obtained from three independent experiments. The *p* value was calculated using Student's two-tailed *t* test. *Error bars* indicate S.D. *D,* equal amounts of the cell lysates (20 μg) were immunoprecipitated (*IP*) with anti-β-catenin antibody. The immunoprecipitates were subjected to 7.5% SDS-PAGE, and the membranes were probed with anti-phospho-Smad2 (*upper panel*) or anti-β-catenin (*middle panel*) antibody. The relative intensities showed ratios of p-Smad2 *versus* β-catenin (*lower panel*). The quantitative data were obtained from three independent experiments. The *p* value was calculated using Student's two-tailed *t* test. *Error bars* indicate S.D.

complex formation might be due to a decrease in pY654-β-catenin levels in the *GnT-III* expressing cells. Nevertheless, the detailed mechanism for the down-regulation of complex formation by *GnT-III* overexpression is required for further study.

Effects of GnT-III Expression on TGF-β-induced Cell Motility—TGF-β-induced EMT is known to be closely related to enhancement of cell motility. To examine whether GnT-III expression affected TGF-β-induced cell motility, we performed a transwell assay. As expected, treatment with TGF-β1 greatly enhanced cell migration in control cells (Fig. 6). However, overexpression of *GnT-III* significantly suppressed the enhancement. The mechanism for the suppression could be ascribed to two aspects. One is that GnT-III expression decreased the breakdown of cell-cell junctions, as described above; the other is that GnT-III expression down-regulated integrin-mediated cell migration, as reported previously (36–39).

DISCUSSION

In this study, TGF-β1 induced EMT and specifically down-regulated the expression of GnT-III and its products in both epithelial GE11 cells and human nontumorigenic immortalized breast epithelial MCF-10A cells. In contrast to *GnT-III*, the expression of *GnT-V* was enhanced by TGF-β1 treatment, although the treatment did not affect *Fut8* expression. Most importantly, TGF-β-induced EMT could be influenced by overexpression of *GnT-III*, via prolongation of E-cadherin turnover and suppression of the β-catenin·p-Smad complex formation.

In the EMT process, the phenotype conversion from less mobile epithelial cells to fibroblastic mesenchymal cells with higher motility is accompanied by declining expression of epithelia cell molecules and enhanced expression of mesenchymal molecules. This process is essential for normal development during embryogenesis and is also implicated in the promotion of tumor invasion and metastasis. However, the *N*-glycosylation status of proteins is known to play a key role in early neoplastic transformation, tumor invasion, and cancer metastasis (8, 9). The overexpression of *GnT-III* in highly metastatic B16 melanoma cells reduces GnT-V's products, β1,6-GlcNAc-branched *N*-glycans, and it increases bisected *N*-glycans, which ultimately result in a significant decrease in metastatic potential in experimental models of lung metastasis (18, 19). In fact, GnT-III has been clearly shown to induce an anti-metastatic phenotype, although GnT-V enhances tumor formation and metastatic phenotype, as well as poor prognosis (40, 41). This

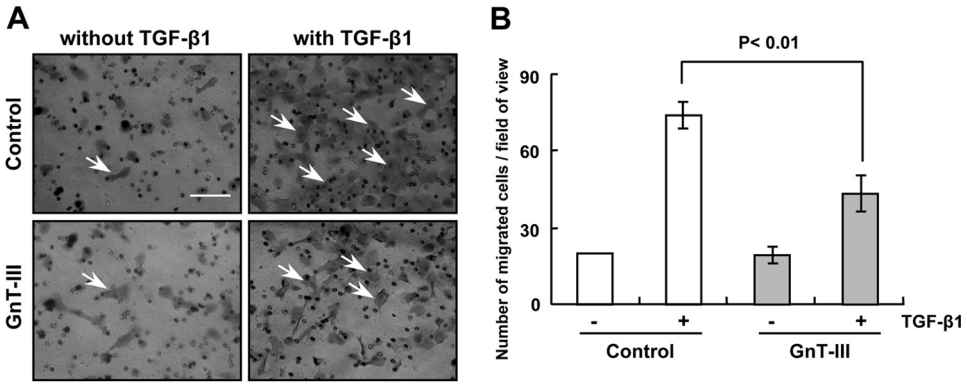

FIGURE 6. **Effects of overexpression of *GnT-III* on FN-mediated migration induced by TGF-β1.** Cell migration toward FN was determined using the transwell assay as described under "Experimental Procedures." Cells that migrated were stained with 0.5% crystal violet. *A,* representative example. *Scale bar,* 100 μm. *B,* migrated cells were counted under a microscope. The quantitative data were obtained from three independent experiments. The *p* value was calculated using Student's two-tailed *t* test. *Error bars* indicate S.D.

study clearly shows that GnT-III-expressing cells exhibit enhanced cell-cell adhesion because of a delay in the turnover of E-cadherin on the cell surface and alterations in the expression levels of EMT markers, which could be one of the important underlying mechanisms for the inhibitory functions of GnT-III on cell migration and cancer metastasis. Thus, the general relationship between GnT-III expression and the suppression of cancer metastases can be considered to be due, at least partially, to the modulation of the function of E-cadherin as a tumor suppressor protein preventing cell invasion and metastasis.

One important feature of EMT is increased cell motility via a decrease in cell-cell adhesion and increase in integrin-mediated cell migration. In fact, the overexpression of *GnT-III* has been shown to suppress α3β1 integrin-mediated cell migration on laminin 332 and to inhibit α5β1 integrin-mediated cell spreading and migration. Conversely, overexpression of *GnT-V* promotes integrin-mediated cell migration and blocks N-cadherin-mediated cell-cell adhesion (42). Therefore, the results obtained in this study are quite reasonable, *i.e.* down-regulation of GnT-III expression and up-regulation of GnT-V expression during TGF-β-induced EMT. These comprehensive bodies of data suggest that GnT-III plays a role in the suppression of tumor metastasis through at least two mechanisms, enhancement of cell-cell adhesion and down-regulation of extracellular matrix-mediated cell migration.

E-cadherin is a key molecule for EMT. Regulation of E-cadherin-mediated adhesion and associated adherens junctions is thought to underlie the dynamics of the adhesive interaction between cells, which are regulated during tissue development and homeostasis, as well as during the progression of tumor cells (43). In fact, the expression of E-cadherin could be strongly regulated by epithelial cell-cell interaction (44). However, the disruption of E-cadherin-mediated cell adhesion appears to be a central event in the transition from noninvasive tumors to invasive carcinomas. Some studies have focused on identifying and characterizing transcriptional repressors of *E-cadherin* expression in epithelial tumor cells. The most prominent factors to arise from these studies, including the related factors Slug, Snail, SIP1, and Twist, are best known for their roles in early embryogenesis and tumor progression (45). In contrast, results of this study clearly show that E-cadherin-mediated cell-cell adhesion can also be regulated by post-transcriptional modification with GnT-III. Thus, these studies, taken together, demonstrate that the expression of E-cadherin is closely regulated not only by transcriptional factors but also by post-transcriptional modifications.

One limitation of these observations is that it is unclear how the modification of E-cadherin with the bisecting GlcNAc affects its turnover on the cell surface. However, the modification of E-cadherin with complex *N*-glycans has been associated with the formation of dynamic, but weak, adherens junctions, whereas E-cadherin modified by high mannose or less *N*-glycans has been reported to promote the establishment of stable adherens junctions (33). In light of the report showing the importance of ectodomains of E-cadherin in homophilic adhesion, it is tempting to speculate that these *N*-glycans affect the interactions between the ectodomains through stearic hindrance. In addition, Pinho *et al.* (32) reported that E-cadherin undergoes extensive modification of its *N*-glycans with β1,6-branched and sialylation structures during acquisition of the malignant phenotype in a canine mammary tumor cell line model. Therefore, an explanation of the prolongation of turnover of E-cadherin with the bisecting GlcNAc could be that GnT-III inhibits the action of GnT-V, suppresses extensive complex *N*-glycan formation, and then enhances homophilic adhesion. In fact, *N*-glycans also play an important role in supracomplex formation on the cell surface. GnT-III expression decreased GlcNAc β1,6-branching formation and down-regulated galectin-3-mediated integrin·EGF receptor complex formation (38). Nonetheless, further work is needed to decipher the exact underlying exact molecular mechanism.

Different signaling pathways, for example RTK, Notch, Wnt, and TGF-β, are known to provide the necessary stimuli that modulate gene expression and trigger EMT and cell motility (46). We have reported that E-cadherin expression can also interfere with the transcription of the *GnT-III* gene, leading to up-regulation of *GnT-III* transcription, suggesting that there is a bidirectional regulatory mechanism between E-cadherin-mediated cell-cell adhesion and GnT-III expression. However, GnT-IIIs were greatly up-regulated by the knockdown of *β-catenin* or the inhibition of Wnt/β-catenin signaling (22).

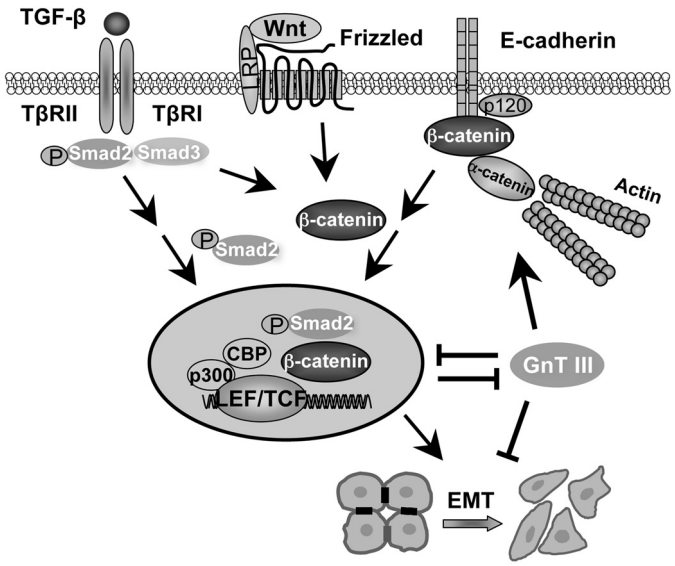

FIGURE 7. **Schematic representation of a model for the regulation of GnT-III expression and its function in EMT and cell-cell adhesion.** It was previously reported that *GnT-III* expression and bisected *N*-glycans were up-regulated by cell-cell adhesion in an E-cadherin-catenin-dependent manner (20, 21) and down-regulated by Wnt/β-catenin signaling (22). The intersection point for the reciprocal regulation of GnT-III is at the β-catenin, which is a central player in both cadherin-mediated cell adhesion and canonical Wnt signaling. In this study, the expression of GnT-III was down-regulated during EMT induced by TGF-β1. Conversely, overexpression of *GnT-III* promoted cell-cell adhesion, and inhibited β-catenin·p-Smad complex formation, EMT, and cell migration induced by TGF-β1. Therefore, GnT-III could be an important mediator for those intracellular signal networks. This study further suggests that the bisecting GlcNAc, a unique structure, plays an important role in cell biological functions during normal development, EMT, and cancer metastasis. →, up-regulation; ⊥, down-regulation.

Curiously, GnT-III expression suppressed p-β-catenin·p-Smad2 complex formation (Fig. 5); the critical promoter targets for this complex are unclear. The activation of TGF-β-mediated phospho-Smad2 did not differ between cells expressed with or without GnT-III, suggesting that a noncanonical signaling mechanism may be at play in EMT formation. GnT-III should be implicated in all these intracellular signal pathways, although it is unclear whether GnT-III affects Notch signaling. In fact, GnT-III expression suppresses RTK signaling, such as EGF receptor- and PDGF receptor-mediated MAPK activation (47). Therefore, GnT-III could be considered an important mediator in the EMT, as shown in Fig. 7. It could also be interesting and important to examine which expression levels of GnT-III can show inhibitory functions for EMT and whether this is a universal observation for epithelial cancer cells.

It is an interesting notion that a glycosyltransferase may specifically regulate a certain set of glycoproteins but not all glycoproteins (48). For example, GnT-III can modify EGF receptor and integrins and then down-regulate their biological functions (17). However, overexpression of GnT-III does not seem to affect the functions of TGF-β receptors, because there was no significant difference in TGF-β receptor-mediated phospho-Smad between cells expressed with or without GnT-III. In contrast to *GnT-III*, the expression of Fut8 is important for the biological functions of TGF-β receptors. In fact, the *Fut8*-deficient mice suffered from emphysema-like changes in the lungs that were mainly due to the lack of core fucosylation of the TGF-β1 receptor, which consequently resulted in a marked dysregulation of TGF-β1 receptor activation and signaling (29).

In conclusion, this is the first clearly delineated description of the regulation of GnT-III expression and its effects in TGF-β1-induced EMT, which further suggests that the bisecting GlcNAc, a unique structure, is of critical importance in the regulation of the biosynthesis of *N*-glycans and plays an important role in cell biological functions during normal development, EMT, and cancer metastasis. We believe that such studies might provide a hint for development of a new concept to regulate EMT, which may contribute to cancer therapy.

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