

III 研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yanyang Zhao, Satsuki Itoh, Xiangchun Wang, Tomoya Isaji, Eiji Miyoshi, Yoshinobu Kariya, Kaoru Miyazaki, Nana Kawasaki, Naoyuki Taniguchi, and Jianguo Gu	Deletion of core fucosylation on $\alpha 3\beta 1$ integrin down-regulates its functions.	J. Biol. Chem.	281	38343-38350	2006
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Deletion of Core Fucosylation on $\alpha 3\beta 1$ Integrin Down-regulates Its Functions*

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The core fucosylation ($\alpha 1,6$ -fucosylation) of glycoprotein is widely distributed in mammalian tissues. Recently $\alpha 1,6$ -fucosylation has been further reported to be very crucial by the study of $\alpha 1,6$ -fucosyltransferase (*Fut8*)-knock-out mice, which shows the phenotype of emphysema-like changes in the lung and severe growth retardation. In this study, we extensively investigated the effect of core fucosylation on $\alpha 3\beta 1$ integrin and found for the first time that *Fut8* makes an important contribution to the functions of this integrin. The role of core fucosylation in $\alpha 3\beta 1$ integrin-mediated events has been studied by using *Fut8*^{+/+} and *Fut8*^{-/-} embryonic fibroblasts, respectively. We found that the core fucosylation of $\alpha 3\beta 1$ integrin, the major receptor for laminin 5, was abundant in *Fut8*^{+/+} cells but was totally abolished in *Fut8*^{-/-} cells, which was associated with the deficient migration mediated by $\alpha 3\beta 1$ integrin in *Fut8*^{-/-} cells. Moreover integrin-mediated cell signaling was reduced in *Fut8*^{-/-} cells. The reintroduction of *Fut8* potentially restored laminin 5-induced migration and intracellular signaling. Collectively, these results suggested that core fucosylation is essential for the functions of $\alpha 3\beta 1$ integrin.

$\alpha 1,6$ -Fucosyltransferase (*Fut8*) catalyzes the transfer of a fucose residue from GDP-fucose to position 6 of the innermost GlcNAc residue of the hybrid and complex types of *N*-linked oligosaccharides on the glycoproteins (Fig. 1) (1). Core fucosylation ($\alpha 1,6$ -fucosylation) of glycoprotein is widely distributed in mammalian tissues and altered under pathological conditions, such as hepatocellular carcinoma and liver cirrhosis (2, 3). A high expression of *Fut8* was observed in 33.3% of papillary carcinoma, and the incidence was directly linked to tumor size and lymph node metastasis, thus *Fut8* expression may be a key

factor in the progression of thyroid papillary carcinomas (4). It has also been reported that the deletion of the core fucose from the IgG1 molecule enhances antibody-dependent cellular cytotoxicity activity by up to 50- to 100-fold. This indicates that the core fucose is an important sugar chain in terms of antibody-dependent cellular cytotoxicity activity (5). Recently, the physiological functions of the core fucose have been further investigated by our group using analysis of core fucose-deficient mice (6). The *Fut8*^{-/-} mice showed severe growth retardation, and 70% died within 3 days after birth. The surviving mice suffered from emphysema-like changes in the lung that appear to be due to the lack of core fucosylation of transforming growth factor- $\beta 1$ receptor, which consequently resulted in a marked dysregulation of transforming growth factor- $\beta 1$ receptor activation and signaling. We also found that the loss of core fucosylation resulted in the down-regulation of EGF³ receptor-mediated signaling pathway (7). These results together suggest that core fucose performs the important physiological functions through modification of some important functional proteins.

Cell-extracellular matrix (ECM) interactions play essential roles during the acquisition of migration and invasive behavior of the cells. The integrin family consists of α and β heterodimeric transmembrane receptors for ECM and connects many biological functions, such as development, the control of cell proliferation, protection against apoptosis, and malignant transformation (8). For example, $\alpha 3\beta 1$ integrin, the major receptor for laminin 5 (LN5), is widely distributed in almost all tissues, and $\alpha 3$ knock-out mice have been reported to show the defects in kidney, lung, and skin (9). It has been reported that G-like repeats of LN5 constitute the favored ligand for $\alpha 3\beta 1$ integrin, triggering haptotaxis (10). Especially, the G3 domain is essential for the unique activity of LN5, such as promotion of cell migration (11). Furthermore, $\alpha 3\beta 1$ integrin has been proposed to be involved in tumor invasion (12, 13): the interaction

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³ The abbreviations used are: EGF, epidermal growth factor; ECM, extracellular matrix; LN5, laminin 5; FN, fibronectin; COL, collagen; mAb, monoclonal antibody; PBS, phosphate-buffered saline; GnT-III, *N*-acetylglucosaminyltransferase III; GnT-V, *N*-acetylglucosaminyltransferase V; MEF, mouse embryonic fibroblast; ERK, extracellular signal-regulated kinase; AAL, *Aleuria aurantia* lectin; LC, liquid chromatography; MS, mass spectrometry; FT, Fourier transform; GM3, NeuAc $\alpha 2,3$ Gal $\beta 1,4$ Glc-ceramide.

Core Fucosylation Regulates $\alpha 3\beta 1$ Integrin-mediated Functions

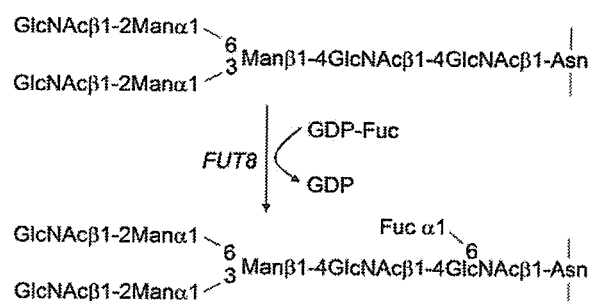


FIGURE 1. Reaction pathway for the biosynthesis of core fucose by *Fut8*. *Man*, mannose; *Fuc*, fucose; *GDP-Fuc*, guanosinediphospho-fucopyranoside; *Asn*, asparagine.

of $\alpha 3\beta 1$ integrin with LN5 in exposed basement membrane provides both a molecular and a structural basis for cell arrest during pulmonary metastasis (14). In some malignant tumors, $\alpha 3\beta 1$ integrin is found to be the most predominant integrin expressed (15), and cell invasion on ECM could be inhibited by antibodies against $\alpha 3$ integrin (13) and $\beta 1$ integrin (14). Thus, $\alpha 3\beta 1$ integrin, which mediates to laminins of basement membrane, preferentially promotes cell migration and metastasis (16–18). Given its various biological functions, $\alpha 3\beta 1$ integrin, as one of most important extracellular adhesive molecules, deserves the more detailed investigation.

It has long been known that various factors can modulate integrin functions, including the status of glycosylation of integrin (19), the partnerships with tetraspanins, growth factor receptors (20–22), and the association with ganglioside GM3 (22), and others. Cell surface integrins are all major carriers of *N*-glycans, therefore *N*-glycosylation of integrins plays an important role in their biological functions (23). For example: the $\alpha 3$ and $\beta 1$ subunits expressed by the metastasis human melanoma cell lines carry $\beta 1,6$ -branched structures, and these cancer-associated glycan chains may modulate tumor cell adhesion by affecting the ligand properties of $\alpha 3\beta 1$ integrin (23). The linkage and expression levels of the terminal sialic acids of $\alpha 3\beta 1$ integrin play an important role in cell-ECM interactions (24, 25). An increase in $\beta 1,6$ -GlcNAc sugar chains of $\beta 1$ integrin resulted in the stimulation of cell migration and the organization of F-actin into extended microfilaments in cells plated on FN-coated plates (26). Moreover, a recent study has shown that introduction of bisecting GlcNAc into $\alpha 5\beta 1$ integrin down-regulates cell adhesion and cell migration (27). These previous papers listed above have shown that the functions of integrins were positively or negatively regulated by *N*-glycans catalyzed by GnT-III, GnT-V, sialyltransferases, and others.

However, until now the effect of core fucosylation on integrin functions remains unclear. Here, we described studies comparing embryonic fibroblasts from wild-type and *Fut8*^{-/-} mice to elucidate the role of core fucosylation in $\alpha 3\beta 1$ integrin-stimulated events, and our finding for the first time showed that core fucosylation is required for the functions of $\alpha 3\beta 1$ integrin.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—A polyclonal antibody against mouse $\alpha 3$ integrin and functional blocking monoclonal antibody (mAb) against $\alpha 2\beta 1$ integrin were obtained from Chemi-

con International, Inc. (Temecula, CA). mAbs against $\alpha 3$ integrin, FAK, FAK (pY397), and functional blocking mAbs against integrin $\alpha 6$ and $\beta 1$ subunits were from BD Transduction Laboratories (Lexington, KY). A polyclonal antibody against rabbit ERK1/2 and peroxidase-conjugated goat antibody against rabbit IgG were obtained from Cell Signaling (Beverly, MA). A mouse control IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A peroxidase-conjugated goat antibody against mouse IgG was obtained from Promega (Madison, WI), and biotinylated *Aleuria aurantia* lectin (AAL) was from Seikagaku Corp., Japan.

Cell Culture—*Fut8*^{+/+} and *Fut8*^{-/-} mouse embryonic fibroblasts (MEFs) and restored cells were previously established in our laboratory (6). *Fut8*^{+/+} and *Fut8*^{-/-} embryonic fibroblasts and restored cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in the presence of 400 μ g/ml Zeocin, and restored cells were maintained in Dulbecco's modified Eagle's medium in the presence of 400 μ g/ml Zeocin and 400 μ g/ml hygromycin.

Western Blot and Lectin Blot Analysis—Cell cultures were harvested in lysis buffer (20 mM Tris-HCl, pH 7.4, 10 mM EGTA, 10 mM MgCl₂, 1 mM benzamide, 60 mM β -glycerophosphate, 1 mM Na₃VO₄, 20 mM NaF, 2 μ g/ml aprotinin, 5 μ g/ml leupeptin, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride). Cell lysates were centrifuged at 15,000 \times *g* for 10 min at 4 °C, the supernatants were collected, and the protein concentrations were determined using a BCA protein assay kit (Pierce). Proteins were then immunoprecipitated from the lysates using a combination of 2 μ g of anti- $\alpha 3$ integrin antibody and Protein G-Sepharose beads. Immunoprecipitates were suspended in nonreducing buffer, heated to 100 °C for 3 min, resolved on 7.5% SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell). The blots were then probed with anti- $\alpha 3$ integrin antibody and biotinylated AAL, respectively. Immunoreactive bands were visualized using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and an ECL kit (Amersham Biosciences).

Cell Surface Biotinylation—Cell surface biotinylation was performed as described previously (28). Briefly, cells were rinsed twice with ice-cold PBS and then incubated with ice-cold PBS containing 0.2 mg/ml sulfo-succinimidobiotin (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, and the cells were washed three times with ice-cold PBS and then solubilized in lysis buffer. The resulting cell lysate was then immunoprecipitated with the anti- $\alpha 3$ integrin antibody as described above. The biotinylated proteins were visualized using a Vectastain ABC kit and an ECL kit.

Migration Assay and Functional Blocking Assay—Transwells (BD Bioscience) were coated with 5 nm of recombinant LN5, as described previously (29), or 15 nm of human plasma FN, 50 μ g/ml collagen I (COL, Sigma) in PBS by an overnight treatment at 4 °C followed by an incubation with 1% bovine serum albumin for 1 h at 37 °C. Serum-starved cells (2×10^5) per well in 500 μ l of fetal calf serum-free medium were seeded in the upper compartment of the plates. After incubation for 3 h, the cells in the upper chamber of the filter were removed with a wet cotton swab. Cells on the lower side of the filter were fixed and

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stained with 0.5% crystal violet. Each experiment was performed in triplicate, and counting was done in three randomly selected microscopic fields within each well. To identify which specific integrin mediates cell migration on LN5, monoclonal antibodies against different types of integrins at concentrations of 10 $\mu\text{g}/\text{ml}$ were preincubated individually with fibroblasts for 10 min at 37 °C. Then cells were transferred into Transwells coated with LN5 and then incubated for 2 h 37 °C. The migrated cells were then quantified as described above.

Construction of Small Interference RNA Vector and Retroviral Infection—Small interfering oligonucleotides specific for integrin $\alpha 3$ subunit were designed on the Takara Bio website, and the oligonucleotide sequences used in the construction of the small interference RNA vector were as follows: 5'-GATCGCTATGGAGAATCACACTGATTCAAGAGATCAGTGTGATTCTCCATAGCTTTTTT-3' and 5'-AATTCAAAAAGCTATGGAGAATCACACTGATCTCTTGAATCAGTGTGATTCTCCATAGCG-3'. The oligonucleotides were annealed and then ligated into BamHI/EcoRI sites of the RNAi-Ready pSIREN-Retro Q vector (Takara Bio). A retroviral supernatant was obtained by transfection of human embryonic kidney 293 cells using a Retrovirus Packaging Kit Eco (Takara Bio) according to the manufacturer's protocol. Embryonic fibroblast cells were infected with the viral supernatant, and the cells were then selected with 15 $\mu\text{g}/\text{ml}$ puromycin for 2–3 weeks. Stable $\alpha 3$ integrin knockdown clones were therefore selected.

Tyrosine Phosphorylation Assay of FAK—Serum-starved cells were detached and held in suspension for 60 min to reduce the detachment-induced activation and then replated on dishes coated with LN5 (5 nm) for the indicated times, and the cell lysates were blotted with anti-phosphotyrosine FAK (pY397) antibody. Then the equal loading was confirmed by blotting with an antibody against total FAK.

Purification of $\alpha 3 \beta 1$ Integrin—The purification of $\alpha 3 \beta 1$ integrin was performed as described previously (30). Briefly, confluent cells were detached with TBS(+) (20 mM Tris-HCl, pH 7.5, 130 mM NaCl, 1 mM CaCl_2 , and 1 mM MgCl_2) and washed with TBS(+). The cell pellets were extracted with 50 mM Tris/HCl containing 15 mM NaCl, 1 mM MgCl_2 , 1 mM MnCl_2 , pH 7.4, and protease inhibitor mixture (Roche Applied Science), 100 mM octyl- β -D-glucopyranoside at 4 °C. The cell extract was applied to an affinity column prepared by coupling 5 mg of GD6 peptide of laminin $\alpha 1$ chain (30) (KQNCLSSRASFRGCVRNLRSLR residues numbered 3011–3032, Peptide Institute, Inc., Osaka, Japan) to 1 ml of activated CH-Sepharose (Sigma). The bound $\alpha 3 \beta 1$ integrin was eluted with 20 mM EDTA in 50 mM Tris/HCl, pH 7.4, containing 100 mM octyl- β -D-glucopyranoside. The elutes containing $\alpha 3 \beta 1$ integrin were further purified on 1 ml of a wheat germ agglutinin-agarose column (Seikagaku Corp.) and eluted with 0.2 M *N*-acetyl-D-glucosamine containing 100 mM octyl- β -D-glucopyranoside. The purity of the integrin was verified by SDS-PAGE by means of a silver staining kit (Daichi Pure Chemicals Co., Ltd., Tokyo, Japan).

Analysis of *N*-Glycan Structure by Liquid Chromatography (LC/Tandem Mass Spectrometry (MS/MS))—Purified $\alpha 3 \beta 1$ integrin was applied to SDS-PAGE and excised from the gel then cut into pieces. The gel pieces were destained and dehydrated with 50% acetonitrile. The protein in the gel was reduced

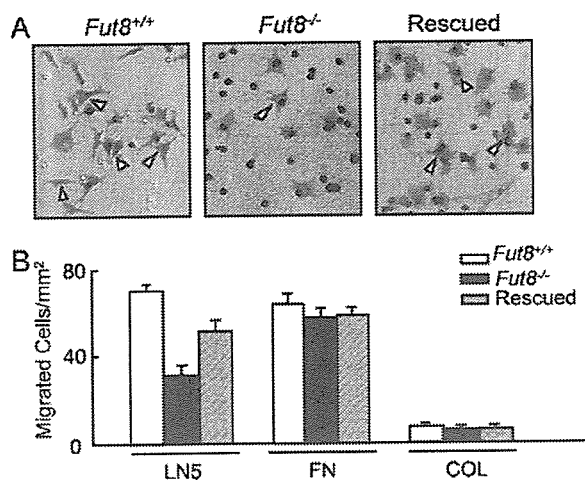


FIGURE 2. Effects of deficient core fucosylation on cell migration on LN5 but not on FN. *Fut8*^{+/+}, *Fut8*^{-/-}, and rescued cells were replated on the upper chamber coated with LN5 (5 nm), FN (15 nm), or 50 $\mu\text{g}/\text{ml}$ COL. Cell migration was determined using the Transwell assay described under "Experimental Procedures." **A**, representative fields on LN5 were photographed using a phase-contrast microscope. The arrowheads indicate migrated cells. **B**, the numbers of migrated cells on LN5, FN, or COL were quantified and expressed as the means \pm S.D. from three independent experiments.

and carboxymethylated with dithiothreitol and monoiodoacetic acid according to the reports described by Kikuchi *et al.* (31) with some modifications. *N*-Glycans were released and extracted from the gel pieces as reported by Kustar *et al.* (32). The extracted oligosaccharides were reduced with NaBH_4 . LC/MS was performed using a quadrupole liner ion trap-Fourier transform (FT) ion cyclotron resonance mass spectrometer (Finnigan LTQ FTTM, Thermo Electron Corp., San Jose, CA) connected to a nanoLC system (Paradigm, Michrom BioResource, Inc., Auburn, CA). The eluents were 5 mM ammonium acetate, pH 9.6/2% CH_3CN (pump A), and 5 mM ammonium acetate, pH 9.6/80% CH_3CN (pump B). The borohydride-reduced *N*-linked oligosaccharides were separated on a Hypercarb (0.1 \times 150 mm, Thermo Electron Corp.) with a linear gradient of 5–20% of B in 45 min and 20–50% of B in 45 min. FT-full MS scan (*m/z* 450–2000) followed by data-dependent MS/MS for the most abundant ions was performed in both negative and positive ion modes as described in the previous report (33).

RESULTS

Impaired $\alpha 3 \beta 1$ Integrin-mediated Cell Migration Was Found in *Fut8*^{-/-} Cells—One of the major functions of $\alpha 3 \beta 1$ integrin is promotion of cell migration. In some malignant tumors, $\alpha 3 \beta 1$ integrin was found to be the most predominant integrin expressed (15), and it has made an important contribution to metastasis (14); therefore, cell motility on different ECMs was firstly examined by utilizing a Transwell assay. Cells were applied into the chambers, the bottoms of which had been coated with LN5, FN, or COL. As shown in Fig. 2 (**A** and **B**), *Fut8*^{-/-} cells showed impaired migration on LN5 by a decrease to 44% relative to *Fut8*^{+/+} cells. Consistently, reintroduction of *Fut8* partly restored cell migration by an increase in the percentage of migrating cells from 44% to 74%, indicating that core fucosylation is required for LN5-stimulated cell migration. But in the case of cell migration on FN, a specific ligand for $\alpha 5 \beta 1$

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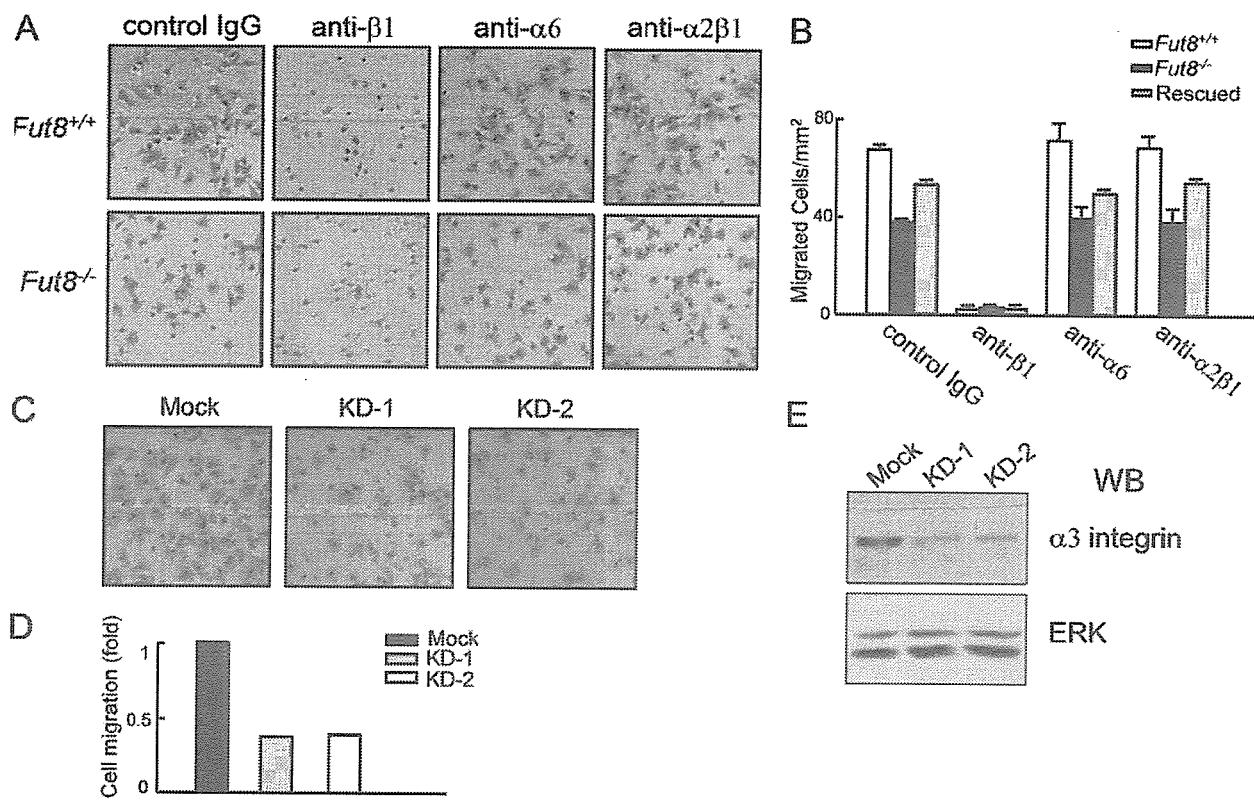


FIGURE 3. Cell migration on LN5 was mediated by $\alpha 3 \beta 1$ integrin. *A*, *Fut8*^{+/+}, *Fut8*^{-/-}, and rescued cells were detached, preincubated with mouse control IgG or function-blocking mAbs against $\beta 1$, $\alpha 6$, or $\alpha 2 \beta 1$ integrin for 10 min, replated on the upper chamber coated with LN5 (5 nM), and checked by Transwell assay. Representative fields were photographed using a phase-contrast microscope. *B*, the numbers of migrated cells were quantified and expressed as the means \pm S.D. from three independent experiments. *C*, cell migration of $\alpha 3$ -knockdown cells on LN5 (5 nM). Representative fields were photographed using a phase-contrast microscope. Arrowheads indicate migrated cells. *D*, quantification of migration of mock and $\alpha 3$ -knockdown cells. The numbers of migrated cells were quantified and expressed as the means \pm S.D. from three independent experiments. *E*, $\alpha 3$ -knockdown was confirmed by blotting total cell lysates with anti- $\alpha 3$ antibody (upper panel), and equal loading was confirmed by probing with an antibody against total protein ERK1/2 (lower panel). KD1 and KD2, $\alpha 3$ -knockdown cells.

integrin, the obvious difference among *Fut8*^{+/+}, *Fut8*^{-/-}, and rescued cells was not found. In addition, the motility of these three types of cells on COL, a ligand for $\alpha 1 \beta 1$ and $\alpha 2 \beta 1$ integrins, was barely detectable (Fig. 2*B*). This suggested that $\alpha 5 \beta 1$, $\alpha 1 \beta 1$ and $\alpha 2 \beta 1$ integrins, unlike receptor of laminin 5, may be not strongly affected by *Fut8*. So MEF cells may favor LN5 as an ECM for cell migration. Furthermore, the cell migration on LN5 was completely blocked by the presence of function-blocking antibodies against $\beta 1$ but not by $\alpha 6$ or $\alpha 2 \beta 1$ integrin antibodies (Fig. 3, *A* and *B*), further excluding the involvement of $\alpha 6$ and $\alpha 2 \beta 1$ integrin on LN5-stimulated cell migration. However, so far the function-blocking antibody against mouse $\alpha 3$ is unavailable. To definitely confirm the important function of integrin $\alpha 3$ subunit for the cell migration on LN5, we utilized an RNA interference strategy to silence $\alpha 3$ in MEF cells. After retroviral infection, the cells were selected based on their resistance to puromycin as described under "Experimental Procedures." Expression of $\alpha 3$ but not $\alpha 5$ (data not shown), or other proteins such as ERK, was effectively down-regulated, compared with those in mock cells (Fig. 3*E*). We then tested cell migration on LN5 and found that $\alpha 3$ -knockdown resulted in a significant decreased cell migration compared with mock cells (Fig. 3, *C* and *D*). Together with the data in Fig. 3 (*A* and *B*), these results provided the evidence that in this study the cell

migration on LN5 was mediated by $\alpha 3 \beta 1$ integrin. This result was consistent with the view of previous study that $\alpha 3 \beta 1$ integrin is distinct from other integrins and preferentially promotes cell migration (16). The result above was also supported by the previous observation that LN5 as well as LN10/11 promoted cell migration is mainly mediated by $\alpha 3 \beta 1$ integrin, but not $\alpha 6 \beta 1$ or $\alpha 6 \beta 4$ integrins (34). However, we cannot definitely exclude the involvement of syndecan-1 and -4, because it has been reported to have an interaction with LN5 (35, 36); therefore, they might regulate integrin functions in an indirect way. Collectively, these results suggested that $\alpha 3 \beta 1$ integrin is a key molecule for cell migration on LN5 in the embryonic fibroblasts and that core fucosylation regulates $\alpha 3 \beta 1$ integrin-mediated cell migration.

Integrin-stimulated Phosphorylation of FAK Was Reduced in *Fut8*^{-/-} Cells—ECM-integrin signaling events are prominently involved in regulating cell migration (16). In particular, the protein-tyrosine kinase FAK plays a prominent role in integrin signaling (37–39). To address the effects of *Fut8* on $\alpha 3 \beta 1$ integrin-mediated signaling, we examined FAK phosphorylation in adherent cells on LN5. As shown in Fig. 4, the level of tyrosine phosphorylation was reduced in the *Fut8*^{-/-} cells compared with *Fut8*^{+/+} cells, moreover the down-regulation of phosphorylation in *Fut8*^{-/-} cells was restored in the rescued cells, suggesting that deficient core fucosylation was able to neg-

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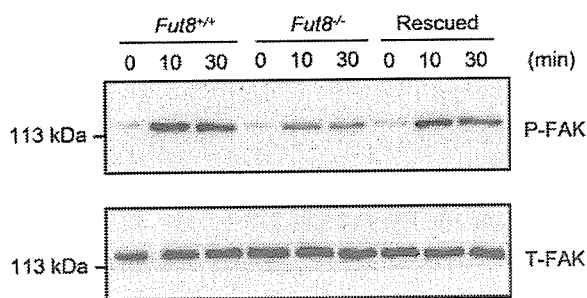


FIGURE 4. Comparison of tyrosine phosphorylation levels of FAK among $Fut8^{+/+}$ and $Fut8^{-/-}$ and rescued cells on LN5. Serum-starved cells were detached and held in suspension for 60 min to reduce the detachment-induced activation and then replated on dishes coated with LN5 (5 nm) for the indicated times, and the cell lysates were blotted with anti-phosphotyrosine FAK antibody to detect the amount of phosphorylation. Then the equal loading was confirmed with an antibody against total protein FAK.

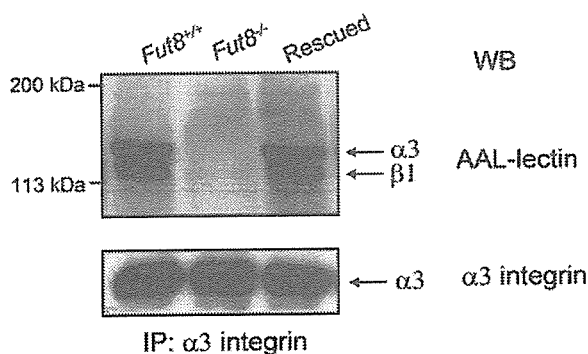


FIGURE 5. Glycosylation analysis of $\alpha 3\beta 1$ integrin from $Fut8^{+/+}$, $Fut8^{-/-}$, and rescued cells. Whole cell lysates were immunoprecipitated (IP) with anti- $\alpha 3$ integrin antibody, and the resulting immunocomplexes were subjected to 7.5% SDS-PAGE under nonreducing condition. After electroblotting, the blots were probed, respectively, by AAL (upper panel) and an anti- $\alpha 3$ integrin antibody (lower panel).

actively regulate $\alpha 3\beta 1$ integrin-mediated signaling pathway. Considerable evidence implicates FAK in the regulation of cell migration. Most notably, FAK-deficient cells exhibit poor migration ability in response to chemotactic and haptotactic migration (40, 41). Therefore, based on such evidence we suggested that the deficient signal transduction may account for the deficient cell migration on LN5 in $Fut8^{-/-}$ cells.

Expression of $\alpha 3\beta 1$ Integrin on the Cell Surface Was Not Influenced by $Fut8$ —Some important glycosyltransferases have been reported to modify and further regulate the functions of integrins by modulating the status of glycosylation on them such as GnT-III and GnT-V; however, there is no such data so far to show the relation of $Fut8$ and integrins. Therefore, in Fig. 5, the fucosylation on $\alpha 3\beta 1$ integrin among $Fut8^{+/+}$, $Fut8^{-/-}$, and rescued cells has been examined by using blotting of $\alpha 3$ integrin-immunoprecipitated lysates with AAL lectin (upper panel). Equal loadings were verified by blotting with $\alpha 3$ integrin antibodies (lower panel). As shown in Fig. 5, the levels of core fucosylation in both $\alpha 3$ and $\beta 1$ subunits were abolished in $Fut8^{-/-}$ cells consistent with no $Fut8$ activity in these cells (7), whereas they were rescued by reintroduction of $Fut8$, suggesting $\alpha 3\beta 1$ integrin is the target of $Fut8$. Furthermore, the effect of deficiency of core fucosylation on the expression of $\alpha 3\beta 1$ integrin on the cell surface was also determined, because *N*-gly-

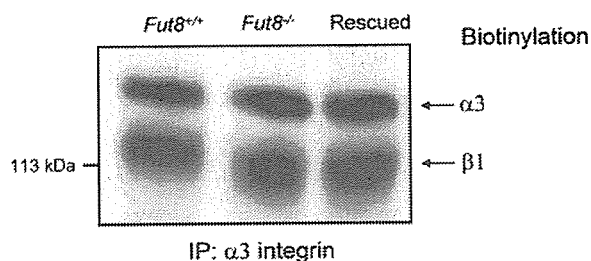


FIGURE 6. Effects of core fucosylation on expression levels of $\alpha 3\beta 1$ integrin on cell surface. $Fut8^{+/+}$, $Fut8^{-/-}$, and rescued cells were biotinylated, whole lysates were immunoprecipitated (IP) with anti- $\alpha 3$ integrin antibody, the samples were subjected to 7.5% SDS-PAGE and transferred to a nitrocellulose membrane, and the biotinylated proteins were then detected as described under "Experimental Procedures."

cosylation plays an important role in the quality control of the expression of glycoproteins. The biotinylation of cell surface proteins followed by immunoprecipitation of $\alpha 3$ integrin was examined by blotting. As shown in Fig. 6, the expression levels of $\alpha 3\beta 1$ integrin on the cell surface remained unchanged among $Fut8^{+/+}$, $Fut8^{-/-}$, and rescued cells, indicating that the expression of $\alpha 3\beta 1$ integrin on cell surface was not influenced by $Fut8$. Collectively, we suggested that the deficiency of core fucosylation resulted in the malfunctions of $\alpha 3\beta 1$ integrin but not its expression level.

Purified $\alpha 3\beta 1$ Integrin, Rich in Core Fucosylation, Was Shown by LC/MS/MS—The analysis of glycan structural alteration in glycoproteins is becoming increasingly important in the discovery of therapies and diagnostic markers (42). To better understand the detailed modification of $Fut8$ for $\alpha 3\beta 1$ integrin, we purified $\alpha 3\beta 1$ integrin from $Fut8^{+/+}$ and $Fut8^{-/-}$ cells by using a GD6 peptide affinity column combined with a wheat germ agglutinin affinity column. The purity was evaluated by SDS-PAGE followed by silver staining. Two major bands, migrating at 150 and 110 kDa on SDS-PAGE under nonreducing conditions (Fig. 7A, inset, right panel), corresponding to the immunoreactivity with the anti- $\alpha 3$ and anti- $\beta 1$ antibodies, were detected, respectively (data not shown). Then we analyzed *N*-glycan profiles of purified $\alpha 3\beta 1$ integrin by LC/MS and LC/MS/MS. The profiles of the *N*-linked oligosaccharides extracted from purified $\alpha 3\beta 1$ integrin of $Fut8^{+/+}$ and $Fut8^{-/-}$, respectively, are shown in Fig. 7A. They were obtained by full MS scan (m/z 450–2000) in the negative ion mode. The FT MS spectra of the peaks 1–7 (from $Fut8^{+/+}$) and peaks 1'–7' (from $Fut8^{-/-}$) are shown in Fig. 7B, respectively. The structures of carbohydrates in these peaks could be deduced from the m/z values of protonated ions obtained by FT MS and data-dependent MS/MS spectra. The oligosaccharides released from $\alpha 3\beta 1$ integrin of $Fut8^{+/+}$ (peaks 1–7) were assigned to fucosylated complex and hybrid type oligosaccharides, whereas those released from $\alpha 3\beta 1$ integrin of $Fut8^{-/-}$ (peaks 1'–7') were nonfucosylated forms. The data correspond to that of AAL lectin blot, revealing that $\alpha 3\beta 1$ integrin derived from $Fut8^{+/+}$ is highly modified by $Fut8$ and suggesting loss of core fucosylation will result in the deficiency of $\alpha 3\beta 1$ integrin function.

DISCUSSION

The physiological importance of fucose modification on proteins has been highlighted by the description of human congen-

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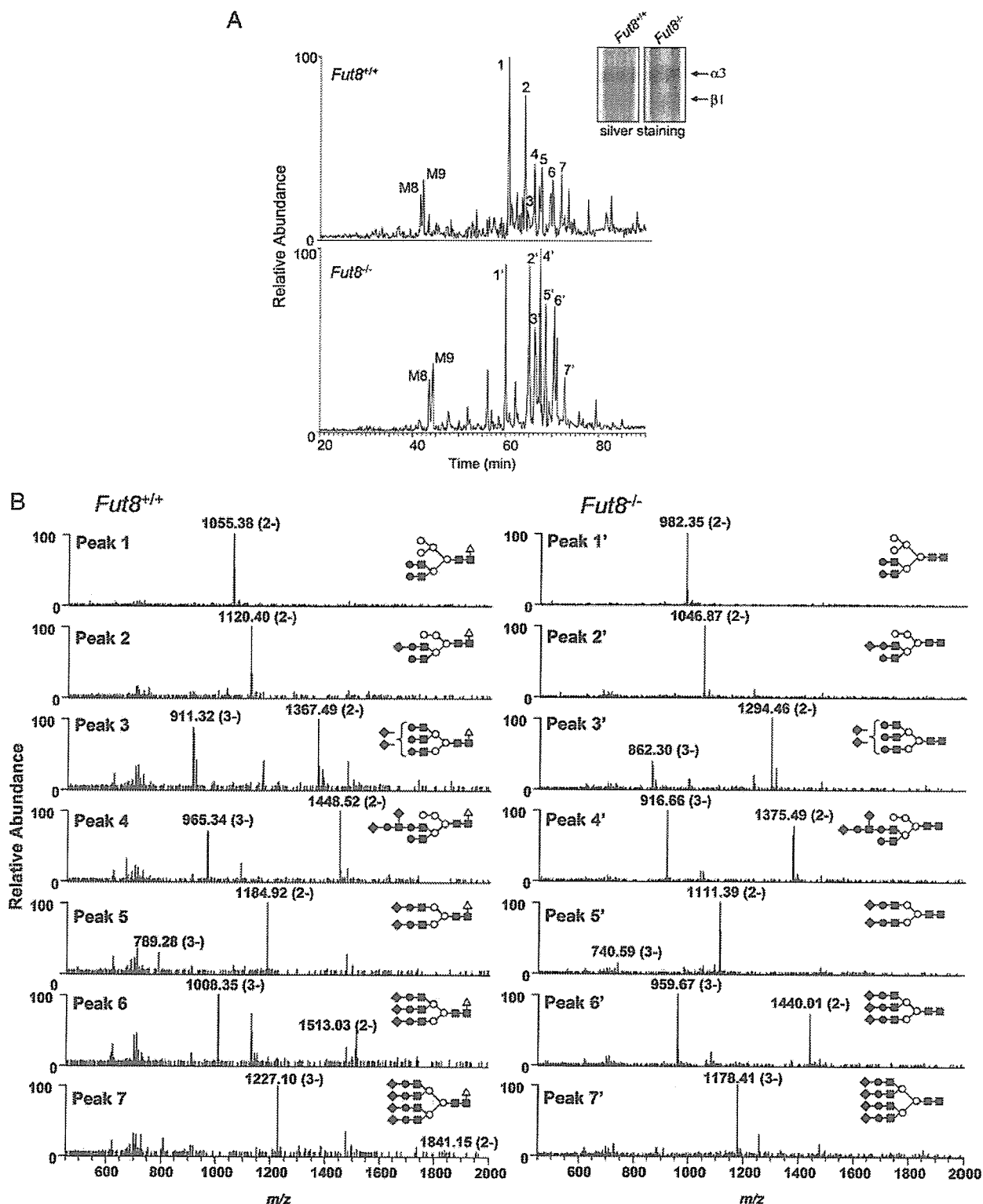


FIGURE 7. Chromatograms of N-linked oligosaccharides extracted from purified $\alpha 3\beta 1$ integrin from *Fut8^{+/+}* and *Fut8^{-/-}* cells. In A: MS, full MS scan (*m/z* 450–2000) in the negative ion mode. LC, pump A, 5 mM ammonium acetate, pH 9.6/2% CH_3CN ; Pump B, 5 mM ammonium acetate, pH 9.6/80% CH_3CN ; column, hypercarb (0.1 \times 150 mm); gradient, 5–20% of B (0–45 min) and 20–50% of B (45–90 min). The purity of $\alpha 3\beta 1$ integrin was verified by silver staining under nonreducing condition as shown in the right panel of the inset. B, FT MS spectra of N-glycans from purified $\alpha 3\beta 1$ integrin from *Fut8^{+/+}* and *Fut8^{-/-}* cells. Peaks 1–7 in *Fut8^{+/+}* cells, peaks 1'–7' in *Fut8^{-/-}* cells, and carbohydrate compositions assigned by *m/z* values of protonated ions and MS/MS spectra. Δ , fucose; \bullet , galactose; \circ , mannose; \blacksquare , N-acetylglucosamine; \blacklozenge , N-acetylneuraminic acid.

ital disorders of glycosylation (6). The congenital disorders of glycosylation-IIc disease is due to lack of the GDP-fucose transporter activity (43, 44), which mainly caused reduced terminal fucosylation of *N*-glycans (45, 46), and the core fucosylation is speculated to be responsible for the phenotype of congenital disorders of glycosylation-IIc (6). Recently, the loss of core fucosylation has been reported to down-regulate transforming growth factor- $\beta 1$ receptor and EGF receptor functions, which is thought to be related to the phenotype of emphysema and growth retardation of *Fut8*^{-/-} mice. In the present study, we found that the deficient core fucosylation results in the blockage of $\alpha 3\beta 1$ integrin-mediated cell migration and cell signaling. These results showed for the first time that in addition to the important physiological functions mentioned above, core fucosylation is also essential for the functions of $\alpha 3\beta 1$ integrin.

Several lines of evidence suggest that *N*-glycans are required for integrin activation. An increase in $\beta 1,6$ -branched sugar chains on $\alpha 5\beta 1$ integrin by GnT-V promotes cell migration on FN (26). Although the overexpression of GnT-III has been reported to inhibit $\alpha 5\beta 1$ integrin-mediated functions in HeLa S3 cells (27). It has also been reported that GnT-III and GnT-V can positively and negatively regulate $\alpha 3\beta 1$ integrin-mediated cell migration on LN5 (47). The modification of $\beta 1$ integrin by sialyltransferase makes this integrin capped with the negatively charged sugar, sialic acid, and contributes to cell motility and invasion (25). We found that cell migration on COL was barely detectable, suggesting that MEFs did not favor COL as an ECM for cell migration. In fact, we found that different cells may favor specific ECM for cell migration (27). We also found that core fucosylation had no significant difference in the cell migration on FN among wild-type, *Fut8*-KO, and rescued cells. This suggests that $\alpha 1,6$ -fucose modification has little or only mild effects on $\alpha 5\beta 1$ integrin, which is a receptor for FN. Actually, we previously reported that the introduction of the bisecting GlcNAc to the $\alpha 5$ subunit resulted in a reduced affinity in the binding of $\alpha 5\beta 1$ integrin to FN, therefore resulting in decreased cell migration (27). Thus, we assumed that the core fucosylation affected $\alpha 3$ subunit in a similar manner, which caused the decreased cell migration on LN5. However, the modification of $\alpha 1,6$ -fucose to $\alpha 5$ subunit may not affect their binding to FN. As described before, only *N*-glycans on some important domains of integrins, can contribute to the regulation of their functions (48). For example, the addition of a glycan at the $\beta 1$ or $\beta 3$ subunit I-like domains caused an increase in the distance between the head and stalk domains, therefore inducing the integrin dimer to adopt a more activated integrin conformation. Furthermore, it has recently been reported that the *N*-glycans only located on some specific sites of integrin $\alpha 5$ subunit play key roles in functional expression (49).

It has been reported that purified $\alpha 5\beta 1$ integrin from human placenta and purified $\alpha 3\beta 1$ integrin from the human ureter epithelium cell line HCV29 exhibited a highly heterogeneous glycosylation pattern, and >50% of these were fucosylated (50, 51). In this study, the $\alpha 3\beta 1$ integrin we purified from mouse embryonic fibroblast carried the bi-, tri-, and tetra-antennary complex types, and the majority of these were core-fucosylated. So it is easily postulated that core fucosylation may be important to integrin functions due to the abundance of it. However,

to our knowledge, no reports showing that core fucosylation regulates integrin functions have appeared to date. The fact that integrin-mediated migration and cell signaling were decreased in *Fut8*^{-/-} cells, and such inhibition was partly rescued by re-introduction of the *Fut8* gene to *Fut8*^{-/-} cells, strongly suggested that core fucosylation is important to $\alpha 3\beta 1$ integrin, and *Fut8*, like other important glycosyltransferases, plays an essential role in the regulation of integrin functions.

Although the precise reason for why the core fucosylation modifies these molecular functions remains to be elucidated, we proposed some possible mechanisms: *Fut8* may affect the cross-talk between growth factor receptors and integrin. It is well known that integrin mediated functions cooperatively with growth factor receptors in the control of cell proliferation, cell differentiation, cell survival, and cell migration in epithelial cells and fibroblasts (52), because integrins and growth factor receptors share many common elements in their signaling pathway (19). PC12 cells in a serum-free medium were plated on the plates without ECM coating and, when treated with EGF alone, failed to induce neurite formation (53), suggesting that the integration of the signaling pathway triggered by receptor and integrins is required for the regulation of PC12 cell differentiation. In our study, the association of integrin with EGF receptor was indicated by co-precipitation, and we found that the complex of $\alpha 3\beta 1$ integrin and EGF receptor in *Fut8*^{-/-} cells was decreased compared with *Fut8*^{+/+} cells.⁴ This may affect the signal integration of both partners and, thus, further affect the $\alpha 3\beta 1$ integrin-stimulated signal and cell migration, or deficient core fucosylation may cause the conformation of integrin to change. Luo *et al.* (48) have suggested that the changes in the glycan structures of integrin can affect its conformation and activity. They reported that in Chinese hamster ovary-K1 cells, the addition of a glycan at $\beta 1$ I-like domain caused an increase in the distance between the $\beta 1$ head and stalk domains, therefore inducing the integrin dimmer to be a more extended (activated) integrin conformation (48). Consistently, the affinity of the binding of $\alpha 5\beta 1$ integrin to fibronectin was significantly reduced by the introduction of the bisecting GlcNAc (27). So we supposed that core fucosylation contributes to stable conformation and normal activity of $\alpha 3\beta 1$ integrin to its ligand. However, we cannot exclude additional reasons that still remain to be determined.

The $\alpha 3$ integrin gene is expressed during the development of many epithelial organs, including the kidney (54), lung (55), and others. As a major basement membrane receptor in both kidney and lung during embryogenesis, $\alpha 3\beta 1$ integrin is likely to be involved in mediating signals between the mesenchyme and epithelial cells in the kidney and lung. The glomeruli of $\alpha 3$ -KO mice showed the abnormality in kidney, including disorganized glomerular basement membrane and a dramatic absence of foot process formation by podocytes (9). Therefore, it could be worthy to extensively examine the effects of core fucosylation on $\alpha 3\beta 1$ integrin *in vivo* in the future.

In conclusion, we demonstrate here some aspects of the biological significance of the core fucosylation of $\alpha 3\beta 1$ integrin-mediated

⁴ Y. Zhao, S. Itoh, X. Wang, T. Isaji, E. Miyoshi, Y. Kariya, K. Miyazaki, N. Kawasaki, N. Taniguchi, and J. Gu, unpublished data.



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ated cell migration and signaling. This study provides new insights into the biological functions of core fucosylation and the significance of the modification of *N*-glycans for $\alpha 3 \beta 1$ integrins.

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N-Acetylglucosaminyltransferase III Antagonizes the Effect of N-Acetylglucosaminyltransferase V on $\alpha 3\beta 1$ Integrin-mediated Cell Migration*

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N-Acetylglucosaminyltransferase V (GnT-V) catalyzes the addition of $\beta 1,6$ -GlcNAc branching of N-glycans, which contributes to metastasis. N-Acetylglucosaminyltransferase III (GnT-III) catalyzes the formation of a bisecting GlcNAc structure in N-glycans, resulting in the suppression of metastasis. It has long been hypothesized that the suppression of GnT-V product formation by the action of GnT-III would also exist *in vivo*, which will consequently lead to the inhibition of biological functions of GnT-V. To test this, we draw a comparison among MKN45 cells, which were transfected with GnT-III, GnT-V, or both, respectively. We found that $\alpha 3\beta 1$ integrin-mediated cell migration on laminin 5 was greatly enhanced in the case of GnT-V transfectant. This enhanced cell migration was significantly blocked after the introduction of GnT-III. Consistently, an increase in bisected GlcNAc but a decrease in $\beta 1,6$ -GlcNAc-branched N-glycans on integrin $\alpha 3$ subunit was observed in the double transfectants of GnT-III and GnT-V. Conversely, GnT-III knockdown resulted in increased migration on laminin 5, concomitant with an increase in $\beta 1,6$ -GlcNAc-branched N-glycans on the $\alpha 3$ subunit in CHP134 cells, a human neuroblastoma cell line. Therefore, in this study, the priority of GnT-III for the modification of the $\alpha 3$ subunit may be an explanation for why GnT-III inhibits GnT-V-induced cell migration. Taken together, our results demonstrate for the first time that GnT-III and GnT-V can competitively modify the same target glycoprotein and furthermore positively or negatively regulate its biological functions.

Malignant transformation is accompanied by increased $\beta 1,6$ -GlcNAc branching of N-glycans attached to Asn-X-Ser/Thr sequences in mature glycoproteins (1–3). N-Acetylglucosaminyltransferase V (GnT-V)³ catalyzes the addition of $\beta 1,6$ -linked GlcNAc (see Fig. 8) and defines this subset of N-glycans (4, 5). A relation between GnT-V and cancer metastasis has been reported by Dennis *et al.* (6) and Yamashita *et al.* (1). Studies on transplantable tumors in mice indicate that the product of GnT-V directly contributes to the growth of cancer and subsequent metastasis (7, 8). On the other hand, somatic tumor cell mutants that are deficient in GnT-V activity produce fewer spontaneous metastases and grow more slowly than wild-type cells (6, 9). The suppression of tumor growth and metastasis has been reported in GnT-V-deficient mice (3). Moreover, Partridge *et al.* (10) reported that GnT-V-modified N-glycans with poly-N-acetyllactosamine, the preferred ligand for galectin-3, on surface receptors oppose their constitutive endocytosis and result in promoting intracellular signaling and consequently cell migration and tumor metastasis. These results indicate that inhibition of GnT-V might be useful in the treatment of malignancies by targeting their roles in metastasis.

N-Acetylglucosaminyltransferase III (GnT-III) participates in the branching of N-glycans (see Fig. 8), catalyzing the formation of a unique sugar chain structure-bisecting GlcNAc (11). GnT-III is generally regarded to be a key glycosyltransferase in the N-glycan biosynthetic pathway, since *in vitro* the introduction of the bisecting GlcNAc results in the suppression of further processing and the elongation of N-glycans as the result of catalysis by other glycosyltransferases, which are unable to use the bisected oligosaccharide as a substrate (12, 13). It is interesting to note that the metastatic capabilities of B16 mouse melanoma cells are down-regulated by introduction of the GnT-III gene (14). E-cadherin, a homophilic type of adhesion molecule (15), is highly associated with the prevention of metastasis (16), and E-cadherin on GnT-III-transfected cell

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³ The abbreviations used are: GnT-V, N-acetylglucosaminyltransferase V; GnT-III, N-acetylglucosaminyltransferase III; ECM, extracellular matrix; LN5, laminin 5; FN, fibronectin; COL, collagen I; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; LC, liquid chromatography; MS, mass spectrometry; FT-ICR, Fourier transform ion cyclotron resonance.

surfaces was found to be resistant to proteolysis, resulting in an extended half-life of turnover (17). Thus, GnT-III, contrary to GnT-V, has long been thought to inhibit cancer metastasis.

Cell-extracellular matrix (ECM) interactions play essential roles during the acquisition of migration and invasive behavior of cells. Cell surface transmembrane glycoprotein-integrin is a major receptor for ECM and connects many biological functions, such as development, control of cell proliferation, protection against apoptosis, and malignant transformation (18). Integrin $\alpha3\beta1$, the major laminin 5 (LN5) receptor, is widely distributed in almost all tissues, and it has been proposed to be involved in tumor invasion (19–21). In some malignant tumors, $\alpha3\beta1$ integrin was found to be the most predominant integrin expressed (22) and made an important contribution to pulmonary metastasis (23). On the other hand, the glycosylation of integrins contributes to the tumor metastasis. Guo *et al.* reported that an increase in $\beta1,6$ -GlcNAc sugar chains of the integrin $\beta1$ subunit resulted in the stimulation of cell migration (24). Interestingly, it has also been reported that the $\alpha3\beta1$ integrin expressed by the metastasis human melanoma cell lines, contained a higher level of $\beta1,6$ -branched structures than that expressed in a nonmetastasis parent cell line (25).

Although it had been assumed that the reaction of GnT-V can be inhibited by the action of GnT-III, as evidenced by substrate specificity studies *in vitro*, the hypothesis of competition between GnT-III and GnT-V in cell migration and tumor metastasis has not been directly verified so far. In the present study, we examined the functions of $\alpha3\beta1$ integrin, which is believed to be highly associated with tumor metastasis, and found that $\alpha3\beta1$ integrin can be modified by either GnT-III or GnT-V. Our finding clearly shows that GnT-III inhibits the effects of GnT-V on $\alpha3\beta1$ integrin-mediated cell migration by competing with GnT-V for the modification of $\alpha3$ subunit.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Antibodies against integrin $\alpha3$ subunit (P1B5, I-19), monoclonal antibody against β -actin, mouse control IgG, and peroxidase-conjugated rabbit antibody against goat IgG were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Functional blocking antibody against the integrin $\beta1$ subunit was purchased from Chemicon International, Inc. (Temecula, CA). A peroxidase-conjugated goat antibody against mouse IgG was from Promega (Madison, WI). Biotinylated leucoagglutinating phytohemagglutinin (L_4 -PHA), biotinylated erythroagglutinating phytohemagglutinin (E_4 -PHA), and monoclonal antibodies against GnT-III and GnT-V were from Seikagaku Corp.

Cell Culture—Transfected MKN45 Cells were established as previously reported (26). Human gastric cancer cell line MKN45 cells were cultured in RPMI 1640 medium (Sigma) containing 10% fetal bovine serum (Invitrogen), penicillin (100 units/ml), and streptomycin (100 μ g/ml) under a humidified atmosphere containing 5% CO_2 . Human GnT-V cDNA (27) or GnT-III cDNA was inserted into a mammalian expression vector pCXNII (28). Vectors were then transfected into MKN45 cells by means of Lipofectamine (Invitrogen). Selection was performed by the addition of 500 μ g/ml G418 (Sigma). CHP134 cells, a human neuroblastoma cell line expressing endogenous

GnT-III and GnT-V, were cultured in RPMI 1640 medium (Sigma) containing 10% fetal bovine serum and penicillin (100 units/ml) and streptomycin (100 μ g/ml) under a humidified atmosphere containing 5% CO_2 .

Plasmids and Transient Virus Transfection—cDNAs encoding full-length human GnT-III or GnT-III inactive mutant (D317A) were ligated into adenoviral vector, constructed using an adenoviral expression vector kit (Takara Bio). The 3×10^5 MKN45 GnT-V transfectants were then infected with 150 μ l of virus solution (2×10^9 plaque-forming units/ml). After a 24-h incubation, the cultured medium was replaced with a fresh medium. 48 h later after infection, cells were subjected to various experiments.

Construction of Small Interfering RNA Vector and Retroviral Infection—Small interfering oligonucleotides specific for GnT-III were designed on the Takara Bio site on the World Wide Web, and the oligonucleotide sequences used in the construction of the small interfering RNA vector were as follows: 5'-GATCCGTCAACCACGAGTTTCGACCTTCAAGAGAGGTCGAACTCGTGGTTGACTTTTAT-3' and 5'-CGATAAAAAGTCAACCACGAGTTCGACCTCTCTGAAGGTCGAACTCGTGGTTGACG-3'. The oligonucleotides were annealed and then ligated into BamHI/ClaI sites of the pSINsi-hU6 vector (Takara Bio). A retroviral supernatant was obtained by transfection of human embryonic kidney 293 cells using the retrovirus packaging kit Ampho (Takara Bio) according to the manufacturer's protocol. CHP134 cells were infected with the viral supernatant, and the cells were then selected with 500 μ g/ml G418 for 2–3 weeks. Stable GnT-III knockdown clones were selected and confirmed by GnT-III activity and gene expression. Quantitative real time PCR analyses of GnT-III mRNA expression in these clones were performed with a Smart Cycler II System and the SYBR premix Taq (Takara Bio). Reverse transcription was carried out at 42 °C for 10 min, followed by 95 °C for 2 min using random primers, followed by PCR for 45 cycles at 95 °C for 5 s and 60 °C for 20 s with the following primers: 5'-GCGTCATCAACGCCATCAA-3' 5'-TGGACTCGCACACCACAAAG-3'. Normalization of the data were performed using the glyceraldehyde-3-phosphate dehydrogenase mRNA levels.

GnT-III and GnT-V Activity Assay—The activities of GnT-III and GnT-V were assayed as described previously (29, 30). Briefly, cell lysates were homogenized in phosphate-buffered saline (PBS) containing protease inhibitors. The supernatant, after removal of the nucleus fraction by centrifugation for 15 min at 900 $\times g$, was used in the assays, which involved high performance liquid chromatography methods using a pyridylaminated biantennary sugar chain as an acceptor substrate. Protein concentrations were determined using a bicinchoninic acid kit (BCA kit) (Pierce) with bovine serum albumin as a standard.

Western Blot and Lectin Blot Analysis—Cell cultures were harvested in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). Cell lysates were centrifuged at 15,000 $\times g$ for 10 min at 4 °C, the supernatants were collected, and the protein concentrations were determined using a BCA protein assay kit. Proteins were then immunoprecipitated from

GnT-III Counteracts the Effect of GnT-V

the lysates using a combination of 2 μg of anti-integrin $\alpha 3$ subunit antibody and 15 μl of protein G-Sepharose 4 Fast Flow (Amersham Biosciences) for 1 h at 4 °C. Immunoprecipitates were suspended in reducing sample buffer, heated to 100 °C for 3 min, resolved on 7.5% SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell). The blots were then probed with anti- $\alpha 3$ antibody or biotinylated E₄- or L₄-PHA. Immunoreactive bands were visualized using the Vectastain ABC kit (Vector Laboratories, CA) and an ECL kit (Amersham Biosciences). For GnT-III, GnT-V, cell lysate, and actin blotting, an equal amount of cell lysates was subjected to SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were incubated with the corresponding primary antibodies and secondary antibodies for 1 h each, and detection was performed by an ECL kit.

Cell Surface Biotinylation—Cell surface biotinylation was performed as described previously with minor modifications (31). Briefly, various semiconfluent transfected MKN45 cells were washed twice with ice-cold PBS and then incubated with ice-cold PBS containing 0.2 mg/ml sulfo succinimidobiotin (Pierce), for 3 h at 4 °C. After incubation, the cells were washed three times with ice-cold PBS, scraped, and lysed with radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 1 mM sodium orthovanadate, 2 $\mu\text{g}/\text{ml}$ aprotinin, 5 $\mu\text{g}/\text{ml}$ leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The resulting cell lysates were immunoprecipitated with anti- $\alpha 3$ antibody, as described above. The immunocomplex was subjected to 7.5% SDS-PAGE and then transferred to a nitrocellulose membrane. After blocking the membranes with 3% (w/v) skim milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST, pH 7.5), the biotinylated proteins were visualized using a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) and an ECL kit.

Migration Assay—Transwells (BD Biosciences) were coated with 5 nm recombinant LN5 as described previously (32), 10 $\mu\text{g}/\text{ml}$ human plasma FN, and collagen I (COL) (Sigma) in PBS by an incubation overnight at 4 °C. Serum-starved cells (2×10^5 cells/well in 500 μl of 5% fetal calf serum medium) were seeded in the upper chamber of the plates. After incubation overnight at 37 °C, cells in the upper chamber of the filter were removed with a wet cotton swab. Cells on the lower side of the filter were fixed and stained with 0.5% crystal violet. Each experiment was performed in triplicate, and counting was done in three randomly selected microscopic fields within each well.

Functional Blocking Assay—To identify which integrin is involved in cell migration on LN5, functional blocking antibodies against different types of integrins were individually preincubated with cells for 10 min at 37 °C. The preincubated cells were transferred into transwells coated with LN5 and then incubated overnight at 37 °C. The migrated cells were then quantified as described above.

Statistical Analysis—Statistical evaluations were performed using Student's *t* test; differences among experimental groups were considered significant for $p < 0.05$. Data were expressed as mean values \pm S.D.

Purification of $\alpha 3\beta 1$ Integrin—The purification of $\alpha 3\beta 1$ integrin was performed as described previously (33). Briefly,

cells in confluent were detached with TBS(+) (20 mM Tris-HCl, pH 7.5, 130 mM NaCl, 1 mM CaCl₂, and 1 mM MgCl₂) and washed with TBS(+). The cell pellets were extracted with 50 mM Tris/HCl containing 15 mM NaCl, 1 mM MgCl₂, 1 mM MnCl₂, pH 7.4, and protease inhibitor mixture (Roche Applied Science), 100 mM octyl- β -D-glucopyranoside at 4 °C. The cell extract was applied to an affinity column prepared by coupling 5 mg of the GD6 peptide of laminin $\alpha 1$ chain (33) (KQNCLSSRASFRGCVRNLRSLR residues numbered 3011–3032) (Peptide Institute, Inc., Osaka, Japan) to 1 ml of activated CH-Sepharose (Sigma). The bound $\alpha 3\beta 1$ integrin was eluted with 20 mM EDTA in 50 mM Tris/HCl, pH 7.4, containing 100 mM octyl- β -D-glucopyranoside. The elutes containing $\alpha 3\beta 1$ integrin were further purified on a 1-ml wheat germ agglutinin-agarose column (Seikagaku Corp., Tokyo, Japan) and eluted with 0.2 M *N*-acetyl-D-glucosamine containing 100 mM octyl- β -D-glucopyranoside.

Analysis of *N*-Glycan Structures by Mass Spectrometry (LC/MSⁿ)—Purified $\alpha 3\beta 1$ integrin was applied to SDS-PAGE, and the $\alpha 3$ subunit was excised from the gel and then cut into pieces. The gel pieces were destained and dehydrated with 50% acetonitrile. The protein in the gel was reduced and carboxymethylated by the incubation with dithiothreitol and sodium monoiodoacetate (34). *N*-Glycans were extracted from the gel pieces as reported by Kustar *et al.* (35) and reduced with NaBH₄. Half of the extracted oligosaccharides were incubated with α -neuraminidase from *Arthrobacter ureafaciens* in 50 mM phosphate buffer, pH 5.0, at 37 °C for 18 h and desalted with Envi-carb (Supelco, Bellefonte, PA). LC/MS and LC/multistage MS (MSⁿ) was carried out on a quadrupole liner ion trap-Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS; Finnigan LTQ FTTM, Thermo Electron Corp., San Jose, CA) connected to a nano-LC system (Paradigm, Michrom BioResource, Inc., Auburn, CA). The eluents were 5 mM ammonium acetate, pH 9.6, 2% CH₃CN (pump A) and 5 mM ammonium acetate, pH 9.6, 80% CH₃CN (pump B). The borohydride-reduced *N*-linked oligosaccharides were separated on a Hypercarb (0.1 \times 150 mm, Thermo Electron Corp.) with a linear gradient of 5–20% B in 45 min and 20–50% B in 45 min. A full MS¹ scan (m/z 450–2000) by FT-ICR MS followed by data-dependent MS^{2,3} for the most abundant ions was performed in both negative and positive ion modes as previously reported (36).

RESULTS

Overexpression of GnT-V Stimulated $\alpha 3\beta 1$ Integrin-mediated Cell Motility—It has been reported that overexpression of GnT-V in epithelial cells results in a loss of contact inhibition, increased cell motility in athymic nude mice (7), and an enhanced metastasis (8). In this study, experiments were first designed to determine whether GnT-V overexpression could affect cell migration on different ECMs. The extent of haptotaxis toward LN5, FN, and COL, specific ligands for $\alpha 3\beta 1$, $\alpha 5\beta 1$, and $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrin, respectively, was observed in MKN45 cells transfected with mock, GnT-III, or GnT-V. In the case of the GnT-V transfectants on LN5, the number of transwell cells migrating to the lower surface of the membrane was considerably increased ($p = 0.001$), the overexpression of

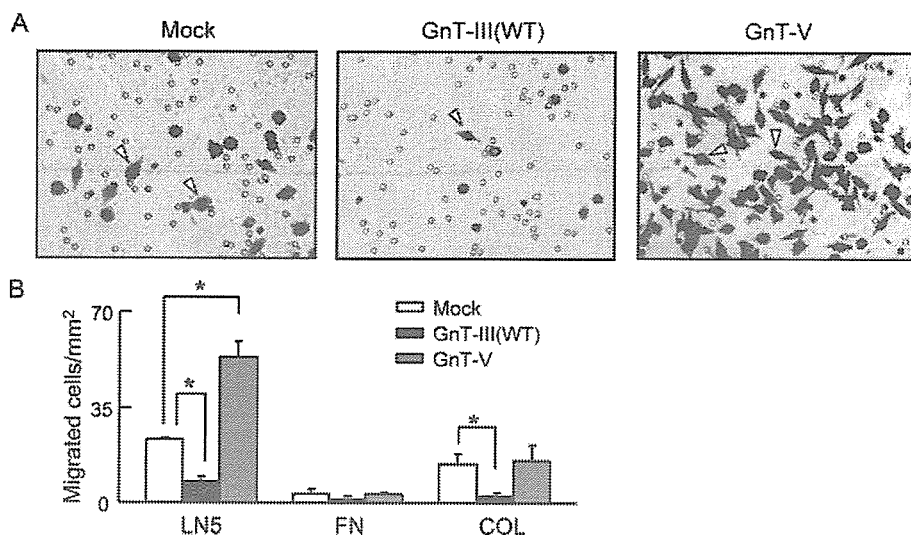


FIGURE 1. Increased cell migration induced by GnT-V on LN5. MKN45 cells were replated on the upper chamber in the presence of 5% fetal bovine serum. Cell migration was determined using the Transwell assay as described under "Experimental Procedures." After incubation overnight, the cells that had migrated to the lower surface of the membrane were fixed and stained with 0.3% Crystal Violet. *A*, cell migration on LN5 (5 nM). Representative fields were photographed using a phase-contrast microscope. The arrowheads indicate migrated cells. *B*, quantification of migration on LN5 (5 nM), COL (15 nM), and FN (15 nM). The numbers of migrated cells were quantified and expressed as the means \pm S.D. from three independent experiments. WT, wild type.

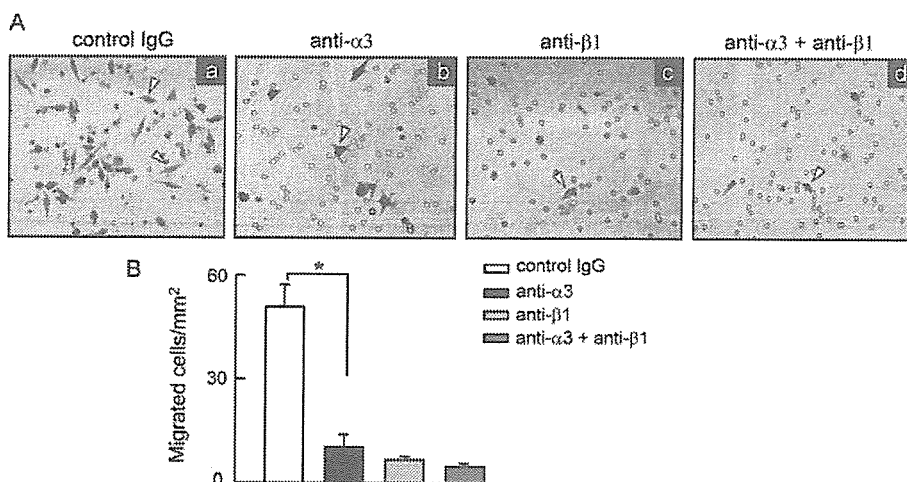


FIGURE 2. GnT-V induced cell migration was mediated by α 3 β 1 integrin. *A*, GnT-V-transfected MKN45 cells were detached, preincubated with mouse control IgG (*a*) or function-blocking monoclonal antibodies against α 3 (*b*) or β 1 (*c*) or both (*d*) for 10 min, and then replated on the upper chamber coated with LN5 (5 nM) and checked by Transwell assay. Representative fields were photographed using a phase-contrast microscope. The arrowheads indicate migrated cells. *B*, quantification of migration on LN5 (5 nM). The numbers of migrated cells were quantified and expressed as the means \pm S.D. from three independent experiments.

GnT-III resulted in a decrease in cell migration on LN5 compared with mock ($p = 0.0013$) (Fig. 1*A*). However, the migration of these three types of cells on FN was barely detectable. Although GnT-III transfection resulted in a decreased cell migration on COL compared with mock ($p = 0.007$), GnT-V transfection failed to induce a significant increase in cell migration on COL (Fig. 1*B*), suggesting that MKN45 cells may favor LN5 as an ECM for cell migration induced by GnT-V. These results further supported the view that α 3 β 1 integrin, one of the most abundant integrins in epithelial cells, is distinct from other integrins, such as α 5 β 1 integrin, and preferentially pro-

motes cell migration (37). Moreover, the cell migration of GnT-V transfectant on LN5 was strongly inhibited by the presence of function-blocking antibodies against integrin α 3 or/and β 1 subunit, suggesting that the GnT-V-induced cell migration on LN5 was mainly mediated by α 3 β 1 integrin (Fig. 2). These results indicated that overexpression of GnT-V resulted in an increase in α 3 β 1 integrin-mediated cell motility.

Overexpression of GnT-III Inhibited α 3 β 1 Integrin-mediated Cell Migration Induced by GnT-V—The Overexpression of GnT-III has been reported to inhibit cell migration by enhancement of E-cadherin-mediated homotypic adhesion (17) and by inhibiting α 5 β 1 integrin-mediated cell migration (38). In addition, *in vitro* GnT-V cannot use the product of GnT-III, a bisected oligosaccharide, as a substrate (12), so experiments were then designed to determine whether the introduction of GnT-III prevents α 3 β 1 integrin-mediated cell migration enhanced by GnT-V. The efficiency of transfection was confirmed by immunostaining with anti-GnT-III antibody and determined to be more than 80% (data not shown). As shown in Fig. 3, the transfection of GnT-III into the GnT-V transfectant resulted in a significant decrease in cell migration compared with the GnT-V transfectant ($p = 0.002$). However, the inhibition was not observed after transfection of the GnT-III-inactive mutant, suggesting that the activity of GnT-III was essential for the negative regulation of GnT-V-induced cell migration. Therefore, we proposed that GnT-III directly counteracted

the effect of GnT-V on α 3 β 1 integrin-mediated cell migration.

Transfection of GnT-III Had No Effect on the Expression of GnT-V and Integrin α 3 Subunit—To explore the possible mechanisms involved in the inhibition of GnT-III- to GnT-V-induced cell migration, we first attempted to determine whether the overexpression of GnT-III affected the expression of GnT-V and α 3 subunit expressed on the cell surface by means of blotting a total cell lysate with the GnT-III antibody and the biotinylation of cell surface proteins followed by immunoprecipitation of α 3 using the corresponding antibody, since *N*-glycosylation plays an important role in the quality control of