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Figure caption

Figure 1

Relative quantities of transferrin oligosaccharides from sample A.

The amounts of all glycoforms in total oligosaccharides are presented. Abbreviations are those presented in Table 1. (a) The laboratory numbers for the data are not the same as the numbers given to the authors. (*) The error bars representing standard deviation (SD) from repeated measurements by lab-12 (n=7) and lab-16 (n=2) show intra-assay variance. The SDs of lab-12 were quite small. (b) Comparison of the quantitation by different methods. The data from different laboratories were averaged: chromatography (n=4), a combination of permethylation and MALDI MS (n=7), and LC/ESI MS (n=2). Lab-5 was excluded from the calculation (see text). The error bars representing SD indicate inter-laboratory, or inter-assay, variance.

Figure 2

Relative quantities of IgG oligosaccharides from sample B.

Three bars on the left, for each laboratory, show the relative abundance of differently galactosylated species as a percentage of the total. That for monogalactosylated biantennary oligosaccharide bearing bisecting GlcNAc represents the content of total oligosaccharides identified. (a) The laboratory numbers for the data are not the same as the numbers given to the authors. (*) The error bars representing standard deviation (SD) from repeated measurements by lab-12 (n=7) and lab-16 (n=2) show intra-assay variance. The SDs of lab-12 were quite small. (b) Comparison of the quantitation by different methods. The data from different laboratories were averaged: chromatography (n=4), a combination of permethylation and MALDI MS (n=5), and LC/ESI MS (n=2). Lab-4 was excluded from the calculation. The error bars representing SD indicate inter-laboratory, or inter-assay, variance.

Figure 3

MALDI mass spectrum of permethylated oligosaccharides from sample A transferrin.

Oligosaccharides released by PNGase F were permethylated, and analyzed by MALDI TOF MS in positive ion and reflectron mode. The signals from polyhexose as an internal calibrant are indicated by asterisks. The mass spectrum was provided by lab-7.

Figure 4

MALDI mass spectra of permethylated oligosaccharides from sample B IgG.

The mass spectrum was provided by lab-8.

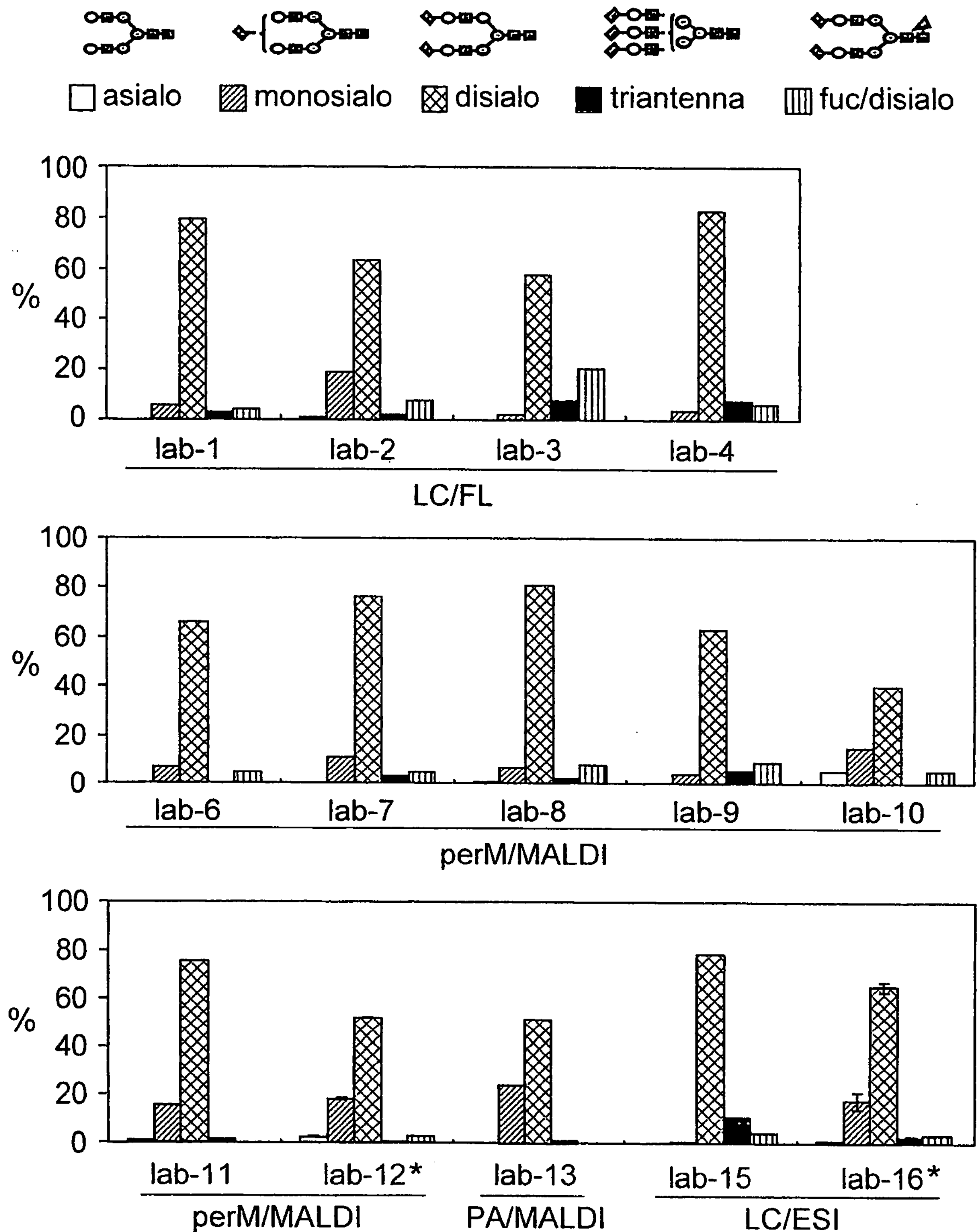
Figure 5

Mass spectrometric analysis of glycopeptides for site-specific glycan profiling of sample A

transferrin.

(a) Relative abundances of the oligosaccharides at each *N*-glycosylation site measured by RP-LC/ESI MS/MS (lab-15) or MALDI linear TOF MS of tryptic glycopeptides (lab-17). (*) The error bars representing standard deviation (SD) from repeated measurements by lab-17 (n=5) show intra-assay variance. (b, c) MALDI mass spectra of tryptic glycopeptides. The glycopeptides containing Asn-432 (b) or Asn-630 (c) were isolated by reversed phase chromatography and analyzed by MALDI linear TOF MS (lab-17). The ions indicated by asterisks are derived from the glycosidic cleavage during measurements.

Figure 1a (Wada)



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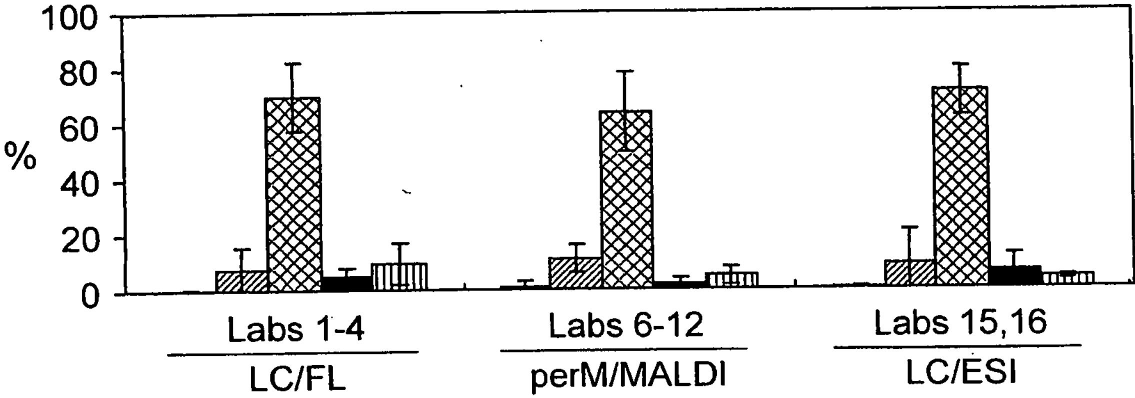

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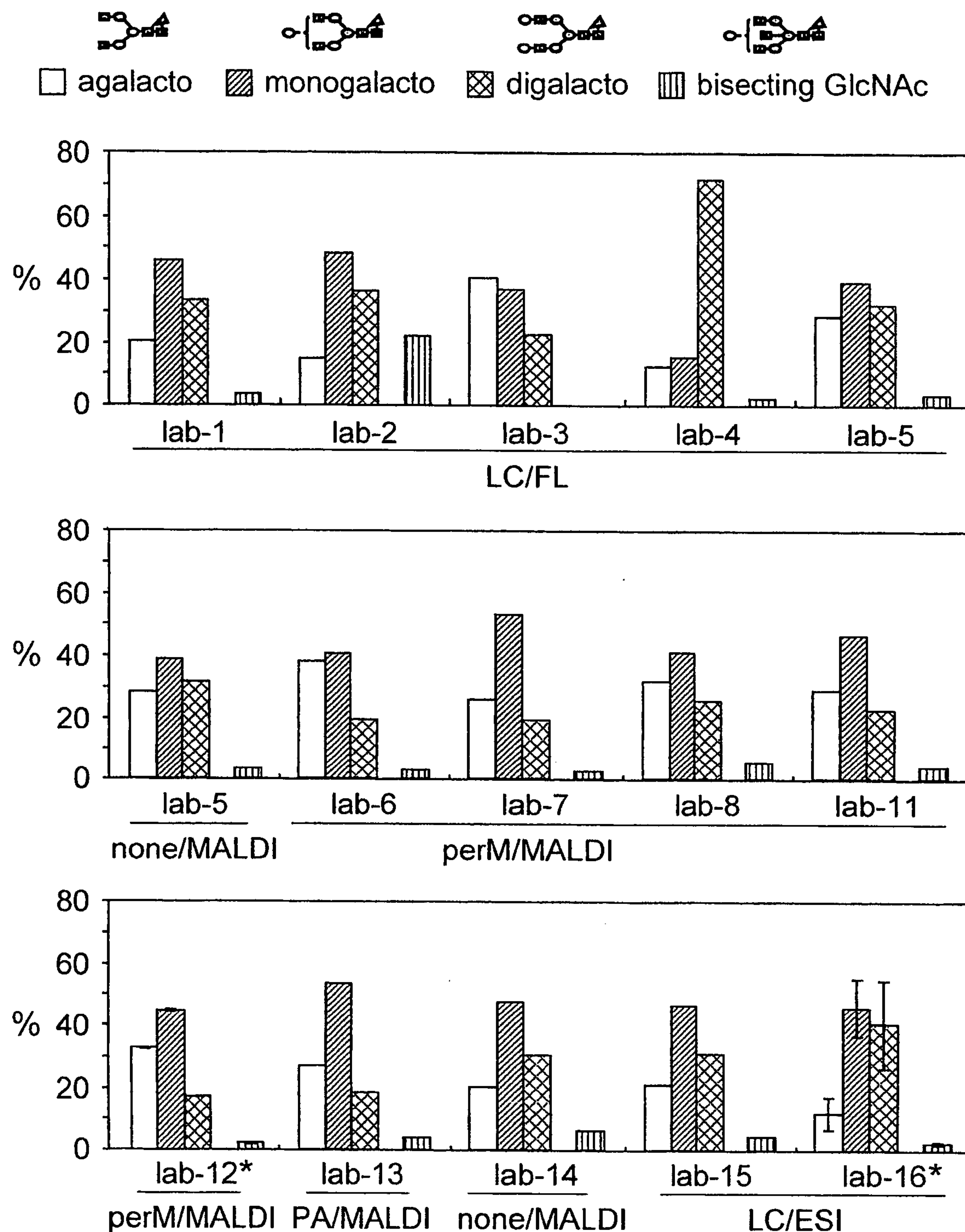

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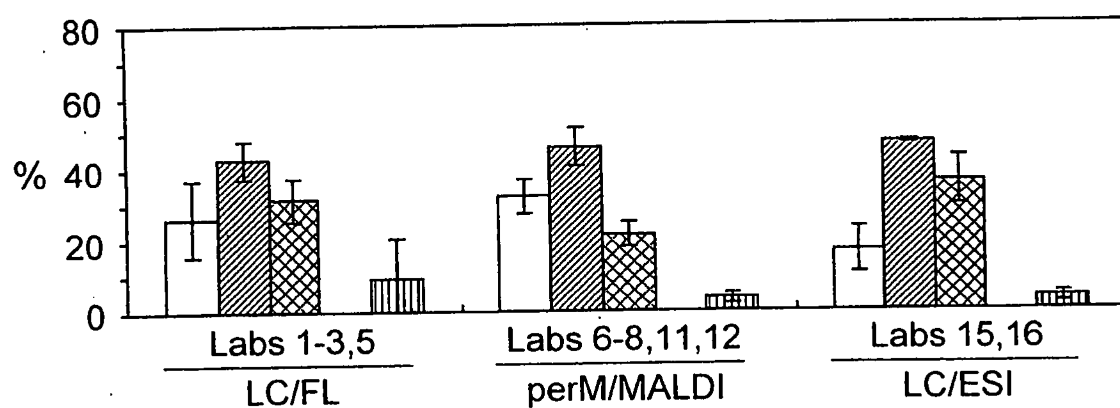

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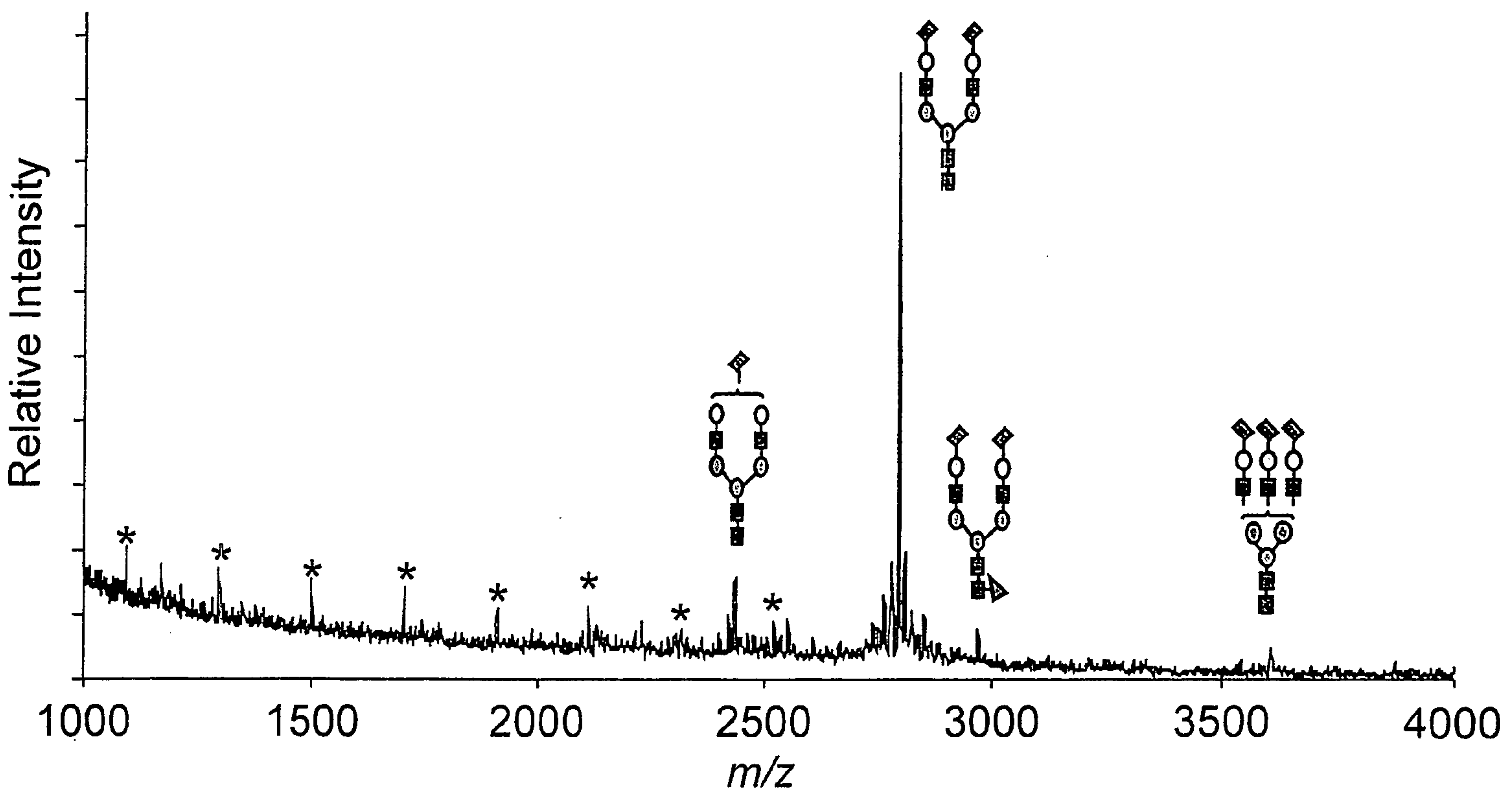

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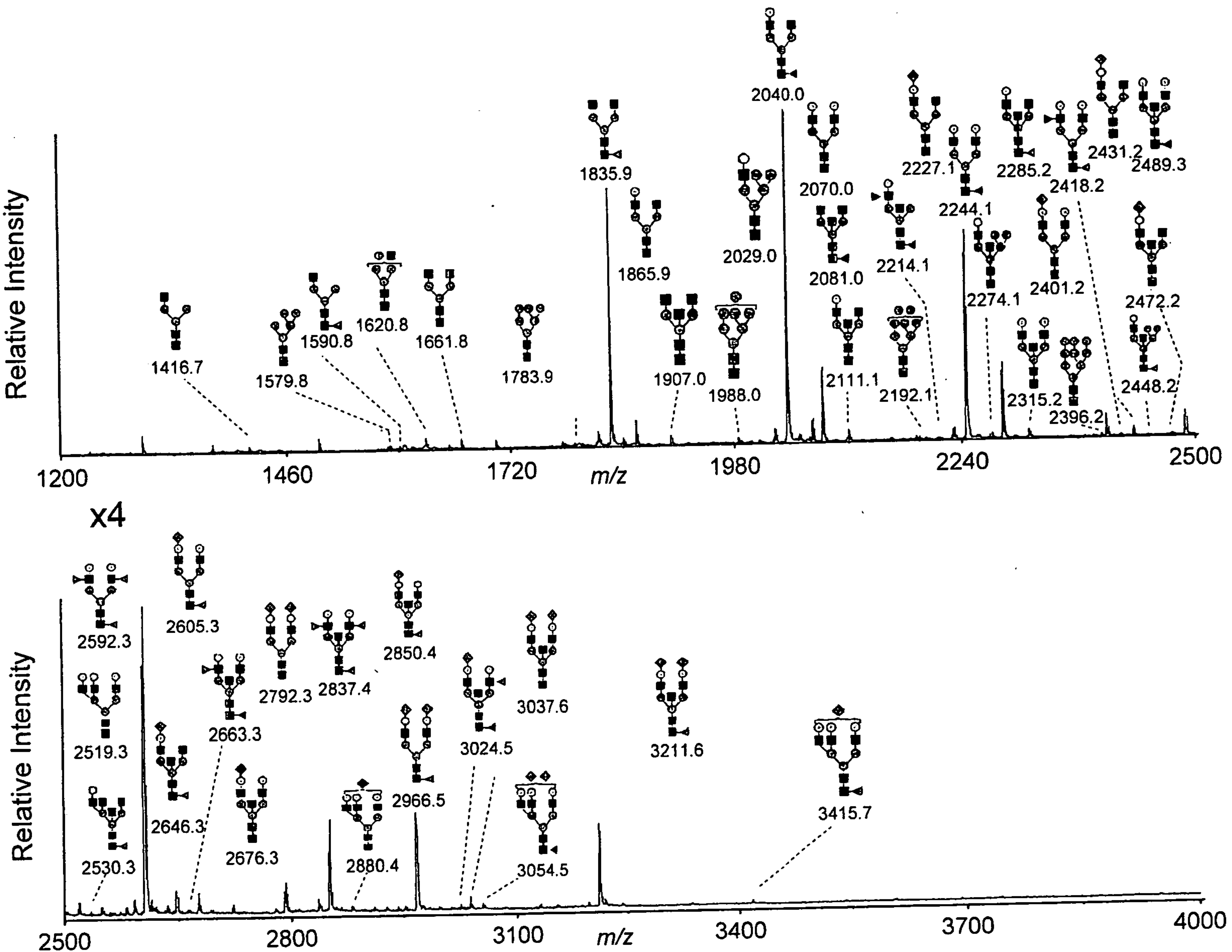

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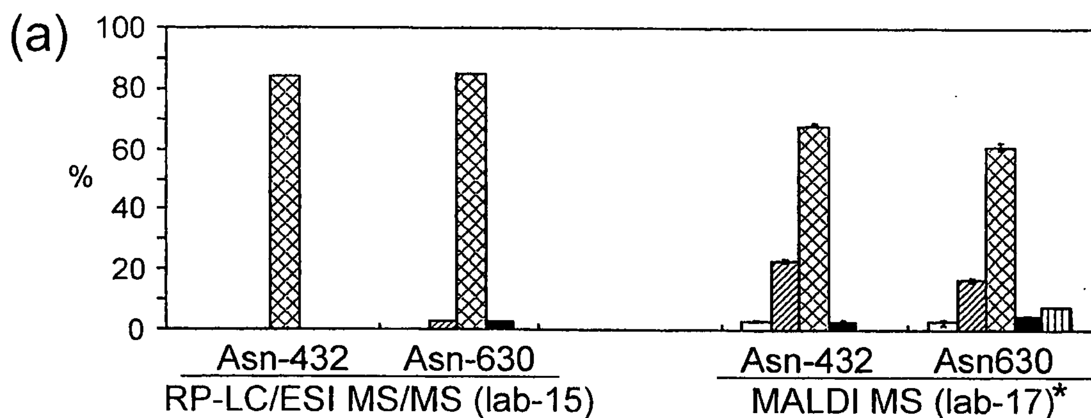

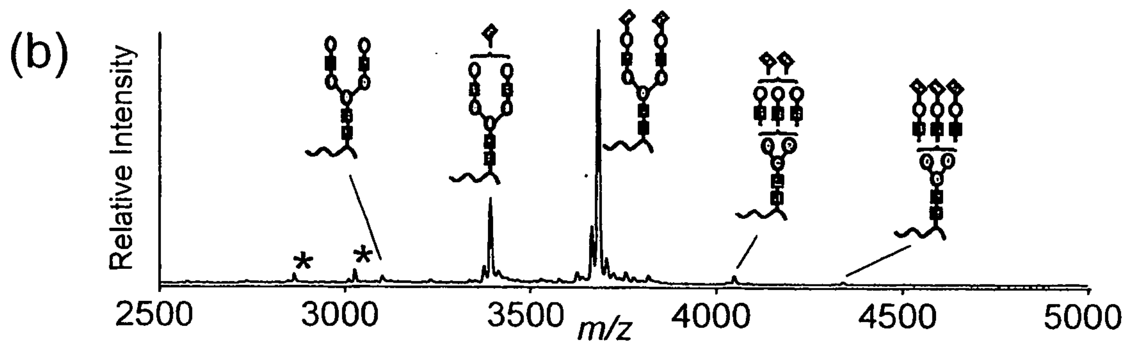

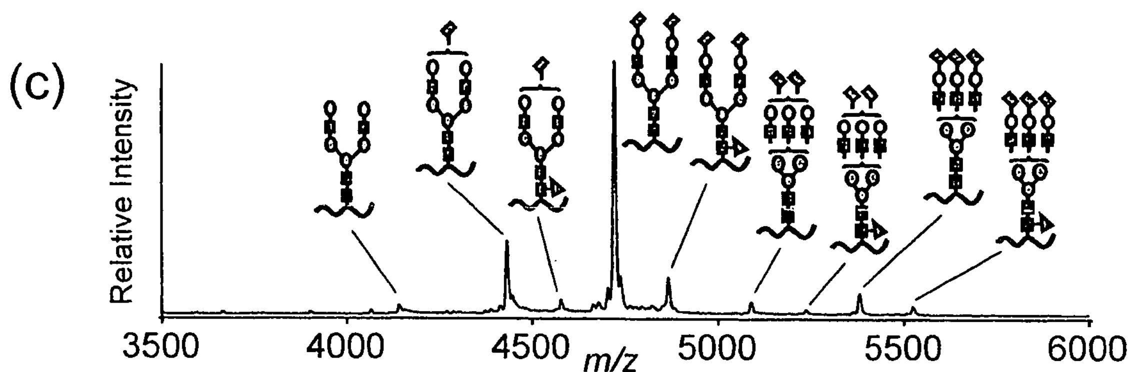

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Deletion of Core Fucosylation on α3β1 Integrin Down-regulates Its Functions*

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The core fucosylation (α1,6-fucosylation) of glycoprotein is widely distributed in mammalian tissues. Recently α1,6-fucosylation has been further reported to be very crucial by the study of α1,6-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 α3β1 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 α3β1 integrin-mediated events has been studied by using $Fut8^{+/+}$ and $Fut8^{-/-}$ embryonic fibroblasts, respectively. We found that the core fucosylation of α3β1 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 α3β1 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 α3β1 integrin.

α1,6-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 (α1,6-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-β1 receptor, which consequently resulted in a marked dysregulation of transforming growth factor-β1 receptor activation and signaling. We also found that the loss of core fucosylation resulted in the down-regulation of EGF[3] 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, α3β1 integrin, the major receptor for laminin 5 (LN5), is widely distributed in almost all tissues, and α3 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 α3β1 integrin, triggering haptotaxis (10). Especially, the G3 domain is essential for the unique activity of LN5, such as promotion of cell migration (11). Furthermore, α3β1 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, NeuAcα2,3Galβ1,4Glc-ceramide.

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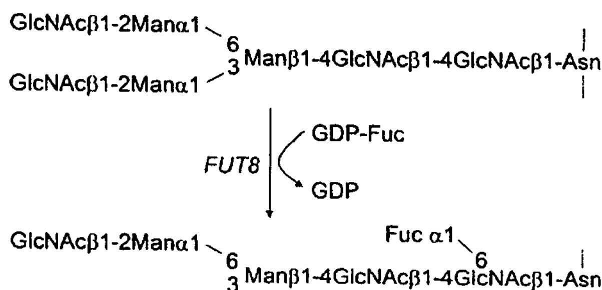

FIGURE 1. **Reaction pathway for the biosynthesis of core fucose by *Fut8*.** *Man*, mannose; *Fuc*, fucose; *GDP-Fuc*, guanosinediphospho-fucopyranoside; *Asn*, asparagine.

of α3β1 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, α3β1 integrin is found to be the most predominant integrin expressed (15), and cell invasion on ECM could be inhibited by antibodies against α3 integrin (13) and β1 integrin (14). Thus, α3β1 integrin, which mediates to laminins of basement membrane, preferentially promotes cell migration and metastasis (16–18). Given its various biological functions, α3β1 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 α3 and β1 subunits expressed by the metastasis human melanoma cell lines carry β1,6-branched structures, and these cancer-associated glycan chains may modulate tumor cell adhesion by affecting the ligand properties of α3β1 integrin (23). The linkage and expression levels of the terminal sialic acids of α3β1 integrin play an important role in cell-ECM interactions (24, 25). An increase in β1,6-GlcNAc sugar chains of β1 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 α5β1 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 α3β1 integrin-stimulated events, and our finding for the first time showed that core fucosylation is required for the functions of α3β1 integrin.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—A polyclonal antibody against mouse α3 integrin and functional blocking monoclonal antibody (mAb) against α2β1 integrin were obtained from Chemicon International, Inc. (Temecula, CA). mAbs against α3 integrin, FAK, FAK (pY397), and functional blocking mAbs against integrin α6 and β1 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 μg/ml Zeocin, and restored cells were maintained in Dulbecco's modified Eagle's medium in the presence of 400 μg/ml Zeocin and 400 μg/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 $MgCl_2$, 1 mM benzamidine, 60 mM β-glycerophosphate, 1 mM Na_3VO_4, 20 mM NaF, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride). Cell lysates were centrifuged at 15,000 × *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 μg of anti-α3 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-α3 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 sulfosuccinimidobiotin (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-α3 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 μg/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 μl 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 μg/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 α3 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'-GATCCGCTATGGAGAATCACACTGATTCAAGAGATCAGTGTGATTCTCCATAGCTTTTTTG-3' and 5'-AATTCAAAAAAGCTATGGAGAATCACACTGATCTCTTGAATCAGTGTGATTCTCCATAGCG-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 fibroblasts cells were infected with the viral supernatant, and the cells were then selected with 15 μg/ml puromycin for 2–3 weeks. Stable α3 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 α3β1 Integrin—The purification of α3β1 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 α1 chain (30) (KQNCLSSRASFRGCVRNLRLSR residues numbered 3011–3032, Peptide Institute, Inc., Osaka, Japan) to 1 ml of activated CH-Sepharose (Sigma). The bound α3β1 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 α3β1 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 α3β1 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 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 FT™, 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 × 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).

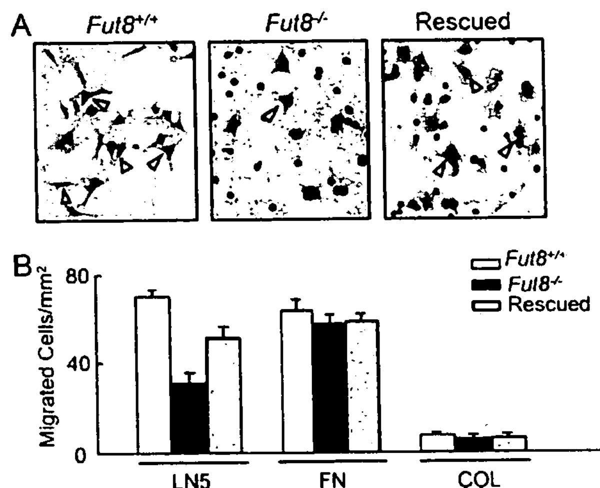

FIGURE 2. **Effects of deficient core fucosylation on cell migration on LN5 but not on FN.** *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells were replated on the upper chamber coated with LN5 (5 nM), FN (15 nM), or 50 μg/ml COL. Cell migration was determined using the Transwell assay described under "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 ± S.D. from three independent experiments.

RESULTS

Impaired α3β1 Integrin-mediated Cell Migration Was Found in Fut8$^{-/-}$ *Cells*—One of the major functions of α3β1 integrin is promotion of cell migration. In some malignant tumors, α3β1 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 α5β1

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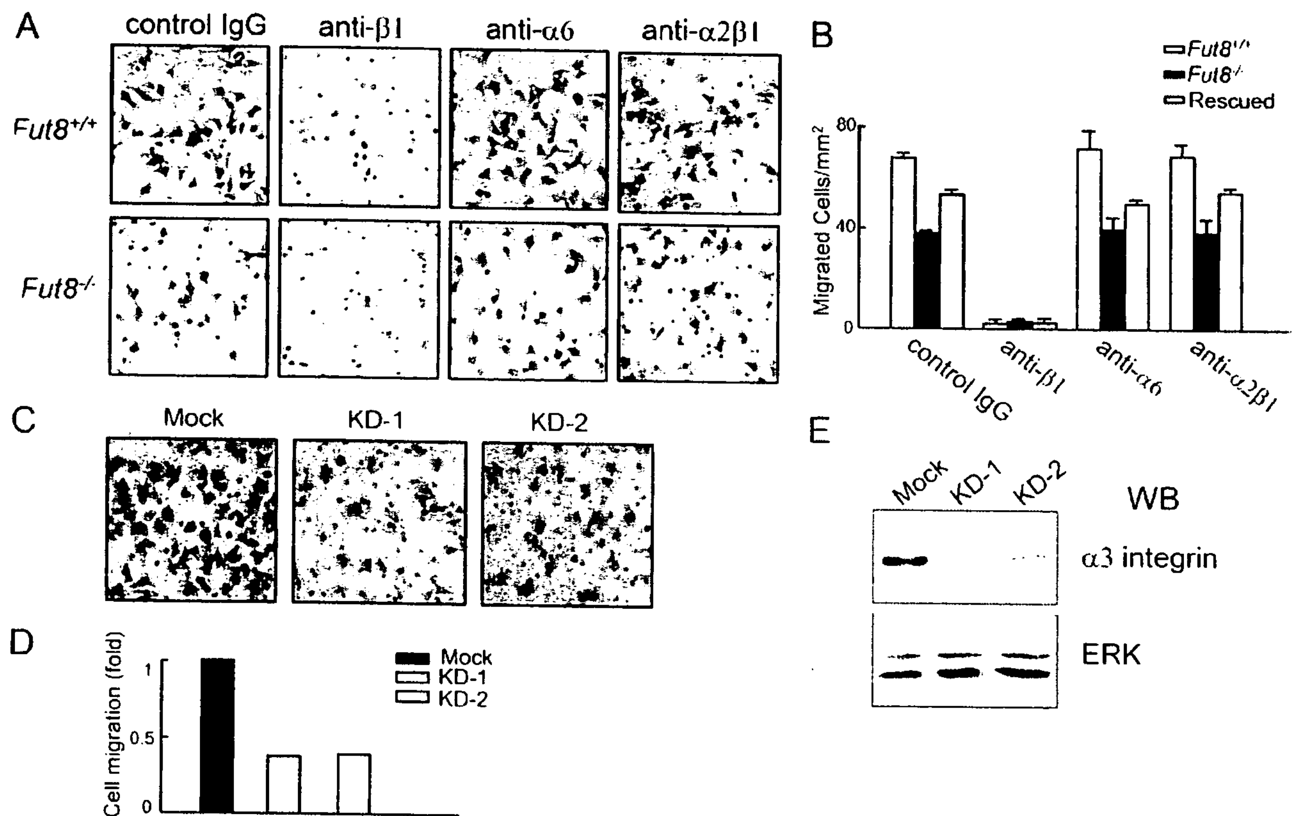

FIGURE 3. **Cell migration on LN5 was mediated by α3β1 integrin.** *A*, *Fut8*, *Fut8*, and rescued cells were detached, preincubated with mouse control IgG or function-blocking mAbs against β1, α6, or α2β1 integrin for 10 min, replated on the upper chamber coated with LN5 (5 nM), and checked by Transwell assay. Representative fields were photographed using a phase-contract microscope. *B*, the numbers of migrated cells were quantified and expressed as the means ± S.D. from three independent experiments. *C*, cell migration of α3-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 α3-knockdown cells. The numbers of migrated cells were quantified and expressed as the means ± S.D. from three independent experiments. *E*, α3-knockdown was confirmed by blotting total cell lysates with anti-α3 antibody (*upper panel*), and equal loading was confirmed by probing with an antibody against total protein ERK1/2 (*lower panel*). *KD1* and *KD2*, α3-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 α1β1 and α2β1 integrins, was barely detectable (Fig. 2*B*). This suggested that α5β1, α1β1 and α2β1 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 β1 but not by α6 or α2β1 integrin antibodies (Fig. 3, *A* and *B*), further excluding the involvement of α6 and α2β1 integrin on LN5-stimulated cell migration. However, so far the function-blocking antibody against mouse α3 is unavailable. To definitely confirm the important function of integrin α3 subunit for the cell migration on LN5, we utilized an RNA interference strategy to silence α3 in MEF cells. After retroviral infection, the cells were selected based on their resistance to puromycin as described under "Experimental Procedures." Expression of α3 but not α5 (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 α3-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 α3β1 integrin. This result was consistent with the view of previous study that α3β1 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 α3β1 integrin, but not α6β1 or α6β4 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 α3β1 integrin is a key molecule for cell migration on LN5 in the embryonic fibroblasts and that core fucosylation regulates α3β1 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 α3β1 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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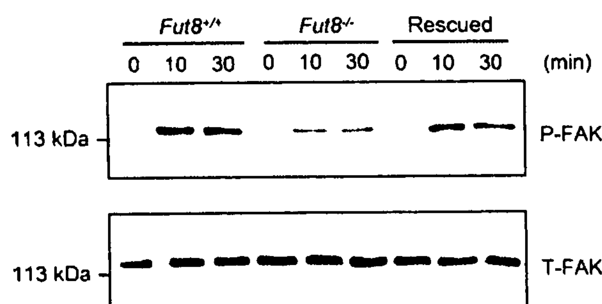

FIGURE 4. **Comparison of tyrosine phosphorylation levels of FAK among $Fut8^{+/+}$ and $Fut8^{-/-}$ and rescued cells on LN5.** Serum-starved cells were detached and held in suspension for 60 min to reduce the detachment-induced activation and then replated on dishes coated with LN5 (5 nM) for the indicated 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.

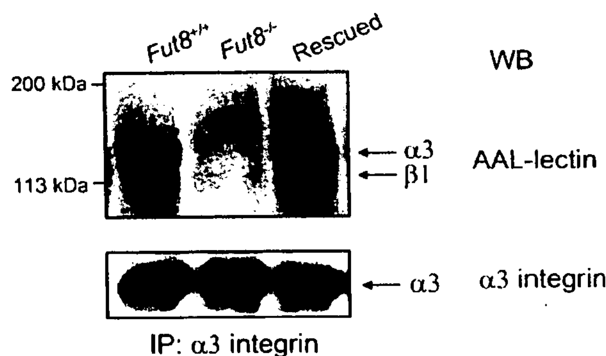

FIGURE 5. **Glycosylation analysis of α3β1 integrin from $Fut8^{+/+}$, $Fut8^{-/-}$, and rescued cells.** Whole cell lysates were immunoprecipitated (*IP*) with anti-α3 integrin antibody, and the resulting immunocomplexes were subjected to 7.5% SDS-PAGE under nonreducing condition. After electroblotting, the blots were probed, respectively, by AAL (*upper panel*) and an anti-α3 integrin antibody (*lower panel*).

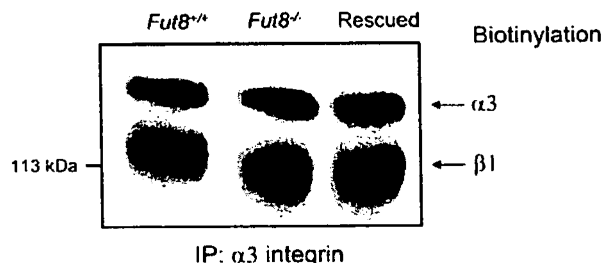

FIGURE 6. **Effects of core fucosylation on expression levels of α3β1 integrin on cell surface.** $Fut8^{+/+}$, $Fut8^{-/-}$, and rescued cells were biotinylated, whole lysates were immunoprecipitated (*IP*) with anti-α3 integrin antibody, the samples were subjected to 7.5% SDS-PAGE and transferred to a nitrocellulose membrane, and the biotinylated proteins were then detected as described under "Experimental Procedures."

atively regulate α3β1 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 α3β1 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 α3β1 integrin among $Fut8^{+/+}$, $Fut8^{-/-}$, and rescued cells has been examined by using blotting of α3 integrin-immunoprecipitated lysates with AAL lectin (*upper panel*). Equal loadings were verified by blotting with α3 integrin antibodies (*lower panel*). As shown in Fig. 5, the levels of core fucosylation in both α3 and β1 subunits were abolished in $Fut8^{-/-}$ cells consistent with no *Fut8* activity in these cells (7), whereas they were rescued by reintroduction of *Fut8*, suggesting α3β1 integrin is the target of *Fut8*. Furthermore, the effect of deficiency of core fucosylation on the expression of α3β1 integrin on the cell surface was also determined, because *N*-glycosylation plays an important role in the quality control of the expression of glycoproteins. The biotinylation of cell surface proteins followed by immunoprecipitation of α3 integrin was examined by blotted. As shown in Fig. 6, the expression levels of α3β1 integrin on the cell surface remained unchanged among $Fut8^{+/+}$, $Fut8^{-/-}$, and rescued cells, indicating that the expression of α3β1 integrin on cell surface was not influenced by *Fut8*. Collectively, we suggested that the deficiency of core fucosylation resulted in the malfunctions of α3β1 integrin but not its expression level.

Purified α3β1 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 α3β1 integrin, we purified α3β1 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. 7*A*, *inset*, *right panel*), corresponding to the immunoreactivity with the anti-α3 and anti-β1 antibodies, were detected, respectively (data not shown). Then we analyzed *N*-glycan profiles of purified α3β1 integrin by LC/MS and LC/MS/MS. The profiles of the *N*-linked oligosaccharides extracted from purified α3β1 integrin of $Fut8^{+/+}$ and $Fut8^{-/-}$, respectively, are shown in Fig. 7*A*. 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. 7*B*, 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 α3β1 integrin of $Fut8^{+/+}$ (*peaks 1–7*) were assigned to fucosylated complex and hybrid type oligosaccharides, whereas those released from α3β1 integrin of $Fut8^{-/-}$ (*peaks 1′–7′*) were nonfucosylated forms. The data correspond to that of AAL lectin blot, revealing that α3β1 integrin derived from $Fut8^{+/+}$ is highly modified by *Fut8* and suggesting loss of core fucosylation will result in the deficiency of α3β1 integrin function.

DISCUSSION

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

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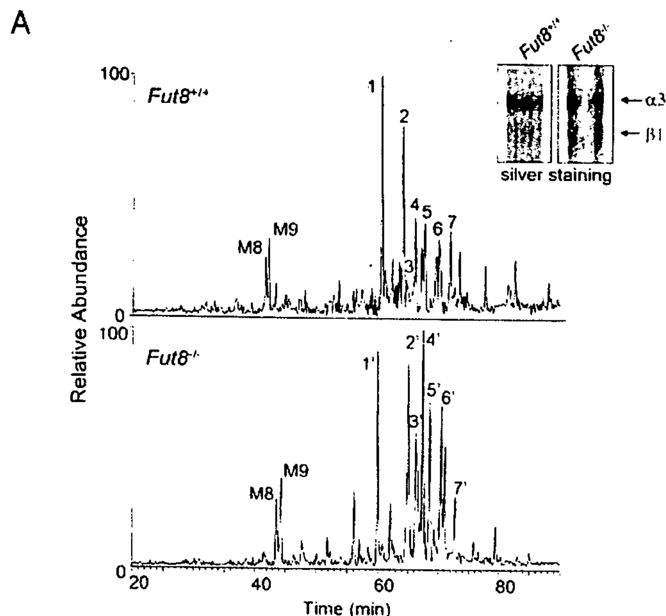

FIGURE 7. **Chromatograms of *N*-linked oligosaccharides extracted from purified α3β1 integrin from *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ cells.** In *A*: MS, full MS scan (*m/z* 450–2000) in the negative ion mode. LC, pump A, 5 mM ammonium acetate, pH 9.6/2% CH_3CN; Pump B, 5 mM ammonium acetate, pH 9.6/80% CH_3CN; column, hypercarb (0.1 × 150 mm); gradient, 5–20% of B (0–45 min) and 20–50% of B (45–90 min). The purity of α3β1 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 α3β1 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. △, fucose; ●, galactose; ○, mannose; ■, *N*-acetylglucosamine; ♦, *N*-acetylneuraminic acid.

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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-β1 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 α3β1 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 α3β1 integrin.

Several lines of evidence suggest that *N*-glycans are required for integrin activation. An increase in β1,6-branched sugar chains on α5β1 integrin by GnT-V promotes cell migration on FN (26). Although the overexpression of GnT-III has been reported to inhibit α5β1 integrin-mediated functions in HeLa S3 cells (27). It has also been reported that GnT-III and GnT-V can positively and negatively regulate α3β1 integrin-mediated cell migration on LN5 (47). The modification of β1 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 α1,6-fucose modification has little or only mild effects on α5β1 integrin, which is a receptor for FN. Actually, we previously reported that the introduction of the bisecting GlcNAc to the α5 subunit resulted in a reduced affinity in the binding of α5β1 integrin to FN, therefore resulting in decreased cell migration (27). Thus, we assumed that the core fucosylation affected α3 subunit in a similar manner, which caused the decreased cell migration on LN5. However, the modification of α1,6-fucose to α5 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 β1 or β3 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 α5 subunit play key roles in functional expression (49).

It has been reported that purified α5β1 integrin from human placenta and purified α3β1 integrin from the human ureter epithelium cell line HCV29 exhibited a highly heterogenous glycosylation pattern, and >50% of these were fucosylated (50, 51). In this study, the α3β1 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 α3β1 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 α3β1 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 α3β1 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 β1 I-like domain caused an increase in the distance between the β1 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 α5β1 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 α3β1 integrin to its ligand. However, we cannot exclude additional reasons that still remain to be determined.

The α3 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, α3β1 integrin is likely to be involved in mediating signals between the mesenchyme and epithelial cells in the kidney and lung. The glomeruli of α3-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 α3β1 integrin *in vivo* in the future.

In conclusion, we demonstrate here some aspects of the biological significance of the core fucosylation of α3β1 integrin-medi-

[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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