Glycoconj J (2012) 29:599–607
DOI 10.1007/s10719-012-9386-1

Potential roles of *N*-glycosylation in cell adhesion

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Received: 12 January 2012 / Revised: 13 April 2012 / Accepted: 24 April 2012 / Published online: 8 May 2012

Abstract The functional units of cell adhesion are typically multiprotein complexes made up of three general classes of proteins; the adhesion receptors, the cell-extracellular matrix (ECM) proteins, and the cytoplasmic plaque/peripheral membrane proteins. The cell adhesion receptors are usually transmembrane glycoproteins (for example E-cadherin and integrin) that mediate binding at the extracellular surface and determine the specificity of cell-cell and cell-ECM recognition. E-cadherin-mediated cell-cell adhesion can be both temporally and spatially regulated during development, and represents a key step in the acquisition of the invasive phenotype for many tumors. On the other hand, integrin-mediated cell-ECM interactions play important roles in cytoskeleton organization and in the transduction of intracellular signals to regulate various processes such as proliferation, differentiation and cell migration. ECM proteins are typically large glycoproteins, including the collagens, fibronectins, laminins, and proteoglycans that assemble into fibrils or other complex macromolecular arrays. The most of these adhesive proteins are glycosylated. Here, we focus mainly on the modification of *N*-glycans of integrins and laminin-332, and a mutual regulation between cell adhesion and bisected *N*-glycan expression, to address the important roles of *N*-glycans in cell adhesion.

Keywords Bisected *N*-glycan · Cell adhesion · E-cadherin · *N*-glycosylation · Integrin

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Introduction

Cell adhesion is crucial for assembly of individual cells into the three-dimensional tissues of multicellular organisms. Cells do not simply stick together to form tissues, but rather are organized into very diverse and highly distinctive patterns. A variety of cell adhesion mechanisms are responsible for assembling cells together and, along with their connections to the internal cytoskeleton, determine the overall architecture of the tissue [1]. Thus, cell adhesion systems should be regarded as mechanisms that help to translate basic genetic information into the complex three-dimensional patterns of cells in tissues. On the other hand, an increasing body of evidence suggests that *N*-glycosylation is also important for the cell adhesion. Changes in the *N*-glycan structures of those adhesive molecules can affect cell-cell and cell-ECM interactions, thereby affecting cell adhesion, migration and tumor invasion [2–4].

The oligosaccharides of glycoproteins are produced via catalysis by various glycosyltransferases. As a result, a specific structure is determined by the expression pattern of these enzymes and their associated molecules under physiological and pathological events, including cell growth, migration, differentiation, and tumor invasion. It is known that some malignant phenotypes are highly associated with *N*-glycans containing β1-6*N*-acetylglucosamine

branching, which is an association catalyzed by *N*-acetylglucosaminyltransferase V (GnT-V). By contrast, *N*-acetylglucosaminyltransferase III (GnT-III) has been found to play an important role in the suppression of metastasis. Therefore, it is not difficult to speculate that aberrant glycosylation patterns can serve as markers for certain disease states including cancer metastasis.

Integrins are cell surface transmembrane glycoproteins that function as adhesion receptors in cell-ECM interactions and link matrix proteins to the cytoskeleton. E-cadherin is the main epithelial cell-cell adhesion molecule, which is involved in Ca^{2+}-dependent cell–cell adhesion. It is well known that epithelial-to-mesenchymal transition (EMT), a process associated with normal development, wound healing, cancer progression and metastasis, is associated with loss of E-cadherin expression [5]. This review reports recent advances in the potential roles of *N*-glycans in integrin-mediated biological functions, effect of E-cadherin-mediated cell adhesion on expression of GnT-III, which, in turn, regulates the cell adhesion as well as EMT.

Relationship between GnT-III and GnT-V

GnT-III transfers *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to a β1-4 mannose in *N*-glycans to form a "bisecting" GlcNAc linkage, as shown in Fig. 1. Bisecting GlcNAc linkages are found in various hybrid and complex

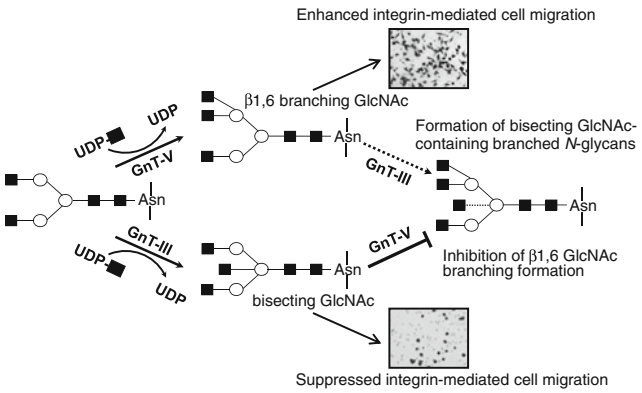

Fig. 1 Glycosylation reactions of GnT-III and GnT-V and their relationships and functions. GnT-III transfers GlcNAc from UDP-GlcNAc to the core mannose with a β-4 linkage to form the bisecting GlcNAc in *N*-glycans. GnT-V catalyzes the formation of β1-6 GlcNAc branched structures. GnT-III could be considered to have an antagonistic role to GnT-V, because bisecting GlcNAc renders the biantennary substrate inaccessible to GnT-V. The reaction pathway represented by a dash line may not be predominant *in vivo*. ○, *mannose*; ■, *N*-acetylglucosamine. The interaction of integrin with its ECM can activate intracellular signaling pathways and cytoskeletal formation, and regulate cell adhesion and migration. Enhanced expression of GnT-V in various cell types results in an increase in integrin-mediated cell migration examined with a transwell assay. In contrast, overexpression of GnT-III down-regulates integrin-mediated cell migration

N-glycans on glycoproteins. GnT-III is generally regarded as a key glycosyltransferase in *N*-glycan biosynthetic pathways. Introduction of a bisecting GlcNAc suppresses β1-6 GlcNAc branching formation catalyzed by GnT-V, which is strongly associated with cancer metastasis, since GnT-V cannot utilize the bisected oligosaccharide as an acceptor substrate [6–8]. The β1-6 GlcNAc-branched *N*-glycans can be preferentially modified by β1,4 GalT and β1,3 GlcNAcT to form poly-*N*-acetyllacotosamine for elongation of *N*-glycans, which are further processed to form other sugar motifs such as sialyl Lewis X, which may contribute to promotion of cancer metastasis. Furthermore, it is known that the β1,6 GlcNAc-branched *N*-glycans, which may enhance the complex formation such as integrins, galectins and growth factor receptors on cell surface, resulting in an increase in cell motility and cellular signaling [9, 10]**.** It has also been reported that GnT-V activity and β1-6 GlcNAc-branched *N*-glycans levels are increased in highly metastatic tumor cell lines [4, 11]. Consistently, cancer metastasis is greatly suppressed in GnT-V knockout mice [12]. Therefore, GnT-III may have an antagonistic role to GnT-V, thereby contributing to the suppression of cancer metastasis.

On the other hand, the studies based on overexpression of GnT-III in cell lines, have shown the involvement of GnT-III and its products in a variety of biological events, but the growth and development of the GnT-III-deficient mice were apparently normal [13], or mice carrying the GnT-III gene inactivating mutation were reported to have a subtle phenotype that appears to have a neurological basis [14]. In pathological conditions, retardation of diethynitrosamine/phenobarbital-induced liver tumor progression was obtained in GnT-III-deficient mice, suggesting that GnT-III may aid tumor progression [15]. However, GnT-III expression was not induced during hepatocarcinogenesis in the mice model. Furthermore, transgenic GnT-III mice did not get tumors earlier or possess more or bigger tumor than wild-type controls [16]. Therefore, the physiological and pathological roles of GnT-III remain unclear, and analysis of molecular mechanisms for GnT-III action is quite important.

N-glycans regulate integrin functions

Several research groups, including our lab, reported that alternations in the oligosaccharide portion of integrins, that are modulated by the expression of each glycosyltransferase gene such as GnT-III, GnT-V and α2,6 sialyltransferase (ST6GalI), regulate cell malignant phenotypes such as integrin-mediated cell migration and cell spreading [17]. Overexpression of GnT-V resulted in an increase in cell migration and invasion. GnT-V-null cells displayed enhanced cell adhesion to fibronectin (FN)-coated plates with the concomitant inhibition of cell migration. The restoration

of GnT-V in the null cells reversed these abnormal characteristics, indicating the direct involvement of *N*-glycosylation events in these phenotypic changes [18]. When NIH3T3 cells were transformed with the oncogenic *Ras* gene, the cell spreading on FN was greatly enhanced due to an increase of β1-6 GlcNAc branched tri- and tetra-antennary oligosaccharides in α5β1 integrins [4]. Similarly, characterization of carbohydrate moieties of integrin α3β1 from non-metastatic and metastatic human melanoma cell lines showed that β1-6 GlcNAc branched structures were highly expressed in metastatic cells compared with non-metastatic cells [11].

In contrast to GnT-V, the overexpression of GnT-III resulted in an inhibition of α5β1 integrin-mediated cell spreading and migration, and the phosphorylation of the focal adhesion kinase [19, 20]. The affinity of the binding of integrin α5β1 to FN was significantly reduced as a result of the introduction of a bisecting GlcNAc to the α5 subunit. The opposing effects of GnT-III and GnT-V have been observed for the same target protein, integrin α3β1 [21]. GnT-V stimulated α3β1 integrin-mediated cell migration, while overexpression of GnT-III inhibited GnT-V-induced cell migration. The modification of the α3 subunit by GnT-III supersedes modification by GnT-V. As a result, GnT-III inhibits GnT-V-induced cell migration. Furthermore, a knockdown of endogenous expression of GnT-III results in an increase in expression level of β1 6 branching GlcNAc. These results indicate that GnT-III counteracts GnT-V, and strongly suggest that remodeling of glycosyltransferase-modified *N*-glycan structures either positively or negatively modulates cell adhesion and migration. Of course, besides antagonistic effects to GnT-V as described above, other mechanisms for GnT-III action cannot be excluded out. For example, it could also be explained that the bisecting GlcNAc might be recognized by an unidentified endogenous lectin, which is similar to E4-PHA, to regulate cell behavior of animal cells. Alterations of *N*-glycans on integrins could also regulate their *cis*-interactions with membrane-associated proteins including the epidermal growth factor receptor (EGFR) [22, 23], and the tetraspanin family of proteins in microdomain [24, 25] as well as endocytosis [10, 26, 27]. In addition, GnT-III also contributes to suppress some other important glycoproteins, such as EGFR. The overexpression of GnT-III significantly reduces the ability of EGF to bind to its receptor, reduces EGFR autophosphorylation, and subsequently blocks EGFR-mediated Erk activation [28, 29].

Roles of *N*-glycosylation on α5β1 integrin

Although alteration of the oligosaccharide portion on integrin α5β1 could affect *cis*- and *trans*-interactions caused by GnT-III and GnT-V, as described above, the molecular mechanism remains unclear. Since integrin α5β1 contains 26 potential *N*-linked glycosylation sites (14 in the α subunit and 12 in the β subunit), the determination of those crucial *N*-glycosylation sites for its biological function is therefore, quite important for an understanding of the underlying mechanism. We sequentially mutated either one or a combination of asparagine residues in the putative *N*-glycosylation sites to glutamine residues as shown in Fig. 2. We found that *N*-glycosylation on the β-propeller domain of the α5 subunit (S3-5) is essential for its heterodimer formation and its biological functions such as cell spreading, cell migration and cytoskeletal formation, as well as for the proper folding of the α5 subunit [30]. Seales *et al*. reported that the I-like domain on the β1 subunit, which could be the partner of the β-propeller of the α5 subunit [31], supporting the importance of *N*-glycans on the β-propeller. The crystal structure of integrin αVβ3 has been successfully revealed, and the main contact between the αV and β3 subunit is the β-propeller on the αV and A domain on β3 with hydrophobic, ionic, and other interactions [32, 33]. Since the α5 subunit has a 47 % homology to αV, Mould, *et al.* speculate that the structural environment of the αβ interfaces could be affected by the presence of *N*-glycans by a homology modeling structure of α5β1 [34]. To further investigate the underlying molecular mechanism of GnT-III-regulated biological functions, we characterized the *N*-glycans on the α5 subunit in detail, and found that site-4 was a key site that could be specifically modified by GnT-III [19]. Furthermore, the deletion of site-4 abolished the suppression of cell spread induced by GnT-III in the transfectant.

It could be of interest to understand why GnT-III specifically and effectively modifies site-4 of the 14 putative *N*-glycosylation sites in the α5 subunit. There is currently no detailed information available regarding this observation, but several explanations could be proposed. First, *N*-glycosylation occurs on site-4 because it provides the easiest access for GnT-III. Second, GnT-III may associate with some other molecules, which define the specificities for protein or peptide substrates. Several studies have shown that the glycosyltransferase complex formation may play a crucial role in the determination of both activity and substrate specificity [35, 36]. Third, each glycosyltransferase may have its own pathway to operate glycosylation in Golgi apparatus. Indeed, it has been reported that caveolin-1 may co-localize with GnT-III to regulate its localization and activity [37].

We also found three *N*-glycosylation sites on I-like domain of integrin β1 subunit, which are important for α5 and β1 dimer formation and its expression on the cell surface [38]. Although the involvement of *N*-glycan in the αβ interaction remains unclear, it could be explained that an unknown lectin domain may exist on the each subunit, since

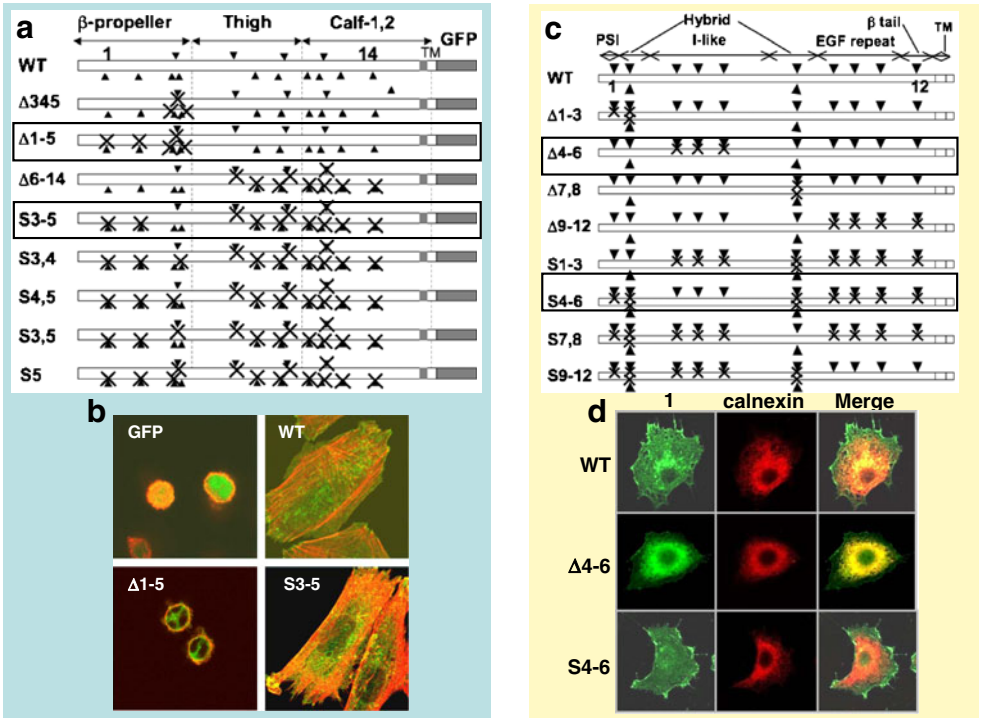

Fig. 2 Important *N*-glycosylation sites on α5β1 integrin. The various underglycosylated mutants of α5 (**a**) and β1 (**c**) subunits were constructed. The cross indicates mutation of Asn residue to Gln residue. **b** Actin stress fiber formation of GFP-tagged α5 unglycosylated mutants spread on FN stained with Phalloidin-Alexa 549. **d** The localization of β1 by immunostaining. Both the WT and the S4-6 of the β1 subunit were expressed mainly on the cell surface as usual, while the Δ4-6 accumulated mainly in the ER colocalized with calnexin

the lectin domain of αMβ2 integrin is associated with GlcNAc on the non-reducing terminal of sugar chains on chilled platelets for its phagocytosis [39, 40].

Appropriate intermolecular interaction through *N*-glycan is important for integrin-medaited supracomplex formation and its cellular signaling

Laminins (Lms) are large heterotrimeric glycoproteins that are prominent components of basement membranes, and are involved in important biological roles including tissue development, cell differentiation, survival, adhesion and migration [41]. Laminin-332 (Lm332), is composed of α3, β3, and γ2 chains. Lm332 is expressed in the skin and other stratified squamous epithelial tissues where it is associated with hemidesmosomes via integrin α6β4. Most of the studies have identified the functional domains of Lm332 and revealed the relationship between its activities and the processing of its subunits [42]. In fact, *N*-glycans on Lm332 also affect its activities [43]. To examine the effects of *N*-glycans of Lm332 on its activities, we purified Lm332s from the conditioned media of GnT-III and GnT-V overexpressing MKN45 cells. GnT-III modification of Lm332 caused a decrease in its keratinocyte cell adhesion and migration activities as shown in Fig. 3. Furthermore, we found that galectin-3, which is a β-galactoside binding protein, strongly bound to unmodified Lm332 but not to the Lm332 modified by GnT-III, and that binding of galectin-3 was completely blocked by lactose [23]. Co-immunoprecipitation revealed that galectin-3 associated with both β4 integrin and EGFR, thereby cross-linking the two molecules. The associations were inhibited by either the presence of lactose or expression of GnT-III. These results clearly identify the molecular mechanism responsible for the inhibitory effects of GnT-III on ECM-integrin-meditated cell adhesion, migration and signal transduction, address the importance of *N*-glycosylation-mediated supramolecular complex formation on cell surfaces.

N-glycan on β4 integrin not only controlled its association with other molecules such as EGFR, but also modulated

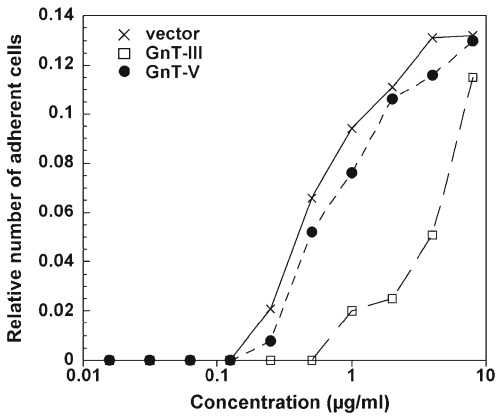

Fig. 3 Effects of bisected *N*-glycans of laminin332 on the cell adhesion. Various Lm332s were purified from the conditioned media of GnT-III and GnT-V overexpressing MKN45 cells. Cell adhesion activity of Lm332 modified by GnT-V toward Lm332 null keratinocytes was similar to that of vector-Lm332, but was decreased in the Lm332 modified by GnT-III

its activation and cellular signal transduction [9]. The glycan-mediated soft interactions could be more important than the stronger associations by protein-protein interactions. The deletion of all 5 potential *N*-glycosylation sites on β4 integrin promoted the EGFR-β4 integrin association, but the intracellular signaling was blocked. Therefore, *N*-glycans, which would generate the space between two molecules, may have a suppressive effect on protein-protein interaction. Since sialylation plays important roles in intermolecular interactions either by its negative charge or by the sialic acid binding of Ig-like lectins [44], the enhanced associations between β4 integrin and EGFR, could be also explained by the significant decrease of sialic acid on β4 integrin [9]. Similarly, tetraspanin CD82 with incomplete *N*-glycosylation exhibits an enhanced association with the α3 and α5 integrin subunits [45]. A modest association between Lm332 and α6β4 integrin mediated by galectin-3 through the *N*-glycans on both molecules promoted cell adhesion and migration on Lm332 as well as α6β4 integrin clustering on Lm332 [23, 43]. Accordingly, an appropriate intermolecular interaction through *N*-glycan is important for the efficient cellular signaling and the following cellular function.

A mutual regulation between GnT-III expression and E-cadherin-mediated cell adhesion, and potential roles in EMT

The Adherens junction provides important adhesive contacts between neighboring epithelial cells, and forms intracellular links to the actin cytoskeleton and signaling pathways including the regulation of gene transcription [46]. E-cadherin is the core transmembrane protein of the adherens junction and is required for binding and localization of a number of important cytoplasmic proteins, termed catenins, that connect the cadherin complex to the actin cytoskeleton and several signaling pathways [47]. The catenin family comprises α-catenin, β-catenin and γ-catenin (plakoglobin). The regulation of cadherin-mediated adhesion and associated adherence junctions is thought to underlie the dynamics of inter-cellular adhesive interactions, which are regulated during tissue development and homeostasis, as well as during tumor cell progression. In epithelial derived tumors, loss of cell–cell adhesion is correlated with down-regulation of E-cadherin as well as increased proliferation and tumor invasiveness. Therefore, most studies have focused on the identification and characterization of transcriptional repressors of E-cadherin expression in epithelial tumor cells. The most prominent factors identified in these studies included the related factors, Slug, Snail, SIP1 and Twist, which are best known for their roles in early embryogenesis and tumor progression [48].

On the other hand, E-cadherin can be post-translationally modified by phosphorylation, *O*-glycosylation and *N*-glycosylation. Casein kinase II, a serine-threonine kinase, phosphorylates the cytosolic tail of E-cadherin and enhances binding to β-catenin [49]. Cytoplasmic *O*-glycosylation of the E-cadherin cytosolic tail has been shown to occur in response to endoplasmic reticulum stress and inactivate E-cadherin-mediated intercellular adhesion by preventing its transport to the cell membrane[50]. In addition, E-cadherin can be *N*-glycosylated and the *N*-glycosylation at Asn-633 is essential for E-cadherin expression, folding and trafficking [51, 52]. In an earlier study, Yoshimura, *et al.* reported that E-cadherin-mediated cell adhesion was regulated by GnT-III. Overexpression of GnT-III increased the retention of E-cadherin at the cell border, which might result in an enhancement of E-cadherin-mediated homotypic adhesion [53, 54]. Thus, expression of E-cadherin may be regulated, not only by transcriptional factors, but also by post-transcriptional processing, maturation and modifications.

Conversely, the expression of GnT-III was up-regulated by E-cadherin-mediated cell adhesion [55]. GnT-III activity was increased under dense culture conditions compared with sparse culture conditions [56]. A significant up-regulation of GnT-III expression was observed only in epithelial cells that express E-cadherin, but not in E-cadherin-deficient cells. Furthermore, disruption of actin polymerization by treatment with cytochalasin D or by lack of α-catenin expression, interfered with regulation of GnT-III [57]. It is worth mentioning that sialylation is also associated with cell density. Wieser, *et al.* reported that cell-contact mediated hyposialylation of contactinhibin [58], which was involved in contact-dependent inhibition of growth. It could be explained that introduction of a bisecting GlcNAc by GnT-III expression induced by a dense culture, suppressed GlcNAc branching formations catalyzed by GnT-IV or/and GnT-V, which resulted in a decrease in sialylation on non-reducing terminal of *N*-glycans. The detailed molecular mechanism remains further study.

To further explore the molecular mechanism of this regulation, the roles of β-catenin, an essential molecule in both cadherin-mediated cell adhesion and canonical Wnt signaling, were investigated. Unexpectedly, shRNA knockdown of β-catenin resulted in a dramatic increase in GnT-III expression and its product, the bisected *N*-glycans [59]. Stimulation of the Wnt signaling pathway by the addition of exogenous Wnt3a or BIO, a GSK-3β inhibitor, consistently and significantly inhibited GnT-III expression and its products. Conversely, the inhibition of β-catenin translocation into the nuclei increased GnT-III activation. Therefore, GnT-III expression might be precisely regulated by the interplay of E-cadherin-catenin complex-mediated cell-cell adhesion and Wnt/β-catenin signaling, which were both crucial in the process of EMT and important for integrin-mediated cell adhesion (Fig. 4).

E-cadherin is a key molecule for EMT. One important feature of EMT is increased cell motility via a decrease in cell-cell adhesion and increase in integrin-mediated cell migration. Different signaling pathways, for example, receptor tyrosine kinase (RTK), Notch, Wnt and TGF-β, are known to provide the necessary stimuli that modulate gene expression and trigger EMT and cell motility [60]. In fact, there is a bidirectional regulatory mechanism between E-cadherin-mediated cell-cell adhesion and GnT-III expression. Overexpression of GnT-III partially inhibited EMT induced by TGF-β1 [61]. Similar to previous studies [53, 54], GnT-III modified E-cadherin, which served to prolong E-cadherin turnover on the cell surface. Furthermore, GnT-III expression consistently inhibited β-catenin translocation from cell-cell contact into the cytoplasm and nucleus. GnT-III expression suppressed p-β-catenin/p-Smad2 complex formation, which was essential for initiation of EMT [62, 63]. Taken together, these observations clearly demonstrate that GnT-III plays important roles in EMT, and to explain a molecular mechanism for the inhibitory effects of GnT-III on cancer metastasis.

It remains unclear how the modification of E-cadherin with the bisecting GlcNAc affects its turnover on the cell surface. However, the modification of E-cadherin with complex *N*-glycans has been associated with the formation of dynamic, but weak, adherens-junctions, whereas E-cadherin modified by high mannose or less *N*-glycans has been reported to promote the establishment of stable adherens-junctions [51]. In addition, Pihno, *et al.* reported that E-cadherin underwent extensive modification of its *N*-glycans with β1-6 branched and sialylated structures during acquisition of the malignant phenotype in a canine mammary tumor cell line model [64]. Therefore, an explanation of the prolongation of turnover of E-cadherin with the bisecting GlcNAc could be that GnT-III inhibits the action of GnT-V, suppresses extensive complex *N*-glycan formation, and then enhances homophilic adhesion. In fact, up-regulation of GnT-V expression, in contrast to GnT-III, was observed in TGF-β-induced EMT [61]. Recently, Terao, *et al.* reported that overexpression of GnT-V promoted EMT and keratinocyte migration in GnT-V-transgenic mouse [65].

Future perspectives

In this review, we mainly introduced our studies to address the importance of *N*-glycans, especially potential roles of GnT-III in cell adhesion and EMT. It would be quite important to further examine whether the phenomena *in vitro* can also be observed *in vivo* study using animal models such as GnT-III knockout mice and transgenic mice, to elucidate the

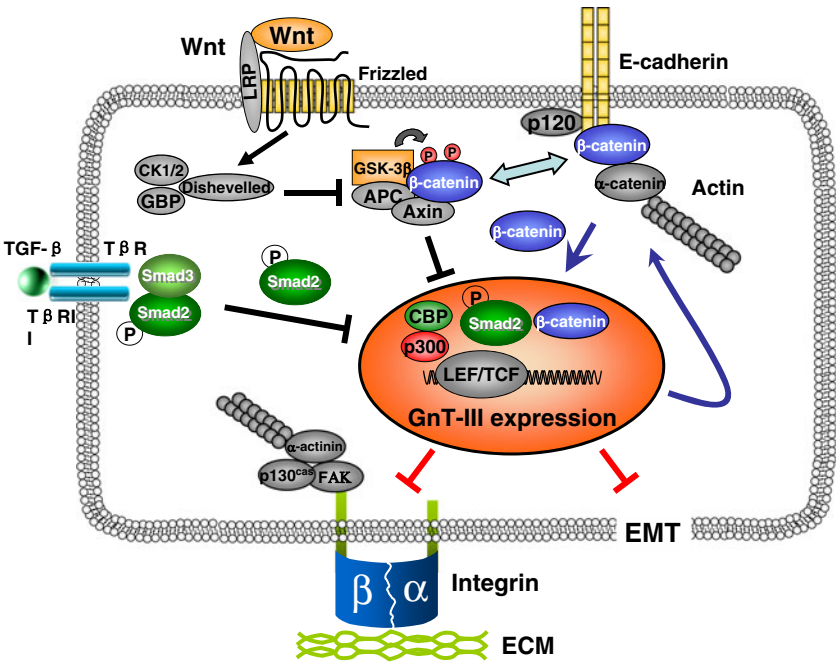

Fig. 4 A working model for the regulation of GnT-III expression and its function in cell adhesion and EMT. The GnT-III expression and bisected *N*-glycans were up-regulated by cell-cell adhesion in an E-cadherin-catenin-dependent manner, and down-regulated by Wnt/β-catenin signaling. The intersection point for the reciprocal regulation of GnT-III is at the β-catenin, which is a central player in both cadherin-mediated cell adhesion and canonical Wnt signaling. Enhanced expression of GnT-III by E-cadherin-mediated cell adhesion could reversely promote cell-cell adhesion, and inhibited integrin-mediated cell migration and EMT. Recently, we also found that TGF-β signaling pathway inhibited GnT-III expression. Therefore, GnT-III could be an important mediator for those networks. →, up-regulation; ⊥, down-regulation

relationship between GnT-III expression and cell adhesion. Since the GnT-III-deficient mice are viable and reproduce normally, suggesting that GnT-III and the bisected *N*-glycans apparently are not essential for normal development as described above, the physiological roles of GnT-III are yet to be identified [66]. Therefore, further work is needed to decipher the exact roles of GnT-III and bisected *N*-glycans.

Acknowledgments This work was partly supported by a Grant-in-Aid for Scientific Research NO. 21370059 (JG), for Challenging Exploratory Research No. 23651196 (JG) from the Japan Society for the Promotion of Science and by the Academic Frontier Project for Private Universities from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and Mizutani Foundation for Glycoscience (JG).

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