invading viruses, and bacteria. Many secreted molecules such as hormones and toxins have also been reported to bind to carbohydrate receptors on the cell surface. In addition, most receptors on the cell surface are *N*-glycosylated, including epithelial growth factor receptor (EGFR), integrins and transforming growth factor β receptor (TGFβR). Modified oligosaccharides affect protein folding and stability, and have the ability to interfere with carbohydrate-carbohydrate, carbohydrate-protein, and glycoprotein-glycoprotein interactions, and as a result, regulate many physiological and pathological events, including cell growth, migration, differentiation and tumor invasion, host-pathogen interactions, cell trafficking and transmembrane signaling. Therefore, it is not surprising that aberrant glycosylation patterns can serve as markers for certain disease states including cancer metastasis, development and differentiation.(9) In this review, we mainly focus on the modification of *N*-glycans of receptors on the cell surface to further address the important roles of *N*-glycans in cancer science.

Important functions of *N*-acetylglucosaminyltransferases

1. GnT-V

N-Acetylglucosaminyltransferase V (GnT-V)(10-12) has been thought to have a close relationship with cancer metastasis.(13, 14) GnT-V catalyzes the formation of β1,6 GlcNAc branching structures, which play important roles in tumor metastasis (Fig. 1).(15) GnT-V deficient mice were generated to assess the functions of GnT-V products in normal development and cancer progression.(16) The mice appeared to be normal at birth, lacking any detectable GnT-V enzyme activity and L_4-PHA reactive glycoproteins. Adult GnT-V deficient mice differed in their responses to various extrinsic conditions, including cancer progression, T cell hypersensitivity, autoimmune disease, and nurturing responses following birth. More importantly, a relationship between GnT-V and cancer metastasis has been reported, i.e. that polyomavirus middle T antigen (PyMT)-induced tumor growth and metastasis were suppressed in GnT-V deficient mice to a considerable extent, compared with in their PyMT-transgenic littermates expressing GnT-V. In addition, the products of GnT-V promoted focal adhesion turnover, which enhanced the PyMT-dependent activation of phosphatidylinositol 3 (PI3) kinase-PKB, and promoted tumor growth and metastasis.(17)

On the other hand, it has been shown that the forced expression of GnT-V in epithelial cells results in a loss of contact inhibition, increased cell motility, and morphological transformation in culture (Fig. 2).(18) It has also been reported that *N*-glycans of EGFR, as well as other cytokine receptors modified by GnT-V, play an important role in the endocytosis of EGFR to regulate its

expression level on the cell surface.(19) Moreover, the upregulation of GnT-V in the liver of a rodent model of hepatocarcinogenesis as well as in regenerative liver has also been reported.(20) A different underlying mechanism for cancer metastasis may be operative but the β1,6 GlcNAc branching on specified glycoproteins may cause functional changes of metastatic potential.(17, 21) Matriptase in the GnT-V transfectants was found to be resistant to auto-digestion as well as exogenously added trypsin.(22, 23) Interestingly, a secreted type of GnT-V induces tumor angiogenesis without mediation of glycosylation, which is a novel function of GnT-V distinct from the original glycosyltransferase.(24)

GnT-V also appears to be involved in the regulation of apoptosis.(25) GnT-V expression was quantitatively analyzed by utilizing a neuroblastoma, one of the most common pediatric solid tumors. High expression levels of GnT-V were found to be associated with favorable stages. Conversely, the downregulation of GnT-V expression by small interfering RNA resulted in a decrease in susceptibility to cell apoptosis induced by retinoic acid in a neuroblastoma.

Thus GnT-V is associated with the prognosis of the disease and the inhibition of GnT-V might be useful in the treatment of malignancies by targeting their roles in metastasis.(26)

2. GnT-III

Contrary to the function of GnT-V, *N*-acetylglucosaminyltransferase III (GnT-III)(27, 28) is a key glycosyltransferase in *N*-glycan biosynthetic pathways and is involved in the inhibition of metastasis. GnT-III catalyzes the addition of *N*-acetylglucosamine in ß1–4 linkage to the ß-linked mannose of the trimannosyl core of *N*-linked sugar chains to produce a a "bisecting" GlcNAc linkage which is found in various hybrid and complex *N*-glycans (Fig. 1). The introduction of a bisecting GlcNAc catalyzed by GnT-III results in the suppression of further processing and the elongation of *N*-glycans, which is catalyzed by other glycosyltransferases in vitro, which are not able to utilize the bisected oligosaccharide as a substrate (Fig. 1).(29) When GnT-III transfected melanoma B16 cells were injected into syngeneic mice via tail vein, lung metastasis was minimal whereas many lung metastatic foci were observed in control transfected melanoma cells. Sugar analyses involving lectin blotting of the cells indicated that the GnT-V product, a β1,6 GlcNAc branching structure, found originally in the parental cells was no longer present in the GnT-III transfectants.(30)

E-cadherin mediates homotypic adhesion and suppression of the phosphorylation of the E-cadherin-β-catenin complex on the cell-cell adhesion.(21, 31) When located on the cell surface, E-cadherin was found to be resistant to proteolysis and remained at the cell-cell border as a result

of the overexpression of GnT-III (Fig. 2). The increased GnT-III product on E-cadherin leads to a reduced level of phosphorylation of β-catenin through EGFR or Src signaling, and therefore β-catenin remained in a tight complex with E-cadherin and is not translocated into the nuclei. β-Catenin otherwise enhances the expression of various genes that are related to cell growth or oncogenesis. The suppression of the phosphorylation of β-catenin, therefore, permits it to remain on the cell surface and not be released from the complex, and this may also enhance the homophilic interactions of E-cadherin and contribute to the suppression of cancer metastasis.

Conversely, GnT-III was recently reported to be regulated through E-cadherin-mediated cell-cell adhesion (Fig. 2).(32) In other words, GnT-III activity has been found to be increased under dense culture conditions compared with sparse culture conditions. The regulation of cadherin-mediated adhesion and the associated adherens junctions is thought to underlie the dynamics of the adhesive interaction between cells, which is regulated during tissue development and homeostasis, as well as during the development of tumor cells. In fact, the expression of E-cadherin could be greatly regulated by epithelia cell-cell interactions.(33) However, significant and obvious regulation of E-cadherin through GnT-III was only observed in epithelial cells that expressed basal levels of E-cadherin and GnT-III, i.e. not in MDA-MB231 cells, an E-cadherin-deficient cell line, and not in MDCK, in which GnT-III expression is undetectable, as well as not in fibroblasts, which lack E-cadherin. Considering the up-regulation of GnT-III in the densely culture model, to a certain extent, cells under sparse and dense culture conditions can be interpreted as being cells in the proliferation and differentiation maintenance states, respectively. In that study, GnT-III expression was reported to be significantly upregulated by cell-cell interactions, which might be reasonable for the maintenance of cell differentiation rather than cell proliferation. In fact, the results of several studies have suggested that E-cadherin has the ability to induce ligand-independent activation of EGFR and subsequent activation of Rac1 as well as MAP kinase, which appears to be involved in cell migration and proliferation.(34) Thus, it is possible that the up-regulation of GnT-III through cell-cell interactions might neutralize signals responsible for the maintenance of the cell differentiation phenotype.

On the other hand, the overexpression of GnT-III results in alteration of the functions of EGFR and integrins, which will be discussed in detail below. However, GnT-III deficient mice that lacked GnT-III activity have been produced and found to be viable and to reproduce normally.(35) These mice also exhibited normal cellularity and morphology of organs, including the brain and kidneys. No alterations were apparent in circulating leukocytes or erythrocytes, or in serum metabolite levels that reflect kidney function. Thus GnT-III and the bisecting GlcNAc in

N-glycans appear to be dispensable for normal development, homeostasis and reproduction in the mouse.

GnT-III has also been reported to affect antibody-dependent cellular cytotoxicity (ADCC) activity (Fig. 1). A number of mechanisms for the anti-tumor activities of therapeutic antibodies have been proposed, and include an extended half-life, the blockage of signaling pathways, activation of apoptosis and effector-cell-mediated cytotoxicity. Fcγ receptors on effector cells have been reported to be the major components for the in vivo activities of antibodies against tumors.(36) Fc-receptor-dependent mechanisms are important components for the actions of cytotoxic antibodies against tumors, and indicate than an optimal antibody against tumor would preferentially bind to an activated Fc receptor and minimally to the inhibitory partner Fcγ receptor IIB. Umana *et al.* reported that the expression of antibodies with altered glycoforms, i.e., addition of bisecting GlcNAc, leads to increase in ADCC through a higher affinity for Fcγ receptor III of up to 10-20 fold.(37) They concluded that the increase in ADCC activity is therefore probably due to increased affinity of the modified antibody for Fcγ receptor III.

Thus, GnT-III catalyzes the formation of bisecting GlcNAc, a unique structure, and consequently contributes to anti-metastatic functions and ADCC.

3. Fut8

α1,6 Fucosyltransferase (*Fut8*) (38-40) catalyzes the transfer of a fucose residue from GDP-fucose to position 6 of the innermost GlcNAc residue of hybrid and complex types of *N*-linked oligosaccharides on glycoproteins to produce core fucosylation in mammals (Fig. 1).

The physiological importance of deletion of core fucose in proteins has been highlighted by the description of human congenital disorders of glycosylation (CDG).(41, 42) CDG comprises a rapidly growing group of inherited disorders in which the glycosylation of glycoproteins is defective due to mutations in genes that are required for the assembly of lipid-linked oligosaccharides, their transfer to nascent glycoproteins (CDG-I), or the processing of protein-bound glycans (CDG-II). Besides the effects on CDG-IIc, the level of core fucosylation has also been found to be elevated in both the liver and serum during the process of hepatocarcinogenesis.(43) The core fucosylation of α-fetoprotein, a well-known tumor marker for hepatocellular carcinomas (HCC), is known to distinguish patients with HCC from those with chronic hepatitis and liver cirrhosis.(44, 45) It has also been reported that deletion of the core fucose from the IgG1 molecule enhances ADCC activity by up to 50-100 fold (Fig. 1),(46, 47) and therefore is thought to have considerable potential for use in antibody therapy against cancer.

To define the physiological role of *Fut8* much more clearly, *Fut8*-null mice were recently generated. The appearance of *Fut8*$^{-/-}$ mice could not be distinguished from that of *Fut8*$^{+/-}$ and *Fut8*$^{+/+}$ mice within 3 d of age, but approximately 70% of them died during this period. Most of the survivors exhibited severe growth retardation and emphysema-like changes in the lungs.(48) The down-regulation of TGFβ receptor (TGFβR), and EGFR as well as platelet-derived growth factor receptor (PDGFR) activation are plausible factors that may be responsible for the emphysema-like changes and growth retardation, respectively (Fig. 3). (48, 49) It has also been revealed that core fucose modulates low density lipoprotein (LDL) receptor-related protein-1 (LRP-1) function; the loss of core fucosylation of LRP-1 significantly impairs the LRP-1 scavenging function, leading to an increase of insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3), which may be involved in growth retardation in *Fut8*$^{-/-}$ mice as well.(50)

Sugar remodeling regulates protein functions

The remodeling of cell surface growth factor receptors through modification of their oligosaccharide structures is associated with the functions and biological behavior of tumor cells. Nerve growth factor has been shown to bind to its receptor, TrkA, on the surface of PC12 cells, resulting in TrkA dimerization and phosphorylation.(51) TrkA-mediated neurite outgrowth and its tyrosine phosphorylation are blocked as the result of the transfection of GnT-III into PC12 cells, suggesting that bisecting structures may participate in the regulation of TrkA functions.(52)

EGFR-mediated cellular responses to EGF and transforming growth factor-α stimulation regulate several biological functions including cell growth and cell differentiation. The extracellular domain of EGFR contains 12 potential *N*-glycosylation sites,(53) and the remodeling of *N*-glycans on EGFR can modulate EGFR-mediating functions.(54-60) It has been reported that the binding of EGF to EGFR is significantly reduced by treatment with some *N*-glycosylation inhibitors,(54) or EGF binding as well as tyrosine kinase activity is reduced in the presence of certain lectins.(55-57) In addition, the glycosylation site on Asn-420 of EGFR was reported to suppress ligand-independent spontaneous oligomerization,(58) indicating that *N*-glycans are required for ligand binding. Interestingly, similar effects of deletion of the *N*-glycan in domain III have been observed for other ErbB family members.(61-63) On the other hand, the overexpression of GnT-III, a pivotal glycosyltransferase that plays a major role in the biosynthesis of hybrid and complex types of *N*-linked oligosaccharides,(27) significantly reduces the ability of EGF to bind to its receptor, reduces EGFR autophosphorylation, and subsequently blocks EGFR-mediated Erk phosphorylation in U373 MG glioma cells(57) and PC12 cells(60). It

was also revealed that endocytosis of EGFR is upregulated in GnT-III transfected HeLaS3 cells.(59) Partridge *et al.* reported that GnT-V-modified *N*-glycans containing poly-*N*-acetyllactosamine, the preferred ligand for galectin-3, on surface receptors avoid their constitutive endocytosis, resulting in promotion of intracellular signaling, and consequently cell migration and tumor metastasis.(19) They found that GnT-V deficient tumor cells were less responsive to EGF, insulin-like growth factor (IGF), PDGF, basic FGF (bFGF), and fetal calf serum than wild type cells. These cytokine receptors are all highly *N*-glycosylated with 8 to 16 *N*-glycosylation sites. EGFR in carcinoma cells was reported to be expressed at 10 to 12 occupied sites, and a subset of *N*-glycans are GnT-V-modified and carry extensions of poly-*N*-acetyllactosamine.(64) However, TGFβRI and TGFβRII contain only one and three potential *N*-glycosylated sites, respectively. GnT-V deficient cells consistently exhibited a two- to three-fold decrease in sensitivity to TGFβ compared with the ~100- fold decrease in sensitivity to EGF, PDGF, IGF-1, and FGF, supporting the conclusion that both Golgi processing (i.e., that of GnT-V and poly-*N*-acetyllactosamine) and the number of *N*-glycans per receptor are important.(65) Moreover, EGFR was found to be associated with galectin-3 on the surface of wild type cells whereas this interaction was greatly reduced in GnT-V deficient cells. Such associations result in delayed removal of EGFR through constitutive endocytosis in wild type cells. It is possible that galectin-3 binds to poly-*N*-acetyllactosamine (i.e., a polymer of Galβ1,4GlcNAcβ1,3) with higher affinity than to the more ubiquitous *N*-acetyllactosamine antennae,(66) that GnT-V controls the production of these larger polymers by producing the preferred intermediate for their addition,(67) and that the nonlectin *N*-terminal domain of galectin-3 mediates pentamer formation in the presence of multivalent ligands, thereby cross-linking glycoproteins in proportion to the ligand concentration.(68) The resulting superstructures of galectins and glycoproteins on the cell surface generate a molecular lattice. The receptors are anchored to the cell surface by such a lattice, resulting in positive regulation of receptors signals, such as those of Ras, PI-13 kinase, and Smad2 and 3, and the loss of cell-cell adhesion junctions.(19) 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.(13) Thus, *N*-linked oligosaccharides on EGFR appear to be important factors in receptor function.

Another important receptor family is the integrins, which comprise α and β subunits. Each subunit has a large extracellular region, a single transmembrane domain and a short cytoplasmic tail (except for β4 integrin). The *N*-terminal domains of the α and β subunits associate to form the integrin headpiece, which contains the extracellular matrix binding site, whereas the C-terminal

segments traverse the plasma membrane and mediate interactions with the cytoskeleton and with signaling molecules. Integrin engagement during cell adhesion leads to intracellular phosphorylation, such as phosphorylation of focal adhesion kinase (FAK), thereby regulating gene expression, cell growth, differentiation and survival from apoptosis.(69)

A growing body of evidence indicates that the presence of an appropriate oligosaccharide can modulate integrin activation. When human fibroblasts were cultured in the presence of l-deoxymannojirimycin, an inhibitor of α-mannosidase II, which prevents *N*-linked oligosaccharide processing, immature α5β1 integrin appeared in the cell surface, and FN-dependent adhesion was greatly reduced.(70) In fact, the treatment of purified integrin α5β1 with *N*-glycosidase F, also known as PNGase F, which cleaves between the innermost GlcNAc and asparagines residues of *N*-glycans in *N*-linked glycoproteins, resulted in the blocking of α5β1 binding to FN and inherent association of the two subunits,(71) suggesting that *N*-glycosylation is essential for functional integrin α5β1. An alteration in the expression of *N*-glycans in α5β1 integrin could contribute to the adhesive properties of tumor cells and tumor formation. When NIH3T3 cells were transformed with the oncogenic Ras gene, cell spreading on FN was greatly enhanced due to an increase in β1, 6 GlcNAc branched tri- and tetra-antennary oligosaccharides in α5β1 integrins.(72) Similarly, characterization of the carbohydrate moieties of integrin α3β1 from non-metastatic and metastatic human melanoma cell lines showed that β1, 6 GlcNAc branched structures were expressed at high levels in metastatic cells compared with in non-metastatic cells,(73) confirming the notion that the β1, 6 GlcNAc branched structure lead to cancer invasion and metastasis properties. These cancer-associated glycan chains may modulate tumor cell adhesion by affecting the ligand binding properties of these integrins.

Furthermore, when exploring the possible mechanisms involved in the increased β1,6 branched *N*-glycans on the surface of metastatic cancer cells, Guo *et al.*, found that cell migration toward FN and invasion through the Matrigel were both substantially stimulated in cells in which the expression of GnT-V was induced.(74) Increased branched sugar chains inhibited the clustering of integrin α5β1 and the organization of F-actin into extended microfilaments in cells plated on FN-coated plates, confirming the hypothesis that the degree of adhesion of cells to their ECM substrate is a critical factor as to regulation of the rate of cell migration, i.e., migration is maximal under conditions of intermediate levels of cell adhesion.(75) Conversely, the progression of PyMT oncoprotein-induced mammary carcinomas in GnT-V null mice was significantly retarded compared with that observed in wild-type mice. The adhesion of mouse embryonic fibroblasts (MEF) to the matrix in GnT-V null and wild type mice was investigated to elucidate the

mechanism by which the deletion of GnT-V retards tumor progression. GnT-V null MEF exhibited enhanced adhesion to and spreading on fibronectin-coated plates with the concomitant inhibition of cell migration. GnT-V null MEF also showed increased focal adhesion kinase tyrosine phosphorylation, consistent with the decreased cell motility on fibronectin-coated plates. The expression of GnT-V cDNA in the null MEF reversed these abnormal characteristics, indicating the direct involvement of *N*-glycosylation events in these phenotypic changes. α5β1 integrin exhibited increased clustering on null MEF cell surfaces, consistent with previous studies that revealed decreased integrin clustering in cells overexpressing GnT-V. More surprisingly, GnT-V null MEF exhibited increased expression levels of both the α5 and β1 subunits in lysates and on the cell surface. The increased α5β1 integrin expression in the null MEF was due to increased α5β1 integrin transcript levels that decreased after the re-expression of GnT-V cDNA, confirming that the increase in α5β1 integrin expression in null MEF was due to changes in GnT-V expression. The increased null MEF transcripts were shown to be caused, at least in part, by increased integrin promoter activity. Moreover, the increased α5β1 integrin transcripts in GnT-V null MEF were not due to a different response to fibronectin; rather, they appeared to be mediated through activation of a protein kinase C signaling pathway. These results demonstrate that the deletion of MEF GnT-V resulted in enhanced integrin clustering and the activation of α5β1 integrin transcription through protein kinase C signaling, which in turn up-regulated the levels of cell surface α5β1 integrin, resulting in increased matrix adhesion and inhibition of migration.(76)

In addition, sialylation at the non-reducing termini of *N*-glycans of α5β1 integrin plays an important role in cell adhesion. It has been reported that the hyposialylation of β1 integrin contributed to an increase in the extent of FN binding in myeloid cells, in which the expression of ST6Gal I sialyltransferase was downregulated on treatment with phorbol ester.(77) A similar phenomenon has been observed for hematopotic or epithelial cells. The increased sialylation of the β1 integrin subunit was correlated with decreased adhesiveness and metastatic potential.(78-80) However, on the other hand, the enzymatic removal of α2,8-linked oligosialic acids from the α5 integrin subunit expressed in G361 melanoma cells inhibited cell adhesion to FN,(81) supporting the observation that the *N*-glycans of the α and β integrin subunits play distinct roles in cell-ECM interactions.(82) Collectively, these findings suggest that the interaction of integrin α5β1 with FN is dependent on its *N*-glycosylation and the processing status of *N*-glycans.

Interestingly, the overexpression of GnT-III resulted in inhibition of α5β1 integrin-mediated cell spreading and migration, and the phosphorylation of focal adhesion kinase.(83) The affinity of the binding of integrin α5β1 to fibronectin was significantly reduced as

a result of the introduction of a bisecting GlcNAc to the α5 subunit. Thus, the overexpression of GnT-III inhibits tumor metastasis through at least two mechanisms: enhancement of cell-cell adhesion and down-regulation of cell-ECM adhesion.

As mentioned above, bisecting structures may participate in the regulation of TrkA functions.(52) However, this is not always the case. We recently found that the overexpression of GnT-III of Neuro2a cells, which lack TrkA expression, resulted in enhancement of neurite outgrowth under serum-deprivation conditions.(84) In that study, the biological significance of the bisecting GlcNAc structure on *N*-glycans introduced by GnT-III in Neuro2a cell differentiation were clearly demonstrated. The overexpression of GnT-III in the cells led to the induction of axon-like processes with numerous neurites and swellings, in which β1 integrin was localized, under conditions of serum starvation. This enhancement of neuritogenesis was suppressed by the addition of either a bisecting GlcNAc-containing *N*-glycan or E_4-PHA, which preferentially recognizes the bisecting GlcNAc. GnT-III-promoted neuritogenesis was also significantly perturbed by treatment with a functional blocking anti β1 integrin antibody. In fact, β1 integrin was found to be one of the target proteins of GnT-III, as confirmed by a pull down assay with E_4-PHA. These findings suggest that *N*-glycans with a bisecting GlcNAc on target molecules, such as β1 integrin, play important roles in the regulation of neuritogenesis. All these findings provide new aspects of the involvement of GnT-III and integrin in neuritogenesis.

Future perspective

As described above, modulation of the *N*-glycans of the receptors could significantly alter their important functions in cancer science. Since they have multiple potential sites for N-glycosylation, it is important to identify the N-glycans which are required for the receptor functions. With powerful genetic methods involving such as knock-out, knock-in and RNA silencing, studies on the physiological regulation of *N*-glycosylation on glycoproteins and identification of their target proteins will be involving a highlight of this stage of cancer science.

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Figure legends

Fig. 1 Glycosylation reactions catalyzed by the actions of glycosyltransferase GnT-III, GnT-V and Fut8, and their biological functions.

Fig. 2 Increased expression of GnT-V in epithelial cells results in a loss of contact inhibition and increased cell motility. Overexpression of GnT-III resulted in resistance of E-cadherin to proteolysis, and the E-cadherin remained on the cell-cell borders. Conversely, GnT-III can be upregulated through cell-cell interactions, therefore signals responsible for the maintenance of the cell differentiation phenotype being neutralized.

Fig. 3 Lack of core fucosylation of EGFR leads to the suppression of EGF signaling and cell growth.

EGF binding to high-affinity type of EGFR is significantly reduced in $Fut8^{-/-}$ cells, and that leads to dysfunction of EGF signaling and cell growth.

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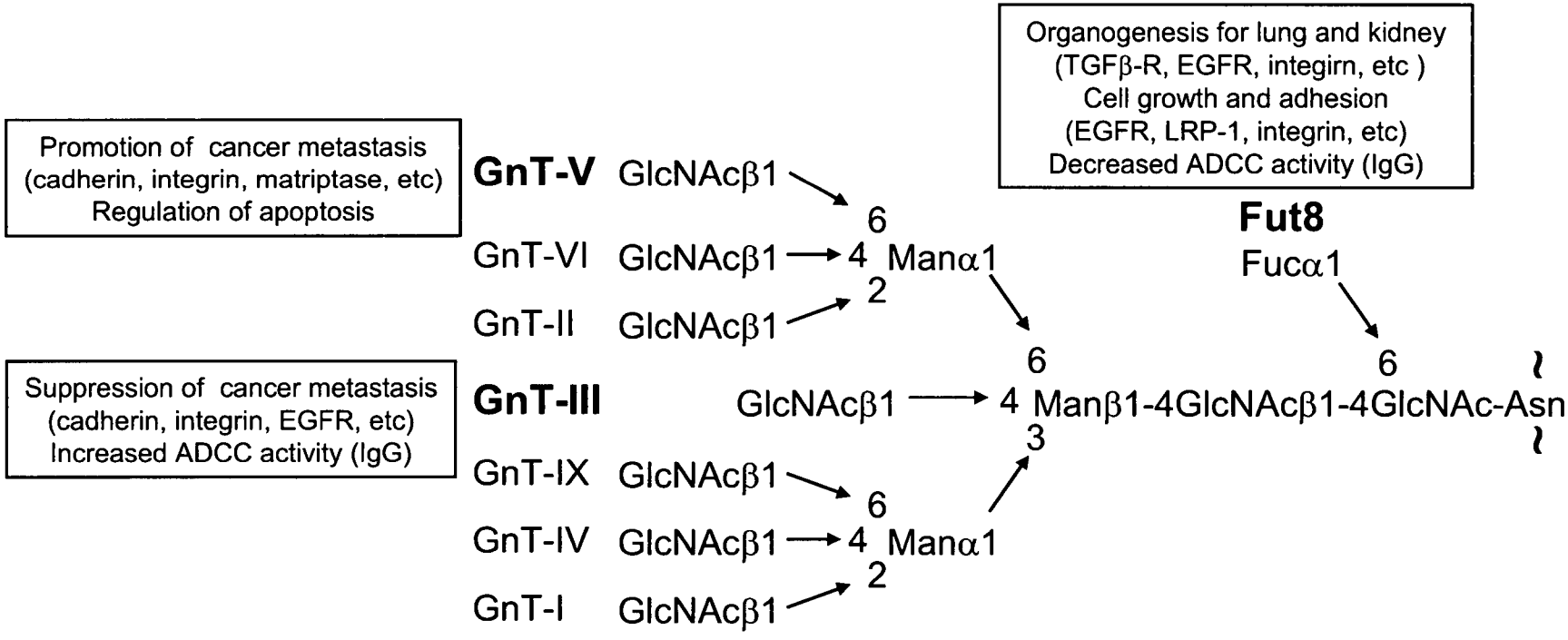

Fig. 2 Taniguchi N. et al.