Published in final edited form as:

Trends Mol Med. 2012 April ; 18(4): 224–232. doi:10.1016/j.molmed.2012.02.001.

Two opposing roles of O-glycans in tumor metastasis

Shigeru Tsuboi^{1,2}, Shingo Hatakeyama³, Chikara Ohyama^{2,3}, and Minoru Fukuda⁴

¹Department of Biochemistry, Oyokyo Kidney Research Institute, 90 Kozawa Yamazaki, Hirosaki, 036-8243 JAPAN

²Department of Urology, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, 036-8243 JAPAN

³Department of Advanced Transplant and Regeneration Medicine, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, 036-8243 JAPAN

⁴Glycobiology Unit, Tumor Microenvironment Program, Sanford-Burnham Medical Research Institute, 10901 North Torrey Pines Road, La Jolla CA 92037 USA

Abstract

Despite the high prevalence and poor outcome of patients with metastatic cancers, the processes of tumor metastasis still remain poorly understood. It has been shown that cell-surface carbohydrates attached to proteins through the amino acids serine or threonine (O-glycans) are involved in tumor metastasis, with the roles of O-glycans varying depending on their structures. Core2 O-glycans allow tumor cells to evade natural killer (NK) cells of the immune system and survive longer in the circulatory system, thereby promoting tumor metastasis. Core3 O-glycans or O-mannosyl glycans suppress tumor formation and metastasis by modulating integrin-mediated signaling. Here, we highlight recent advances in our understanding of the detailed molecular mechanisms by which O-glycans promote or suppress tumor metastasis.

Two opposing roles of carbohydrates in tumor metastasis

Extensive investigation of cell-surface carbohydrates in tumor cells has revealed two opposing roles in tumor metastasis: depending on their structures, carbohydrates can either promote or suppress metastasis. Cell-surface carbohydrates attached to proteins are classified by the nature of their linkages to the protein as either N-glycans (N-acetylglucosamine attached to asparagine) or O-glycans (N-acetylgalactosamine attached to serine or threonine).

N-glycans have been shown to play several important roles in tumor metastasis [1]. For example, upregulation of the N-glycan branching enzyme β -1,6-N-acetylglucosaminyltransferase V reduced cell-cell interactions within a tumor, promoting cell detachment and invasion by tumor cells [2]. β -1,6-N-acetylglucosaminyltransferase III suppressed lung metastasis of melanoma cells by inhibiting tumor cell attachment to extracellular matrices in the extravasation stage of metastasis [3]. Recently, it was reported that HNK-1 glycan suppressed astrocytic tumor invasion and metastasis [4].

© 2012 Elsevier Ltd. All rights reserved.

Corresponding authors: Tsuboi, S. (tsuboi@oyokyo.jp) and Fukuda, M. (minoru@sanfordburnham.org).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Although it has been suggested that *O*-glycans are also involved in the process of tumor metastasis [5], the detailed molecular mechanisms have been largely unknown. It has turned out that the roles of *O*-glycans in tumor metastasis vary depending on their structures. In this review, we summarize the roles of three different *O*-glycans in tumor metastasis.

Structures of *O*-glycans involved in tumor metastasis

In mammalian cells, biosynthesis of *O*-glycans starts with the transfer of *N*-acetylgalactosamine (GalNAc) or mannose (Man) to a polypeptide (Figure 1). GalNAc is transferred to a serine or threonine residue in the polypeptide by peptide GalNAc transferase (GalNAc-T). The first GalNAc can then be extended with additional sugars including galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose, or sialic acid (Neu5Ac). There are four common *O*-glycan core structures, core1–core4 in mammalian tissues, that depend on the combination of sugars added (Figure 1).

To generate the core1 or core 2 structures the initial GalNAc α 1-Ser/Thr is first converted by core1 synthase to Gal β 1-3GalNAc α 1-Ser/Thr (core1). Core1 is then converted to core2 by β -1,6-*N*-acetylglucosaminyltransferase (core2 β 1-6 GlcNAc transferase, or C2GnT). There are three enzymes that can synthesize core2, C2GnT-1, -2 and -3 (Figure 1). Core2 *O*-glycan is a scaffold for the subsequent production of lactosamine repeats (poly-*N*-acetylglucosamine) on *O*-glycans. The poly-*N*-acetylglucosamine formation is a critical step for the terminal modification by sialic acid and fucose [7-9]. The carbohydrates that contain sialic acid or fucose perform important roles in the various steps of tumor formation and tumor progression [10, 11]. Enhanced expression of certain sialic acid types or their linkages often correlates with poor prognosis for many human malignancies [12, 13]. For instance, fibrosarcoma cells use α 2-6-linked sialic acid to evade tumor immune surveillance systems [14].

Core3 *O*-glycan is synthesized onto the initial GalNAc α 1-Ser/Thr by core3 synthase (β 3GnT-6), which adds β -1,3-linked *N*-acetylglucosamine to *N*-acetylglactosamine at the reducing terminus [15]. Core4 *O*-glycan is then synthesized by the addition of β -1,6-*N*-acetylglucosamine to the core3 acceptor by C2GnT-2 [16, 17] (Figure 1).

The importance of *O*-mannosylation is illustrated by the roles *O*-mannosyl glycans play in a variety of processes including viral entry into cells, metastasis, cell adhesion, and neuronal development [18], despite the fact that *O*-mannosyl glycans are rare in mammals and found in a limited number of glycoproteins [19, 20]. α -dystroglycan (α -DG) is a highly glycosylated cell-surface molecule that functions as a linker between extracellular matrix (ECM) proteins and epithelial cells by binding to both laminin and β -dystroglycan. Binding of α -DG to laminin is mediated through a unique *O*-mannosyl glycan referred as laminin-binding glycan [21, 22]. Biosynthesis of laminin-binding glycan is initiated by the transfer of mannose to a Ser or Thr residue that is catalyzed by protein *O*-mannosyltransferase 1 and 2 (POMT1 and 2) [23]. Protein *O*-mannose β -1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) [24] and LARGE [25] then catalyze the transfer of GlcNAc to Man α 1-Ser/Thr. GlcNAc β -1,2-Man α 1-Ser/Thr is finally converted to *O*-mannosyl glycan containing Neu5Ac and phosphorylated mannose, which is apparently required for α -DG binding to laminin [21] (Figure 1). Recent studies showed that LARGE has α -xylosyltransferase and β -glucuronic acid transferase activities, suggesting that laminin-binding glycan is composed of polymer of α -xylose and β -glucuronic acid disaccharide units [26].

The expression of core2 *O*-glycans has been detected in malignant tumors [27-32], whereas core3 and core4 *O*-glycans are synthesized in normal cells but downregulated in cancer cells [33, 34]. In addition, it was demonstrated that *O*-mannosyl glycans are dramatically decreased in aggressive prostate and breast carcinomas [22]. These observations led to the

hypothesis that core2 *O*-glycans are metastasis-promoting but that core3 and core4 *O*-glycans and *O*-mannosyl glycans are metastasis-suppressing. We explain below in more detail the recently identified mechanisms by which these *O*-glycans promote or suppress tumor metastasis.

Metastasis-promoting core2 *O*-glycans

The roles of cell-surface carbohydrates in promoting tumor metastasis have been investigated so far by focusing on selectin-mediated interactions between tumor cells and other cells (Box 1). Sialyl Lewis x (sLe(x)) expressed on tumor cells interacts with E-selectin on endothelial cells, and core2 *O*-glycan-based sLe(x) interacts with P-selectin on platelets. These interactions are the critical events for successful extravasation of tumor cells into metastasis-target organs [35, 36]. Fucosylation is a key step for the synthesis of selectin ligands and also plays a critical role in tumor progression [37, 38].

Core2 *O*-glycan was originally found in activated T cells, and aberrant expression of core2 *O*-glycan was shown to be associated with immunodeficiency [39]. In addition, aberrant *O*-glycosylation associated with cancer alters the effector functions mediated by cytotoxic T lymphocytes and NK cells [40, 41]. Core2-based *O*-glycans also play an important role in tumor metastasis by contributing to survival of circulating tumor cells [32].

It had been reported that the expression of C2GnT-1 (written as C2GnT hereafter), a key enzyme for core2 *O*-glycan expression, was closely correlated with highly metastatic phenotypes of several tumor types. Yousefi et al. [27] made a comparison of C2GnT activity between highly and poorly metastatic tumor cell lines, and in all the experimental models of malignancy C2GnT activity was elevated in highly metastatic cell lines compared with poorly metastatic cell lines, while several other glycosyltransferase activities were not significantly different. The correlation between C2GnT expression and the malignant phenotypes implies that C2GnT expression might promote tumor metastasis.

Molecular cloning of C2GnT provided useful research tools such as a DNA probe and anti-C2GnT antibody [6, 42]. To validate the above observations from tumor cell lines, several research groups examined specimens from cancer patients by immunohistochemical analysis using anti-C2GnT and *in situ* hybridization using C2GnT cDNA as a probe. The analyses revealed that C2GnT expression was detected in high grade or highly metastatic tumors but not low grade tumors or normal tissues in patients. Furthermore, C2GnT expression was closely correlated with the vessel invasion of colorectal cancer [28]. Statistical analysis between the expression of C2GnT in pulmonary adenocarcinoma and clinicopathological variables revealed that C2GnT expression was significantly associated with vessel invasion and lymph node metastasis [29]. These results indicate that C2GnT expression is correlated with the progression of colorectal cancer and lung cancer. Thereafter, it was found that C2GnT expression also positively correlates with the progression of prostate cancer, testicular germ cell tumor, and bladder cancer [30-32].

Statistical analysis of the relationship between C2GnT expression, measured by immunohistochemical staining, and the prognosis showed prostate cancer, testicular germ cell tumor, and bladder cancer patients with C2GnT-positive tumor cells lived a significantly shorter time than patients with C2GnT-negative tumor cells, indicating that C2GnT expression was an excellent prognostic indicator [30-32]. The above observations strongly suggested that C2GnT expression promotes tumor metastasis. However, the detailed molecular mechanisms underlying highly metastatic phenotypes of C2GnT-expressing tumors were unknown.

Evasion of NK cell immunity by C2GnT-expressing tumor cells

The first attempt to understand the mechanism by which C2GnT expression promotes metastasis was made for bladder cancer. Tumor formation assays using immunodeficient mice (nude mice, SCID/beige mice, and NK cell-depleted nude mice) revealed that C2GnT-expressing cells were more resistant to NK cell attack than C2GnT-non-expressing cells. These results suggest that C2GnT-expressing bladder tumor cells possess a high ability to evade NK cell attack, resulting in longer survival after tumor cells disseminate into the host blood circulation [32]. The NK cell receptor-tumor ligand interaction mediates tumor cell rejection by host NK cells in circulation. Among those NK receptors, the NK-activating receptor natural killer group 2 member D (NKG2D) plays a critical role in eliminating tumor cells. NKG2D interacts with the tumor cell-expressed ligand MHC Class I-related chain A (MICA), and NK cells are activated by the interaction. Activated NK cells secrete several apoptosis-inducing substances such as granzyme B and perforin, thereby killing target tumor cells [43] (Figure 2A). The mechanism by which C2GnT-expressing tumor cells evade NK cell attack was investigated by focusing on the NKG2D-MICA interaction. The core2 branch is a scaffold for the subsequent production of lactosamine disaccharide repeats, poly *N*-acetylglucosamine (Gal β 1-4GlcNAc)_n, on *O*-glycans (Figure 1) [7]. Galectins are β -galactoside-binding proteins that have been implicated in numerous biological processes including immunity and tumor progression [44-46]. Galectin-1, 3 and 9 suppress tumor rejection responses by regulating T-cell functions [47-52]. In addition, it was previously suggested that galectin-3 is involved in the survival of disseminating cancer cells in host circulation during metastasis [53]. Considering poly *N*-acetylglucosamine is present on core2 *O*-glycans on MICA and galectin-3 binds MICA through this poly *N*-acetylglucosamine, galectin-3 is likely to contribute to survival of the C2GnT-expressing bladder tumor cells in circulation. Modification of the MICA *O*-glycans with poly *N*-acetylglucosamine and galectin-3 occurs on the NKG2D binding site in MICA, thereby reducing the affinity of MICA for NKG2D, severely impairing NK cell activation, and preventing the NK killing of C2GnT-expressing bladder tumor cells [32] (Figure 2B). This masking of the tumor cells promotes immune evasion of C2GnT-expressing bladder tumor cells. When tumor cells were intravenously injected into nude mice, C2GnT-expressing tumor cells produced several metastatic foci in the lungs, although C2GnT-non-expressing tumor cells produced few foci. After poly *N*-acetylglucosamine was cleaved by endo β -galactosidase and galectin-3 was removed from MICA in C2GnT-expressing tumor cells, the tumor cells became susceptible to NK cell attack and produced few metastatic foci [32]. Thus both *in vitro* and *in vivo* evidence confirms that modification of MICA with poly *N*-acetylglucosamine and galectin-3 allows C2GnT-expressing tumor cells to evade NK cell attack, resulting in longer survival of those tumor cells in host circulation and promoting tumor metastasis. Considering that this novel evasion system was demonstrated in bladder tumors [32], and that C2GnT expression correlates with a highly metastatic phenotype in several other types of tumor [27-31], it is highly likely that those tumors also use this evasion system.

Analyses of NKG2D knockout mice reveal that the NKG2D-MICA interaction is critical to tumor rejection responses [54, 55]. Previous studies reported that some tumor cells modulate the NKG2D-mediated tumor immunosurveillance in the following three ways: a large amount of soluble MICA shed by tumor cells downregulate NKG2D expression [56-58]; tumor cells sustain localized expression of the NKG2D ligands [59]; and tumor cells decrease the cell-surface expression of MICA by retaining MICA within cells [60]. Modification of MICA with poly *N*-acetylglucosamine and galectin-3 is the fourth and newly identified mechanism by which tumor cells modulate the NKG2D-MICA interaction to evade NK cell immunity by using cell-surface carbohydrates [32].

It has been reported that core2 *O*-glycans are abundantly present in normal breast tissues and were decreased in tumor formation [5, 61], suggesting that core2 *O*-glycans do not necessarily play important roles in breast tumor formation. However, the roles of the breast tumor cell-surface *O*-glycans in metastasis remain poorly understood. Future studies on the relationship between the decrease in core2 *O*-glycans and breast tumor metastasis will provide new insights into the role of cell-surface carbohydrates in tumor metastasis.

Metastasis-suppressing core 3 and 4 *O*-glycans

In contrast to C2GnT, core3 synthase expression is generally observed in normal tissues, but its expression is downregulated in cancer cells, including gastric and colorectal carcinomas [15, 33, 34]. The expression of C2GnT-2, which is responsible for the formation of core4 *O*-glycan, is also downregulated in cancer cells [62]. Based on these observations, it was speculated that core3 and core4 *O*-glycans suppress tumor metastasis.

To determine the roles of core3 *O*-glycans in tumor metastasis, two metastatic prostate cancer cell lines, PC3 and LNCaP, were transfected with core3 synthase cDNA and examined for various phenotypes. Forced expression of core3 synthase in PC3 and LNCaP cells reduced cell migration and invasion *in vitro* [63]. Core3 synthase-expressing PC3 cells produced much smaller tumors in mouse prostates without metastasizing to surrounding lymph nodes, as compared with mock-transfected PC3 cells, when tumor cells were inoculated into immunodeficient mouse prostates. Moreover, core3 synthase-expressing LNCaP cells barely produced subcutaneous tumors, in contrast with mock-transfected LNCaP cells, when tumor cells were subcutaneously inoculated into immunodeficient mice [63]. These results from both *in vitro* and *in vivo* systems suggested that core3 synthase expression suppresses tumor formation and tumor metastasis. However, the mechanisms by which core3 and core4 *O*-glycans decrease cell invasion and suppress tumor metastasis were not understood.

Integrins, a class of important cell-surface receptors (Box 2), carry various types of glycans. $\alpha 2\beta 1$ integrin carrying *O*-glycans is a collagen-receptor and one of the major players in promoting tumor metastasis by mediating tumor cell adhesion, migration, and invasion [64] (Figure 3a). Thus, $\alpha 2\beta 1$ integrin was the focus for determining the role of core3 *O*-glycans in tumor metastasis. Analyses of the prostate cancer cells discussed above revealed that the cell-surface expression of $\alpha 2\beta 1$ integrin was significantly decreased in core3 synthase-expressing tumor cells [63], and the integrin $\beta 1$ subunit was shown to carry mucin-type *O*-glycans [65]. Taken together, these results indicated that core3 *O*-glycans on the $\beta 1$ subunit reduced the cell-surface expression levels of $\alpha 2\beta 1$. There are two possible causes for reduced levels of $\alpha 2\beta 1$ (Figure 3). One is that the attachment of core3 *O*-glycans hindered proper *N*-glycosylation of the $\beta 1$ subunit, resulting in expression of the immature glycoform and reduced cell-surface levels of $\alpha 2\beta 1$ [63]. It has been observed that protein glycosylation state influences cell-surface expression levels of integrins and their downstream signal transduction pathways. For instance, loss of *N*-glycan on the β -propeller domain of the integrin $\alpha 5$ subunit decreases the cell-surface expression of $\alpha 5\beta 1$ [66, 67], and lack of *N*-glycosylation on $\beta 4$ integrin suppresses ERK- and EGFR-mediated signaling [68]. The second cause could be that core3 *O*-glycans inhibit the association of the $\beta 1$ subunit with the $\alpha 2$ subunit, resulting in reduced cell-surface levels of the mature $\alpha 2\beta 1$ complex [63].

One of the $\alpha 2\beta 1$ integrin-mediated signaling pathways that regulate cell migration involves focal adhesion kinase (FAK) [64]. The reduced cell-surface expression of $\alpha 2\beta 1$ subsequently decreases FAK phosphorylation, impairing FAK-mediated signal transduction [63]. Impaired signaling led to reduced lamellipodia formation and cell migration. Reduced cell migration led to decreased cell invasion, suppressing tumor formation and metastasis

(Figure 3b). This is the currently accepted model for how core3 synthase expression suppresses tumor metastasis.

It is likely that core3 synthase expression plays important roles in maintaining tissue homeostasis by blocking tumorigenesis in normal tissues and that its downregulation in cancer cells promotes both tumor formation and metastasis by increasing cell migration and invasion. Forced expression of core3 synthase and C2GnT-2 in tumor cells significantly decreases cell invasion and tumor formation *in vivo* [15, 62]. In core3 synthase-deficient mice, formation of colon carcinoma after treatment with the colon carcinoma-inducing chemicals azoxymethane and dextran sodium sulfate was greatly promoted compared with wild-type mice [69]. These studies using *in vivo* models strongly support the tumor-suppressing functions of core3 O-glycans.

Metastasis-suppressing O-mannosyl glycans

Discovery of another metastasis-suppressing O-glycan has unexpectedly come from studies of the muscular dystrophy. α -DG is a highly glycosylated cell-surface molecule that is expressed in the epithelial cell-basement membrane (BM) interface and functions as a linker between ECM proteins and epithelial cells by binding to both laminin and β -dystroglycans (Figure 4). Binding of α -DG to laminin is mediated through a unique O-glycan carried by α -DG, the laminin-binding glycan, which has been shown to contain the core portion of sialic acid, α -xylose- β -glucuronic acid disaccharide unit repeats and phosphorylated mannose [19, 26] (Figure 1). Laminin interaction with α -DG through the laminin-binding glycan is critical for assembling BM. Therefore, defects in the glycosylation of this glycan, which requires several glycosyltransferase-like genes including POMT1, POMT2, POMGnT1 and LARGE [23], cause muscular dystrophy [24, 25, 70].

LARGE is a causative gene product for muscular dystrophy in both mice and humans [71-73] and contains a domain homologous with β 3-N-acetylglucosaminyltransferase 1 (β 3GnT1) [71]. β 3GnT1 synthesizes i-antigen, a linear poly N-acetyllactosamine on human fetal red blood cells [74]; however, the physiological role of β 3GnT1 was unknown. Based on the structural similarity of β 3GnT1 to LARGE, a role for β 3GnT1 in the biosynthesis of laminin-binding glycans was investigated.

The important role for laminin-binding glycans in tumor metastasis was first demonstrated in prostate and breast cancers [22]. In aggressive human prostate and breast cancer cells, laminin-binding glycans of α -DG are dramatically decreased, although the amount of α -DG core protein is not changed [22]. The decrease in laminin-binding glycans was associated with decreased expression of β 3GnT1. Forced expression of β 3GnT1 in the aggressive cancer cells restored synthesis of laminin-binding glycans and reduced cell migration and invasion *in vitro* [22]. Moreover, β 3GnT1-expressing PC3 cells inoculated into the prostates of immunodeficient (SCID) mice produce much smaller tumors than mock-transfected PC3 cells [22]. These results suggest that laminin-binding glycans synthesized by β 3GnT1 are involved in suppressing tumor formation and metastasis.

The β 1 integrins such as α 2 β 1 and α 9 β 1 mediate ECM-induced cell motility by activating several signaling pathways including the ERK-AKT pathway. Downregulation of β 3GnT1 in prostate cancer cells increases phosphorylation of AKT and ERK, promoting ECM-mediated cell migration [22]. Conversely, phosphorylation of ERK and AKT was much lower in β 3GnT1-expressing tumor cells than mock-transfected cells [22], suggesting a molecular mechanism by which β 3GnT1 expression suppresses tumor migration and metastasis (Figure 4a). In aggressive tumor cells with downregulated β 3GnT1 expression, the laminin globular domains 4 and 5 of laminin α -chain are unable to bind to α -DG due to lack of laminin-binding glycan (denoted by broken arrow in Figure 4), but laminin globular

domains 1, 2, and 3 as well as other ECM proteins can still bind to the $\beta 1$ integrin (denoted by bold and solid arrow), activating the ERK-AKT signaling pathway and leading to high levels of tumor migration and metastasis (Figure 4a) [75, 76]. By contrast, the laminin-binding glycan is synthesized in $\beta 3\text{GnT1}$ -expressing tumor cells and a portion of laminin population binds to α -DG as well as the integrins (denoted by thin and solid arrows in Figure 4b), with the result being decreased laminin binding to the $\beta 1$ integrin. Laminin binding to α -DG therefore counteracts the ERK-AKT signaling pathway, leading to low tumor migration and metastasis (Figure 4b) [22]. Future studies will examine the mechanism of $\beta 3\text{GnT1}$ downregulation in normal tissues and low-metastatic tumor cells. The above results also indicate that glycosyltransferases which synthesize laminin-binding glycans are coordinately regulated, as seen for LARGE and $\beta 3\text{GnT}$. By using siRNA library screening, recent studies revealed that Fer kinase downregulates the expression of LARGE and $\beta 3\text{GnT1}$ [77]. As Fer kinase also function as an oncogene, this finding supports the conclusion that laminin-binding glycan function as tumor suppressor.

Other O-glycosylated proteins involved in tumor metastasis

There are several other observations suggesting that *O*-glycosylation plays important roles in tumor metastasis. Two studies of human colon cancer cells implicate sialylated *O*-glycans in tumor metastasis. Removal of sialic acids from the $\beta 4$ integrin *O*-glycans by the sialidase NEU1 decreased phosphorylation of the integrin, resulting in suppression of migration and metastasis [78], and removal of sialic acids from mucin *O*-glycans by another sialidase, NEU4, also suppressed adhesion and migration of colon cancer cells [79]. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) stimulates cancer cell death through the proapoptotic receptors DR4 and DR5. *O*-glycosylation of the death-receptors is critical in controlling apoptotic signaling [80]. Finally, increased *O*-glycosylation of oncofetal fibronectin is required for the epithelial-mesenchymal transition (EMT) process, a key step in tumor progression and metastasis [81].

Concluding remarks

This short review, with an emphasis on the most recent advances, provides several examples that support the significance of *O*-glycans in tumor metastasis. *O*-glycans play two opposing roles in tumor metastasis depending on their structures. *O*-glycans containing a core2 branch promote tumor metastasis by impairing NK cell activation [32]. By contrast, *O*-glycans containing core3 or phosphorylated *O*-mannosyl glycans suppress tumor metastasis by modulating integrin-mediated signaling [22, 63].

Our current understanding of the molecular mechanisms by which those *O*-glycans promote or suppress tumor metastasis may lead to the development of new therapeutic methods and agents for preventing tumor metastasis. Reduction of C2GnT activity or downregulation of C2GnT expression using specific inhibitors or siRNAs in tumor cells could restore tumor cell susceptibility to NK cell attack, promoting tumor rejection and suppressing tumor metastasis. Increased expression of core3 synthase and $\beta 3\text{GnT1}$ in tumor cells would reduce integrin-mediated signaling, suppressing both tumor migration and metastasis.

Further studies on the structures and functions of *O*-glycans, focused on the process of tumor metastasis, will provide a new insight into the roles of cell-surface carbohydrates in tumor metastasis.

Acknowledgments

We thank the Japan Society for the Promotion of Science for their support (grant 22570131 to S. T. and B22390301 to C. O.), as well as the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant 21791483

to C. O.), the Japan Science and Technology Agency (CREST to C. O.), and the National Institutes of Health (grants P01CA71932 and R01CA3000 to M. F.).

References

1. Fuster MM, Esko JD. The sweet and sour of cancer: glycans as novel therapeutic targets. *Nat Rev Cancer*. 2005; 5:526–542. [PubMed: 16069816]
2. Dennis JW, et al. UDP-N-acetylglucosamine:alpha-6-D-mannoside beta1,6 N-acetylglucosaminyltransferase V (Mgat5) deficient mice. *Biochim Biophys Acta*. 2002; 1573:414–422. [PubMed: 12417426]
3. Yoshimura M, et al. Suppression of lung metastasis of B16 mouse melanoma by N-acetylglucosaminyltransferase III gene transfection. *Proc Natl Acad Sci U S A*. 1995; 92:8754–8758. [PubMed: 7568011]
4. Suzuki-Anekoji M, et al. HNK-1 Glycan Functions as a Tumor Suppressor for Astrocytic Tumor. *J Biol Chem*. 2011; 286:32824–32833. [PubMed: 21784847]
5. Brockhausen I. Mucin-type O-glycans in human colon and breast cancer: glycodynamics and functions. *EMBO Rep*. 2006; 7:599–604. [PubMed: 16741504]
6. Bierhuizen MF, Fukuda M. Expression cloning of a cDNA encoding UDP-GlcNAc:Gal beta 1-3-GalNAc-R (GlcNAc to GalNAc) beta 1-6GlcNAc transferase by gene transfer into CHO cells expressing polyoma large tumor antigen. *Proc Natl Acad Sci U S A*. 1992; 89:9326–9330. [PubMed: 1329093]
7. Maemura K, Fukuda M. Poly-N-acetylglucosaminyl O-glycans attached to leukosialin. The presence of sialyl Le(x) structures in O-glycans. *J Biol Chem*. 1992; 267:24379–24386. [PubMed: 1447188]
8. Lofling J, Holgersson J. Core saccharide dependence of sialyl Lewis X biosynthesis. *Glycoconj J*. 2009; 26:33–40. [PubMed: 18607721]
9. Stone EL, et al. Glycosyltransferase function in core 2-type protein O glycosylation. *Mol Cell Biol*. 2009; 29:3770–3782. [PubMed: 19349303]
10. Gilbert SF. Ageing and cancer as diseases of epigenesis. *J Biosci*. 2009; 34:601–604. [PubMed: 19920344]
11. Varki A, et al. Glycosylation Changes in Cancer. 2009
12. Hedlund M, et al. alpha 2-6-Linked sialic acids on N-glycans modulate carcinoma differentiation in vivo. *Cancer Res*. 2008; 68:388–394. [PubMed: 18199532]
13. Wang FL, et al. High expression of alpha 2, 3-linked sialic acid residues is associated with the metastatic potential of human gastric cancer. *Cancer Detect Prev*. 2009; 32:437–443. [PubMed: 19232843]
14. Cohen M, et al. Sialylation of 3-methylcholanthrene-induced fibrosarcoma determines antitumor immune responses during immunoediting. *J Immunol*. 2010; 185:5869–5878. [PubMed: 20956342]
15. Iwai T, et al. Core 3 synthase is down-regulated in colon carcinoma and profoundly suppresses the metastatic potential of carcinoma cells. *Proc Natl Acad Sci U S A*. 2005; 102:4572–4577. [PubMed: 15755813]
16. Yeh JC, et al. Molecular cloning and expression of a novel beta-1, 6-N-acetylglucosaminyltransferase that forms core 2, core 4, and I branches. *J Biol Chem*. 1999; 274:3215–3221. [PubMed: 9915862]
17. Schwientek T, et al. Control of O-glycan branch formation. Molecular cloning of human cDNA encoding a novel beta1,6-N-acetylglucosaminyltransferase forming core 2 and core 4. *J Biol Chem*. 1999; 274:4504–4512. [PubMed: 9988682]
18. Stalnakier SH, et al. Mammalian O-mannosylation: unsolved questions of structure/function. *Curr Opin Struct Biol*. 2011; 21:603–609. [PubMed: 21945038]
19. Chiba A, et al. Structures of sialylated O-linked oligosaccharides of bovine peripheral nerve alpha-dystroglycan. The role of a novel O-mannosyl-type oligosaccharide in the binding of alpha-dystroglycan with laminin. *J Biol Chem*. 1997; 272:2156–2162. [PubMed: 8999917]
20. Endo T. O-mannosyl glycans in mammals. *Biochim Biophys Acta*. 1999; 1473:237–246. [PubMed: 10580142]

21. Yoshida-Moriguchi T, et al. O-mannosyl phosphorylation of alpha-dystroglycan is required for laminin binding. *Science*. 2010; 327:88–92. [PubMed: 20044576]
22. Bao X, et al. Tumor suppressor function of laminin-binding alpha-dystroglycan requires a distinct beta3-N-acetylglucosaminyltransferase. *Proc Natl Acad Sci U S A*. 2009; 106:12109–12114. [PubMed: 19587235]
23. Barresi R, Campbell KP. Dystroglycan: from biosynthesis to pathogenesis of human disease. *J Cell Sci*. 2006; 119:199–207. [PubMed: 16410545]
24. Yoshida A, et al. Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. *Dev Cell*. 2001; 1:717–724. [PubMed: 11709191]
25. Kanagawa M, et al. Molecular recognition by LARGE is essential for expression of functional dystroglycan. *Cell*. 2004; 117:953–964. [PubMed: 15210115]
26. Inamori K, et al. Dystroglycan function requires xylosyl- and glucuronyltransferase activities of LARGE. *Science*. 2012; 335:93–96. [PubMed: 22223806]
27. Yousefi S, et al. Increased UDP-GlcNAc:Gal beta 1-3GalNAc-R (GlcNAc to GalNAc) beta-1, 6-N-acetylglucosaminyltransferase activity in metastatic murine tumor cell lines. Control of polylactosamine synthesis. *J Biol Chem*. 1991; 266:1772–1782. [PubMed: 1824844]
28. Shimodaira K, et al. Carcinoma-associated expression of core 2 beta-1,6-N-acetylglucosaminyltransferase gene in human colorectal cancer: role of O-glycans in tumor progression. *Cancer Res*. 1997; 57:5201–5206. [PubMed: 9393734]
29. Machida E, et al. Clinicopathological significance of core 2 beta1,6-N-acetylglucosaminyltransferase messenger RNA expressed in the pulmonary adenocarcinoma determined by in situ hybridization. *Cancer Res*. 2001; 61:2226–2231. [PubMed: 11280791]
30. Hagiwara S, et al. Expression of core 2 beta1,6-N-acetylglucosaminyltransferase facilitates prostate cancer progression. *Glycobiology*. 2005; 15:1016–1024. [PubMed: 15932919]
31. Hatakeyama S, et al. Core 2 N-acetylglucosaminyltransferase-1 expression induces aggressive potential of testicular germ cell tumor. *Int J Cancer*. 2010; 127:1052–1059. [PubMed: 20017138]
32. Tsuboi S, et al. A novel strategy for evasion of NK cell immunity by tumours expressing core2 O-glycans. *EMBO J*. 2011; 30:3173–3185. [PubMed: 21712812]
33. Vavasseur F, et al. O-glycan biosynthesis in human colorectal adenoma cells during progression to cancer. *Eur J Biochem*. 1994; 222:415–424. [PubMed: 8020479]
34. Vavasseur F, et al. Synthesis of O-glycan core 3: characterization of UDP-GlcNAc: GalNAc-R beta 3-N-acetyl-glucosaminyltransferase activity from colonic mucosal tissues and lack of the activity in human cancer cell lines. *Glycobiology*. 1995; 5:351–357. [PubMed: 7655172]
35. Laubli H, Borsig L. Selectins promote tumor metastasis. *Semin Cancer Biol*. 2010; 20:169–177. [PubMed: 20452433]
36. St Hill CA. Interactions between endothelial selectins and cancer cells regulate metastasis. *Front Biosci*. 2012; 17:3233–3251. [PubMed: 21622232]
37. Sozzani P, et al. Is Sialyl Lewis x antigen expression a prognostic factor in patients with breast cancer? *Int J Surg Pathol*. 2008; 16:365–374. [PubMed: 18977761]
38. Yusa A, et al. Epigenetic silencing of the sulfate transporter gene DTDST induces sialyl Lewisx expression and accelerates proliferation of colon cancer cells. *Cancer Res*. 2010; 70:4064–4073. [PubMed: 20460514]
39. Tsuboi S, Fukuda M. Roles of O-linked oligosaccharides in immune responses. *Bioessays*. 2001; 23:46–53. [PubMed: 11135308]
40. Lakshminarayanan V, et al. Immune recognition of tumor-associated mucin MUC1 is achieved by a fully synthetic aberrantly glycosylated MUC1 tripartite vaccine. *Proc Natl Acad Sci U S A*. 2012; 109:261–266. [PubMed: 22171012]
41. Benson V, et al. Glycosylation regulates NK cell-mediated effector function through PI3K pathway. *Int Immunol*. 2010; 22:167–177. [PubMed: 20089585]
42. Skrinkosky D, et al. Altered Golgi localization of core 2 beta-1,6-N-acetylglucosaminyltransferase leads to decreased synthesis of branched O-glycans. *J Biol Chem*. 1997; 272:22695–22702. [PubMed: 9278427]

43. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. *Nature Immunology*. 2008; 9:495–502. [PubMed: 18425106]
44. Rabinovich GA, et al. An emerging role for galectins in tuning the immune response: lessons from experimental models of inflammatory disease, autoimmunity and cancer. *Scand J Immunol*. 2007; 66:143–158. [PubMed: 17635792]
45. Yang RY, et al. Galectins: structure, function and therapeutic potential. *Expert Rev Mol Med*. 2008; 10:e17. [PubMed: 18549522]
46. Saegusa J, et al. Galectin-3 is critical for the development of the allergic inflammatory response in a mouse model of atopic dermatitis. *Am J Pathol*. 2009; 174:922–931. [PubMed: 19179612]
47. Rubinstein N, et al. Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection; A potential mechanism of tumor-immune privilege. *Cancer Cell*. 2004; 5:241–251. [PubMed: 15050916]
48. Demotte N, et al. Restoring the association of the T cell receptor with CD8 reverses anergy in human tumor-infiltrating lymphocytes. *Immunity*. 2008; 28:414–424. [PubMed: 18342010]
49. Dardalhon V, et al. Tim-3/galectin-9 pathway: regulation of Th1 immunity through promotion of CD11b+Ly-6G+ myeloid cells. *J Immunol*. 2010; 185:1383–1392. [PubMed: 20574007]
50. Peng W, et al. Tumor-associated galectin-3 modulates the function of tumor-reactive T cells. *Cancer Res*. 2008; 68:7228–7236. [PubMed: 18757439]
51. van Kooyk Y, Rabinovich GA. Protein-glycan interactions in the control of innate and adaptive immune responses. *Nat Immunol*. 2008; 9:593–601. [PubMed: 18490910]
52. Zwirner NW, et al. Overcoming the hurdles of tumor immunity by targeting regulatory pathways in innate and adaptive immune cells. *Curr Pharm Des*. 2010; 16:255–267. [PubMed: 20109135]
53. Zaia Povegliano L, et al. Immunoexpression of Galectin-3 in Colorectal Cancer and its Relationship with Survival. *J Gastrointest Cancer*. 2010
54. Guerra N, et al. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity*. 2008; 28:571–580. [PubMed: 18394936]
55. Zafirova B, et al. Altered NK cell development and enhanced NK cell-mediated resistance to mouse cytomegalovirus in NKG2D-deficient mice. *Immunity*. 2009; 31:270–282. [PubMed: 19631564]
56. Groh V, et al. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature*. 2002; 419:734–738. [PubMed: 12384702]
57. Doubrovina ES, et al. Evasion from NK cell immunity by MHC class I chain-related molecules expressing colon adenocarcinoma. *J Immunol*. 2003; 171:6891–6899. [PubMed: 14662896]
58. Clayton A, et al. Human tumor-derived exosomes down-modulate NKG2D expression. *J Immunol*. 2008; 180:7249–7258. [PubMed: 18490724]
59. Oppenheim DE, et al. Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance. *Nat Immunol*. 2005; 6:928–937. [PubMed: 16116470]
60. Fuertes MB, et al. Intracellular retention of the NKG2D ligand MHC class I chain-related gene A in human melanomas confers immune privilege and prevents NK cell-mediated cytotoxicity. *J Immunol*. 2008; 180:4606–4614. [PubMed: 18354183]
61. Storr SJ, et al. The O-linked glycosylation of secretory/shed MUC1 from an advanced breast cancer patient's serum. *Glycobiology*. 2008; 18:456–462. [PubMed: 18332077]
62. Huang MC, et al. C2GnT-M is downregulated in colorectal cancer and its re-expression causes growth inhibition of colon cancer cells. *Oncogene*. 2006; 25:3267–3276. [PubMed: 16418723]
63. Lee SH, et al. Core3 O-glycan synthase suppresses tumor formation and metastasis of prostate carcinoma PC3 and LNCaP cells through down-regulation of alpha2beta1 integrin complex. *J Biol Chem*. 2009; 284:17157–17169. [PubMed: 19395705]
64. Basson MD. An intracellular signal pathway that regulates cancer cell adhesion in response to extracellular forces. *Cancer Res*. 2008; 68:2–4. [PubMed: 18172287]
65. Clement M, et al. Expression of sialyl-Tn epitopes on beta1 integrin alters epithelial cell phenotype, proliferation and haptotaxis. *J Cell Sci*. 2004; 117:5059–5069. [PubMed: 15383613]

66. Isaji T, et al. N-glycosylation of the beta-propeller domain of the integrin alpha5 subunit is essential for alpha5beta1 heterodimerization, expression on the cell surface, and its biological function. *J Biol Chem*. 2006; 281:33258–33267. [PubMed: 16959765]
67. Gu J, Taniguchi N. Potential of N-glycan in cell adhesion and migration as either a positive or negative regulator. *Cell Adh Migr*. 2008; 2:243–245. [PubMed: 19262156]
68. Kariya Y, Gu J. N-glycosylation of ss4 integrin controls the adhesion and motility of keratinocytes. *PLoS One*. 2011; 6:e27084. [PubMed: 22073258]
69. An G, et al. Increased susceptibility to colitis and colorectal tumors in mice lacking core 3-derived O-glycans. *J Exp Med*. 2007; 204:1417–1429. [PubMed: 17517967]
70. Muntoni F, et al. Muscular dystrophies due to glycosylation defects. *Neurotherapeutics*. 2008; 5:627–632. [PubMed: 19019316]
71. Peyrard M, et al. The human LARGE gene from 22q12.3-q13.1 is a new, distinct member of the glycosyltransferase gene family. *Proc Natl Acad Sci U S A*. 1999; 96:598–603. [PubMed: 9892679]
72. Grewal PK, et al. Mutant glycosyltransferase and altered glycosylation of alpha-dystroglycan in the myodystrophy mouse. *Nat Genet*. 2001; 28:151–154. [PubMed: 11381262]
73. Longman C, et al. Mutations in the human LARGE gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of alpha-dystroglycan. *Hum Mol Genet*. 2003; 12:2853–2861. [PubMed: 12966029]
74. Sasaki K, et al. Expression cloning of cDNA encoding a human beta-1,3-N-acetylglucosaminyltransferase that is essential for poly-N-acetyllactosamine synthesis. *Proc Natl Acad Sci U S A*. 1997; 94:14294–14299. [PubMed: 9405606]
75. Larsen M, et al. The matrix reorganized: extracellular matrix remodeling and integrin signaling. *Curr Opin Cell Biol*. 2006; 18:463–471. [PubMed: 16919434]
76. Ido H, et al. Molecular dissection of the alpha-dystroglycan- and integrin-binding sites within the globular domain of human laminin-10. *J Biol Chem*. 2004; 279:10946–10954. [PubMed: 14701821]
77. Yoneyama T, et al. Fer kinase regulates cell migration through alpha-dystroglycan glycosylation. *Mol Biol Cell*. 2012 in press.
78. Uemura T, et al. Contribution of sialidase NEU1 to suppression of metastasis of human colon cancer cells through desialylation of integrin beta4. *Oncogene*. 2009; 28:1218–1229. [PubMed: 19151752]
79. Shiozaki K, et al. Regulation of sialyl Lewis antigen expression in colon cancer cells by sialidase NEU4. *J Biol Chem*. 2011; 286:21052–21061. [PubMed: 21521691]
80. Wagner KW, et al. Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. *Nat Med*. 2007; 13:1070–1077. [PubMed: 17767167]
81. Freire-de-Lima L, et al. Involvement of O-glycosylation defining oncofetal fibronectin in epithelial-mesenchymal transition process. *Proc Natl Acad Sci U S A*. 2011; 108:17690–17695. [PubMed: 22006308]

Box 1. Selectin

Selectins, a family of cell adhesion molecule, are expressed by leukocytes, endothelial cells, and platelets. All selectins are single-chain transmembrane glycoproteins that interact with cell-surface glycoconjugates and mediate tethering, rolling, and adhesion of several types of cells. There are three subsets of selectins: L-selectin is constitutively expressed by leukocytes, E-selectin by activated endothelial cells, and P-selectin by platelets and activated endothelial cells. Sialyl Lewis X (sLe(x)) and sialyl Lewis A (sLe(a)) tetrasaccharides are carbohydrate motifs displayed on protein or lipid scaffolds that are critical components of functional selectin ligand glycoconjugates.

Box 2. Integrin and integrin-mediated signaling

Integrins are cell-surface receptors that recognize and bind to extracellular matrix proteins and counter receptors. Integrins are heterodimers composed of α and β subunits. There are 18 α subunits and 8 β subunits that assemble in 24 different combinations with overlapping substrate specificity and cell-type-specific expression patterns. Binding of activated integrins to their ligands mediates a large number of structural and signaling changes within the cell. Immediate intracellular changes are increased tyrosine phosphorylation of specific substrates such as Src, focal adhesion kinase (FAK), and p130Cas as well as an increase in the concentrations of lipid second messengers such as phosphatidylinositol-4,5-bisphosphate. The immediate effects initiate activation of signaling pathways via serine/threonine protein kinases such as ERK, AKT, and JNK and GTPases such as Rho. These signaling pathways ultimately lead to cell survival, proliferation, differentiation, and migration.

Figure 1. O-glycan biosynthesis pathways that promote or suppress tumor metastasis

(a) To begin synthesis of core2 and core4 O-glycans N-acetylgalactosamine (GalNAc) is transferred to a serine (Ser) or threonine (Thr) residue in a polypeptide by the peptide GalNAc transferase (GalNAc-T), as shown on the left side of the figure. GalNAcα1-Ser/Thr is then converted by core1 synthase

Figure 2. Core2 O-glycans promote tumor metastasis by evading NK cell attack

The molecular mechanism by which C2GnT-expressing tumor cells evade NK cells. **(a)** On the left is an illustration of a major mechanism by which tumors are rejected by NK cells. NK cells are activated by the interaction of NKG2D with MICA on the surface of the tumor cells. Activated NK cells secrete perforin and granzyme B to induce apoptosis of target tumor cells. **(b)** On the right is the mechanism by which C2GnT-expressing tumor cells evade NK cell attack. The protein galectin-3 binds to poly-*N*-acetyllactosamine in the NKG2D-binding site of MICA. Galectin-3 reduces the affinity of MICA for NKG2D, severely impairing NK cell activation. This masking of the tumor cells increases survival of the tumor cells in host blood circulation, promoting tumor metastasis.

Figure 3. The role of core3 O-glycan in suppressing migration and tumor metastasis
(a) In tumor cells with low core3 synthase expression, ligand binding to $\alpha 2\beta 1$ integrin induces phosphorylation of FAK. Phosphorylated FAK transduces signals to mediate tumor cell migration and invasion, promoting metastasis. **(b)** In cells that express core3 synthase, core3 O-glycans on the $\beta 1$ subunit of $\alpha 2\beta 1$ integrin prevent the association of the $\alpha 2$ and $\beta 1$ subunits, reducing the amount of functional $\alpha 2\beta 1$ complex on the cell-surface. The reduced levels of $\alpha 2\beta 1$ limit FAK phosphorylation, impairing signal transduction. Impaired signaling reduces cell migration and invasion and suppresses tumor formation and metastasis.

Figure 4. The role of phosphorylated *O*-mannosyl glycan (laminin-binding glycan) in suppressing tumor metastasis

(a) In tumor cells with downregulated $\beta 3\text{GnT1}$ expression, laminin (LN) globular domains 4 and 5 are unable to bind to α -DG because the α -DG laminin-binding glycan is not synthesized (denoted by broken arrow), but laminin globular domains 1, 2, and 3 still bind to $\beta 1$ integrin (denoted by bold and solid arrow), activating ERK-AKT signaling. This activated signaling mediates high tumor migration and invasion, resulting in metastasis. **(b)** In $\beta 3\text{GnT1}$ -expressing tumor cells, laminin-binding glycan containing α -xylose and β -glucuronic acid disaccharide unit repeats and phosphorylated mannose is synthesized on α -DG. Laminin globular domains 4 and 5 bind to α -DG through laminin-binding glycan (denoted by solid arrow), decreasing the amount of laminin available to bind $\beta 1$ integrin (denoted by thin and solid arrows) and counteracting the ERK-AKT signaling. This results in suppressed tumor migration and metastasis. The globular domains 1 through 5 of laminin α -chain are indicated by the circled numbers.