Tadashi Suzuki · Kazuaki Ohtsubo
Naoyuki Taniguchi *Editors*

Sugar Chains

Decoding the Functions of Glycans

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Preface

When the title of this book, *Sugar Chains*, was first proposed by the publisher, for a moment I thought that it may sound a bit too non-scientific, but after pondering it, I found that this may be a perfect title for the book, as it somewhat reflects how glycoscience or glycobiology has been perceived by those who are non-experts. It is just an enigmatic "sugar" thing (in a strict sense, it actually represents sucrose!) attached to lipids or proteins that gives all of us, whether experts or not, a headache because of its incredible structural diversity as well as the lack of simple analytical methods.

It has been more than two decades since the word "glycobiology" was coined. While this research area, due to its unique methodology not compatible with most biochemists, has long been regarded as a very specialized field with limited interest, the recent explosive research progress has provided countless examples of critical roles for glycan chains in various important biological phenomena. For instance, cell surface glycans can be regarded as a "face" of cells, and their structures are known to change depending on developmental stages or environment. Therefore, cell surface glycans are utilized for identification of stem cells such as induced pluripotent stem (iPS) cells or embryonic stem (ES) cells, or as valuable biomarkers in diagnosis/detection of cancer.

In this book, recent breakthrough results have been introduced regarding the roles of glycans in quality control or intracellular trafficking of proteins, immunology, viral infection, stem cell biology, neuroscience, and various diseases such as cancer, diabetes, chronic obstructive pulmonary disease (COPD), muscular dystrophy, or schizophrenia. In each chapter, outstanding "glyco-related" questions are also posed, so that researchers not familiar with glycoscience will have a clearer idea about what the future direction for further clarification of the role of glycans in respective research fields will be. We are proud of the fact that quite an impressive line-up of articles is gathered here. We do hope that this book will serve as a good "textbook" especially for those who are not familiar with, but nevertheless interested in, sugar chains in diverse research fields.

We editors would like to thank Mr. Kaoru Hashimoto and Mr. Yasutaka Okazaki (both of Springer Japan), for giving us the opportunity of editing this book; Ms. Momoko Asawa and Ms. Yuko Matsumoto (both of Springer Japan); and Ms. Kotoko Ueno (Glycometabolome Team, RIKEN) for their devoted help in the editing process.

Wako, Japan Tadashi Suzuki (for the editors)

Contents

Chapter 1
N-Glycans and Quality Control of Proteins

Nobuko Hosokawa and Tadashi Suzuki

Abstract Glycosylation is one of the most ubiquitous posttranslational modifications for eukaryotic proteins. There are numerous examples of the attachment of glycans to carrier proteins resulting in changes in their physicochemical properties, such as solubility or heat stability, as well as physiological properties, such as bioactivity or intra- or intercellular trafficking. In addition, recent studies have revealed that *N*-glycans can act as a readout for the folding status of glycoproteins in the endoplasmic reticulum (ER), so that only proper amounts of functional proteins are made and delivered to their respective destinations. This process is often called the glycoprotein quality control system, as a part of the ER protein homeostasis machinery. After misfolded glycoproteins are targeted for destruction in the ER, they are eventually retrotranslocated into the cytosol for proteasomal degradation. In the cytosol, glycans are again used for recognition by ubiquitin ligases but are eventually removed from glycoproteins in order to efficiently degrade misfolded glycoproteins. In the present review, a particular focus will be on the mannose-trimming processes, whereby *N*-glycan-dependent ER-associated degradation signals are created and recognized in the ER, as well as on the function of Fbs proteins and PNGase that recognize *N*-glycans in the cytosol.

Keywords Endoplasmic reticulum (ER) • ER-associated degradation (ERAD) • EDEM • Fbs protein • Glycoprotein • Mannose trimming • *N*-glycans • PNGase • Protein quality control • Ubiquitin ligase

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1.1 *N*-Glycans as Signals for Glycoprotein Quality Control in the Endoplasmic Reticulum

The endoplasmic reticulum (ER) is an organelle where the majority of secretory and membrane proteins are synthesized, with approximately one-third of genes encoding proteins that enter the secretory pathway. Most of these proteins are modified with glycosylation, resulting in changes in their solubility and stability, or biological activities. An *N*-glycan comprised of 14 sugars ($Glc_3Man_9GlcNAc_2$) is covalently attached en bloc to the Asn residue in the consensus sequence (Asn-Xaa-Ser/Thr) of newly synthesized proteins in mammalian or yeast ER (Fig. 1.1a) (Kornfeld and Kornfeld 1985). However, immediately after the addition, processing of glucose from the *N*-glycan begins, followed by mannose processing (Helenius and Aebi 2004; Parodi 2000). Normally, it is believed that glycoproteins leave the ER after the removal of a (and potentially more) mannose residue(s) from the *N*-glycans. The specific sugar moieties are recognized by lectins in the ER (Fig. 1.1b). Nascent polypeptides are eventually folded with the assistance of chaperone proteins, oxidoreductases and other enzymes in the ER. The ER quality control mechanism strictly monitors the folding states of proteins, ensuring that only correctly folded or assembled proteins are sorted further to the secretory pathway

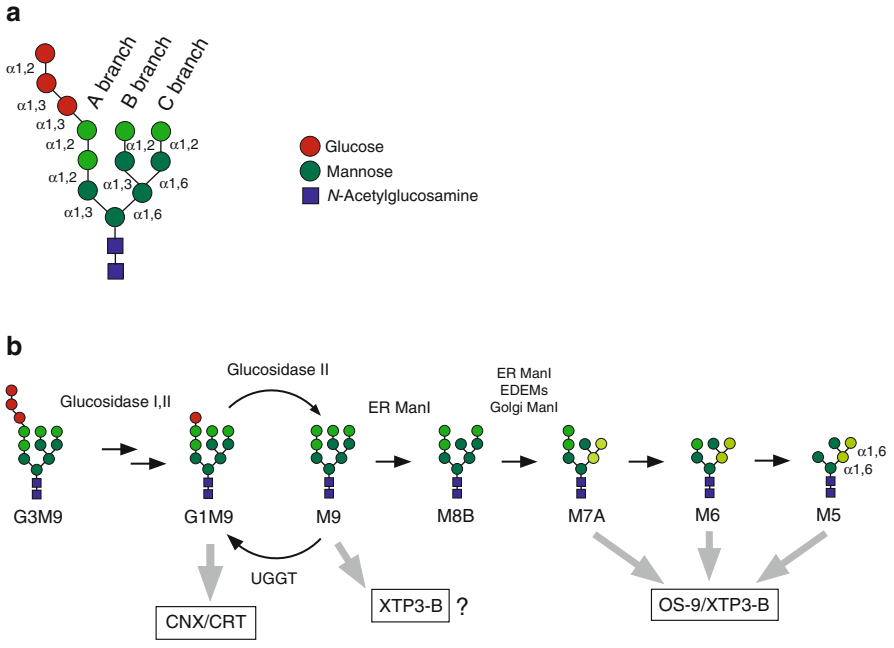

Fig. 1.1 Structure of *N*-glycan (**a**) and processing/recognition of *N*-glycans during quality control of glycoproteins (**b**). α1,2-Linked mannoses are shown in *light green*, and α1,6-linked mannoses are shown in *olive green* in **b**

(Ellgaard and Helenius 2003; Sitia and Braakman 2003). Polypeptides that fail to obtain their native conformations or have unfolded structures under stress conditions are prevented from being secreted. They reenter the folding cycle, whereupon terminally misfolded polypeptides are eventually degraded. ER-associated degradation (ERAD) is a mechanism whereby misfolded ER proteins are retrotranslocated to the cytosol and degraded by proteasomes (McCracken and Brodsky 2003; Plemper and Wolf 1999). During these processes, *N*-glycans act as signals for the quality control of glycoproteins.

In the ER, monoglucosylated *N*-glycan ($Glc_1Man_9GlcNAc_2$) is recognized by the lectin calnexin (CNX) and its soluble homologue calreticulin (CRT), thereby assisting protein folding (Helenius and Aebi 2001; Maattanen et al. 2010). Once the terminal glucose is removed by glucosidase II, glycoproteins are released from CNX/CRT. UDP-glucose:glycoprotein-glucosyltransferase (UGGT) is an enzyme that adds monoglucose back to *N*-glycans on the polypeptides that still have not acquired their native conformations, thus, directing the nonnative conformer back to CNX/CRT (CNX/CRT cycle or monoglucose cycle) (Caramelo and Parodi 2008; D'Alessio et al. 2010). Since *S. cerevisiae* possesses calnexin (Cne1p) but lacks UGGT, glycoproteins do not enter this extensive folding cycle in this organism.

Glycoproteins that fail to obtain their native conformations are removed from the ER, thereby preventing deleterious effects such as aggregation formation or interference with the folding of newly synthesized polypeptides. Mannoses are processed from *N*-glycans on the polypeptides while remaining in the ER, and hence unfolded glycoproteins with mannose-processed *N*-glycans are targeted for degradation (mannose timer model) (Helenius 1994; Jakob et al. 1998). Removal of the mannose from the A branch terminates reentry to the CNX/CRT cycle. Recently, *N*-glycans lacking the terminal mannose of the C branch, or the exposure of the α1,6-linked mannose, have been identified as the signal for ERAD (Aebi et al. 2010; Hosokawa et al. 2010a; Lederkremer 2009; Smith et al. 2011). Yos9p in *S. cerevisiae* and OS-9 and XTP3-B/Erlectin in mammals are resident ER proteins that contain sugar recognition domains for mannose 6-phosphate homology (MRH) (Castonguay et al. 2011; Munro 2001) and recognize *N*-glycan signals for ERAD.

Membrane-embedded ubiquitin ligases (E3) play central roles in ERAD. The Hrd1p-Hrd3p ubiquitin ligase complex in *S. cerevisiae* and its mammalian homologue HRD1-SEL1L form large membrane complexes by the association of various ERAD components including ubiquitination enzymes (E2) and Der1p (Derlins in mammals) (Claessen et al. 2012; Hampton and Sommer 2012; Smith et al. 2011). In the luminal side of the complex, a large domain of Hrd3p/SEL1L acts as a scaffold for the recognition of misfolded cargo by the association of lectins and chaperone proteins. Hrd3p/SEL1L also directly binds to the misfolded proteins; thus, in coordination with lectins and chaperones, the client proteins are transported to the cytosol through the retrotranslocation channel.

In the following sections, we will review the current knowledge of the role of various mannosidases/mannose-binding lectins for the creation/recognition of "degradation signals" on misfolded glycoproteins.

1.2 Enzymes That Process Mannose Residues from the *N*-Glycans for ERAD: ER Mannosidase, Golgi Mannosidases, and EDEM Proteins

In mammals, three subfamilies of Class I α-mannosidases (glycosyl hydrolase family 47 (Herscovics 1999a; Mast and Moremen 2006)) are known: ER processing α1,2-mannosidase (ER ManI), Golgi α1,2-mannosidases (Golgi ManIA, IB, and IC), and ER degradation enhancing α-mannosidase-like proteins (EDEM1, 2, and 3) (Fig. 1.2a). This class of α-mannosidases trims α1,2-linked mannoses, although the mannose-processing activity of EDEM proteins remains controversial. *S. cerevisiae* possesses ER mannosidase (Mns1p) and EDEM homologues (Htm1p/Mnl1p and Mnl2p) but lacks Golgi α1,2-mannosidase.

The mannose-processing specificity of ER ManI and Golgi ManI has been well characterized using purified recombinant proteins in vitro. ER ManI (Mns1p in yeast) preferentially removes mannose from the B branch, creating $Man_8GlcNAc_2$ isomer B (Man8B) (Gonzalez et al. 1999; Herscovics 1999b; Tremblay and Herscovics 1999). However, ER ManI is also able to remove mannoses from other branches when high concentrations of this enzyme are incubated with *N*-glycans for a prolonged time period (Aikawa et al. 2012; Herscovics et al. 2002). This suggests that *N*-glycans that are recognized as degradation signals can be created by the sole function of ER ManI. In agreement with this, overexpression of ER ManI in cultured cells enhances glycoprotein ERAD (Hosokawa et al. 2003; Wu et al. 2003) and increases mannose trimming from ERAD substrates, whereas knockdown of ER ManI greatly inhibits mannose processing (Avezov et al. 2008). Under normal conditions, ER ManI is concentrated in a specific ER subcompartment termed the ER quality control compartment (Avezov et al. 2008), in which misfolded proteins destined for degradation are enriched. ER ManI is short-lived and is degraded in the lysosome (Wu et al. 2007). This mechanism is thought to prevent clearance of nascent polypeptides during the folding process by the presence of excess ER ManI, which would otherwise impair normal cellular function.

Golgi α1,2-mannosidases preferentially process mannoses from the A and C branches once the B-branch terminal mannose is removed (Lal et al. 1998). Thus, it is also possible that Golgi α1,2-mannosidases create the *N*-glycan tags recognized as ERAD signals during the recycling of misfolded substrates between the ER and Golgi (Hammond and Helenius 1994; Hosokawa et al. 2007). It was recently reported that ER ManI resides in the Golgi apparatus (Pan et al. 2011), and a model was proposed whereby misfolded glycoproteins that escaped the ER quality control checkpoint are sorted to the Golgi apparatus, where ER ManI captures, demannosylates, and assists the substrates sent back to the ER for degradation (Pan et al. 2013b). In this scenario, the question is which enzyme processes mannoses from the folded proteins in the ER? Is there another α1,2-mannosidase in the ER that normally creates *N*-glycan degradation signals? Several issues remain unresolved regarding mannose processing in the early secretory pathway.

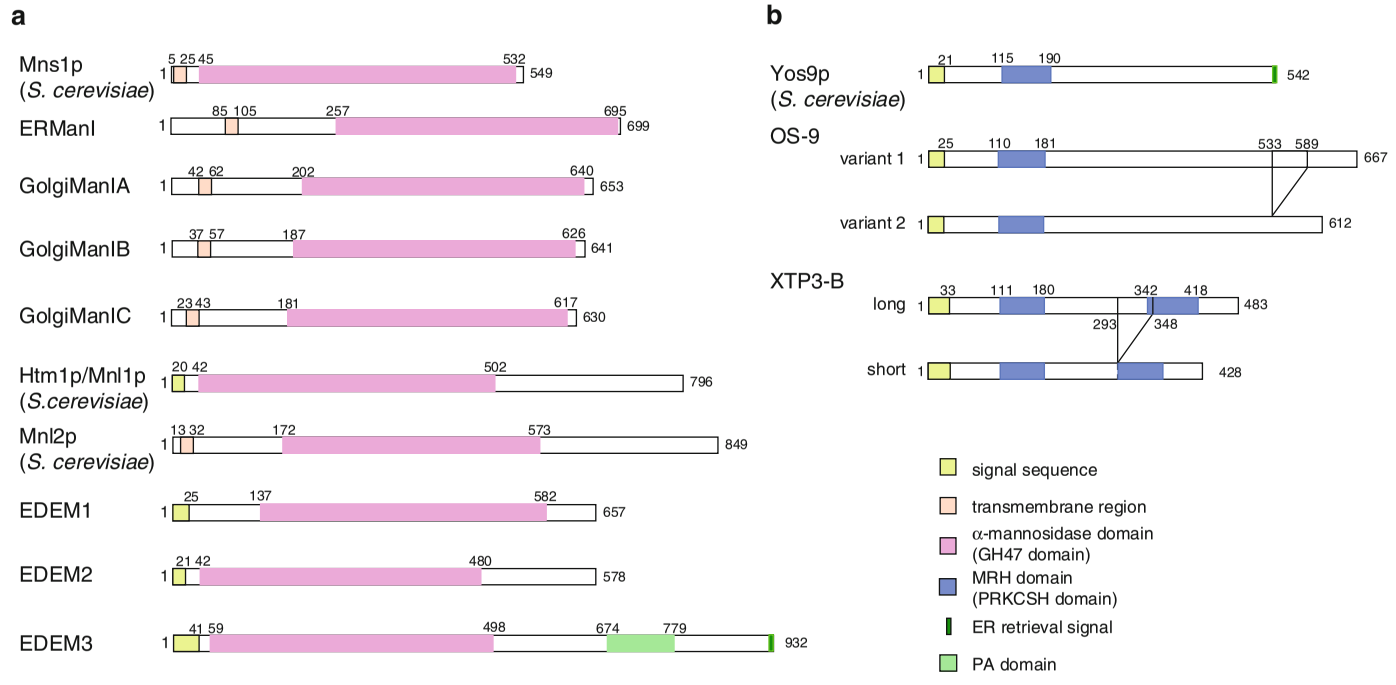

Fig. 1.2 Schematic representation of the primary structures of Class I α-mannosidases (**a**) and MRH domain-containing lectins in the ER (**b**). Accession numbers for the protein sequences used are (**a**) *S. cerevisiae* Mns1, P32906; *H. sapiens* ER ManI (MAN1B1), Q9UKM7; Golgi ManIA (MA1A1), P33908; Golgi ManIB (MA1A2), O60476; Golgi Man1C (MA1C1), Q9NR34; *S. cerevisiae* Htm1/Mnl1, P38888; *H. sapiens* EDEM1, Q92611; EDEM2, Q9BV94; and EDEM3, Q9BZQ6 and (**b**) *S. cerevisiae* Yos9, Q99220; *H. sapiens* OS9, Q13438; XTP3-B, Q96DZ1. GH47, glycosyl hydrolase family 47; PRKCSH, protein kinase C substrate 80 K-H; and PA, protease-associated domain. The domain structure is based on the conserved domain database of NCBI (http://www.ncbi.nlm.nih.gov/cdd)

ER ManI (gene name *MAN1B1*) was recently revealed to be one of the genes that cause autosomal recessive cognitive disorders (Najmabadi et al. 2011; Rafiq et al. 2011). Homozygous nonsense mutations or missense mutations in both alleles were identified. Furthermore, using exome analysis, *MAN1B1* was identified as one of the genes that cause congenital disorders of glycosylation (CDG) (Rymen et al. 2013), producing developmental delay, facial dysmorphism, and obesity in addition to intellectual disability. Involvement of ER ManI in liver disease (Pan et al. 2009) or in liver carcinogenesis, independent of its enzyme activity, was also reported (Pan et al. 2013a).

Mammalian EDEM1 and yeast Htm1p/Mnl1p were identified as homologues of the ER α-mannosidase required for glycoprotein ERAD (Hosokawa et al. 2001; Jakob et al. 2001; Nakatsukasa et al. 2001). There are three EDEM proteins in mammals and two in yeast, although little is known about the recently identified Mnl2p (Martinez Benitez et al. 2011). Several mechanisms are proposed for the requirement of EDEM proteins in ERAD. Initially, they were proposed to be lectins since mannose-processing activity was not detected, although lectin activity has not been shown in vitro either. The second mechanism is that EDEMs process mannose to generate *N*-glycan signals for degradation. A third mechanism is the glycan-independent recognition of ERAD substrates by EDEMs. Recently, the α-mannosidase activity of Htm1p/Mnl1p was shown to process mannose from the C branch in yeast cells (Clerc et al. 2009; Quan et al. 2008) and was followed by an in vitro experiment using recombinant protein purified in complex with an oxidoreductase Pdi1p (PDI in mammals) (Gauss et al. 2011). As for mammalian EDEMs, however, in vitro activity assays have not been successful to date. Transient cellular expression of EDEM1 processes terminal mannose from the C branch (Hosokawa et al. 2010b) or from the A branch (Olivari and Molinari 2007) of *N*-glycans on model ERAD substrates. Also, transient expression of EDEM3 enhances mannose processing, creating M7-M6 *N*-glycans on total cellular glycoproteins as well as on misfolded ERAD substrates (Hirao et al. 2006). However, EDEM2 mannose-processing activity appears lacking (Mast et al. 2005). Enzyme activity and its specificity require future in vitro experimentation. Mammalian EDEMs are also reported to recognize misfolded proteins in a glycan-independent manner. EDEM1 point mutants lacking mannosidase activity (Cormier et al. 2009) or deletion mutants that lack the mannosidase domain (Ron et al. 2011) are able to associate and degrade ERAD substrates. An intrinsically disordered region close to the N-terminus of EDEM1 is suggested to associate with the misfolded polypeptides (Marin et al. 2012).

In the case of EDEM1 binding to misfolded proteins independently of glycans, its mannosidase domain is used to associate with SEL1L, which has five *N*-glycosylation sites. Thus, a model was proposed whereby EDEM1 acts as a quality control receptor that links ERAD substrate to dislocation machinery (Cormier et al. 2009). This is similar to the proposed function of OS-9 and XTP3-B, in which the lectin domains of OS-9 and XTP3-B recognize *N*-glycans on SEL1L (Christianson et al. 2008) (discussed further in the next section). Morphological study has revealed that EDEM1, in addition to its ER localization, is concentrated in vesicles that lack the COPII coat

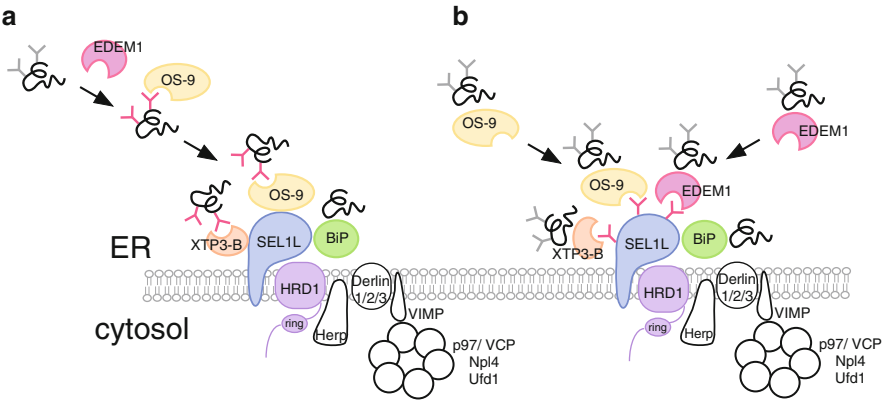

Fig. 1.3 Two distinct models of *N*-glycan recognition by ER lectins and EDEM1 in the ERAD process. Recognition of *N*-glycans on misfolded cargos (**a**) or on SEL1L protein (**b**)

(Zuber et al. 2007). These vesicles are termed EDEMosomes (Cali et al. 2008) or ERAD tuning vesicles (Bernasconi et al. 2012) and have been demonstrated to contain OS-9 and SEL1L in addition to EDEM1. Hence, these vesicles are proposed to regulate the ERAD machinery by removing ERAD factors through lysosomal degradation (Bernasconi et al. 2012). Based on these observations, *N*-glycans are suggested to act as signals for docking with other ER resident proteins (Hebert and Molinari 2012) (Fig. 1.3b), which awaits further verification.

A number of EDEM partner proteins have been elucidated. Yeast Htm1p/Mnl1p tightly associates with Pdi1p through intermolecular disulfide bonding (Gauss et al. 2011; Sakoh-Nakatogawa et al. 2009), which is required for its mannosidase activity. In mammals, ERdj5, another PDI family member that functions as a disulfide reductase in the ER, forms a trimeric complex including BiP and EDEMs for ERAD (Ushioda et al. 2008). The association of ER ManI with EDEM1 is also reported, which enhances glycoprotein ERAD by suppressing the degradation of ERManI under ER stress conditions (Termine et al. 2009). SEL1L is another partner of EDEMs (Bernasconi et al. 2012; Cormier et al. 2009). Thus, the function of EDEMs could be modified by these partner proteins or may represent the coordinated roles of proteins incorporated in different functional complexes.

1.3 Lectins That Recognize *N*-Glycan Tags for ERAD: Yos9p, OS-9, and XTP3-B, the MRH Domain-Containing Lectins in the ER

Misfolded polypeptides that have *N*-glycan tags for degradation are recognized by specific lectins. *S. cerevisiae* Yos9p (yeast OS-9) was found to associate with Hrd3p and Kar2p and discriminates ERAD cargo for degradation (Bhamidipati et al. 2005;

Buschhorn et al. 2004; Kim et al. 2005; Szathmary et al. 2005). OS-9 (osteosarcoma amplified 9) (Kimura et al. 1998; Su et al. 1996) and XTP3-B (XTP3-transactivated gene B protein)/Erlectin (Cruciat et al. 2006) are the mammalian homologues (Fig. 1.2b). Yos9p and OS-9 have one MRH domain, while XTP3-B has two. While several splice variants are reported in mammals, their functional differences remain unclear.

Using recombinant proteins, both yeast Yos9p and mammalian OS-9 have been shown to recognize *N*-glycan structures in which the C-arm α1,2-mannose residue is trimmed (Hosokawa et al. 2009; Mikami et al. 2010; Quan et al. 2008), revealing that the mechanisms are conserved in budding yeast and mammals. Recently, the human OS-9 MRH domain was crystallized in a complex with M5 *N*-glycan, which revealed the characteristic P-type lectin fold, similar to the mannose 6-phosphate receptor domain, as well as the structural basis for the recognition of specific mannose linkage (Satoh et al. 2011). In yeast, mannose residues are sequentially processed from *N*-glycans by Mns1p and Htm1p/Mnl1p as described above; however, the equivalent mammalian enzymes remain unidentified. In yeast, the lectin domain of Yos9p recognizes *N*-glycans on misfolded cargo. Yos9p also assists the degradation of non-glycosylated proteins, which is conducted in an MRH domain-independent manner (Benitez et al. 2011; Jaenicke et al. 2011). In mammals, OS-9 is required for glycoprotein ERAD; however, two mechanisms are suggested: OS-9 MRH domain recognizes *N*-glycans on the ERAD substrates (Hosokawa et al. 2008; Mikami et al. 2010; Satoh et al. 2011; Tyler et al. 2012) or *N*-glycans on SEL1L (Christianson et al. 2008) (Fig. 1.3). *N*-glycan structures on SEL1L remain unidentified; however, mannose trimming would proceed from *N*-glycans on long-lived ER resident proteins. We estimate that approximately one-half of the OS-9 associates with SEL1L and that all endogenous XTP3-B binds to SEL1L under steady-state conditions (Fujimori et al. 2013; Hosokawa et al. 2008). Further study will reveal the dynamic regulation of how chaperones and lectins act in the recognition and sorting of misfolded glycoproteins to the retrotranslocation machinery.

Relatively little is known about the function of XTP3-B. XTP3-B has two MRH domains and despite its sequence homology, only the C-terminal MRH domain exhibits lectin activity (Cruciat et al. 2006; Fujimori et al. 2013; Yamaguchi et al. 2010). XTP3-B was shown to recognize cell surface-expressed M5-6 glycans, the same species that OS-9 associates with (Yamaguchi et al. 2010). The function of OS-9 and XTP3-B is considered to be redundant, since simultaneous knockdown of both OS-9 and XTP3-B inhibits the degradation of soluble ERAD substrates (Bernasconi et al. 2010), whereas knockdown of XTP3-B normally does not affect glycoprotein ERAD (Christianson et al. 2008; Hosokawa et al. 2008). However, for some ERAD substrates, such a redundancy is not observed (Fujimori et al. 2013; Tyler et al. 2012). Instead, another mechanism is suggested whereby XTP3-B recognizes M9-glycans and inhibits the degradation of misfolded cargo bearing M9 glycans (Fujimori et al. 2013), which requires further study.

1.4 Cytosolic Events of Glycoprotein ERAD; Fbs Proteins

Once in the cytosol, misfolded proteins destined for elimination are ultimately degraded by the ubiquitin-proteasome-dependent pathway. For most ERAD substrates, polyubiquitination serves as a targeting signal, and occurs by the concerted action of activating E1, conjugating E2 and ligating E3 enzymes (Hershko et al. 2000; Pickart 2001). Among the various E3 enzymes, one of the most interesting is the SCF complex (complex containing Skp1, Cul1, and Roc1) containing Fbs1 (Fbg1/NFB42/Fbx2/Fbxo2/OCP1) or Fbs2 (Fbg2/Fbx6) as *N*-glycan-recognizing ubiquitin ligases (Yoshida and Tanaka 2010). Fbs1/Fbs2 has carbohydrate-binding properties, preferably binding to high mannose-type glycans (Yoshida et al. 2002, 2003), and structural studies have revealed that the core $Man_3GlcNAc_2$ structure of *N*-glycans is important for recognition by Fbs1 (Mizushima et al. 2004, 2007). Therefore, this complex can serve as an *N*-glycoprotein-specific ubiquitin ligase. While this protein shows high homology with other F-box proteins, i.e., Fbg3 (Fbx44), Fbg4 (Fbx17), and Fbg5 (Fbx27) (Ilyin et al. 2002; Yoshida and Tanaka 2010), the carbohydrate-binding properties of Fbg3 have not been demonstrated. On the other hand, Fbg4 and Fbg5 appear to bind to glycoproteins/glycans with lower efficiency (Glenn et al. 2008). A recent study indicated that, by co-expressing with Skp1 proteins, Fbg5 appears to show significant binding to concanavalin A-reactive glycoproteins (Yoshida et al. 2011), while evidence for carbohydrate binding of Fbs4 was lacking, even in the presence of Skp1. Thus, the nature of carbohydrate binding to Fbg3/4 requires clarification in future studies.

1.5 Physiological Functions of Fbs Proteins

Of the Fbs protein family, Fbs1 is the most well-characterized protein in terms of its physiological function. The expression of Fbs1 is mainly restricted to the adult brain and testes, whereas Fbs2 exhibits wide tissue distribution (Yoshida et al. 2003). Fbs1 is shown to be involved in glycoprotein ERAD (Shenkman et al. 2013; Yoshida et al. 2002), and, consistent with this observation, Fbs1 is shown to interact with CHIP, a co-chaperone with ubiquitin ligase activity (Nelson et al. 2006). Furthermore, overexpression of Fbs1 in Alzheimer disease model mice reduced BACE1 levels and led to reduced synaptic deficits (Gong et al. 2010), indicating that Fbs1 may have a protective role in the progression of this devastating disease. Most recently Nogo receptor 2 was identified as a binding protein for Fbs1 protein (Kern et al. 2012). This is of particular interest as Nogo receptors are known to bind amyloid-beta precursor protein (APP) (Park et al. 2006; Park and Strittmatter 2007; Zhou et al. 2011) and Alzheimer disease model mice lacking Nogo receptor 2 show reduced amyloid plaque formation (Zhou et al. 2011).

While known to have a limited tissue distribution, Fbs1 was previously identified as a protein highly expressed in the organ of Corti and named organ of

Corti protein 1 (OCP1; (Thalmann et al. 1997)). Another protein abundant in organ of Corti, OCP2, is Skp1 (Thalmann et al. 1997). Mice lacking Fbx2/Fbs1 developed age-related hearing loss (Nelson et al. 2007). It was found that OCP1 (Fbs1) and OCP2 (Skp1) appear to form a complex, which co-localizes with the epithelial gap-junction system (Henzl et al. 2001). Moreover, Fbs1 was found to bind with connexin 26 (Henzl et al. 2004). As mutations in the connexin26 gene are the most common cause of hereditary deafness (Denoyelle et al. 1997; Kelsell et al. 1997), the interaction of Fbs1-Skp1-connexin26 may somehow be critical for the hearing system.

While Fbs1 is known to be a component of the SCF^{fbs1} complex, the majority of Fbs1 was shown to exist as a monomer or alternatively as an Fbs1-Skp1 heterodimer (Yoshida et al. 2007). As Fbs1 can prevent the aggregation of glycoproteins, Fbs1 may have an E3 ubiquitin ligase-independent function, assisting the clearance of aberrant glycoproteins in neuronal cells by suppressing their aggregation (Yoshida et al. 2007). On the other hand, SCF^{fbs1} or SCF^{fbs2} complexes can associate with a p97/VCP/Cdc48, an AAA ATPase, and a protein that plays a central role in extracting proteins from the ER into the cytosol during the ERAD process (Yoshida et al. 2005).

1.6 Cytoplasmic PNGase

For substrates to efficiently enter the interior of the cylinder-shaped 20S proteasome, where the active sites of proteases reside, the bulky modification of polypeptide side chains such as glycans or polyubiquitin may need to be removed prior to proteolysis. The cytoplasmic peptide: *N*-glycanase (PNGase, Png1 in yeast (Suzuki et al. 2000), Ngly1 in mammalian cells (Suzuki et al. 2003)) is responsible for the removal of *N*-glycans from glycoproteins (Suzuki 2007; Suzuki et al. 2002). This enzyme is found ubiquitously, from yeast to mammals, and was demonstrated to be involved in the glycoprotein ERAD process (Hirsch et al. 2003; Hosomi et al. 2010; Kim et al. 2006; Masahara-Negishi et al. 2012; Tanabe et al. 2006; Wiertz et al. 1996a, b).

The removal of glycans by PNGase is required for the efficient degradation of at least a subset of misfolded glycoproteins in yeast. Considering its biochemical activity, this enzyme may also constitute an *N*-glycan-specific component for ERAD. Interestingly, like Fbs1/Fbs2, cytoplasmic PNGase in mice can bind to p97/VCP/Cdc48 (Li et al. 2005, 2006), in addition to other ERAD or ubiquitin-proteasome pathway-related proteins (Katiyar et al. 2004, 2005; Park et al. 2001; Suzuki et al. 2001a). Moreover, recent studies suggest that, at least in vitro, Fbs1 protects the misfolded glycoproteins from the action of cytoplasmic PNGase (Yamaguchi et al. 2007). These protein-protein interactions may be critical in establishing complex protein networks and regulates the sequential reactions in a concerted manner, as formation of such a complex may greatly enhance the efficiency of ERAD by carrying out a number of reactions, i.e., ubiquitination, deglycosylation, deubiquitination, and proteolysis in one place (Suzuki and Lennarz 2003).

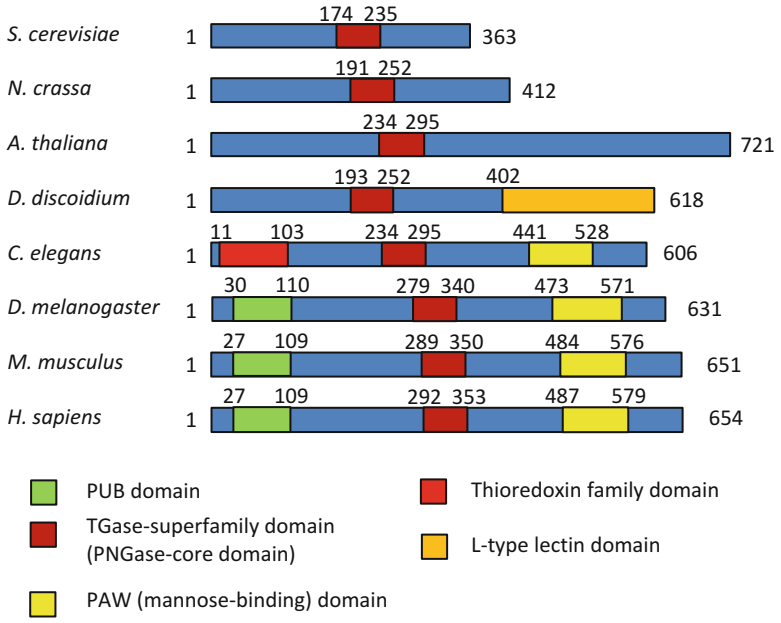

Fig. 1.4 Schematic representation of the primary structure of various eukaryotic PNGase orthologues. Accession numbers for the protein sequences used are *S. cerevisiae*, Q02890; *N. crassa*, BAI53085; *A. thaliana*, Q9FGY9; *D. discoideum*, Q55FC8; *C. elegans*, Q9TW67; *D. melanogaster*, Q7KRR5; *M. musculus*, Q9JI78; and *H. sapiens*, Q96IV0. The domain structure is based on the conserved domain database of NCBI (http://www.ncbi.nlm.nih.gov/cdd), and common regions aligned in all PNGase orthologues are shown; PUB domain (domain ID, cd10459; residue 11–93), TGase superfamily domain (domain ID, pfam01841; residue 37–107), PAW (mannose-binding) domain (domain ID, smart00613; residue 1–89), thioredoxin family domain (domain ID, cd02947; residue 2–92), and L-type lectin domain (domain ID, cd01951; residue 11–223). TGase transglutaminase (This figure is reproduced from Suzuki 2015)

It is also interesting to note that, while the gene encoding the cytoplasmic PNGase is highly conserved from yeast to human, its structural organization differs significantly (Fig. 1.4). For instance, the mammalian Ngly1 protein retains the N-terminal PUB domain, which is shown to be critical for protein-protein interactions (Allen et al. 2006; Kamiya et al. 2012; Madsen et al. 2009; Suzuki et al. 2001b), and the C-terminal PAW domain (Doerks et al. 2002), which contains a mannose-binding module (Zhou et al. 2006). This observation may imply that this protein acquired diverse domains as an evolutional consequence.

In addition to enzyme activity, cytoplasmic PNGases also exhibit a carbohydrate-binding property (Suzuki et al. 1994, 1995). Subsequently, it was found that the core PNGase domain, besides the mannose-binding module found in C-termini of orthologues in higher eukaryotes, binds with high affinity to glycans bearing *N,N'*-diacetylchitobiosyl structures (Suzuki et al. 2006; Zhao et al. 2009) (Fig. 1.4). This carbohydrate-binding property is conserved, even in *Neurospora* PNG1 protein, while this protein is enzymatically inactive (Maerz et al. 2010) (see below). It is

therefore tempting to speculate that the carbohydrate-binding property of the cytoplasmic PNGase orthologue may, at least to some degree, play a role in their enzyme-independent function.

1.7 Physiological Functions of the Cytoplasmic PNGases

While the Png1 mutant in budding yeast (Suzuki et al. 2000) or in *Arabidopsis thaliana* (Diepold et al. 2007) did not show any significant phenotypic consequences, more recent studies suggest that it may play important physiological roles in other eukaryotes. For instance, a defect in the orthologue of *PNG1* in *Caenorhabditis elegans* was shown to result in an increase of axon branching of specific neurons during morphogenesis of the vulval egg-laying organ (Habibi-Babadi et al. 2010). While the *C. elegans* PNG1 protein is a dual enzymatic protein (PNGase and thioredoxin) (Kato et al. 2007; Suzuki et al. 2007), it appears that PNGase activity, but not the thioredoxin domain, is critical for normal axonal branching (Habibi-Babadi et al. 2010). Moreover, a defect in Pngl, an orthologue of Ngly1, was shown to result in severe developmental defects in the fruit fly (Funakoshi et al. 2010). The PNGase orthologue in *Dictyostelium discoideum* was also shown to have a significant physiological role in its aggregation during multicellular development (Gosain et al. 2012). Most recently, exome analysis identified human patients with mutations in the Ngly1 gene (Enns et al. 2014; Need et al. 2012). These patients exhibited multiple symptoms that included developmental delay, movement disorder, and hypotonia (Enns et al. 2014; Need et al. 2012). Other common symptoms include alacrima and abnormal liver function. Mechanistic insight into these phenotypic consequences remains elusive. Whatever the mechanism may be, however, these observations clearly indicate the functional importance of cytoplasmic PNGases in various organisms, including humans.

It was also shown that a PNG1 mutant (*PNG1 orthologue*) in *Neurospora crassa* showed temperature sensitive growth with strong polarity defects (Seiler and Plamann 2003). Interestingly, the PNG1 in *N. crassa* contains intrinsic mutations in two out of three essential amino acids in the catalytic triad (i.e., Cys-to-Ala and His-to-Tyr mutations) (Maerz et al. 2010), which consists of Cys, His, and Asp (Katiyar et al. 2002; Makarova et al. 1999). It is therefore predicted that, at least in some organisms, PNGase orthologues will also have enzyme-independent functions.

1.8 Future Perspectives

As reviewed in this article, it is now clear to cell biologists that *N*-glycans play pivotal roles in the homeostasis and quality control of glycoproteins in the ER. One should note, however, that the situation is not as simple as originally envisaged. For example, the importance of mannosidases or lectins on ERAD is

now known to be substrate-specific (Christianson et al. 2011; Hosomi et al. 2010), and we currently have little information on the molecular mechanism behind this substrate dependency. Moreover, even the possibilities of carbohydrate-binding-independent functions of lectins (Benitez et al. 2011; Jaenicke et al. 2011) or enzyme-independent functions of enzymes (Cormier et al. 2009; Maerz et al. 2010; Ron et al. 2011) are currently proposed. Efforts should therefore be directed at accumulating comprehensive data on the effects of mannosidases/lectins on the degradation of varied ERAD substrates, rather than simplifying the scheme based on the results of a limited number of substrates. The extensive efforts of numerous researchers are imperative for a complete understanding of the functional importance of the players involved in recognition/degradation of misfolded glycoproteins in the ERAD process.

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Note Recently, it is reported that EDEM2 processes mannose from the middle (B) branch of N-glycans in the vertebrate ER (Ninagawa et al. 2014).

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Chapter 2
Glycan-Mediated Protein Transport from the Endoplasmic Reticulum

Morihisa Fujita, Xiao-Dong Gao, and Taroh Kinoshita

Abstract Protein sorting in the secretory pathway is an essential step to route proteins to their proper locations. A number of secretory proteins contain intrinsic sorting signals that are recognized by transport machineries. Efficient sorting of several secretory proteins requires cargo receptors that recognize the sorting signals of specific proteins and sort them into transport vesicles. In the early secretory pathway from the endoplasmic reticulum (ER), the cargo receptors function as linkers between the secretory proteins and coat protein complex II (COPII). Most secretory proteins are posttranslationally modified by the addition of sugar chains in the ER. Several lines of evidence indicate that these glycan structures act as signals for transport of the modified proteins and determine their final destinations. In the secretory pathway, there are several lectin-type cargo receptors that recognize specific glycan structures on proteins. The interactions between glycoproteins and cargo receptors are dependent upon the environment, such as the pH in organelles, as well as the glycan structures. Impairment of cargo receptors causes several diseases through inefficient delivery of their target molecules. In this review, we describe the current understanding of the sorting and transport mechanisms of glycoproteins from the ER in mammalian cells.

Keywords Asparagine (*N*)-linked oligosaccharide • Cargo receptor • Endoplasmic reticulum (ER) • ER exit site • ERGIC-53 • ER-Golgi intermediate compartment (ERGIC) • Glycosylphosphatidylinositol (GPI) • Golgi apparatus • Lectin • p24 family

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2.1 Introduction

Secretory protein synthesis begins in the endoplasmic reticulum (ER), where proteins receive posttranslational modifications including glycosylation and disulfide bond formation and are prompted to undergo correct folding and assembly. After proteins achieve their proper conformations, they exit from the ER to the ER-Golgi intermediate compartment (ERGIC) or Golgi apparatus via coat protein complex II (COPII)-coated transport vesicles (Bonifacino and Glick 2004; D'Arcangelo et al. 2013; Jensen and Schekman 2011). Some proteins are retrieved from the Golgi to the ER by coat protein complex I (COPI)-coated vesicles (Lee et al. 2004). In the Golgi, the glycans on the proteins and the proteins themselves are further modified and sorted to vesicles or carriers toward their proper destinations. During the transport steps, proteins must be correctly selected and sorted into transport carriers. A number of secretory proteins contain intrinsic sorting signals in their polypeptide sequences (Apodaca et al. 2012; Barlowe 2003). Some membrane proteins are directly associated with coat proteins or secretory machineries via these signals. However, soluble secretory proteins that are luminally localized in the membrane and even some membrane proteins cannot directly bind to coat proteins that are localized in the cytoplasm. Instead, cargo receptors act as adaptors to link the secretory proteins and coat proteins (Braulke and Bonifacino 2009; Dancourt and Barlowe 2010). The cargo receptors are usually transmembrane proteins that shuttle between organelles. The cargo receptors for soluble secretory proteins have binding sites for cargo proteins in their luminal regions, while their cytoplasmic regions contain binding motifs for coat proteins (Dancourt and Barlowe 2010). Therefore, the cargo receptors sort their target proteins into the transport carriers.

In addition to the polypeptide sequences, posttranslational modifications are used for protein sorting. For example, mono-ubiquitination of proteins on the plasma membrane acts as a transport signal for internalization (Mukhopadhyay and Riezman 2007). Ubiquitination also functions as a sorting signal for substrate proteins from endosomes to the interior of multivesicular bodies, which is the first step in the degradation of substrate proteins in lysosomes. Several lines of evidence indicate that carbohydrates on proteins also function as sorting signals in the early and late secretory pathways (Fujita and Kinoshita 2012; Kim et al. 2009; Potter et al. 2006). The interactions between specific carbohydrates and their cargo receptors are essential for efficient delivery of the modified proteins to their target organelles.

In the late secretory pathway, it has been well characterized that mannose (Man)-6-phosphate (Man-6-P) residues on asparagine (*N*)-linked oligosaccharides act as protein sorting signals to lysosomes (Braulke and Bonifacino 2009; Kim et al. 2009). There are two types of Man-6-P receptors, cation dependent and cation independent, on the trans-Golgi network (TGN), which bind to Man-6-P residues on lysosomal proteins and sort them into clathrin-coated vesicles bound for late endosomes. Impairment of Man-6-P formation caused by mutations in *N*-acetylglucosamine (GlcNAc) phosphotransferase, which transfers phospho-GlcNAc to the 6-position of Man residues in *N*-linked oligosaccharides, causes missorting of

lysosomal proteins. It is known that mutations in GlcNAc phosphotransferase cause I-cell disease (mucolipidosis II), one of the lysosomal storage diseases (Kollmann et al. 2010). Apical protein trafficking mediated by galectins is another example of glycan-mediated protein transport in the late secretory pathway (Delacour et al. 2009). Galectins possess carbohydrate recognition domains for β-galactose or lactose but have oligosaccharide specificities, as described previously (Hirabayashi et al. 2002). In polarized epithelial cells, the plasma membranes are separated by tight junctions into two distinct domains, termed the apical and basolateral membranes. It has been reported that some galectins (galectin-3, galectin-4, and galectin-9) directly influence the apical sorting of proteins from the TGN or endosomes (Delacour et al. 2006; Mishra et al. 2010). Galectin-4 seems to be required for the recruitment of proteins into lipid rafts for apical transport, whereas galectin-3 is involved in apical sorting of lipid raft-independent proteins (Delacour et al. 2009). In addition to the late secretory pathway, oligosaccharides also function as sorting signals for proteins in the early secretory pathway between the ER and Golgi. In this review, we focus on two different types of glycan-mediated transport in mammalian cells. In particular, the interactions of *N*-linked glycoproteins and glycosylphosphatidylinositol (GPI)-anchored proteins with their cargo receptors are described.

2.2 ERGIC-53 Family Members for the Transport of High-Man-Type *N*-Linked Oligosaccharides

N-linked oligosaccharide synthesis starts from dolichol-linked oligosaccharides (DLOs) in the ER (Aebi 2013). The dolichol-diphosphate-linked oligosaccharide in DLO structure prior to transfer to proteins in yeast and mammalian cells consists of three glucose (Glc) residues, nine Man residues, and two GlcNAc residues, here named Glc3-Man9-GlcNAc2. This structure is transferred to nascent proteins by the oligosaccharyltransferase (OST) complex. The terminal Glc of the three residues in Glc3-Man9-GlcNAc2 is removed by α-glucosidase I (Fig. 2.1) (Roth et al. 2010; Ruddock and Molinari 2006). The remaining two Glc residues are trimmed by α-glucosidase II. The structure Glc1-Man9-GlcNAc2 is recognized by two lectin-type chaperones, calnexin and calreticulin (Helenius and Aebi 2004). These chaperones form complexes with the co-chaperone ERp57, a member of the protein disulfide isomerase family, and facilitate protein folding. The calnexin/calreticulin chaperones cannot bind to the Man9-GlcNAc2 structure after complete trimming of Glc by α-glucosidase II. When the protein folding is incomplete even after Glc trimming, one Glc is retransferred to the Man9-GlcNAc2 structure by uridine diphosphate (UDP)-Glc:glycoprotein glucosyltransferase (UGGT), generating Glc1-Man9-GlcNAc2, which is again recognized by calnexin/calreticulin to prompt the glycoprotein folding (D'Alessio et al. 2010). This folding process series involving calnexin/calreticulin is termed the calnexin cycle (Fig. 2.1). When the glycoproteins are properly folded, α-glucosidase II trims a Glc. Thereafter, trimming of a Man on the middle arm of the oligosaccharide by ER mannosidase I

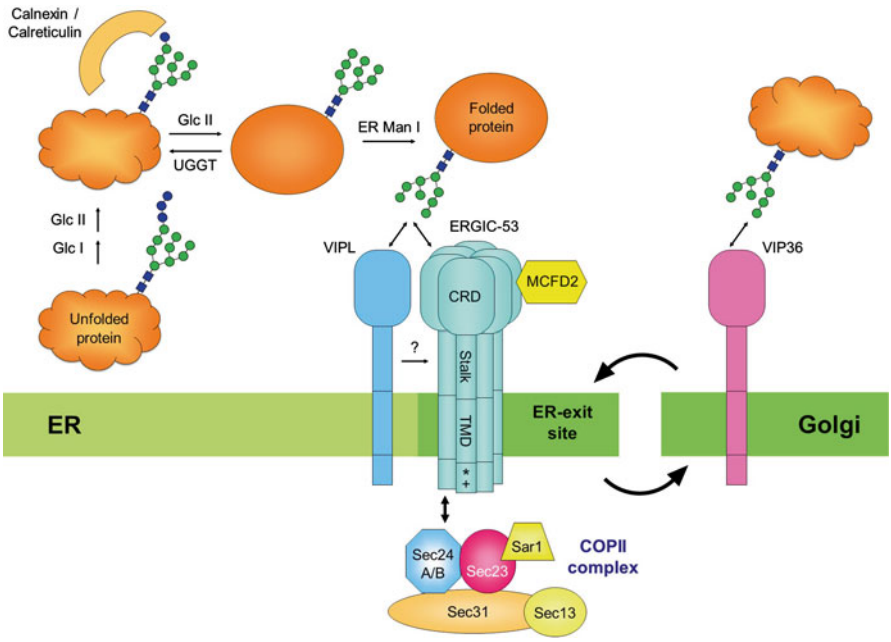

Fig. 2.1 Folding and sorting of *N*-glycosylated proteins in the ER. DLO is synthesized through a series of reactions and transferred to nascent proteins by the OST complex, forming *N*-glycosylated proteins. After protein transfer, three Glc residues are trimmed by α-glucosidase I (Glc I) and II (Glc II). The Glc1-Man9-GlcNAc2 structure on the protein is recognized by calnexin and calreticulin, which are lectin-type molecular chaperones, to prompt the protein folding. Calnexin and calreticulin are associated with ERp57, a protein disulfide isomerase family protein. After complete trimming of the Glc residues by Glc II, the chaperones dissociate from *N*-linked oligosaccharides. When the protein folding is incomplete, however, a Glc residue is retransferred to the Man9-GlcNAc2 structure by UGGT, regenerating Glc1-Man9-GlcNAc2 that is again recognized by calnexin/calreticulin. ER mannosidase I (ER Man I) usually trims a Man residue from the Man9-GlcNAc2 structure after Glc trimming and before the exiting of glycoproteins from the ER. Proteins that are correctly folded are then transported from the ER to the Golgi. ERGIC-53 acts as a cargo receptor for several *N*-glycosylated proteins in their transport from the ER. The cytoplasmic tail of ERGIC-53 binds to Sec24A or B, components of COPII. MCFD2 is associated with ERGIC-53 and contributes to the transport of coagulation factors V and VIII through protein-protein interactions and enhancement of ERGIC-53 recognition. After glycoproteins reach the ERGIC or cis-Golgi, they dissociate from ERGIC-53. VIP36 is probably involved in the retrieval of some glycoproteins. VIPL is localized in the ER and may have some interaction with ERGIC-53. *CRD*, carbohydrate recognition domain; *TMD* transmembrane domain; *, COPII binding site; +, COPI binding site

usually occurs. The oligosaccharide after the Man trimming ceases to be a substrate for UGGT (Sousa et al. 1992). In the folding process, proteins that fail to become correctly folded are recognized as misfolded proteins and subsequently degraded by a process known as ER-associated degradation (ERAD) (Olzmann et al. 2013). Several glycan-processing enzymes and lectins including EDEMs and OS-9 are also involved in the ERAD pathway for the recognition of misfolded

glycoproteins (Clerc et al. 2009; Hosokawa et al. 2010), as described in other excellent reviews (Araki and Nagata 2011; Lederkremer 2009). Glycoproteins correctly folded in the ER possess oligosaccharide(s) consisting of Man8-GlcNAc2 or Man9-GlcNAc2.

The secretory glycoproteins are then transported from the ER to the Golgi. Cargo receptors containing leguminous (L)-type lectin domains are involved in the trafficking of some proteins containing *N*-linked oligosaccharides between the ER and Golgi (Fig. 2.1). ERGIC-53, VIP36, VIPL, and ERGL are type I single-spanning membrane proteins with an L-type lectin domain (Nufer et al. 2003). ERGIC-53 is the best-characterized member of the family (Hauri et al. 2000). It is a 53-kDa protein originally found in the ERGIC (Schweizer et al. 1988). ERGIC-53 has a signal sequence for ER insertion, a large N-terminal luminal domain, a transmembrane domain, and a short C-terminal sequence. The short C-terminal sequence faces the cytoplasmic side and has a di-phenylalanine motif (FF) that preferentially associates with Sec24A and B, subunits of the COPII complex (Nyfeler et al. 2008), and a di-lysine motif (KKxx) that binds with the COPI complex. Using these motifs, ERGIC-53 recycles between the ER, ERGIC, and Golgi. The N-terminal domain contains a carbohydrate recognition domain (CRD) and an α-helical stalk domain. The α-helical stalk domain contains conserved cysteine residues and forms intermolecular disulfide bonds that mediate the dimerization or hexamerization of ERGIC-53 (Lahtinen et al. 1999; Schweizer et al. 1988). Crystal structure analyses indicated that the CRD domain of ERGIC-53 is similar to other L-type lectins that have a β-sandwich structure with a concave face comprising seven β-strands and a convex face comprising six β-strands forms (Velloso et al. 2002). ERGIC-53 is a Ca^{2+}-binding protein and its cargo binding is pH and Ca^{2+} dependent. A conserved histidine residue at the carbohydrate-binding site may serve as a pH sensor for cargo binding (Appenzeller-Herzog et al. 2004). In the ER, where the pH is neutral, the histidine residue is deprotonated, and Ca^{2+} in the pocket of the carbohydrate recognition site of ERGIC-53 contributes to the carbohydrate binding. In contrast, in the environment of the ERGIC and Golgi, which is more acidic, the histidine residue is protonated and the Ca^{2+} in ERGIC-53 is released, leading to dissociation of the cargo.

In mammalian cells, several specific proteins including coagulation factors V and VIII, cathepsin Z, cathepsin C, and α1-antitrypsin have been determined as ERGIC-53-dependent cargo proteins (Appenzeller et al. 1999; Kamiya et al. 2008; Nichols et al. 1998). It has been identified that mutations in ERGIC-53 cause autosomal recessive bleeding disorders with combined deficiency of coagulation factors V and VIII (Nichols et al. 1998). Loss of function of ERGIC-53 causes inefficient ER export of glycosylated coagulation factors V and VIII. In patients with defective ERGIC-53, the plasma levels of factors V and VIII are significantly decreased. ERGIC-53 recognizes both the *N*-linked oligosaccharide and polypeptide of the coagulation factors. In addition to the loss of function of ERGIC-53, mutations in MCFD2 have been found to cause deficiency in factors V and VIII (Zhang et al. 2003). MCFD2 encodes a 16-kDa soluble protein with a signal sequence and two EF-hand motifs that bind to Ca^{2+} (Fig. 2.1). MCFD2 is likely to bind to the

polypeptide segments of coagulation factors (Nishio et al. 2010). Additionally, MCFD2 associates with ERGIC-53 and enhances its sugar-binding ability, particularly for the Man8B structure, which is a Man8-GlcNAc2 structure obtained after Man trimming by ER mannosidase I (Kawasaki et al. 2008). The association between MCFD2 and ERGIC-53 is pH independent and Ca^{2+} dependent. Cathepsin Z and cathepsin C are other examples of cargo proteins with transport mediated by ERGIC-53 (Appenzeller et al. 1999). Similar to factor VIII, these cathepsins interact with ERGIC-53 via both their oligosaccharides and peptide β-hairpin loop structures (Appenzeller-Herzog et al. 2005). MCFD2 is dispensable for the binding of cathepsin Z and cathepsin C to ERGIC-53 (Nyfeler et al. 2006). The reason why only limited proteins are dependent on ERGIC-53 is that protein-protein interactions as well as protein-oligosaccharide interactions are required for the binding between ERGIC-53 and its cargo proteins.

As mentioned above, there are three cargo receptor-like L-type lectins, VIP36, VIPL, and ERGL, other than ERGIC-53 in mammalian cells (Nufer et al. 2003). VIP36 is localized in the ERGIC and cis-Golgi (Reiterer et al. 2010). VIPL stands for VIP36-like, and the protein is ER resident (Nufer et al. 2003). It is widely expressed in mammalian cell lines. It has been reported that VIPL associates with ERGIC-53 (Qin et al. 2012). Although ERGIC-53 has low affinity and broad specificity for high-Man oligosaccharides and does not distinguish between monoglucosylated and deglucosylated forms, VIPL and VIP36 display a preference for deglucosylated forms of high-Man oligosaccharides (Kamiya et al. 2008). VIPL and VIP36 exhibit distinct pH profiles in binding assays. VIPL binds optimally at about pH 7.5, while VIP36 binds optimally at around pH 6.5 (Kamiya et al. 2008). ERGL is not well characterized but has cell type and tissue specificities in its expression (Yerushalmi et al. 2001). Based on their localizations and carbohydrate specificity, a model has been proposed for the function of L-type lectins in mammalian cells (Fig. 2.1) (Dancourt and Barlowe 2010; Kamiya et al. 2008). VIPL localizes in the ER, binds to glycoproteins that are deglucosylated and have completed their folding in the ER, and delivers them to ERGIC-53. The cargo glycoproteins associated with ERGIC-53 are then sorted and incorporated into COPII vesicles. In the post-ER compartment, the lower pH causes dissociation between ERGIC-53 and its cargo proteins. VIP36 is involved in the retrieval of misfolded proteins from the Golgi to the ER.

In yeasts, there are two homologues of ERGIC-53, named Emp46p and Emp47p. In a double-mutant yeast for these homologues, secretion of glycoproteins to the culture medium is decreased (Sato and Nakano 2002). Based on a structural analysis, the CRDs of Emp46p and Emp47p have a β-sandwich fold that is similar to that of ERGIC-53 (Satoh et al. 2006). However, these homologues do not bind Ca^{2+}, while Emp46p binds K^+ at a distinct position from the corresponding site for Ca^{2+} binding in ERGIC-53. This K^+ binding is essential for Emp46 functions. Emp47 does not bind to any metal ions.

2.3 p24 Family of Proteins for Sorting and Transport of GPI-Anchored Proteins (GPI-APs) from the ER

GPI anchoring of proteins is one of the conserved posttranslational modifications occurring in the ER (Ikezawa 2002; Kinoshita et al. 2008; Orlean and Menon 2007). About 60 proteins in budding yeasts and about 150 different proteins in mammalian cells are modified by GPI, respectively. Proteins that receive GPI anchoring contain a signal sequence for ER insertion at their N-terminus and a GPI-attachment signal at their C-terminus. GPI is biosynthesized from phosphatidylinositol (PI) by stepwise addition of sugars, an acyl chain, and phosphoethanolamine (EtNP) on the ER membrane (Kinoshita 2014). The structure of the mammalian GPI complete precursor is EtNP-Man-(EtNP)Man-(EtNP)Man-GlcN-(acyl)PI or EtNP-(Man)Man-(EtNP)Man-(EtNP)Man-GlcN-(acyl)PI, where GlcN stands for glucosamine. The GPI complete precursor is transferred to nascent proteins with a GPI-attachment signal at the C-terminus, thereby generating GPI-APs. The transfer is mediated by the GPI transamidase complex, which consists of five subunits, PIGK, GPAA1, PIGS, PIGT, and PIGU (Fig. 2.2). After GPI attachment to proteins, GPI-APs are transported from the ER to the Golgi. During their transport, the GPI moieties are

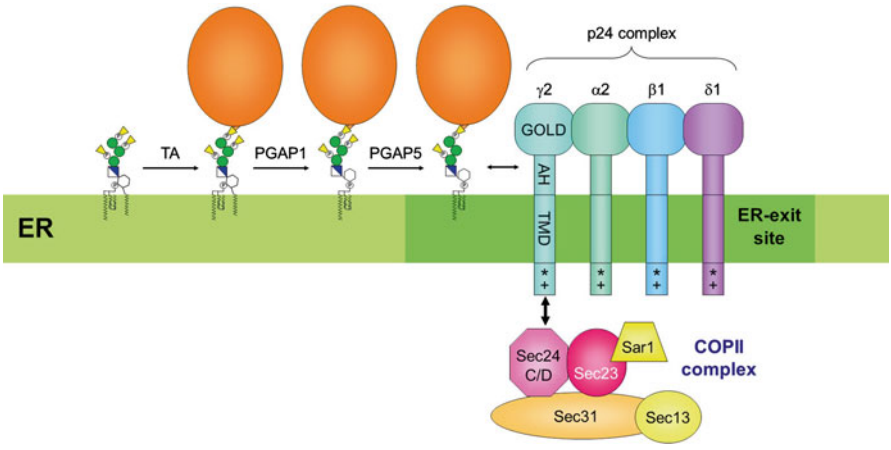

Fig. 2.2 Structural remodeling and sorting of GPI-APs in the ER. GPI is biosynthesized in the ER through an enzymatic reaction pathway consisting of ten steps. A preformed GPI complete precursor is attached to the C-terminus of a newly synthesized protein by GPI transamidase (TA), generating a GPI-AP. After GPI attachment to a protein, an acyl chain linked to inositol is removed by PGAP1, a GPI deacylase. A side-chain EtNP attached to the second mannose is then removed by PGAP5, a GPI-EtNP phosphodiesterase. These two GPI-remodeling reactions in the ER are critical for sorting of GPI-APs to the ER exit sites. The remodeled GPI-APs are efficiently recognized by a p24 complex that concentrates GPI-APs into COPII-coated vesicles. The α-helical region (AH) of p24γ2 may specify the GPI-AP recognition. The p24 proteins form hetero-oligomeric complexes. Each p24 family member protein has an ER export signal that is recognized by Sec24C or D, components of COPII. *GOLD* Golgi dynamics domain; *TMD* transmembrane domain; *, COPII binding site; +, COPI binding site

remodeled. Before exiting from the ER, GPI-APs receive two remodeling reactions in mammalian cells (Fig. 2.2). One is removal of an acyl chain from the inositol moiety of the GPI anchors, and the other is removal of the EtNP from the second Man of the GPI (Fujita and Kinoshita 2010, 2012). The first reaction is mediated by PGAP1, which is a multi-spanning membrane protein containing a lipase motif, GxSxG (Tanaka et al. 2004). The second reaction is carried out by PGAP5, which is a membrane protein containing a metal-dependent phosphodiesterase motif in the luminal side of the ER (Fujita et al. 2009). Actually, PGAP5 requires manganese for its activity. The remodeling reactions by both PGAP1 and PGAP5 are prerequisite for efficient transport of GPI-APs from the ER, particularly for their sorting into the ER exit sites (Fujita et al. 2011).

Since GPI-APs are luminally localized and do not span the membrane to the cytosol, they cannot directly bind with COPII. Instead, they associate with cargo receptors for linking with COPII. A hetero-oligomeric complex of proteins from p24 family is involved in efficient transport of GPI-APs from the ER and seems to function as a cargo receptor (D'Arcangelo et al. 2013; Herzig et al. 2012). The p24 proteins are type I membrane proteins of about 24 kDa, which can be divided into four subfamilies (α, β, γ, and δ) based on the primary sequences (Strating and Martens 2009; Strating et al. 2009). In most vertebrates, ten of the p24 paralogs [α1, α2, α3, β1, γ1, γ2, γ3, γ4, γ5, and δ1 (p24α1 is a pseudogene in humans)] are encoded. All p24 proteins share similar domain architectures consisting of a signal sequence, a GOLD domain, and an α-helical region, followed by a transmembrane region and a short C-terminal tail (Fig. 2.2). The C-terminal tail faces the cytoplasm, and some of the tails of the family members possess the ER exit signal recognized by COPII and the ER retrieval signal recognized by COPI. Therefore, they are mainly recycled between the ER and Golgi. The p24 proteins form hetero-oligomeric complexes with proteins from other subfamilies. Although it has been reported that they exist as monomers and dimeric or larger complexes, several reports suggest that they form heterotetrameric complexes consisting of proteins from each of four subfamilies as the basic units (Fullekrug et al. 1999; Hirata et al. 2013; Marzioch et al. 1999). It seems possible that the compositions of p24 complexes vary in different locations, such as the ER and Golgi (Jenne et al. 2002). Further studies are needed to clarify this point and to determine the exact compositions of the p24 complexes. Knockdown of p24δ1 in mammalian cells and deletion of *EMP24* (p24β) or *ERV25* (p24δ) in yeast cells result in destabilization of all the other p24 family members, suggesting that they stabilize one another, probably by forming complexes (Marzioch et al. 1999; Theiler et al. 2014). On the other hand, overexpression of each p24 family member causes mislocalization and accumulation in the ER (Blum and Lepier 2008; Blum et al. 1999; Rojo et al. 2000), suggesting that the balance of the protein expression levels is important for the correct complex formation.

Several studies have revealed that the transport of GPI-APs is delayed in yeast *emp24*Δ (p24β), *erv25*Δ (p24δ), *erp1*Δ (p24α), or *erp2*Δ (p24γ) cells (Belden and Barlowe 1996; Copic et al. 2009; Marzioch et al. 1999; Schimmoller et al. 1995). Furthermore, Emp24p can be chemically cross-linked with several GPI-APs

(Castillon et al. 2011; Muniz et al. 2000), suggesting their direct association. In mammalian cells, knockdown of p24δ1 or p24β1 results in delayed transport of GPI-APs, supporting the findings in yeast cells (Bonnon et al. 2010; Takida et al. 2008). In p24δ1-knockdown cells, sorting of GPI-APs into the ER exit sites is impaired (Fujita et al. 2011), suggesting that the p24 proteins act as cargo receptors to concentrate GPI-APs at the ER exit sites for efficient packaging into COPII vesicles in mammalian cells. In yeast cells, however, sorting and concentration of GPI-APs to the ER exit sites are independent of Emp24p (Castillon et al. 2011). It seems that the yeast p24 complex may act as a linker that facilitates and stabilizes vesicle formation by recruiting COPII components to sites with pre-concentrated GPI-APs.

Two GPI-anchor remodeling reactions mediated by PGAP1 and PGAP5 in the ER, which occur after the transfer to proteins, are crucial for interaction with the p24 proteins and efficient sorting into the ER exit sites (Fujita et al. 2011). Therefore, only correctly processed GPI-APs can be associated with a p24 complex and transported from the ER. Additionally, it has been reported that p24α2, p24β1, p24γ2, and p24δ1 are associated with GPI-APs depending on the pH. Under mild alkali and neutral pH conditions corresponding to the ER, p24 proteins are associated with GPI-APs, whereas they dissociate in mild acidic conditions that reflect the pH in the Golgi. Recently, it has been reported that p24γ2 among the five p24γ subfamily members is primarily involved in the efficient transport of GPI-APs (Theiler et al. 2014). In particular, the α-helical region of p24γ2 is pivotal for the recognition and transport of GPI-APs. The p24 complex containing p24γ2 recognizes GPI-APs and seems to sort them into COPII vesicles (Fig. 2.2).

The cytosolic regions of p24 proteins are used to bind with COPII. Sec24 isoforms, components of COPII, directly associate with the sorting signals of cargo proteins (Mancias and Goldberg 2008; Miller et al. 2002, 2003). There are three Sec24p isoforms (Sec24p, Sfb2p, and Lst1p) in yeasts and four isoforms (Sec24A, B, C, and D) in mammalian cells (Bonifacino and Glick 2004). While these isoforms have overlapping functions for cargo binding, the p24 complex seems to be preferentially recognized by Lst1p in yeasts and Sec24C and D in mammalian cells (Bonnon et al. 2010; Castillon et al. 2011). Conversely, ERGIC-53 is recognized by Sec24A and B via the di-phenylalanine motif, when proteins are sorted into COPII vesicles as described above (Nyfeler et al. 2008).

Although the p24 proteins play key roles in maintaining the fidelity of GPI-AP transport in the ER, the functions of the p24 family members are diverse. There are many reports regarding other functions, including the unfolded protein response in the ER, retrograde transport from the Golgi, and maintenance of Golgi structures (Bremser et al. 1999; Strating and Martens 2009). In particular, it appears interesting that the p24 family proteins are involved in the transport of other proteins, such as invertase in yeasts and Wnt proteins in *Drosophila* and mammalian cells (Buechling et al. 2011; Port et al. 2011; Schimmoller et al. 1995). In HEK293T cells, knockdown of p24γ2, which is also utilized in GPI-AP transport, results in inefficient secretion of Wnt1, suggesting that a similar p24 protein complex may be used for ER-to-Golgi transport. Further analyses are required to clarify how these distinct cargo proteins are selected and recognized by the p24 complex.

2.4 Concluding Remarks

We have described two types of glycan-mediated protein transport in the early secretory pathway. The protein sorting and transport processes are regulated by the structures of *N*-linked oligosaccharides or GPI anchors on proteins and by recognition molecules. The sorting mechanisms of proteins in the ER are closely linked to the protein folding. There are several checkpoints for monitoring the folding status of proteins, and only properly folded proteins are delivered to the subsequent sorting steps. Glycan processing participates in both glycoprotein folding and sorting. We still do not know how ERGIC-53 recognizes specific glycoproteins in vivo and how p24 family proteins switch between GPI-APs and other cargo proteins.

Both ERGIC-53 and p24 proteins form oligomers, but the oligomer formation appears to be dynamic. It remains unknown how the oligomers are formed, maintained, and dissociated. Recently, it has been reported that p24β1 binds with sphingomyelin containing a specific acyl chain (C18) via the cytoplasmic face of the transmembrane region, which induces dimerization of p24β1 and COPI-dependent retrograde transport from the Golgi (Contreras et al. 2012). Several transmembrane proteins possess a consensus motif (VxxTLxxIY) found in the transmembrane region of p24β1. Interactions between specific (glyco)sphingolipids and these transmembrane and juxtamembrane regions may be involved in regulating the protein oligomerization and localization.

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Chapter 3
Gangliosides and T-Cell Immunity

Masakazu Nagafuku and Jin-ichi Inokuchi

Abstract Gangliosides are sialic acid-containing glycosphingolipids separated into several series on the basis of the absence or presence of one or more sialic acid residues linked to the galactose residue in the second position from the ceramide backbone. Gangliosides are fundamental constituents of lipid rafts in eukaryotic cell membranes and are considered to play a variety of roles in cell physiology such as modulation of signal transduction. As with gangliosides, T cells, key players of the adaptive immune response, are phenotypically divided into several subpopulations, and each of them preferentially expresses differential series of gangliosides. Recent studies have shown that the differential ganglioside expression is likely to contribute to the appropriate lipid raft structures for T-cell activation via the antigen receptor, T-cell receptor. Furthermore, the abnormal ganglioside levels in T cells are associated with the pathogenesis of autoimmune and allergic disorders. Therefore, a variety of lipid rafts with different gangliosides are conceivably formed on the plasma membrane of individual T-cell subsets, suggesting the regulation of gangliosides is a therapeutic target for immune system diseases.

Keywords Gangliosides • Glycosphingolipids • Lipid rafts • T cells • T-cell receptor • Autoimmune diseases • Allergic diseases

3.1 Introduction

Gangliosides are a family of glycosphingolipids (GSLs) which contain sialic acid (SA) and are separated into several series on the basis of the absence (o-series) or presence of one (a-series) or two (b-series) SA residues linked to the galactose residue in the second position from ceramide backbone (Tidhar and Futerman 2013; Hakomori 2008) (Fig. 3.1a). Gangliosides are ubiquitous components of eukaryotic cell membranes and have important regulatory functions such as cell recognition,

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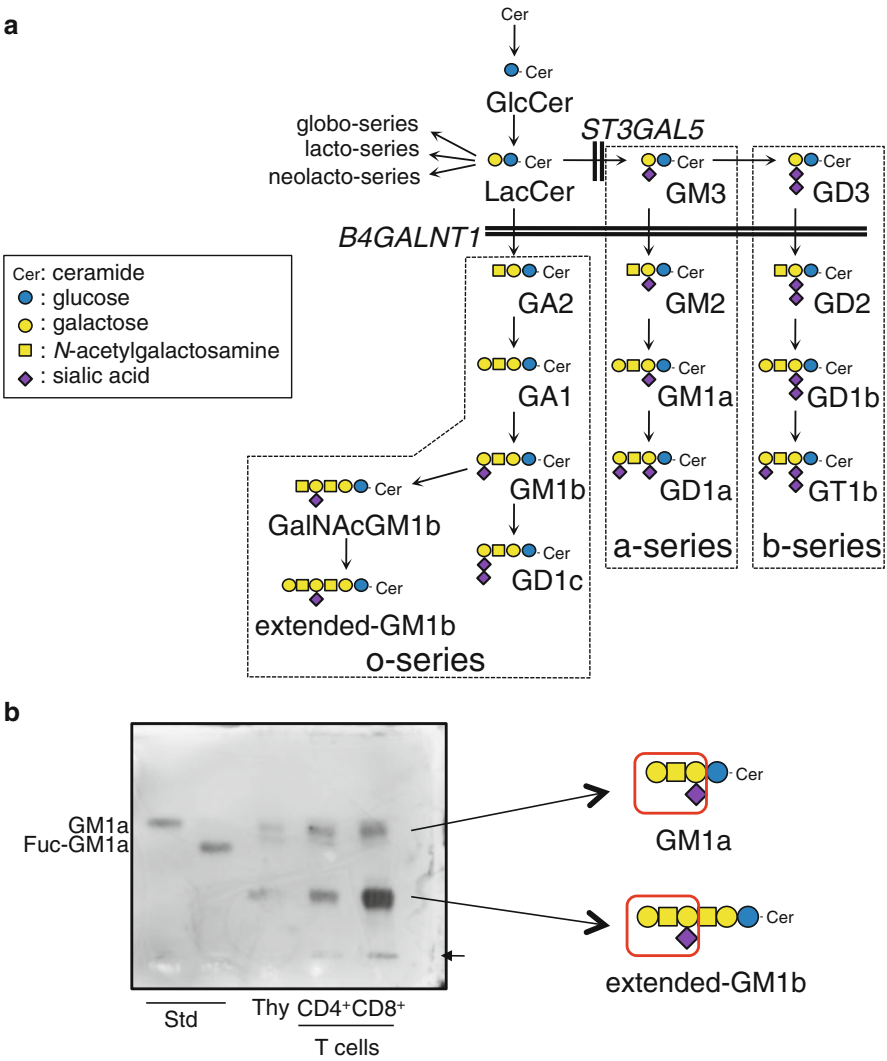

Fig. 3.1 Ganglio-series ganglioside biosynthetic pathways. (**a**) Gangliosides, SA-containing GSLs, are enzymatically synthesized from ceramide and are divided into o-, a-, and b-series species. These pathways consist of a highly ordered consecutive and stepwise process catalyzed by specific glycosyltransferases which can transfer one monosaccharide residue (glucose, galactose, *N*-acetylgalactosamine, or sialic acid) at a time. *GlcCer* glucosylceramide, *LacCer* lactosylceramide. (**b**) Acidic lipids obtained from thymocytes (Thy), $CD4^+$ T cells, and $CD8^+$ T cells of normal mice were separated on TLC plates and were stained with HRP-conjugated CTx-B. An *arrow* indicates the origin for TLC. *Std* standard lipids, *Fuc-GM1a* fucosylated-GM1a

ligand–receptor interactions, cell adhesion, and cell growth control (Regina Todeschini and Hakomori 2008). Membrane lipids are not distributed uniformly within the outer leaflet of the plasma membrane, but gangliosides are thought to segregate together with cholesterol and sphingomyelin to form highly organized membrane microdomains, called lipid rafts (Simons and Ikonen 1997; Brown and London 1998). Lipid rafts are involved in a variety of cell functions, including serving as receptors for bacterial toxins, mediators of cell adhesion, and modulators of signal transduction. Although lipid rafts have catapulted gangliosides into the limelight, many studies only utilize gangliosides (usually GM1a, which can be easily detected with cholera toxin) as a raft marker. It is, however, fortunate for ganglioside biology that numerous membrane gangliosides have been found to be altered in situations such as cell differentiation and cell activation in many cell types. These revelations have encouraged the development of new concepts for the treatment of cancers, metabolic diseases, and immune system disorders by modification of ganglioside levels (Inokuchi 2011; Liu et al. 2013; Kidani and Bensinger 2014).

T cells are lymphocytes that have differentiated under the influence of the thymus and are responsible for cell-mediated immunity. T cells are functionally divided into T helper (Th) cells and T cytotoxic (Tc) cells that generally express specific cell surface molecules CD4 and CD8, respectively. $CD4^+$ Th cells and $CD8^+$ Tc cells employ closely similar mechanisms in T-cell antigen receptor (TCR)-mediated signaling despite playing distinct immune functions (Rudolph et al. 2006; Li et al. 2013). Interestingly, the two T-cell subsets express different levels of gangliosides as well as different kinds of ganglioside species, which provide the appropriate distinct intracellular signaling events for immune function of each T-cell subset (Nagafuku et al. 2012). T-cell activation via TCR together with costimulatory receptors requires the recruitment of both extracellular and intracellular molecules into lipid rafts (Dykstra et al. 2003; Tuosto et al. 2001; Harder et al. 2007) (Fig. 3.2). Immunological synapses are dynamic structures formed at the T-cell–antigen-presenting cell (APC) interface during antigen recognition (Fooksman et al. 2010; Monks et al. 1998; Davis and Dustin 2004; Saito and Yokosuka 2006) and are proposed to be incredibly diverse both in structure and function (Alarcon et al. 2011; Thauland and Parker 2010). Although lipid rafts have been thought to be involved in the immunological synapse formation, the composition and behavior of membrane gangliosides during the immunological synapse formation remain controversial (Harder et al. 2007; Bi et al. 2001; Alarcon et al. 2011; Hashimoto Tane et al. 2010).

3.2 T-Cell Development and Activation

3.2.1 T-Cell Development

T cells, also called T lymphocytes, are central components of adaptive immunity, which is characterized by high specificity for distinct molecules and diversity of the response based on the presence of an antigen receptor. T cells arise from bone

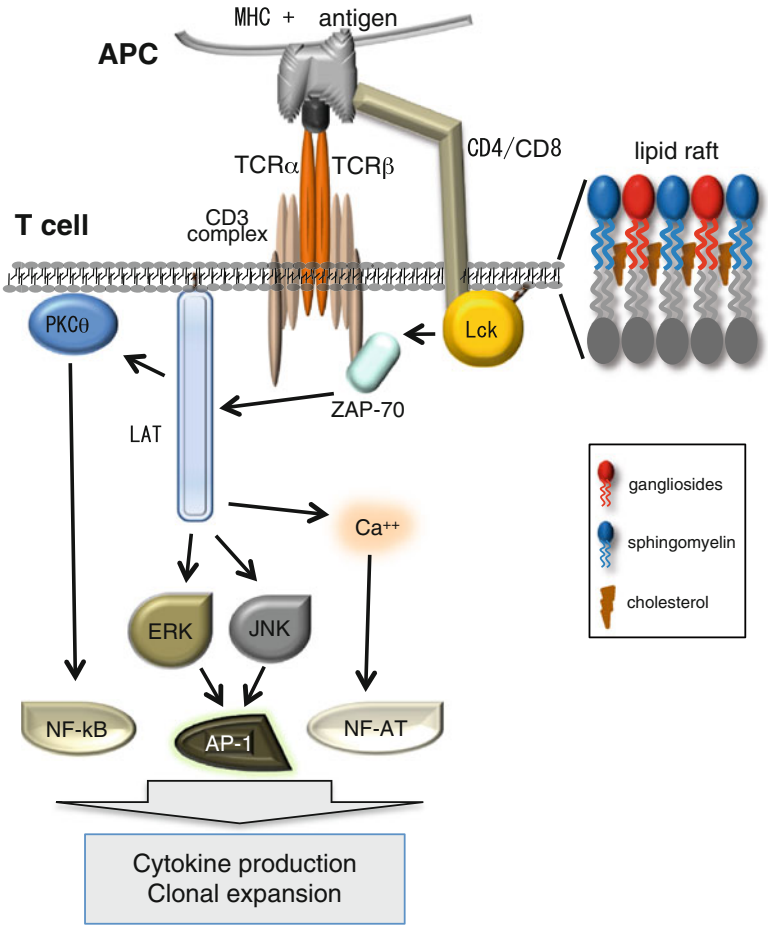

Fig. 3.2 TCR signal transduction and lipid rafts. Antigen recognition by TCR leads to rapid activation in a variety of intracellular signaling molecules, resulting in cytokine gene expression and clonal expansion. Lipid rafts are specialized microdomains of the plasma membrane, consisting of concentrated amounts of gangliosides, sphingomyelin, and cholesterol. The signals mediated through TCR are thought to depend on raft integrity. Some raft-associated proteins such as Lck and LAT are indispensable for TCR signaling, indicating the importance of lipid rafts as platforms on the plasma membrane

marrow precursors, which migrate to the thymus and differentiate under the influence of the thymic microenvironment (Boehm and Bleul 2006; Ladi et al. 2006). The most important biological purpose of T-cell development is to generate a fully competent T-cell population with differentiated effector functions, capable of quickly identifying and sufficiently eliminating pathogens. Thymic immature T cells (called thymocytes) develop in an ordered manner, characterized by the expression of TCR and specific surface markers (Rothenberg and Taghon 2005) (Fig. 3.3a). The early T-cell

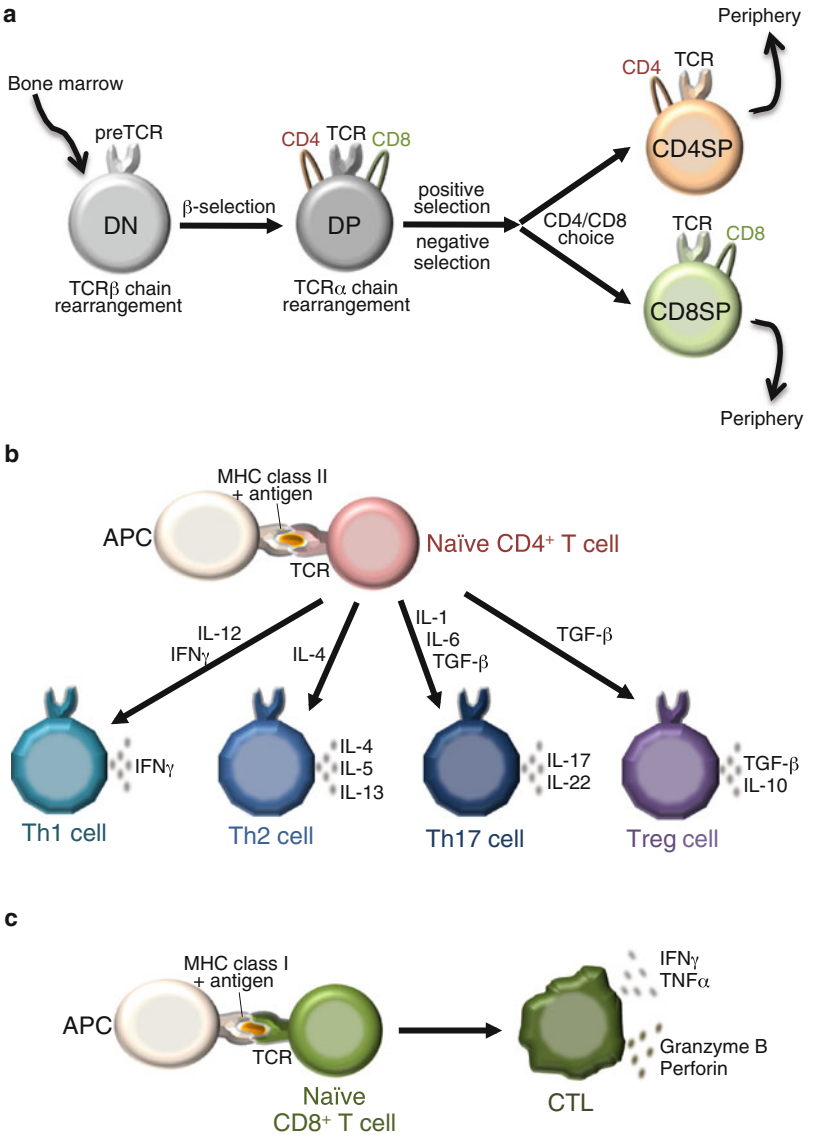

Fig. 3.3 Stages of T-cell development and functional maturation. (**a**) T-cell development in the thymus: landmark stages, checkpoints, and developmental choices. T-cell precursors come from bone marrow to the thymus where they differentiate into mature T-cell subsets. Potentially harmful cells that strongly recognize self-antigen as well as cells that fail to express useful antigen receptors can be eliminated during maturation stages. Mature $CD4^+$ and $CD8^+$ T cells are released into the circulation and reside in the peripheral lymphoid tissues. *APC* antigen-presenting cell. (**b**) Functional maturation of $CD4^+$ T cells. Naïve $CD4^+$ T cells differentiate into distinct subsets of effector Th cells by recognizing an antigen presented by APC. Cytokines produced by self and neighboring cells promote differentiation steps by activating specific transcription factors that stimulate production of the cytokines of each subset. Note that these cytokines can promote the development of each Th subset and suppress the development of the other subset. (**c**) Naïve $CD8^+$ T cells differentiate into CTLs. Both the appropriate antigen recognition and cytokines from Th cells are required for the generation of effector CTLs

progenitors express neither CD4 nor CD8 and are therefore termed double negative (DN) thymocytes. In the DN stage productive rearrangement of TCRβ chain genes takes place and leads to the expression of a functional pre-TCR. The pre-TCR signaling induces cell survival, proliferation, and both CD4 and CD8 coreceptor expressions. The resulting cells are termed double positive (DP) thymocytes. In this stage DP cells rearrange their TCRα genes and express a mature TCR. Only cells that successfully express a TCR on their cell surface subsequently undergo positive and negative selection to generate a T-cell repertoire that responds to foreign antigens but not self-antigens (termed repertoire selection). The surviving cells ultimately become mature CD4 and CD8 single positive (SP) thymocytes. SP cells maintain the expression of one coreceptor and are referred to as lineage-committed $CD4^+$ or $CD8^+$ mature T cells, which exit the thymus to circulate to the periphery. It is well known that sphingosine 1-phosphate, a metabolite of ceramide, and its receptors are required for the emigration of mature SP thymocytes from the thymus as well as the trafficking and migration of lymphocytes into the follicular areas of secondary lymphoid organs (spleen and lymph nodes) (Rosen et al. 2003; Allende et al. 2004).

3.2.2 Mature T-Cell Subsets and Their Activation

$CD4^+$ T cells participate in a wide variety of immune functions including humoral immunity, allergic responses and autoimmune diseases. $CD4^+$ T cells are also called Th cells because they can recognize foreign antigens presented by MHC class II molecules on APCs (Fig. 3.3b) and turn on B cells to produce antibodies that act against these antigens. On the other hand, $CD8^+$ T cells possess a killing ability against cancer cells and virus-infected cells, so they are also called killer T cells or Tc cells. $CD8^+$ T cells can recognize intracellular antigens, such as viral components and mutated proteins, presented by MHC class I molecules on virtually all nucleated cells (Fig. 3.3c). The initial activation of naïve $CD4^+$ and $CD8^+$ T cells is triggered by the recognition of antigens by their TCR. TC R interacts with APC that bears a cognate antigen peptide–MHC. This interaction is responsible for the formation of a unique "immunological synapse" (Monks et al. 1998; Fooksman et al. 2010; Yokosuka and Saito 2010; Kupfer and Kupfer 2003). T-cell activation via TCR together with costimulatory receptors requires the recruitment of both extracellular and intracellular molecules into the specific cell membrane regions known as lipid rafts (Dykstra et al. 2003). $CD4^+$ T cell and $CD8^+$ T cell share closely similar mechanisms in the TCR-mediated signaling despite playing different immune functions. Interestingly, the two T-cell subsets express different levels of gangliosides as well as different kinds of ganglioside species, which may provide the appropriate distinct intracellular signaling events for each T-cell subset (Nagafuku et al. 2012).

The combination of TCR and costimulatory signaling induces several immune responses: cytokine secretion (primarily IL-2) and proliferation of T cells, leading to an increase in the cell numbers (called clonal expansion), and differentiation of

the naive T cells into effector T cells (Smith-Garvin et al. 2009). Naïve $CD4^+$ T cells are activated and differentiate into polarized effector Th cell subsets (Th1, Th2, and Th17 cells) and regulatory T cells (Treg) (Zhu et al. 2010) (Fig. 3.3b). The defining characteristics of these subsets are the cytokine production: IFN-γ for Th1; IL-4, IL-5, and IL-13 for Th2; IL-17 and IL-22 for Th17; and TGF-β and IL-10 for Treg. These cytokines determine their effector functions and pathophysiological roles. Although the mechanisms that allow naïve T cells to generate distinct effector T-cell subsets remain poorly understood (Gerlach et al. 2010; Tubo et al. 2013; Plumlee et al. 2013), several studies have shown that asymmetric division is one of the mechanisms that generate this diversity by regulating the differentiation of $CD4^+$ T cells and $CD8^+$ T cells (Chang et al. 2007; King et al. 2012). More than one decade ago, it was discovered that plasma membrane ganglioside levels and de novo sphingolipid biosynthesis are increased during the differentiation from naïve to effector T cells (Tuosto et al. 2001). The expression patterns and levels of ganglioside species also alter among Th subsets, which may provide the appropriate membrane microenvironments for each Th subset development and activation (Balamuth et al. 2001; Leitenberg et al. 2001; Izsepi et al. 2013). In addition, recent studies suggest that ganglioside composition in T cells is associated with immune system disorders such as autoimmune diseases and allergy, as will hereinafter be described in detail.

3.3 Ganglioside Biosynthesis and Lipid Rafts

All eukaryotic cells are surrounded with a plasma membrane that defines their shape and acts as a selective barrier against diffusion of ions, proteins, and charged molecules, into and out of the cytoplasm. In the plasma membrane there are specific microdomains, called lipid rafts, in which GSLs, sphingomyelin, and cholesterol are major structural components. Lipid rafts are thought to be involved in multiple cellular functions such as receptor-mediated signal transduction, endocytic processes, membrane trafficking, and protein and lipid sorting (Simons and Ikonen 1997; Brown and London 1998).

Sphingolipids are a family of membrane lipids that have a long-chain base (called the sphingoid base) with an amide-linked fatty acid that is synthesized de novo from serine and palmitate, consisting of free sphingoid bases (sphingosine and sphinganine), ceramide, sphingomyelin, and GSLs (Tidhar and Futerman 2013) (Fig. 3.1a). GSLs are divided into multiple subcategories according to their characteristic structures: globo-series, lacto-series, neolacto-series, and ganglio-series (Hakomori 2008). Ganglio-series gangliosides contain one or more SA residues, which are negatively charged in the physiological pH range; therefore, they could contribute to the cell surface charge and related properties of the plasma membrane microenvironments by interacting with positively charged molecules (Kabayama et al. 2007; Sonnino et al. 2007). Thus, gangliosides play an important role in the formation and stabilization of lipid rafts (Hakomori 2002; Sonnino et al. 2007). Gangliosides are also subdivided into several groups on the basis of the absence (o-series) or

presence of one (a-series) or two (b-series) SA residues linked to the galactose residue in the second position from ceramide (Fig. 3.1a).

GM3, the simplest of the "a-series" gangliosides, is synthesized by ST3Gal5 (also named GM3S), which catalyzes the transfer of SA to the nonreducing terminal galactose (Gal) of lactosylceramide (LacCer). GM3 can be altered by β4Galnt1 (also named GM2/GD2S), which transfers *N*-acetylgalactosamine (GalNAc) to GM3, to form GM2 (a downstream a-series ganglioside), or by GD3 synthase to form GD3, the simplest of the "b-series" gangliosides. β4Galnt1 also elongates LacCer to form GA2, the simplest precursor of the "o-series" gangliosides. Each branch of GSL biosynthesis is a committed pathway (Fig. 3.1a); competition between enzymes at a key branch point determines the relative expression levels of o-, a-, and b-series gangliosides. Gangliosides are known to be preferentially packaged with cholesterol to form lipid rafts in specific cell membrane regions (Simons and Ikonen 1997). As gangliosides expose SA residues to the exterior of outer leaflet membranes and membrane fluidity is directly determined by the physicochemical characteristics of lipid molecules composing the lipid bilayer, it is very interesting to investigate their true physiological counterparts for electrostatic interactions in lipid rafts. It has been proposed that metabolic disorders, such as type 2 diabetes, are membrane microdomain disorders caused by aberrant expression of gangliosides (Inokuchi 2011).

3.4 Ganglioside Expression in T-Lineage Cells

3.4.1 Ganglioside Analysis with Cholera Toxin

Cholera toxin is a protein secreted by the bacterium *Vibrio cholerae*. The B subunit of cholera toxin (CTx-B) has long been used for the detection, visualization, and functional analysis of GM1a (considered a representative for gangliosides and lipid rafts) because CTx-B, compared to other antibodies against gangliosides, is able to bind to GM1a with much higher affinity (Cuatrecasas 1973; Masserini et al. 1992; Kuziemko et al. 1996). Staining with CTx-B has demonstrated that $CD8^+$ T cells express higher levels of GM1a in rafts than $CD4^+$ T cells (de Mello Coelho et al. 2004). Although CTx-B is widely known to specifically recognize ganglioside GM1a (a-series species), this toxin also reacts with other types of gangliosides including fucosylated-GM1a and extended-GM1b, both of which have a monosialo-ganglio-triose structure, Galβ1-3GalNAcβ1-4(SAα2-3)Galβ1- (Fig. 3.1b) (Nakamura et al. 1987). In fact, two CTx-B-reactive gangliosides, GM1a (a-series) and extended-GM1b (o-series), are expressed in different quantities in individual mouse T-cell subsets (Nagafuku et al. 2012): GM1a was expressed in both thymocytes and $CD4^+$ T cells but only trace amounts in $CD8^+$ T cells, and extended-GM1b was expressed much more than GM1a in $CD8^+$T cells (Fig. 3.1b). The presence in a single cell of a variety of rafts with different gangliosides has been suggested (Kovacs et al. 2002; Gomez-Mouton et al. 2001). It has been reported

that cross-linking of gangliosides using CTx-B or a homologue, the heat-labile enterotoxin of *Escherichia coli*, can induce apoptosis in $CD8^+$ T cells but not in $CD4^+$ T cells (Trambley et al. 1999). The apoptosis is caused by the activation of NF-κB and c-Myc via the induction of caspase-dependent signaling (Sorice et al. 1997). While this process is known to proceed in a Fas- and p55 tumor necrosis factor receptor-independent pathway (Salmond et al. 2002), the events in rafts following the ganglioside cross-linking remain undetermined. Considering the difference in the expression of CTx-B-binding gangliosides between $CD4^+$ T cells and $CD8^+$ T cells, it seems possible that the apoptosis caused by CTx-B cross-linking may involve extended-GM1b rafts but not GM1a rafts (Nagafuku et al. 2012). This suggests that each T-cell subset has a unique raft in the plasma membrane and that the raft provides a distinct function in different intracellular events following receptor-mediated stimulation. Taken together, the raft structure recognized by CTx-B represents only a part of them, and rafts must be heterogeneous and grouped into subclasses. Thus, to understand the role of rafts in the differentiation, maturation, and activation processes of $CD4^+$ T cells and $CD8^+$ T cells, it is critical to define the ganglioside composition in each respective T-cell lineage. It is, however, noted that there are apparent differences in the ganglioside composition among primary T cells freshly isolated from lymphoid organs and cultured T cells (cloned cell lines and blasted cells stimulated with activators), as well as among animal species (Suzuki et al. 1987; Potapenko et al. 2007).

3.4.2 Ganglioside Expression in T-Cell Subsets

Ganglioside expression in whole T-cell populations (a mixture of all T-lineage cells) has previously been examined using biochemical analyses, thin-layer chromatography, and high-performance liquid chromatography (Marusic et al. 2004; Kiguchi et al. 1990; Tani-ichi et al. 2005). Whole T-cell populations are, however, truly a "mixed population"; therefore, any such results would be of limited value to study specific T-cell subsets. Since flow cytometry allows multiparameter analysis of cells at a single-cell level, it is a valuable tool to determine the amounts of a specific ganglioside species in individual T-cell subsets. FACS analyses with monoclonal antibodies (mAbs) against several ganglioside species previously determined that mature $CD4^+$ T cells and $CD8^+$ T cells express differential species of gangliosides (Marusic et al. 2004; Nakamura et al. 1995). More recently, the structures of gangliosides in immature thymocytes and $CD4^+$ and $CD8^+$ T cells isolated from mouse lymphoid organs were investigated by LC-MS/MS analysis (Nagafuku et al. 2012). All T-cell subsets commonly express the six distinct species (GM1a, GM1b, GD1b, GD1c, GalNAcGM1b, and extended-GM1b), but their expression levels are remarkably different in each subset. The expression of o-series gangliosides (GalNAcGM1b and extended-GM1b) is greatly enhanced by the differentiation from thymocytes to $CD4^+$ T cells and $CD8^+$ T cells. It is noteworthy that almost all gangliosides expressed in $CD8^+$ T cells are o-series species. The expression of

GM1b is maintained among T-cell subsets, but GM1a is expressed in both thymocytes and $CD4^+$ T cells but only in trace amounts in $CD8^+$ T cells. Human $CD4^+$ T cells isolated from peripheral blood mainly express a-series gangliosides (GM3 and GM1a), as is the case in murine $CD4^+$ T cells expressing the same series gangliosides (GM1a) (Garofalo et al. 2002; Sorice et al. 1997; Misasi et al. 1993). Human $CD8^+$ T cells also express a-series gangliosides (GM3 and GM1a) in contrast to murine $CD8^+$ T cells expressing o-series gangliosides.

β4Galnt1 gene expression is markedly increased in both $CD4^+$ T cells and $CD8^+$ T cells compared to thymocytes. ST3Gal5 gene expression is increased in $CD4^+$ T cells and is decreased in $CD8^+$ T cells compared to the expression in thymocytes (Nagafuku et al. 2012). These gene expression patterns could partially explain the abovementioned distinct expression profile of gangliosides during the differentiation processes from immature thymocytes to mature $CD4^+$ T cells and $CD8^+$ T cells. In the case of GD1c, Nakamura et al. reported that $CD4^+$ T cells can be separated into GD1c-positive IL-2-producing Th1-like cells and GD1c-negative IL-4-producing Th2-like cells (Nakamura et al. 1995). In non-obese diabetic (NOD) mice, both resting $CD4^+$ effector T cells and $CD8^+$ effector T cells contain GM1a and GD1a as the major gangliosides detected by TLC analysis and CTx-B staining assay; $CD8^+$ effector cells express higher levels of GD1a than $CD4^+$ effector cells, and both GM1a and GD1a are upregulated by the activation with anti-CD3 and anti-CD28 (Misasi et al. 1993; Wang et al. 2009). Although in these studies the possibility that GD1a assigned in their analysis might contain extended-GM1b, this possibility is supported by the following lines of evidence: extended-GM1b is sialidase resistant, migrates like GD1a on TLC, exhibits CTx-B binding activity very similar to GM1a, and is detected in a higher amount in $CD8^+$ T cells than $CD4^+$ T cells (Nakamura et al. 1987; Nagafuku et al. 2012). These findings suggest that T-cell subsets can be classified into the distinct functional subpopulations based on the differences of ganglioside expression profiles.

Polarization of the ganglioside GM1a occurs only in $CD4^+$ T cells after TCR clustering (Kovacs et al. 2002). In addition, GM1a and GM3 gangliosides define different types of raft membrane domains that segregate either to the leading pole or the trailing uropod of the polarized human T cell, respectively (Gomez-Mouton et al. 2001). In the surface of a T cell, there may be two or more types of lipid rafts that are formed by distinct ganglioside species according to different developmental stages and activation status.

3.5 T-Cell Gangliosides and T-Cell Activation

3.5.1 T-Cell Activation and Lipid Rafts

T-cell activation is initiated and sustained by engagement of the TCR with MHC–peptide complex on APC as well as engagement of costimulatory molecules (e.g., CD28 on T cells with CD80/86 on APC). CD4 and CD8 are coreceptors that

bind to nonpolymorphic regions of MHC and facilitate signaling by the TCR during T-cell activation. Cross-linking of TCR and CD28 with anti-CD3 and anti-CD28 mAbs can also activate T cells without APC. In each case T-cell activation requires the recruitment of both extracellular and intracellular molecules into the specific cell membrane regions known as lipid rafts (Dykstra et al. 2003; Harder et al. 2007) (Fig. 3.2).

Lipid rafts are highly enriched in cholesterol, gangliosides, and sphingomyelin (Simons and Toomre 2000). Among these lipid components, cholesterol is thought to have the critical function of maintaining raft structure. Cholesterol can fill the space between the hydrocarbon chains of the sphingolipids and functions as glue that keeps the raft assembly together. In fact, cholesterol depletion from cell membranes using drugs, such as methyl-β-cyclodextrin, generally results in the disruption of raft-mediated cellular functions (Simons and Toomre 2000). Thus, despite some concerns regarding side effects of the drug on signaling events (Pizzo et al. 2002), this experimental approach has been widely used to verify the importance of cholesterol in rafts. In contrast to many studies manipulating the cholesterol level in lipid rafts, reports that address a role for gangliosides in raft function are much fewer.

3.5.2 T-Cell Activation via TCR and Ganglioside Composition in T-Cell Subsets

ST3Gal5 is responsible for a-series and b-series ganglioside synthesis; therefore, *ST3GAL5*-null mice lack all of a- and b-series gangliosides (GM3, GD3, GM2, and GM1a), resulting in LacCer accumulation and compensatory increase of o-series gangliosides (GA1, GM1b, GalNAcGM1b, and extended-GM1b) (Fig. 3.1) (Yoshikawa et al. 2009; Nagafuku et al. 2012). $CD4^+$ T cells from control mice express reliable levels of a-series GM1a compared to $CD8^+$ T cells. $CD4^+$ T cells (but not $CD8^+$ T cells) from *ST3GAL5*-null mice exhibit severe impairments in TCR-mediated proliferation and cytokine production (Nagafuku et al. 2012; Zhu et al. 2011). However, the defect is rescued by preincubation of the cells with a-series gangliosides (GM3 and GM1a), but with neither of b-series gangliosides. Human $CD4^+$ T cells isolated from peripheral blood mainly express a-series ganglioside GM3 (Garofalo et al. 2002), which forms GM3-containing rafts available as a platform for TCR signal transduction. The importance of GM3-containing rafts may be corroborated by the observation that GM3 forms a complex with both CD4 and Lck (Src family of tyrosine kinases) on plasma membranes and is co-immunoprecipitated with ZAP-70 (Syk family of tyrosine kinases) after cross-linking with anti-CD3 plus anti-CD28 mAbs (Garofalo et al. 2002; Barbat et al. 2007). Taken together, a-series gangliosides are essential for TCR-mediated activation of $CD4^+$ T cells. Consequently, the future challenge will be to examine immune responses in primary T-cell subpopulations separated on the basis of ganglioside species.

Almost all gangliosides expressed in mouse $CD8^+$ T cells are o-series species (GA1, GM1b, GalNAcGM1b, and extended-GM1b). Mice carrying an altered form of *B4GALNT1* lack all other ganglio-series gangliosides except GM3 and GD3 (Fig. 3.1) (Takamiya et al. 1996; Nagafuku et al. 2012). $CD8^+$ T cells (but not $CD4^+$ T cells) from *B4GALNT1*-null mice also exhibit severe impairments in TCR-mediated proliferation and cytokine production (Nagafuku et al. 2012). Correspondingly, they are rescued by preincubation with GA1 and GM1b (o-series), but with neither of the a-series nor b-series gangliosides. FACS analyses show that $CD4^+$ T cells and $CD8^+$ T cells each include subpopulations expressing a particular GSL not observed in the rest of the population; GA1-positive $CD8^+$ T cells exhibit more robust activation via the TCR and play a critical role in CD40-/CD28-costimulated and blockade-resistant allograft rejection, compared with GA1-negative $CD8^+$ T cells (Trambley et al. 1999; Kosaka et al. 2007; Sorice et al. 1997). These findings imply that each subpopulation of $CD4^+$ T cells and $CD8^+$ T cells has a unique ganglioside expression pattern in its rafts, which may be responsible for exerting specific functions of Th and Tc effector cells. GA1-positive mouse $CD8^+$ T cells produce higher levels of IFN-γ in vitro upon TCR stimulation than GA1-negative $CD8^+$ T cells, and both clonal expansions of $CD8^+$ T cells and cytotoxic T-cell (CTL)-dependent allograft rejection are suppressed by the administration of anti-GA1 mAb in vivo (Trambley et al. 1999). It remains unclear whether, in addition to GA1, the other o-series species expressed in primary $CD8^+$ T cells are essential for TCR-mediated activation.

Collectively, o-series ganglioside expression is essential for TCR-mediated activation of $CD8^+$ T cells, and a-series ganglioside is indispensable for that of $CD4^+$ T cells (Fig. 3.4). These results strongly suggest that each T-cell subset has a specific raft microenvironment consisting of different gangliosides and that these rafts provide distinct functions in different events following stimulation via the TCR. Although they share the common mechanisms of TCR-mediated signaling, the two T-cell subsets do have different cellular and molecular modifications (Li et al. 2013; Marrack et al. 2008; Rudolph et al. 2006). CD4 and CD8 can localize to lipid rafts by palmitoylation (a posttranslational lipid modification of proteins for membrane localization), yet raft targeting does not seem to be determined by that process alone (Kroczek et al. 2004; Greenwald et al. 2005). To ensure that CD4 and CD8 are moved to a proper place on the membrane, it might be critical for CD4/CD8 to interact with rafts carrying a specific ganglioside species. CD28, a costimulatory molecule, provides functional differences between $CD4^+$ T cells and $CD8^+$ T cells (Kroczek et al. 2004; Greenwald et al. 2005). In human and murine $CD4^+$ T cells, CD28 promotes the clustering of CTx-B-detectable rafts at the immunological synapse through its downstream signaling molecule protein kinase Cθ (Huppa and Davis 2003; Bi et al. 2001). However, $CD8^+$ T cells do not reorient CTx-B-detectable rafts to the T-cell–APC interface during activation (Kovacs et al. 2002; O'Keefe et al. 2004). Further studies are needed to understand the specific roles of each ganglioside in the regulation of different membrane microenvironments.

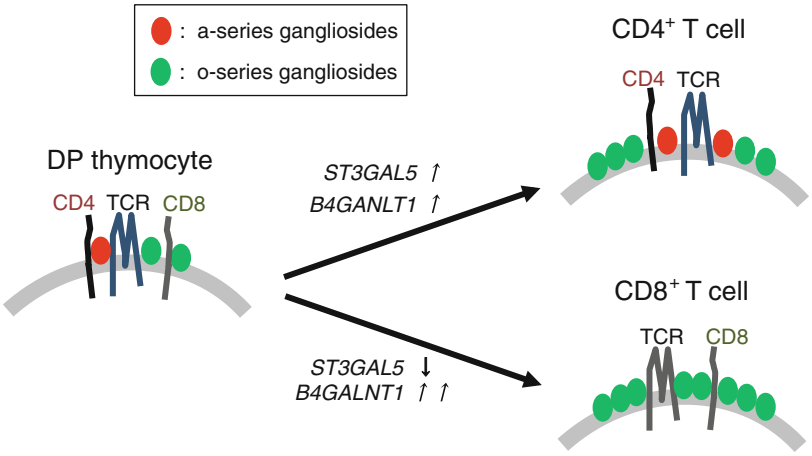

Fig. 3.4 Quantitative and qualitative changes in ganglioside species expressed during T-cell development. The differentiation from immature thymocytes to mature T-cell subsets is accompanied by selective ganglioside expression. The gene expression patterns together with ganglioside analysis confirm that $CD4^+$ T cells express a-series gangliosides due to upregulation of ST3Gal5 gene and $CD8^+$ T cells carry almost only o-series gangliosides due to downregulation of ST3Gal5 gene and upregulation of B4Galnt1 gene expression. This suggests that each T-cell subset has a unique raft composed of different ganglioside species and that these different rafts provide distinct functions in the intracellular events following the TCR- and coreceptor-mediated stimulation. The ganglioside selection process may be indispensable in the formation of distinct and functional lipid rafts in mature T-cell subsets

3.6 Immune System Disorders and T-Cell Gangliosides

Adaptive immunity plays an important role in host defense but can cause tissue injury and disease. Although the T cells that recognize self- or harmless antigens are killed or inactivated in normal individuals, failure of the normal mechanisms of self-tolerance leads to the development of autoimmune diseases and allergy. Each T-cell subpopulation (Th1, Th2, Th17, and Treg) is able to produce different cytokines and display distinct effector functions (Fig. 3.3b), suggesting the different organizations of TCR signaling complexes in lipid rafts in each subpopulation (Leitenberg et al. 2001). The lipid composition of lipid rafts as well as the TCR signaling ability itself differs in Th1 and Th2 cells (Balamuth et al. 2001; Izsepi et al. 2013; Sloan Lancaster et al. 1997; Smith et al. 1998). Recent studies of human cases and mouse models have shown that pathologies of some of these diseases are correlated with ganglioside composition in T cells.

3.6.1 Allergic Asthma and Gangliosides

Allergic asthma is classified as a type 1 hypersensitivity reaction and is a manifestation of immediate and late-phase reactions in the lung. Innate immune cells such as mast cells, basophils, and eosinophils are the effector cells of airway inflammation (Orihara et al. 2010; Holgate 2011). Adaptive immunity contributes to the initiation of allergic reactions. $CD4^+$ T cells play a crucial role via Th2 cytokine (IL-4 and IL-13) production, which then helps B cells to produce IgE antibodies that are specific for the "harmless" antigens (Levine and Wenzel 2010; Hamid and Tulic 2009). An experimental model to study mechanisms of allergic airway inflammation and airway hyperresponsiveness is established by inhalation of ovalbumin (OVA) antigen in OVA-sensitized mice. *ST3GAL5*-null mice dramatically alleviate the allergic airway responses induced by OVA inhalation: extensive mucus hypersecretion, a cardinal feature of asthma, airway infiltration of inflammatory cells, OVA-specific IgE production, and increased Th2 cytokine levels in serum (Nagafuku et al. 2012). Adoptive transfer experiments in which $CD4^+$ T cells isolated from OVA-sensitized control and *ST3GAL5*-null mice were transferred into naïve control mice and the recipient mice were challenged with OVA demonstrate that the immune function of $CD4^+$ T cells in vivo is selectively deficient in the mutant mice. $CD4^+$ T cells from the mutant mice lack GM1a, and self-reactive $CD4^+$ T cells exhibit the enhanced GM1a expression which causes persistence of abnormal cell activation (Dong et al. 2010; Kabouridis and Jury 2008). A recent report shows that airway inflammation is suppressed by the administration of antisense oligonucleotides against the ST3Gal5 gene (Karman et al. 2010). These findings strongly suggest that a-series ganglioside GM1a is essential for T-helper-cell function. Recently, novel $CD4^+$ T-cell subsets, Th17 cells and Treg cells, have been described. In allergic airway inflammation, the balance between effector Th2 cells and suppressive Treg cells is skewed toward Th2 predominance (Shalaby and Martin 2010). Th17 cells have been suggested to contribute to neutrophilic, steroid-resistant, severe asthma and to enhance Th2-mediated airway inflammation, although a role for the cells in asthma remains to be determined (Lloyd and Hessel 2010). Reportedly, *ST3GAL5*-null mice exhibit a decreased number of Th17 cells skewed by in vitro culture (Zhu et al. 2011).

3.6.2 Systemic Lupus Erythematosus and Gangliosides

Systemic lupus erythematosus (SLE) is an autoimmune disease and develops multisystem clinical manifestations such as rashes, arthritis, glomerulonephritis, hemolytic anemia, thrombocytopenia, and central nervous system involvement (Rahman and Isenberg 2008; Tsokos 2011). Many different autoantibodies are typically found in SLE patients, and so autoreactive T and B cells are involved in this pathogenesis. T cells from SLE patients have intrinsic alterations in lipid

components of lipid rafts (Jury et al. 2004; Krishnan et al. 2004; Dong et al. 2010). GM1a expression levels in $CD4^+$ T cells (but not in $CD8^+$ T cells) from SLE patients are significantly higher than those from healthy individuals. In addition, quantitative analysis by HPLC reveals that LacCer, GA2, Gb3, GM1a, and GD1a are upregulated in $CD4^+$ T cells from SLE patients compared to healthy individuals (McDonald et al. 2014). Strikingly, the increased expression of GM1a is more marked on CD45RO-positive $CD4^+$ T cells, phenotypically memory cells, from active SLE patients (but not those from inactive SLE patients) (Dong et al. 2010). Activated human T cells show increased GM1a levels (Tani-ichi et al. 2005; Tuosto et al. 2001; Dong et al. 2010; Krishnan et al. 2004). GM1a expression is enhanced in self-reactive $CD4^+$ T cells, causing the persistence of abnormal cell activation (Dong et al. 2010; Kabouridis and Jury 2008). These data implicate gangliosides as key factors in the pathogenesis and pathology of SLE. More recently, $CD4^+$ T cells from SLE patients were stimulated with anti-CD3 plus anti-CD28 mAbs together with a GlcCer synthase inhibitor, NB-DNJ, for 72 h. In NB-DNJ-treated T cell from SLE patients, the expression levels of GM1a and LacCer were normalized to those of healthy individuals, and the defects in TCR signaling in SLE T cells were partially restored (McDonald et al. 2014). Cellular GSL expression levels are controlled through the orchestrated effects of de novo synthesis, turnover, and recycling (Degroote et al. 2004). Interestingly, the increased GSL expression in SLE $CD4^+$ T cells was associated with accelerated internalization of GSLs from the plasma membrane into intracellular compartments and rapid recycling of GSLs back to the plasma membrane, leading to a net increase in plasma membrane expression. SLE T cells exhibit both increased GSL biosynthesis and accelerated trafficking to and from the plasma membrane, perhaps resulting in an aberrant accumulation of gangliosides in lipid rafts (Kidani and Bensinger 2014).

3.6.3 Rheumatoid Arthritis and Gangliosides

Rheumatoid arthritis (RA) is an autoimmune disease, which is characterized by inflammation of the synovium associated with destruction of the joint cartilage and bone (McInnes and Schett 2011). Both cell-mediated and humoral immune responses are thought to contribute to the development of RA. In fact, various inflammatory cells including Th1 and Th17 cells, activated B cells, and macrophages as well as numerous cytokines including IL-1, IL-8, TNF-α, IL-6, IL-17, and IFN-γ are found in the inflamed synovium and joint fluid (Gizinski and Fox 2014; Kobezda et al. 2014). A recent report shows that both GM3 and ST3Gal5 gene expressions are higher in the synovium of RA patients than in that of osteoarthritis (OA), which is not an autoimmune disease, patients (Tsukuda et al. 2012). *ST3GAL5*-null mice accelerate the progression of collagen-induced inflammatory arthritis, a mouse model of RA, and promote the induction of IL-17-producing cells in the regional lymph nodes after collagen immunization (Tsukuda et al. 2012). Contrastingly, it has also been reported that *ST3GAL5*-null mice exhibit a decreased

number of Th17 cells induced by in vitro culture (Zhu et al. 2011). Thus, further studies are needed to determine whether a-series gangliosides expressed in Th17 cells are involved in the development of RA. $CD4^+$ T cells can be separated into GD1c-positive IL-2-producing Th1-like cells and GD1c-negative IL-4-producing Th2-like cells (Nakamura et al. 1995). It is highly possible that each subpopulation of $CD4^+$ T cells has a unique ganglioside expression pattern in its rafts, which may be responsible for exerting specific functions of Th effector cells.

3.7 Future Directions

It is certain that the presence of a variety of rafts with different gangliosides in individual $CD4^+$ T-cell subsets is involved in the pathogenesis of allergic and autoimmune diseases. The selection of specific T cells from the thymocytes (repertoire selection) seems to be accompanied by selective ganglioside expression in individual T-cell subsets. In addition, the different subpopulations of effector $CD4^+$ T cells developed in peripheral lymphoid organs may have a unique ganglioside expression pattern in their rafts, which is responsible for exerting specific Th functions. This suggests that the ganglioside selection process is indispensable for the formation of distinct and functional lipid rafts in mature T cells (Fig. 3.4). All allergic reactions share common features although they differ greatly in the types of antigens. Lowering a-series gangliosides by ST3Gal5 inhibition would be a powerful treatment for immune system disorders by controlling ganglioside expression in lipid rafts. At present there is no selective inhibitor of GM3 synthesis, and so the development of an ST3Gal5 inhibitor is expected.

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Chapter 4
Gangliosides Regulate Tumor Properties: With Focus on the Suppression of Metastasis-Associated ppGalNAc-T13 with GM1

Koichi Furukawa, Yasuyuki Matsumoto, Qing Zhang, and Keiko Furukawa

Abstract Sialic acid-containing glycosphingolipids, gangliosides are expressed in various tissues and cells in our bodies. However, some relatively simple gangliosides are expressed in a tumor-specific manner in neuroectoderm-derived cancers and T-cell leukemias. They are also expressed in small cell lung cancers and osteosarcomas. Not only as tumor markers but as functional molecules on the cell surface membrane, they have been of interest, and indeed their roles in cancer cells have gradually been clarified. Recently, disialyl gangliosides and monosialyl gangliosides have been demonstrated to have opposite functions in the regulation of cancer properties. In particular, ganglioside GM1 showed suppressive effects on cell proliferation, invasion, and cancer metastasis in contrast with cancer-associated disialyl gangliosides such as GD3 and GD2. Based on the gene profiling with DNA array, it was demonstrated that the reduction of GM1 levels resulted in the increased expression of ppGalNAc-T13 and caused increased integrin functions, leading to enhanced metastatic potential of Lewis lung cancers. Trimeric Tn structure on syndecan-1 seems to be a key molecule to cause high metastasis. Both enhancing and suppressing actions of gangliosides on cancer properties have been shown to take place in membrane microdomains named lipid rafts. Therefore, regulatory functions of individual gangliosides in lipid rafts exerted by interacting with membrane molecules should be topics to be investigated now.

Keywords Ganglioside • Metastasis • Microdomain • Signal • Lipid raft • O-glycan

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4.1 Introduction

There have been a number of cancer-associated carbohydrate antigens, and some of them have been used as tumor markers (Brockhausen 1999; Hakomori 2002). While mechanisms for neo-synthesis and expression of cancer-associated carbohydrate antigens have been investigated by a number of researchers (Dall'Olio et al. 2012; Aksoy and Akinci 2004), mechanisms by which these cancer-associated complex carbohydrates play roles in cancer cells have been not well understood.

Among cancer-associated carbohydrate antigens, gangliosides, sialic acid-containing glycosphingolipids, have been considered to be useful markers for various neuroectoderm-derived cancers and some of leukemias (Old 1981; Siddiqui et al. 1984; Furukawa et al. 1993). In particular, ganglioside GD3 was detected as a melanoma-associated antigen (Portoukalian et al. 1979; Dippold et al. 1980; Furukawa and Lloyd 1990), and GD2 was identified as a neuroblastoma-associated ganglioside (Saito et al. 1985). GD2 was also reported as a characteristic antigen for small cell lung cancers (SCLC) (Cheresh et al. 1986), osteosarcomas (Heiner et al. 1987), breast cancers (Cazet et al. 2010), and advanced melanomas (Thurin et al. 1986). GD2 was also reported as one of oncofetal antigens in melanomas (Watanabe et al. 1982; Cahan et al. 1982). On the other hand, monosialyl gangliosides, such as GM1, have been reported to play roles in the suppression of malignant properties of cancer cells (Furukawa et al. 2012b). Virtually, distinct effects between disialyl and monosialyl gangliosides on malignant properties of cancer cells have been defined (Furukawa et al. 2012b), while modes of action of these two main groups of glycolipid antigens have not been well understood to date. The synthetic pathway of these structures is shown in Fig. 4.1.

Cancer metastasis is a major cause of death and the most serious issue in almost all cancers. Unless cancer metastasis is overcome, apparent improvement of prognosis of cancer patients cannot be expected (Yilmaz et al. 2013). Mechanisms for the evolution of cancer metastasis have not been well understood mainly because metastasis consists of multiple steps such as expansion and invasion into surrounding normal tissues, release from primary tumor sites, intravasation, adhesion to vascular walls, extravasation, and formation of new foci (Meng et al. 2012). Although a number of studies on cancer metastasis and ganglioside expression have been reported, definite molecular evidences for the involvement of gangliosides in cancer metastasis have not been demonstrated.

In this review, the involvement of gangliosides in cancer metastasis and its mechanisms elucidated mainly by our own results, and related reports from other laboratories have been summarized.

4.2 Monosialyl Gangliosides Often Suppress Malignant Properties in Cancer Cells

1. GM1 synthase expression resulted in the suppression of PDGF/PDGF receptor-derived signals in mouse Swiss3T3

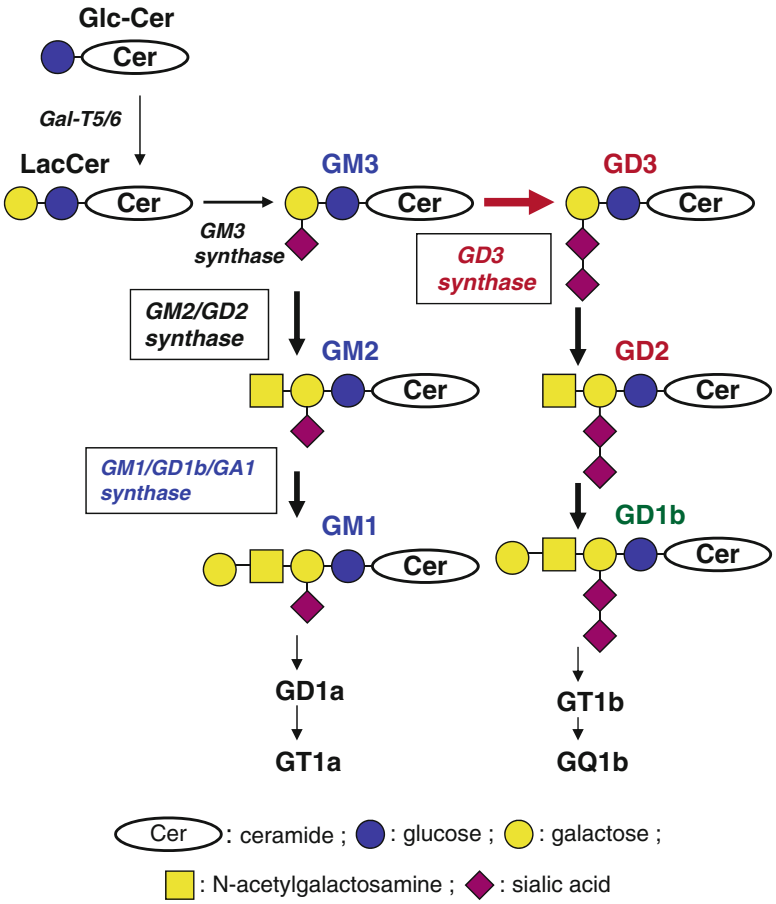

Fig. 4.1 Major gangliosides described in this review and their synthetic pathway. GD3 synthase (ST8SIA1) is a key enzyme for the synthesis of b-series gangliosides, such as GD3 and GD2

Several examples of suppressive functions of GM1 synthase and/or GM1 have been reported by our group using transfectant cell lines of GM1/GD1b synthase cDNA (Miyazaki et al. 1997). The first example to demonstrate that GM1 expression suppressed cancer properties is a study using a mouse fibroblast cell line, Swiss3T3 cell. Overexpression of GM1 synthase resulted in the lowered growth rates and reduced responses to PDGF treatment (Mitsuda et al. 2002). A shift of PDGF receptors from glycolipid-enriched microdomain (GEM)/rafts to non-GEM/rafts with reduced phosphorylation levels was observed in GM1 synthase cDNA-transfectant cells.

2. GM1 synthase in human melanomas

In a human melanoma cell line, SK-MEL-37, overexpression of GM1 synthase also induced reduced cell growth and invasion activity (Dong et al. 2010).

The transfectant cells showed not only neo-expression of GM1 and GD1b but also reduced expression levels of GD3 and GD2. While ganglioside GD3 was enriched in GEM/rafts in the parent cells (Haraguchi et al. 1994), dispersion of GD3 to non-GEM/raft fractions was detected in the cDNA transfectant cells. Consequently, cell growth and invasion activity were suppressed, suggesting the suppressive function of GM1 synthase/GM1. Interestingly, ratios of saturated fatty acids/unsaturated fatty acids in ceramides of GD3 in GEM/rafts were reduced in GM1 synthase cDNA-transfectant cells. Interestingly, intracellular distribution of GD3 was also changed in a melanoma cell line, SK-MEL-28, transfected with caveolin-1 cDNA (Nakashima et al. 2007). Namely, increased ratios of GD3 with unsaturated fatty acids were detected outside of GEM/rafts. Thus, GM1 synthase (or GM1) and caveolin-1 might have a common function in the suppression of signaling transduced via GEM/rafts and also affect intracellular distribution pattern of GD3 with changes in saturated/unsaturated patterns of fatty acids in ceramides.

Consequently, the suppressive function of GM1 synthase/GM1 appears to be similar with that of caveolin-1 (Quann et al. 2013; Razani et al. 2001). It is very interesting that both GM1 and caveolin-1 have been considered as GEM/rafts markers, while neither essential linkages between them nor common actions in these molecules have been demonstrated to date.

4.3 Regulation of Metastatic Potential of Mouse Lewis Lung Cancer (LLC) by Monosialyl Gangliosides

1. Overexpression of GM2/GD2 synthase cDNA resulted in the suppression of metastatic potential of LLC.

   LLC has been used as a useful model for cancer metastasis (Takenaga 1986). To clarify roles of gangliosides in cancer metastasis, an expression vector of GM2/GD2 synthase cDNA was transfected into a low metastatic LLC subline (Chen et al. 2003). An increase of metastatic potential in the transfectants was expected, because GM2 has been considered as a tumor-associated antigen in lung cancers (Hanibuchi et al. 1996) as well as in melanomas (Irie et al. 1989; Yamaguchi et al. 1990). Consequently, GM2/GD2 synthase cDNA-transfectant cells showed reduction in number of metastasis foci in the lung. Phosphorylation levels of focal adhesion kinase (FAK) were also reduced in the transfectant cells compared with the controls. Thus, these results suggested that neo-expression of GM2 suppressed cancer metastasis based on the reduced adhesion signals (Chen et al. 2003).

   Suppression of activation levels of EGF receptor and EGF signals by ganglioside GM3 in A431 cells was also reported (Yoon et al. 2005), suggesting that the suppressive regulation of growth signals by monosialyl gangliosides is a universal phenomenon.

2. GM1 was identified as an altered surface molecule in high metastatic sublines of LLC.

   To identify molecules and genes responsible for the cancer metastatic, low metastatic LLC subclones were repeatedly injected into C57BL/6 mice (iv or sc) (Zhang et al. 2006). In order to examine an alteration in the expression levels of surface molecules, various surface antigens were analyzed, i.e., integrin families, cadherin, CD44, and gangliosides. None of those antigens except GM1 showed definite reduced expression along with increased metastatic potential, i.e., only GM1 showed significant reduction in common among all high metastatic sublines when compared with individual parent lines (Zhang et al. 2006). These results suggested that GM1 suppresses metastatic potential in LLC.

   To examine implication of GM1 in cancer metastasis, knockdown of GM1 synthase was performed, and silencing of GM1 synthase indeed resulted in the establishment of GM1 low LLC sublines with high metastatic potentials. Thus, it was shown that reduction in GM1 expression resulted in a shift of MMP-9 and integrins to GEM/rafts and in promotion of secretion and activation of MMP-9, leading to the enhancement of metastatic property (Zhang et al. 2006).

   All these results suggested that GM1 and/or GM1 synthase induced suppression of cancer properties such as cell growth, invasion, and metastasis, at least partly, by the alteration in the intracellular localization of cognate membrane receptors and by modulation of their functions in GEM/rafts. However, clear modes of action of GM1 and/or GM1 synthase products have not yet been clarified.

3. Identification of responsible genes for the enhancement of LLC metastasis.

   Gene expression profiling using DNA microarray was performed to comprehensively understand the mechanisms for the increased metastatic potentials in high metastatic LLC sublines (Matsumoto et al. 2012). A unique advantageous point of our study was to search metastasis-associated genes by multiple combinations of "high" metastatic lines and "low" metastatic lines on the same basis and to pick up genes that are upregulated commonly among individual combinations along with increased metastatic property (Matsumoto et al. 2012). Thus, we could obtain more universal molecules possibly involved in the cancer metastasis.

   One of the genes upregulated in high metastatic lines both in GM1 synthase gene-silenced lines and in high metastatic sublines to lymph nodes after repeated injection was ppGalNAc-T13 (Matsumoto et al. 2012). ppGalNAc-T13 is a member of GalNAc transferase family involved in the first step of O-glycan synthesis by their activity to transfer alpha-GalNAc to Ser/Thr residues in mucins (Zhang et al. 2003). Twenty family members of ppGalNAc-Ts have been reported so far, and 16 out of them are known to have actual enzyme activities (Brooks et al. 2007). Among them, ppGalNAc-T13 is unique because of its restricted expression in brain tissues (Zhang et al. 2003). One more unique function of ppGalNAc-T13 is the activity to synthesize trimeric Tn antigen.

   Tn antigen is a most famous tumor antigen (Ju et al. 2011, 2013). While Tn has been long known as a tumor antigen, concrete implication of Tn structure in

tumor phenotypes has not been demonstrated so far. Furthermore, its involvement in cancer metastasis has never been well recognized.

4. Mechanisms for the enhancement of metastasis by trimeric Tn.

Trimeric Tn structures were reported first by Nakada et al. (1992; Matsumoto-Takasaki et al. 2012) in colon cancer cell lines as a unique Tn structure detected by a monoclonal antibody LS186. This unique O-glycan structure was distributed in cancer tissues (Ohshio et al. 1995) and was shown to be involved in the malignant properties of colon cancers (Morita et al. 2009), while mechanisms for its function in cancer cells have not been reported to date.

While it has been considered to be a tumor-associated antigen, the real function of Tn antigen has been long unknown. To clarify the meaning of trimeric Tn in cancer metastasis, carrier proteins of trimeric Tn structure was examined by immunoprecipitation with the anti-trimeric Tn antibody. Consequently, syndecan 1 was identified as a representative carrier protein in high metastatic sublines of LLC (Matsumoto et al. 2013).

Syndecan 1 has been known as a typical heparan sulfate proteoglycan involved in various cell events such as cell adhesion, proliferation, and motility (Munesue et al. 2002). In many cases, roles of glycosaminoglycan attached to the molecules have been considered as a key moiety in the interaction with other molecules (Munesue et al. 2007). Since ppGalNAc-T13-transfectant cells exhibited markedly enhanced cell adhesion to fibronectin in an integrin-dependent manner using real-time cell-electronic sensing (RT-CES) (Matsumoto et al. 2013), the interaction between syndecan 1 and integrins was suspected. As expected, it was eventually demonstrated that binding of syndecan 1 to integrins via trimeric Tn markedly enhanced integrin functions.

Tertiary complex consisting of trimeric Tn-carrying syndecan 1, MMP-9, and integrin β1/α5 was demonstrated. This molecular complex could be found in GEM/rafts of high metastatic lines including ppGalNAc-T13-transfectant cells. These results indicated that strong cell adhesion promoted cancer metastasis mainly in GEM/rafts (Simons and Ikonen 1997; Simons and Gerl 2010).

There have been a number of reports on integrin-mediated signaling (Margadant et al. 2011). Upon adhesion of cells to extracellular matrix, activation of FAK and/or Src family kinase(s) occurs, and subsequent activation of p130Cas, ILK, and paxillin is brought about (Margadant et al. 2011). In the case of LLC, strong activation of FAK and subsequent phosphorylation of paxillin were induced based on the expression of ppGalNAc-T13 as shown in Fig. 4.2.

4.4 Interaction Between GM1 and ppGalNAc-T13 in Cancer Metastasis

ppGalNAc-T13 was identified as one of the responsible genes for metastasis of LCC using GM1 synthase-silenced cell lines (Zhang et al. 2006; Matsumoto et al. 2012). Actually, knockdown of GM1 synthase resulted in the upregulation of ppGalNAc-T13 with increased invasion activity. Therefore, GM1 expression-mediated

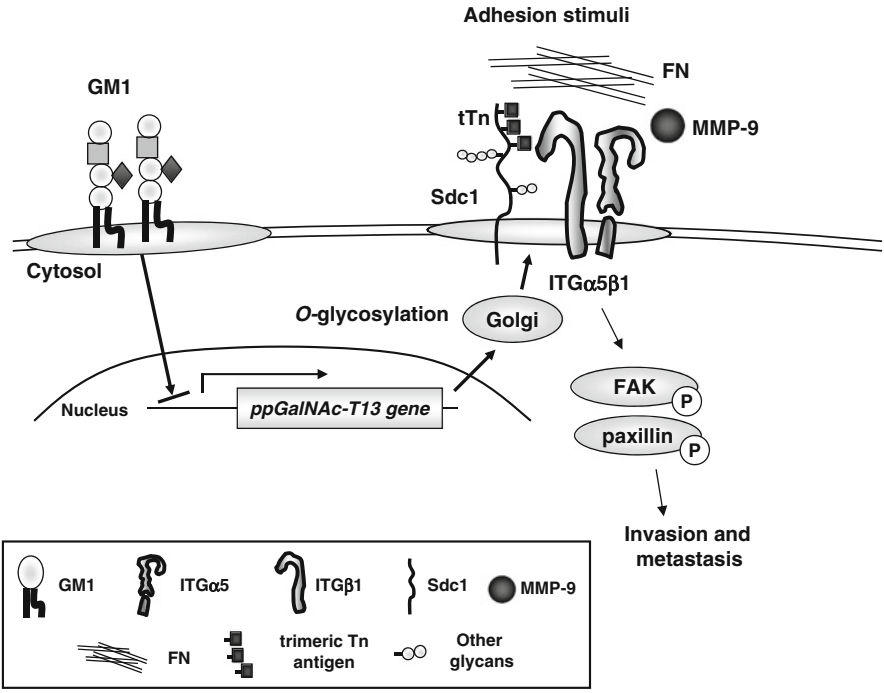

Fig. 4.2 Reduced expression of ganglioside GM1 resulted in the neo-expression of ppGalNAc-T13 and eventually enhanced cancer metastasis. Unique product of ppGalNAc-T13, trimeric Tn is key structure to bind integrins, resulting in the dramatic increase of cell adhesion to ECM. These interactions occur mainly in GEM/rafts and highly activate FAK and paxillin

signals might regulate the gene expression of ppGalNAc-T13, while precise mechanisms are not clear now. In addition, intracellular localization of ppGalNAc-T13 appears to be affected by the presence/absence of GM1 (unpublished data), suggesting the possibility of the direct interaction between GM1 and ppGalNAc-T13.

4.5 ppGalNAc-T13 and Human Diseases

When ppGalNAc-T13 gene was detected, it was found almost exclusively in brain tissues. Interestingly, it was reported that ppGalNAc-T13 was expressed in the bone marrow metastasis of neuroblastomas (Berois et al. 2006). The most interesting point is that ppGalNAc-T13 gene is expressed not only in LLC but also in human lung cancer cell lines including both non-small cell and small cell lung cancers (unpublished data). Moreover, it was detected in the blood of patients with heart diseases (Desai et al. 2012). The fact that ppGalNAc-T13 might be expressed in noncancerous diseases as well as in normal brain tissues suggests that ppGalNAc-T13 is expressed in some particular stage of neuronal differentiation and/or in activated cells in nonneuronal cell lineage. Therefore, induction

mechanisms for ppGalNAc-T13 gene expression should be very interesting and remain to be investigated.

4.6 Disialyl Gangliosides Enhance Cancer Properties in Various Cancers in Contrast with GM1

1. Malignant melanomas: Gangliosides GD3 and GD2 (or GM2) have been considered as melanoma-associated glycolipid antigens (Lloyd and Old 1989), based on early biochemical analysis (Portoukalian et al. 1979; Carubia et al. 1984) and immunological studies using monoclonal antibodies (Pukel et al. 1982; Thurin et al. 1986; Ravindranath et al. 1991). Many human origin mAbs reactive with melanoma gangliosides were generated (Yamaguchi et al. 1987; Irie and Morton 1986, Furukawa and Lloyd 1990). Recently, genetic approaches using cDNAs of glycosyltransferases were performed to analyze gangliosides in cancers (Daniotti et al. 2013). Using a GD3-lacking mutant of SK-MEL-28, GD3 synthase-transfectant cells were compared with control cells, and highly tyrosine-phosphorylated molecules were searched, resulting in the detection of adaptor molecules, p130Cas and paxillin, undergoing strong activation in GD3+ cells (Hamamura et al. 2005). Subsequently, focal adhesion kinase (FAK) was also identified as a critical molecule to be activated in GD3+ cells (Hamamura et al. 2008). Furthermore, a Src family kinase, Yes was identified as a kinase to be constitutively activated under GD3 expression (Hamamura et al. 2011). All these molecules were shown to form a molecular complex in the vicinity of the cell membrane (Furukawa et al. 2006), and these molecules seemed to be candidates for the molecular-targeted therapy of melanomas (Furukawa et al. 2008). In addition to the signaling via growth factor/receptor, adhesion-mediated signaling is also intimately regulated by gangliosides. GD3 expression enhanced cell adhesion to various extracellular matrices (Ohkawa et al. 2010). Gene silencing of integrin (β1) resulted in marked reduction of phosphorylation levels of p130Cas, FAK, and paxillin as well as of adhesion activity (Ohkawa et al. 2008). Then, copresence of adhesion signals and FCS (growth) signals was critical to induce definite activation of p130Cas, FAK, and paxillin. Furthermore, it was demonstrated that integrins shifted to GEM/rafts only in GD3+ cells after serum stimulation, suggesting the interaction of GD3 with integrins and/or these signaling molecules in GEM/rafts to generate signals for malignant properties of melanoma cells (Hakomori et al. 1998; Furukawa et al. 2006; Patra 2008) as shown in Fig. 4.3.
2. Expression analysis of gangliosides on human lung cancer cell lines revealed that small cell lung cancer (SCLC) cells express GD2, while NSCLC cells mainly express GM2 (Yoshida et al. 2001). Although these findings were reported many years ago (Hanibuchi et al. 1996; Grant et al. 1996), the responsible glycosyltransferase gene for each group was demonstrated for the first time in 2001. Namely, GM2/GD2 synthase was expressed commonly in all cell types of lung

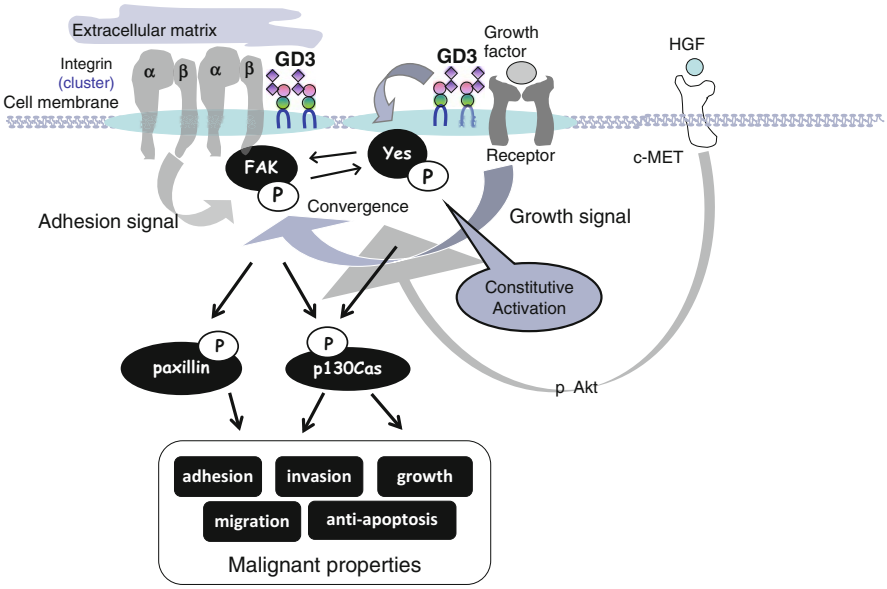

Fig. 4.3 Signaling enhanced by GD3 expression in melanoma cells. Expression of GD3 induced convergence of two major signals mediated by integrins and growth factor/receptors (or HGF/cMET), leading to synergism

cancers, while GD3 synthase was expressed exclusively in SCLC cell lines (Yoshida et al. 2001) as shown in Fig. 4.1. Furthermore, it was shown that anti-GD2 mAb induced apoptosis by reducing tyrosine phosphorylation levels of FAK, leading to anoikis (Aixinjueluo et al. 2005). These results strongly encouraged the application of anti-GD2 mAbs for the treatment of SCLC patients (Yoshida et al. 2002).

3. GD2 expression in breast cancer cell lines resulted in the increased cell proliferation and invasion (Cazet et al. 2010). Recently, GD2 was reported to be a stem cell marker of breast cancers (Battula et al. 2012). As a mechanism for action of GD2, activation of HGF receptor cMET in GD2+ cells was shown (Cazet et al. 2010). In turn, expression of GD3 in melanoma cells did not increase cMET activation after HGF treatment (Furukawa et al. 2014), in which it was shown that GD3 expression resulted in the convergence and synergy between HGF/cMET signals and adhesion signals.
4. Majority of osteosarcoma cell lines expressed high levels of GD2 as reported previously (Heiner et al. 1987). GD2/GD3 expression enhanced cell invasion and motility with increased activation of either FAK or Lyn, leading to the activation of a common target, paxillin (Shibuya et al. 2012). In contrast with melanoma cells, cell growth was not affected by the expression of disialyl gangliosides in osteosarcoma cells (Hamamura et al. 2005). Interestingly, the intensity in the phosphorylation of paxillin and that in cell adhesion was completely opposite. This paradoxical relationship between the weakest adhesion and the strongest activation of paxillin in GD3+/GD2+ is hard to be explained (Furukawa et al. 2012a).

5. Neuroblastoma cells specifically express GD2, and therefore, anti-GD2 antibody therapy has been widely tried (Fukuda et al. 1998). Antibody therapies with human-mouse chimeric antibody or mouse mAb 3F8 have been performed. Consequently, antibody therapies performed during the disease remission can cause an extension of 5-year survival and/or disease-free duration in severe cases (Raffaghello et al. 2003; Matthay et al. 2012; Parsons et al. 2013).
6. Ganglioside expression on human leukemia cells has been reported since a long time ago. GD3 was detected in T-cell acute lymphoblastic leukemia (T-ALL) cells (Siddiqui et al. 1984; Merritt et al. 1987) and also activated T lymphocytes (Welte et al. 1987; Yamashiro et al. 1995). Adult T-cell leukemia cell lines and human lymphotropic virus type I-infected T cells also expressed ganglioside GD2 (Furukawa et al. 1993), while leukemia cells from patients with adult T-cell leukemia expressed significant levels of GD2 only after culture in vitro (Okada et al. 1996).

4.7 Ending Remarks

Many of studies analyzing the roles of monosialyl and disialyl gangliosides suggest that gangliosides regulate natures of GEM/rafts (Furukawa et al. 2012b), resulting in the controlling of cell signaling. Contrastive effects of GD3 synthase and GM1 synthase on cell proliferation and differentiation were demonstrated in PC12 cells by our group (Fukumoto et al. 2000; Nishio et al. 2004). Why so little differences in the carbohydrate structures induce opposite effects as shown in Fig. 4.4 has not been well understood. Molecules interacting with monosialyl gangliosides and those with disialyl gangliosides, particularly with tandem-disialyl structures formed in b-series gangliosides, may belong to distinct protein groups with opposite functions. The former might be suppressive, and the latter may play enhancing roles in the cancer properties. Therefore, identification of molecules interacting with gangliosides in GEM/rafts seems to be crucial for further understanding modes of action of gangliosides (Hashimoto et al. 2012).

There have been a number of studies on cancer-associated carbohydrate antigens (Hakomori S 1985; Zhang et al. 1997). In addition to their roles as tumor markers, functional implications in their cancer phenotypes have been demonstrated (Hollingsworth and Swanson 2004; Furukawa et al. 2012a). Particularly, it is intriguing that the involvement of integrins in the functions of sugar chains has been very frequently found in our studies (Zhang et al. 2006; Ohkawa et al. 2010; Matsumoto et al. 2013) and others' studies (Hakomori and Handa 2002; Cabodi et al. 2010; Hakomori 2010). Furthermore, cell-to-cell interaction in cancer niche via cytokines (Miyata et al. 2014) and other unknown vesicles also seems essential (Peinado et al. 2012). Environmental factors around micro-foci of transforming

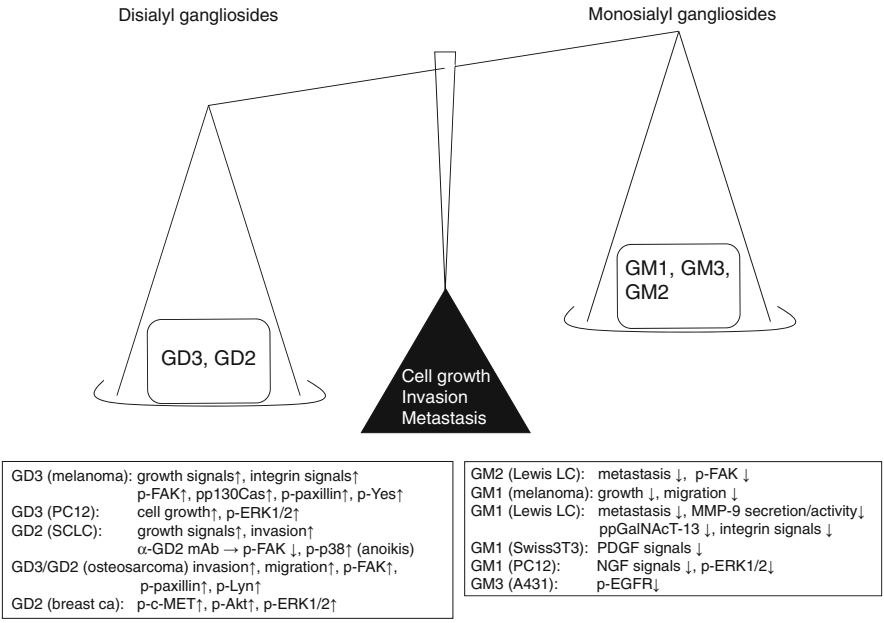

Fig. 4.4 Contrastive effects of monosialyl and disialyl gangliosides on the malignant properties. Generally, disialyl gangliosides are widely considered as cancer-associated glycolipids and enhance malignant properties such as rapid cell growth, increased invasion activity, and metastatic potential. On the other hand, monosialyl gangliosides exert opposite actions

cells could be subjects of studies on carcinogenesis with focus on the alteration in glycosylation machineries.

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Chapter 5
Role of Glycans in Viral Infection

Tadanobu Takahashi and Takashi Suzuki

Abstract A variety of viruses show specific binding to glycans on the cellular surface, such as sialoglycoconjugates, glycosaminoglycans, and histo-blood group antigens. The viral surface proteins recognize terminal sugar chain moieties of glycan and select glycans for binding to specific tissues and hosts. For example, orthomyxoviruses (influenza viruses) and paramyxoviruses recognize terminal moieties of sialic acid linked to galactose for infecting target cells. In most cases, glycans are thought to be involved in cellular surface attachment and cell entry of viruses, as viral receptors and/or coreceptors. Expression of sugar chain moieties is generally dependent on specific tissues, cells, and hosts. Therefore, the specific interactions of viruses with glycans significantly affect tissue tropism and pathogenicity by selection of the viral replication site. For example, human influenza A virus preferentially binds to sialic acid α2,6 linkage to galactose, which is expressed in the human upper respiratory tract. On the other hand, avian influenza A virus preferentially binds to sialic acid α2,3 linkage to galactose, which is expressed in chicken eggs and trachea. The difference in recognition is believed to determine host specificity of influenza A virus. Platforms of the sugar chain are *N*-linked glycan, *O*-linked glycans (containing proteoglycans), and sphingolipid. Difference in these platforms also affects functions of viral receptors. This chapter presents a review about glycans bound and recognized by representative viruses including coronavirus, flavivirus, herpesvirus, norovirus, orthomyxovirus, paramyxovirus, parvovirus, polyomavirus, retrovirus, and reovirus.

Keywords Binding • Heparan sulfate • Histo-blood group antigens • Infection • Glycan • Receptor • Sialic acid • Sugar chain • Sulfatide • Virus

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Abbreviations

AAV	Adeno-associated virus
AAV1	AAV type 1
AAV2	AAV type 2
AAV4	AAV type 4
AAV5	AAV type 5
AAV6	AAV type 6
AAV9	AAV type 9
AIBV	Avian infectious bronchitis virus
ApoE	Apolipoprotein E
BCV	Bovine coronavirus
BKV	BK virus
BPV	Bovine parvovirus
CPV	Canine parvovirus
DEN	Dengue virus
GAG	Glycosaminoglycan
GalCer	Galactosylceramide
HA	Hemagglutinin
HCoV-OC43	Human coronavirus OC43 strain
HCV	Hepatitis C virus
HE	Hemagglutinin-esterase
HN	Hemagglutinin-neuraminidase
HIV	Human immunodeficiency virus
HPAI	Highly pathogenic avian IAV
FPV	Feline parvovirus
hPIV	Human parainfluenza virus
hPIV1	hPIV type 1
hPIV3	hPIV type 3
HSV	Herpes simplex virus
HSV-1	HSV serotype 1
HSV-2	HSV serotype 2
IAV	Influenza A virus
IBV	Influenza B virus
ICV	Influenza C virus
JCV	JC virus
JEV	Japanese encephalitis virus
MHV	Mouse hepatitis virus
MVM	Parvovirus minute virus of mice
MPV	Murine polyomavirus
Neu5Ac	*N*-Acetylneuraminic acid
MuV	Mumps virus
NDV	Newcastle disease virus

Neu5Acα2,3Gal	Neu5Ac α2,3-linked to galactose
$Neu4,5Ac_2$	*N*-Acetyl-4-*O*-acetylneuraminic acid
$Neu5,9Ac_2$	*N*-Acetyl-9-*O*-acetylneuraminic acid
$Neu5,9Ac_2$α2,3Gal	$Neu5,9Ac_2$ α2,3-linked to galactose
$Neu5,9Ac_2$α2,6Gal	$Neu5,9Ac_2$ α2,6-linked to galactose
Neu5Gc	*N*-Glycolylneuraminic acid
NoV	Human norovirus
NSP4	Nonstructural glycoprotein 4
ReV	Reovirus
ReV1	ReV type 1
ReV3	ReV type 3
RoV	Rotavirus
RSV	Human respiratory syncytial virus
SeV	Sendai virus
SV40	Simian virus 40
TGEV	Porcine transmissible gastroenteritis virus
TNF	Tumor necrosis factor
VLP	Virus-like particle
vRNP	Viral ribonucleoprotein complexes
WNV	West Nile virus

5.1 Introduction

All viruses replicate in host cells only and show host (cell) ranges and specificities. Glycans on the cellular surface are highly diverse and species specific. Viral host (cell) ranges and specificities are often dependent on specificity and diversity of glycans on the surface membranes of host cells. In fact, various viruses bind to glycans on the surface membranes of host cells as specific receptors. Typical receptors are sialic acid-containing glycans and sulfated glycans, for example, gangliosides and heparan sulfate, respectively. In many cases, the minus charge of sialic acid and sulfate is likely to play an important role in viral binding with glycans. The typical life cycle of an enveloped virus consists of receptor binding, entry, uncoating of the viral capsid, synthesis of viral components (genomes and proteins), glycosylation of viral proteins, intracellular traffic of viral components, packaging of viral particles, and budding and release of progeny viruses on the cellular surface. Functions of glycans in these steps except for receptor binding mostly remain unknown. This chapter presents a review, mainly in terms of a viral receptor, about glycans recognized by viruses including coronavirus, flavivirus, herpesvirus, norovirus, orthomyxovirus, paramyxovirus, parvovirus, polyomavirus, retrovirus, and reovirus.

5.2 Viruses that Bind to Glycans

5.2.1 Coronavirus

Coronaviruses are positive-stranded RNA viruses and enveloped viruses that are classified within the family *Coronaviridae*. They are a diverse group of viruses that infect various mammalian and avian species. The viruses often affect the respiratory or intestinal tract. It has been shown that many coronaviruses agglutinate erythrocytes (Bingham et al. 1975; Pokorný et al. 1975). Coronaviruses recognize a type of sialic acid as a receptor on cell surface components. Bovine coronavirus (BCV) and human coronavirus OC43 strain (HCoV-OC43) have binding activity to glycoconjugates containing *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac_2), through hemagglutinin-esterase (HE) protein and/or spike (S) protein on the viral surface membrane (Schultze et al. 1991a, b; Künkel and Herrler 1993). The HE protein only agglutinates cells that contain a high content of Neu5,9Ac_2 such as mouse and rat erythrocytes (Schultze et al. 1991b). The S protein is able to agglutinate chicken erythrocytes, but the HE protein cannot (Schultze et al. 1991a). Bovine coronavirus is more efficient in recognizing Neu5,9Ac_2 α2,3-linked to galactose (Neu5,9Ac_2α2,3Gal), whereas HCoV-OC43 is superior with respect to Neu5,9Ac_2 α2,6-linked to galactose (Neu5,9Ac_2α2,6Gal) (Krempl et al. 1995). BCV and HCoV-OC43 use Neu5,9Ac_2 as a receptor to initiate infection of cultured cells (Schultze and Herrler 1992; Künkel and Herrler 1993). These viruses also have esterase activity in the HE protein to cleave the 9-*O*-acetyl group of Neu5,9Ac_2, as does influenza C virus (ICV). The esterase activity is believed to help release of progeny viruses from cellular surfaces of host cells. In contrast to most of the coronaviruses, mouse hepatitis virus (MHV) recognizes *N*-acetyl-4-*O*-acetylneuraminic acid (Neu4,5Ac_2) rather than Neu5,9Ac_2 (Regl et al. 1999; Langereis et al. 2012). Receptor recognition of MHV may reflect change in host tropism from other species to mice.

Porcine transmissible gastroenteritis virus (TGEV) and avian infectious bronchitis virus (AIBV) bind to *N*-acetylneuraminic acid (Neu5Ac) α2,3-linked to galactose (Neu5Acα2,3Gal) (Schultze et al. 1992, 1993) via viral S protein. TGEV infects the porcine small intestine, brush border membranes of which express mucin-like and Neu5Ac-rich glycoprotein. Although TGEV uses aminopeptidase N as the main cellular receptor, TGEV S protein may support viral attachment to the brush border membranes (Schwegmann-Wessels and Herrler 2008). TGEV also recognizes *N*-glycolylneuraminic acid (Neu5Gc) (Schultze et al. 1996), which is expressed in pigs (Suzuki et al. 1997). S protein of AIBV shows much higher binding activity to Neu5Acα2,3Gal than does that of TGEV. AIBV uses only Neu5Ac as the main cellular receptor (Winter et al. 2006; Shahwan et al. 2013). AIBV Beaudette strain shows binding activity to heparan sulfate (HS). This virus is an embryo-adapted virus that has the extended tropism in cell culture. HS may in part contribute to extended tropism of AIBV Beaudette strain (Madu et al. 2007) (Table 5.1).

Table 5.1 Binding activities of coronaviruses to glycans

Virus	Glycan (references)
BCV	Neu5,9Ac_2α2,3Gal (Krempl et al. 1995; Künkel and Herrler 1993; Schultze et al. 1991a, b)
HCoV-OC43	Neu5,9Ac_2α2,6Gal (Krempl et al. 1995; Künkel and Herrler 1993; Schultze et al. 1991a, b)
MHV	Neu4,5Ac_2 (Langereis et al. 2012; Regl et al. 1999)
TGEV	Neu5Acα2,3Gal (Schultze et al. 1993; Schwegmann-Wessels and Herrler 2008)
	Neu5Gc (Schultze et al. 1996)
AIBV	Neu5Acα2,3Gal (Schultze et al. 1992, 1993; Shahwan et al. 2013; Winter et al. 2006)
	HS (Madu et al. 2007)

5.2.2 Flavivirus

Flaviviruses are positive-stranded RNA viruses and enveloped viruses that are classified within the family *Flaviviridae*. Dengue virus (DEN) is the most important mosquito-mediated human pathogen. Clinical manifestations of the virus range from a simple self-limited febrile illness known as dengue fever to a hemorrhagic fever and potentially fatal hemorrhagic shock syndrome. All serotypes (1–4) of DEN recognize nLc_4Cer (Galβ1,4GlcNAcβ1,3Galβ1,4Glc1,1'Cer) from mammalian cells (Aoki et al. 2006). DEN type 2 also recognizes Ar_3Cer (GlcNAcβ1,3Manβ1,4Glcβ1,1'Cer) from mosquito cells (Wichit et al. 2011). It is thought that neutral glycosphingolipids share the important determinant for DEN binding and that the β-GlcNAc residue may play a key role in DEN binding. Chemically synthesized derivatives carrying multiple carbohydrate residues of nLc_4 inhibit binding of DEN type 2, indicating that a binding inhibitor based on nLc_4 could be as a potential DEN drug (Aoki et al. 2006). DEN also binds to some glycosaminoglycans (GAGs) such as HS (Chen et al. 1997; Watterson et al. 2012), heparin (Marks et al. 2001), fucoidan (Hidari et al. 2008), and chondroitin sulfate E (Kato et al. 2010) through the virus envelope E glycoprotein, but does not bind to chondroitin sulfates A, B, C, and D or hyaluronic acid (Kato et al. 2010). DEN infection is inhibited by some GAGs such as heparin (Marks et al. 2001), fucoidan (Hidari et al. 2008), and chondroitin sulfate E (Kato et al. 2010). Most GAGs include GlcA and sulfated GlcA. 3-*O*-Sulfated GlcA inhibits DEV infection, but 2-*O*-sulfated and 3,6-di-*O*-sulfated ones do not (Hidari et al. 2012). It is thought that 3-*O*-GlcA is in part a key structure in DEN binding to GAGs. DEN causes leakage of the vascular endothelium, resulting in dengue hemorrhagic fever. Human endothelial cells are highly susceptible to infection by DEN. The susceptibility may be attributed to DEN attachment directed to HS-containing proteoglycan receptors on endothelial cells (Dalrymple et al. 2011). Two encephalitis flaviviruses, Japanese encephalitis virus (JEV) and West Nile virus (WNV), have a binding activity to heparin (Lee et al. 2004). JEV also binds to and is inhibited by HS (Su et al. 2001).

The binding affinity of WNV and JEV for GAG has been suggested to be a determinant for the neuroinvasiveness of encephalitic flaviviruses (Lee et al. 2004).

E1 and E2 envelope glycoproteins of hepatitis C virus (HCV) recognize HS through an important structure such as 6-*O*-sulfation and *N*-sulfation, not through simple ionic interactions (Barth et al. 2003; Kobayashi et al. 2012). Since HCV strongly binds to HS from liver tissues, HS appears to be one of the molecules that confer the liver-specific tissue tropism of HCV infection (Kobayashi et al. 2012). Binding of HCV to the cell surface is not markedly inhibited by heparin, different from other flaviviruses such as DEN and JEV. Cellular HS may act as an alternative receptor for HCV, not a primary receptor (Heo 2008). However, chondroitin sulfate E from squid cartilage strongly interacts with both E1 and E2 proteins and inhibits the entry of pseudotype HCV into cells, suggesting that chondroitin sulfate E is a potential candidate of an anti-HCV drug (Kobayashi et al. 2012). Apolipoprotein E (ApoE), which has a heparin-binding activity, mediates HCV attachment to the cell surface through specific interactions with cellular HS (Jiang et al. 2012). Syndecan-1, which is a core protein to form HS proteoglycans, serves as the major receptor protein for HCV attachment to cells (Shi et al. 2013).

Sulfated GAGs (especially HS) may serve as receptor proteoglycans for the attachment of flaviviruses to target cells. Elucidation of the mechanism by which flaviviruses bind to sulfated GAGs would contribute to the discovery and development of anti-flavivirus drugs (Table 5.2).

5.2.3 Herpesvirus

Herpesviruses are double-stranded linear DNA viruses and enveloped viruses that are classified within the family *Herpesviridae*. The most common manifestations of herpes simplex virus (HSV) infection are mucocutaneous lesions. The initial contact of HSV serotypes 1 and 2 (HSV-1 and HSV-2) with the cellular surface is

Table 5.2 Binding activities of flaviviruses to glycans

Virus	Glycan (references)
DEN	nLc_4Cer (Aoki et al. 2006)
	Ar_3Cer (Wichit et al. 2011)
	HS (Chen et al. 1997; Watterson et al. 2012)
	Heparin (Marks et al. 2001)
	Fucoidan (Hidari et al. 2008)
	Chondroitin sulfate E (Kato et al. 2010)
JEV	Heparin (Lee et al. 2004)
	HS (Su et al. 2001)
WNV	Heparin (Lee et al. 2004)
HCV	HS (Barth et al. 2003; Kobayashi et al. 2012)
	Chondroitin sulfate E (Kobayashi et al. 2012)

Table 5.3 Binding activities of herpesviruses to glycans

Virus	Glycan (references)
HSV-1, 2	HS (especially 3-*O*-sulfated) (Herold et al. 1991; Shukla et al. 1999; Trybala et al. 2000)

believed to be binding of the virus to HS through the viral envelope glycoproteins gB and gC (Herold et al. 1991; Trybala et al. 2000). However, interactions of gB and gC with HS are not sufficient for HSV entry into cells. After adsorption of HSV with HS on the cellular surface, cell entry requires engagement of the viral envelope glycoprotein gD with one of three classified coreceptors, herpesvirus entry mediator, tumor necrosis factor (TNF) receptor family, and immunoglobulin superfamily (Spear et al. 2000). Additionally, 3-*O*-sulfation of glucosamine residues in HS generated by multiple D-glucosaminyl 3-*O*-sulfotransferase isoforms is a key determinant of the gD binding site. HSV-1 cell entry requires interactions of gD with 3-*O*-sulfated HS or other coreceptors described above (Shukla et al. 1999). 3-*O*-Sulfated HS appears to play an important role in HSV-1 entry into many different cell lines (O'Donnell et al. 2010). The glycoprotein gB has a sequence of a putative fusion activity, suggesting that interactions of gB with cellular surface molecules allow the fusion process for cell entry. However, HS-deficient cells are susceptible to HSV-1 infection (Banfield et al. 1995). HSV-1 bearing gB lacking an HS binding site also maintains cell infectivity (Laquerre et al. 1998). Soluble gB, which was generated by a baculovirus protein expression system, also binds to HS-deficient cells and inhibits HIV-1 infection (Bender et al. 2005). Interaction of gB with other molecules except HS may play an important role in HSV-1 infection. 3-*O*-Sulfated HS and HS-binding peptide have been investigated as anti-HSV agents (Copeland et al. 2008; Ali et al. 2012) (Table 5.3).

5.2.4 Norovirus

Human noroviruses (NoVs) are single-stranded positive-sense RNA viruses and small, round, non-enveloped viruses with a diameter of 38 nm that are classified within the family *Caliciviridae*. These viruses are the major causative pathogens of acute viral gastroenteritis characterized by severe diarrhea. NoV virus-like particles (VLPs) bind to histo-blood group antigens demonstrating A, B, and O phenotypes, through the P domain of viral capsid protein, VP1 (Harrington et al. 2002; Marionneau et al. 2002; Chen et al. 2011). For example, the VLPs derived from Norwalk/68 strain bind to H1 antigen (Fucα1,2Galβ1,3GlcNAc; O phenotype), H2 antigen (Fucα1,2Galβ1,4GlcNAc; O phenotype), Le^b antigen [Fucα1,2Galβ1,3(Fucα1,4) GlcNAc], A1 antigen [GalNAcα1,3(Fucα1,2)Galβ1,3GlcNAc; A phenotype], and A2 antigen [GalNAcα1,3(Fucα1,2)Galβ1,4GlcNAc; A phenotype] but not to B1 antigen [Galα1,3(Fucα1,2)Galβ1,3GlcNAc, B phenotype] or B2 antigen [Galα1,3(Fucα1,2)Galβ1,4GlcNAc, B phenotype] (Harrington et al. 2002;

Table 5.4 Binding activities of noroviruses to glycans

Virus	Glycan (references)
NoV	ABH and Lewis antigens in human blood (Harrington et al. 2002; Marionneau et al. 2002)

Huang et al. 2003, 2005; Hutson et al. 2003; Lindesmith et al. 2003). Humans with O phenotype, but not those with B phenotype, are susceptible to NoV Norwalk/68 strain infection (Hutson et al. 2002; Lindesmith et al. 2003). These studies suggested that histo-blood group antigens are receptors of NoV. However, other NoV VLPs display different ABH and Lewis carbohydrate-binding profiles (Harrington et al. 2002; Huang et al. 2005; Shirato et al. 2008; Shirato-Horikoshi et al. 2007). Indeed, Rockx's epidemiological research indicated that some NoVs can infect individuals with different ABH phenotypes (Rockx et al. 2005). For example, VLPs derived from BUDS strain bind to A and B antigens but not to H antigens. The binding activities of NoVs to histo-blood group antigens vary greatly in a strain-dependent manner. NoVs include at least 36 genotypes in VP1 nucleotide sequence. Various genotype NoVs appear to infect humans with any blood types through binding combinations of some histo-blood group antigens (Table 5.4).

5.2.5 Orthomyxovirus

Representative orthomyxoviruses are influenza A virus (IAV), influenza B virus (IBV), and ICV, which are classified within the family *Orthomyxoviridae*. Influenza viruses are enveloped viruses with a diameter of 100 nm and are respiratory pathogens with strong infection spread. IAVs and IBVs are eight-segmented single-stranded negative-sense RNA viruses, and ICVs are seven-segmented single-stranded negative-sense RNA viruses. Viral hosts are wide species including humans, pigs, birds, and horses for IAVs and mainly humans for IBVs and ICVs. Host receptors on the cellular surface membrane are sialic acid residues existing at the terminal position of glycoconjugates, Neu5Ac for IAVs and IBVs and Neu5,9Ac_2 for ICVs (Rogers et al. 1986; Suzuki et al. 1992). IAVs and IBVs have sialidase activity (an enzyme cleaving Neu5Ac from glycoconjugates), and ICVs also have esterase activity (an enzyme cleaving 9-*O*-acetyl group from Neu5,9Ac_2) to prevent trapping of progeny viruses to sialic acid residues on the cellular surface and on viral glycoproteins. These receptors containing sialic acids are thought to be gangliosides and/or *N*-glycans (Suzuki 1994; Chu and Whittaker 2004). In general, human IAVs show preferential binding to Neu5Acα2,6Gal linkage, whereas avian IAVs show preferential binding to Neu5Acα2,3Gal linkage. Swine IAVs bind to both Neu5Acα2,3Gal and Neu5Acα2,6Gal linkages, equally or with predominance toward Neu5Acα2,6Gal linkage (Ito et al. 1997a; Suzuki et al. 1997). IBVs show preferential binding to Neu5Acα2,6Gal linkage (Suzuki et al. 1992). IAVs and IBVs strongly recognize Neu5Acα2,6(or 3)Galβ1,3GlcNAc and Neu5Acα2,6(or 3)

Galβ1,4GlcNAc through interactions of the viral surface glycoprotein, hemagglutinin (HA) (Suzuki et al. 1992, 2000; Suzuki 1994). The human trachea predominantly expresses Neu5Acα2,6Gal linkage (Baum and Paulson 1990). The pig trachea expresses both Neu5Acα2,3Gal and Neu5Acα2,6Gal linkages (Suzuki et al. 1997, 2000). Chicken eggs and trachea express Neu5Acα2,3Gal linkage (Ito et al. 1997b; Abd El Rahman et al. 2009). Glycoconjugates recognized by respective IAVs coincide with respective virus replication sites expressing their glycoconjugates, strongly suggesting that their glycoconjugates are receptors of IAVs. Some H5N1 highly pathogenic avian IAVs (HPAIs) and H7N9 avian IAVs, isolated from humans, show increased binding activity to Neu5Acα2,6Gal linkage (Yamada et al. 2006; Watanabe et al. 2013; Zhang et al. 2013). Acquisition of Neu5Acα2,6Gal linkage binding activity of H5N1 HPAIs is one of the factors that lead to airborne transmission among ferrets (human infection and transmission model) (Imai et al. 2012; Herfst et al. 2012). Increased binding activity of avian IAVs and animal IAVs other than human IAVs to Neu5Acα2,6Gal linkage could cause a pandemic of a new subtype of IAV among humans. As an alternate pandemic mechanism, a new subtype of IAV could arise by genetic reassortment among segmented viral RNAs from simultaneous infections of human and avian IAVs in pigs, which express both Neu5Acα2,3Gal and Neu5Acα2,6Gal linkages in the trachea. In this way, Neu5Ac binding properties of IAVs may be involved in the pandemic occurrence of a new subtype of IAV.

Since 2008, it has been reported that some IAVs, 2009 pandemic H1N1 IAVs and avian IAVs including H5, H6, H7, and H9 subtypes, show preferential binding to 6-sulfo sialyl Lewis X. These IAVs appear to recognize terminal tri- or tetra-oligosaccharides [Neu5Acα2,3Galβ1,4(6-*O*-SO_3H)GlcNAc and Neu5Acα2,3Galβ1,4(Fucα1,3)(6-*O*-SO_3H)GlcNAc] of 6-sulfo sialyl Lewis X (Gambaryan et al. 2008, 2012; Childs et al. 2009).

Major sialic acids are classified into two types: Neu5Ac and Neu5Gc. Almost all equine IAVs show strong preferential binding to Neu5Gc α2,3-linked to galactose (Neu5Gcα2,3Gal) (Ito et al. 1997a; Suzuki et al. 2000). Almost all avian IAVs also show binding activity to one, although Neu5Gc binding activity is weaker than their Neu5Ac binding activity (Ito et al. 1997a, 2000). Some human and swine IAVs show binding activity to Neu5Gc (preferentially to Neu5Gcα2,6Gal linkage) (Suzuki et al. 1997; Masuda et al. 1999; Takahashi et al. 2009). Neu5Gc and Neu5Gcα2,3Gal linkage is expressed in the horse trachea, duck intestine, and pig trachea, which are natural replication sites of IAVs (Suzuki et al. 1997, 2000; Ito et al. 2000). The function of Neu5Gc is predicted to be an IAV receptor, like Neu5Ac. There is a possibility that human and avian IAVs facilitate transmission to pigs through interactions with Neu5Gc. As described above, pigs are potential intermediate hosts that produce a new subtype of IAV between human IAV and avian IAV. Neu5Gc binding properties of these IAVs may also be involved in a pandemic occurrence.

Sulfatide is a 3-*O*-sulfated galactosylceramide (GalCer). IAV specifically binds to sulfatide, even though it does not contain any sialic acids (Suzuki et al. 1996). Sulfatide is not an IAV receptor for initial infection, different from sialic acids.

Table 5.5 Binding activities of orthomyxoviruses to glycans

Virus	Glycan (references)
Human IAV	Neu5Ac (Neu5Gc) α2,6Gal (Masuda et al. 1999; Suzuki et al. 1992; Suzuki 1994; Takahashi et al. 2009)
Avian IAV	Neu5Ac (Neu5Gc) α2,3Gal (Ito et al. 1997a, 2000; Suzuki et al. 1992; Suzuki 1994)
Swine IAV	Neu5Acα2,6Gal (Suzuki et al. 1997)
	Neu5Ac (Neu5Gc) α2,3Gal (Suzuki et al. 1997)
Equine IAV	Neu5Gcα2,3Gal (Ito et al. 1997a; Suzuki et al. 2000)
IAV	6-Sulfo sialyl Lewis X (Gambaryan et al. 2008, 2012; Childs et al. 2009)
	Sulfatide (Suzuki et al. 1996; Takahashi et al. 2008, 2010, 2013a, b)
IBV	Neu5Acα2,6Gal (Suzuki et al. 1992)
ICV	Neu5,9Ac_2 (Rogers et al. 1986; Suzuki et al. 1992)

Caspase-3-dependent apoptosis enhances IAV replication through enhancement of nuclear export of viral ribonucleoprotein complexes (vRNP) (Wurzer et al. 2003). Sulfatide has interacted with newly synthesized HA transferred to the surface membranes of infected cells. The interaction of HA with sulfatide facilitates formation and replication of progeny virus particles through enhancement of nuclear export of vRNP by inducing caspase-3-independent apoptosis (Takahashi et al. 2008, 2010, 2013b). The binding mechanism of the HA ectodomain with sulfatide is thought to be different from that with Neu5Ac (Takahashi et al. 2013a). An inhibitor of HA binding with sulfatide would become a novel drug that inhibits formation of IAV particles and IAV replication. Sulfatide is involved in various biological properties such as the immune system, nervous system, kidney functions, insulin control, hemostasis/thrombosis, cancer, and other microbes (Takahashi and Suzuki 2012). Further study on sulfatide binding of IAVs would contribute to elucidation of these biological mechanisms and diseases associated with sulfatide (Table 5.5).

5.2.6 Paramyxovirus

Paramyxoviruses are single-stranded negative-sense RNA viruses and enveloped viruses with a diameter of 150–250 nm that are classified within the family *Paramyxoviridae*. Some paramyxoviruses have the envelope glycoprotein, hemagglutinin-neuraminidase (HN), displaying both sialic acid binding activity and sialidase activity. Such viruses that infect humans are human parainfluenza virus (hPIV) and mumps virus (MuV), which are members of the genus *Respirovirus* and *Rubulavirus*, respectively. hPIVs [mainly type 1 (hPIV1) and type 3 (hPIV3)] account for 20 % of causative pathogens isolated from children with pneumonia (Sinaniotis 2004). hPIV1 causes most cases of laryngotracheobronchitis (croup) in children, and hPIV type 3 (hPIV3) often causes pneumonia and bronchiolitis

in infants younger than 6 months of age. hPIV1 shows preferential binding to Neu5Acα2,3Galβ1,3GlcNAc (Suzuki et al. 2001; Tappert et al. 2011), whereas hPIV3 shows binding activity to both Neu5Acα2,3Galβ1,3GlcNAc and Neu5Acα2,6Galβ1,3GlcNAc, in addition to Neu5Gcα2,3Galβ1,3GlcNAc. Higher pathogenicity of hPIV3 may be involved in the broader range of receptor recognition than that of hPIV1. Interestingly, both hPIVs strongly bind to oligosaccharides containing branched *N*-acetyllactosaminoglycans (Suzuki et al. 2001). Blood group I-type polylactosamine antigens may be major receptors of hPIVs. Also, HS binding of hPIV3 suggests that HS may play an important role in cell entry of hPIV3 (Bose and Banerjee 2002). On the other hand, sulfatide, which binds to hPIV3, inhibits hPIV3 infection and multinucleated syncytial giant cell formation of infected cells through suppression of viral fusion activity (Takahashi et al. 2012). MuV is a causative pathogen of childhood disease manifested by swelling of parotid glands and salivary glands, sometimes accompanied by complications such as aseptic meningitis, meningoencephalitis, and orchitis. MuV also has an HN spike protein, which was shown to be sensitive to the sialidase inhibitor 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Waxham and Wolinsky 1986). However, binding of MuV with sialoglycoconjugates remains unknown.

Sendai virus (SeV) is a highly transmissible animal respiratory virus in mice, hamsters, guinea pigs, and rats. SeV is a member of the genus *Respirovirus* possessing HN. Gangliosides and glycophorin were investigated as host cell receptors for SeV (Markwell et al. 1981; Hansson et al. 1984; Suzuki et al. 1985; Wybenga et al. 1996). SeV recognizes ganglio-series gangliosides (GD1a, GT1b, and GQ1b) containing the sequence NeuAcα2,3Galβ1,3GalNAc as viral receptors (Markwell et al. 1981). SeV shows preferential binding to neolacto-series gangliosides containing Neu5Acα2,3Galβ1,4GlcNAc, especially branched blood group I-type and/or linear i-type gangliosides (Suzuki et al. 1985). SeV can also bind to bovine erythrocyte glycoprotein GP-2 containing blood group I-type branched polylactosamine oligosaccharides with Neu5Gcα2,3Gal (Suzuki et al. 1983, 1984). Neu5Gc is expressed in animals other than humans (genetically lacking an active enzyme for synthesis of Neu5Gc in humans). SeV can utilize both species of sialic acid Neu5Ac and Neu5Gc to infect animals.

Newcastle disease virus (NDV) is a transmissible pathogen of bird disease and sometimes of mild conjunctivitis and influenza-like symptoms for human infection. NDV is a member of the genus *Avulavirus* possessing HN. NDV shows preferential binding to gangliosides such as sialylparagloboside (IV^{3}Neu5Acα-nLc$_4$Cer or IV^{3}Neu5Gcα-nLc$_4$Cer) containing Neu5Acα2,3Galβ1,4GlcNAc or Neu5Gcα2,3Galβ1,4GlcNAc and GM3 containing Neu5Acα2,3Gal or Neu5Gcα2,3Gal. NDV also binds to blood group I-type gangliosides, GD3, GM1a, and GD1b, although their binding is weaker than that of sialylparagloboside and GM3 (Suzuki et al. 1985). Gangliosides (GM1, GM2, GM3, GD1a, GD1b, and GT1b) may act as primary receptors, and *N*-linked glycoproteins may function as secondary receptors for NDV entry into cells (Ferreira et al. 2004). On the other hand, pretreatment of chicken East Lansing Line ELL-0 cells with both α2,3- and α2,6-specific sialidases and α2,3(N)- and α2,6(N)-sialyltransferase incubation

Table 5.6 Binding activities of paramyxoviruses to glycans

Virus	Glycan (references)
hPIV1	Neu5Acα2,3Gal (Suzuki et al. 2001; Tappert et al. 2011)
hPIV3	Neu5Acα2,3Gal (Suzuki et al. 2001)
	Neu5Acα2,6Gal (Suzuki et al. 2001)
	Neu5Gcα2,3Gal (Suzuki et al. 2001)
	HS (Bose and Banerjee 2002)
	Sulfatide (Takahashi et al. 2012)
MuV	Sialic acid? (Waxham and Wolinsky 1986)
SeV	Neu5Acα2,3Gal (Suzuki et al. 1985)
	Neu5Gcα2,3Gal (Suzuki et al. 1983, 1984)
NDV	Neu5Acα2,3Gal (Suzuki et al. 1985; Ferreira et al. 2004)
	Neu5Gcα2,3Gal (Suzuki et al. 1985)
RSV	Heparin (Bourgeois et al. 1998; Feldman et al. 1999; Hallak et al. 2000)
	HS (Bourgeois et al. 1998; Feldman et al. 1999; Hallak et al. 2000)
	Chondroitin sulfate B (Hallak et al. 2000)

showed that both α2,3- and α2,6-linked sialic acids containing glycoconjugates may be used for NDV infection (Sánchez-Felipe et al. 2012). Receptor binding properties of NDVs may depend on the viral strain.

Human respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract diseases in infants and young children. RSV is a member of the genus *Pneumovirus* possessing viral surface glycoproteins, attachment G and fusion F proteins, but not including sialidase unlike all paramyxoviruses described above. For virus infection, RSV requires interactions of the G protein and/or the F protein with heparin, HS, and chondroitin sulfate B on the cell surface (Bourgeois et al. 1998; Feldman et al. 1999; Hallak et al. 2000). The G protein and the F protein independently recognize heparin and HS (Feldman et al. 2000). These GAGs and their destroying enzymes also have inhibitory activity against RSV infection (Hallak et al. 2000) (Table 5.6).

5.2.7 Parvovirus

Parvoviruses are non-enveloped viruses that belong to the family *Parvoviridae*. Adeno-associated virus (AAV) is a nonpathogenic human parvovirus with diameters of 20–30 nm. Recombinant AAV has been used for gene transfer to various cells and several organs. AAV type 1 (AAV1), type 4 (AAV4), type 5 (AAV5), and type 6 (AAV6) recognize sialic acids and use them as receptors of infection, but AAV type 2 (AAV2) and type 9 (AAV9) do not. AAV4 specifically recognizes Neu5Acα2,3Gal of *O*-linked glycans, whereas AAV1 and AAV6 specifically recognize both Neu5Acα2,3Gal and Neu5Acα2,6Gal of *N*-linked glycans. Therefore, AAV4 infection is inhibited by mucin that possesses rich *O*-glycans, but AAV1 and

Table 5.7 Binding activities of parvoviruses to glycans

Virus	Glycan (references)
AAV1, AAV6	Neu5Acα2,6Gal (*N*-linked) (Wu et al. 2006)
	Neu5Acα2,3Gal (*N*-linked) (Wu et al. 2006)
AAV2	HS (Summerford and Samulski 1998)
AAV4	Neu5Acα2,3Gal (*O*-linked) (Kaludov et al. 2001)
AAV5	Neu5Acα2,3Gal (Neu5Acα2,6Gal?) (*N*-linked) (Kaludov et al. 2001; Walters et al. 2001)
AAV9	Terminal Gal (*N*-linked) (Shen et al. 2011)
BPV	Neu5Acα2,3Gal (*N*- and *O*-linked) (Johnson et al. 2004)
MVM	Neu5Acα2,3Galβ1,4GlcNAc (Nam et al. 2006)
	Neu5Acα2,8Neu5Ac linkages (Nam et al. 2006)
CPV	Neu5Gc (Löfling et al. 2013)
FPV	Neu5Gc (Löfling et al. 2013)

AAV6 infections are not. AAV5 binds to Neu5Acα2,3Gal of *N*-glycans. Binding of AAV5 to Neu5Acα2,6Gal of *N*-glycans remains unknown. AAV1 efficiently binds to *N*-linked sialylated glycans possessing lactosamine (Galβ1,4GlcNAc) (Walters et al. 2001; Kaludov et al. 2001; Wu et al. 2006). AAV2 infection is strongly or moderately inhibited by heparin or chondroitin sulfate B, respectively. HS mediates AAV2 attachment to the cellular surface and infection (Summerford and Samulski 1998). AAV9 uses the terminal Gal residue of *N*-linked glycans as a receptor (Shen et al. 2011).

Animal parvoviruses sometimes cause fetal diseases for hosts such as dogs and cats. Canine, feline, bovine, and mouse parvoviruses also bind to sialic acids. Bovine parvovirus (BPV) binds to Neu5Acα2,3Gal of both *N*- and *O*-linked glycans for attachment to the cellular surface (Johnson et al. 2004). BPV can strongly bind to glycophorin A through the Neu5Acα2,3Gal moiety of *O*-linked glycans (Blackburn et al. 2005). Parvovirus minute virus of mice (MVM) shows specific binding to terminal moieties, Neu5Acα2,3Galβ1,4GlcNAc such as sialyl Lewis X and Neu5Acα2,8Neu5Ac linkages such as gangliosides GD2, GD3, and GT3 (Nam et al. 2006). Canine parvovirus (CPV) has hemagglutination activity, indicating virus binding to sialic acid (Tresnan et al. 1995). CPV and feline parvovirus (FPV) recognize Neu5Gc but not Neu5Ac. However, Neu5Gc on the cellular surface is unlikely to be a receptor for CPV and FPV infections because overexpression of Neu5Gc has no effect on virus infectivities of some cell lines (Löfling et al. 2013) (Table 5.7).

5.2.8 Polyomavirus

JC virus (JCV) and BK virus (BKV) are non-enveloped viruses with diameters of 40–45 nm that are classified within the family *Polyomaviridae*, closely related to simian virus 40 (SV40) and murine polyomavirus (MPV). Initial JCV infection is

Table 5.8 Binding activities of polyomaviruses to glycans

Virus	Glycan (references)
BKV	Neu5Acα2,3Gal (*N*-linked) (Dugan et al. 2005, 2007)
JCV	Neu5Acα2,6Gal (strong binding, *N*-linked) (Komagome et al. 2002; Liu et al. 1998)
	Neu5Acα2,3Gal (gangliosides) (Komagome et al. 2002)
	LSTc (the strongest binding) (Neu et al. 2010)
MPV	Neu5Acα2,3Gal? (GD1a and GT1b) (Tsai et al. 2003)
SV40	Neu5Acα2,3Gal? (GM1) (Tsai et al. 2003)

thought to occur in childhood and not to cause symptomatic illness but to be a risk factor for progressive multifocal leukoencephalopathy. JCV shows stronger binding to Neu5Acα2,6Gal linkage (of *N*-linked glycans), in addition to binding to Neu5Acα2,3Gal linkage such as gangliosides GM3, GD2, GD3, GD1b, GT1b, and GQ1b, through the major viral capsid protein VP1 (Gee et al. 2004). A linear sialylated pentasaccharide with the sequence LSTc (Neu5Acα2,6Galβ1,4GlcNAcβ 1,3Galβ1,4Glc) binds with JCV and inhibits JCV infection of target cells, strongly suggesting that LSTc is a functional receptor of JCV infection (Neu et al. 2010). JCV binds to an asialoglycolipid, lactosylceramide, but not to GalCer. Therefore, JCV can also bind to GM3 and GD3 after sialidase treatment (i.e., lactosylceramide). JCV weakly binds to GD1a but does not bind to GM1a or GM2 (Liu et al. 1998; Komagome et al. 2002). These studies suggest that both Neu5Acα2,3Gal and Neu5Acα2,6Gal of *N*-linked glycans are also used for cellular surface binding and infection of JCV (Dugan et al. 2008).

BKV infection rarely causes symptom illness in humans but can lead to polyomavirus-associated nephropathy in renal transplant recipients undergoing immunosuppressive therapy. BKV binds to a cellular receptor, Neu5Acα2,3Gal of *N*-linked glycans, via VP1 protein (Dugan et al. 2005, 2007). For nonhuman polyomaviruses, VP1s specifically bind to GD1a and GT1b for MPV and to GM1 for SV40, suggesting that Neu5Acα2,3Gal is a key determinant in the interactions. Gangliosides appear to transport polyoma and SV40 from the cellular surface to the endoplasmic reticulum, and then the viruses enter the nucleus to initiate infection (Tsai et al. 2003) (Table 5.8).

5.2.9 Retrovirus

Retroviruses are single-stranded positive-sense RNA and round enveloped viruses with a diameter of 100 nm that are classified within the family *Retroviridae*. Human immunodeficiency virus (HIV), which is a member of the genus *Lentivirus*, is a pathogen causing long-term and chronic disease that gradually progresses to acquired immunodeficiency syndrome. The viral surface glycoprotein gp120 of HIV binds to some glycolipids containing GalCer (Delézay et al. 1997; Hammache et al. 1998; Harouse et al. 1991), Gb_3Cer (Galα1,4Galβ1,4Glc1,1'Cer) (Mahfoud et al. 2002;

Table 5.9 Binding activities of retroviruses to glycans

Virus	Glycan (references)
HIV	GalCer (Delézay et al. 1997; Hammache et al. 1998; Harouse et al. 1991)
	Gb_3Cer (Lund et al. 2006; Mahfoud et al. 2002)
	GM3 (Hammache et al. 1998)
	Sulfatide (Delézay et al. 1996; van den Berg et al. 1992)
	Heparin (HS?) (Crublet et al. 2008)

Lund et al. 2006), GM3 (Hammache et al. 1998), and sulfatide (Delézay et al. 1996; van den Berg et al. 1992), in addition to heparin (and HS) (Crublet et al. 2008). CD4 is a main primary receptor of HIV for viral attachment to the cellular surface. After interaction of the gp120 with CD4, these glycolipids and HS are thought to interact with gp120 and to act as coreceptors for the fusion process between the cellular membrane and viral membrane of HIV for entry into cells. However, sulfatide may not be a coreceptor for HIV because the fusion process is initiated by mediating binding to GalCer but not to sulfatide (Delézay et al. 1997; Harouse et al. 1991) (Table 5.9).

5.2.10 Reovirus

Reoviruses (ReV) are double-stranded RNA viruses and non-enveloped regular icosahedra non-enveloped viruses with a diameter of 60–80 nm that are classified within the family *Reoviridae*. ReVs can infect the gastrointestinal and respiratory tracts of various mammals. For humans, most children are infected by the age of 5 years. The viral attachment σ1 protein of ReVs recognizes sialic acids of glycoconjugates on the cellular surface. ReV type 1 (ReV1) binds to Neu5Acα2,3Gal and binds strongly to ganglioside GM2, which contains sialic acid linked to the inner galactose residue. The interaction of ReV1 with GM2 is involved in viral infection (Helander et al. 2003; Reiss et al. 2012). ReV type 3 (ReV3) binds to Neu5Acα2,3Gal, Neu5Acα2,6Gal, and Neu5Acα2,8Neu5Ac linkages, in addition to $Neu4,5Ac_2$ (Gentsch and Pacitti 1987; Reiter et al. 2011). Interactions of ReV with sialic acids are believed to act for cellular surface attachment of ReV by rapid but low-affinity adhesion, followed by transition to a higher affinity interaction with an unidentified receptor for cell entry. Therefore, sialic acid is considered to be a coreceptor rather than a main receptor for ReV infection (Barton et al. 2001). ReV1 spreads to the central nervous system via a hematogenous route and infects ependymal cells in the brain, leading to nonlethal hydrocephalus. In contrast, ReV3 spreads to the central nervous system via neural and hematogenous routes and infects neurons, causing lethal encephalitis. These serotype-dependent differences in tropisms and pathogenesis are thought to be involved in the distinct binding with glycochain moieties.

Rotavirus (RoV) is a member of the genus *Rotavirus* and the most important pathogen of severe gastroenteritis in children. There are two groups of RoV in the hemagglutination activity of erythrocytes and sialidase sensitivity of viral infection: sialic acid-dependent and sialic acid-independent RoVs (Isa et al. 2006). A few animal RoVs are sialic acid dependent on the interactions of the viral surface spike VP8* protein, which is formed from the viral VP4 protein by proteolytic cleavage, with sialic acids, whereas human RoVs and the majority of animal RoVs are sialic acid independent. For cell entry, sialic acid-dependent RoVs require gangliosides containing Neu5Ac and/or Neu5Gc, such as GM1(a), GM2, GM3, GD1a, GD1b, GD3, and GT1b, which can inhibit RoV infection (Guo et al. 1999; Martínez et al. 2013; Rolsma et al. 1998; Superti and Donelli 1991; Yu et al. 2012). In addition, some sialic acid-independent RoVs, such as Wa and KUN strains, bind to GM1(a) containing internal Neu5Ac, which can also inhibit infections of these viruses (Guo et al. 1999; Haselhorst et al. 2009; Martínez et al. 2013). These studies suggest that sialic acid-dependent RoVs bind to gangliosides containing terminal Neu5Ac, whereas sialic acid-independent RoVs bind to gangliosides containing internal Neu5Ac. The VP8* protein of human sialic acid-independent RoVs also recognizes histo-blood group antigens, trisaccharide GalNAcα1,3(Fucα1,2)Gal of A antigen for HAL1166 P[11] viral genotype strain (Hu et al. 2012), H1 antigen for P[4] and P[8] viral genotypes, and Le^b antigen for the P[6] viral genotype (Huang et al. 2012). The interactions of RoVs with sialo- or asialo-receptors are dependent on viral strains and genotypes. Nonstructural glycoprotein 4 (NSP4) encoded by RoVs is believed to function as an enterotoxin. NSP4 is secreted as an oligomeric lipoprotein from infected cells and binds to sulfated GAGs (Didsbury et al. 2011). Thus, glycans appear to be involved in the infection and pathogenesis of RoVs and NSP4 through cellular surface attachment (Table 5.10).

Table 5.10 Binding activities of reoviruses to glycans

Virus	Glycan (references)
ReV1	Neu5Acα2,3Gal (Helander et al. 2003)
	GM2 (Reiss et al. 2012)
ReV3	Neu5Acα2,3Gal (Reiter et al. 2011)
	Neu5Acα2,6Gal (Reiter et al. 2011)
	Neu5Acα2,8Neu5Ac (Reiter et al. 2011)
	$Neu4,5Ac_2$ (Gentsch and Pacitti 1987)
RoV	Gangliosides (Neu5Ac and/or Neu5Gc) (Guo et al. 1999; Martínez et al. 2013; Rolsma et al. 1998; Superti and Donelli 1991; Yu et al. 2012)
	Histo-blood group A1 antigen (Hu et al. 2012)
	Histo-blood group H1 antigen (Huang et al. 2012)
	Histo-blood group Le^b antigen (Huang et al. 2012)
RoV NSP4	Sulfated GAGs (Didsbury et al. 2011)

5.3 Conclusion

A variety of viruses recognize glycans such as sialoglycoconjugates, GAGs, and histo-blood group antigens. These glycans are often thought to serve as receptors and/or coreceptors for cellular surface attachment and cell entry of viruses and viral toxins. The interactions of viruses with glycans determine virus-dependent tissue tropism, host, and pathogenicity. In rare cases, the interaction of IAV HA with sulfatide functions as a start switch of progeny virus particle formation, not as a receptor for IAV infection. It may be important to evaluate the interactions of viruses with glycans in terms of insights different from a receptor function. Further studies combining virology and glycobiology should lead to the elucidation and discovery of novel infection and replication mechanisms of a variety of viruses.

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Chapter 6
Discovery and Applications of a Novel Human Pluripotent Stem Cell-Specific Lectin Probe rBC2LCN

Hiroaki Tateno and Jun Hirabayashi

Abstract The cellular glycome of human pluripotent stem cells (hPSCs) such as embryonic stem cells (hESCs) and human-induced pluripotent stem cells (hiPSCs) was exhaustively analyzed using the cutting-edge glycan profiling technology called high-density lectin microarray. Despite the different glycan profiles of the original somatic cells for each tissue, it was found that hiPSCs derived from various origins showed almost the same profile, indicating that the introduction of reprogramming genes caused uniform convergence into glycan structures analogous to those of hESCs. Furthermore, three characteristic features of glycan epitopes expressed in hPSCs were identified: α2-6Sia, α1-2Fuc, and type-1 LacNAc. In addition, a recombinant lectin probe rBC2LCN highly specific to hPSCs was discovered. rBC2LCN is a practical hPSC-specific probe, which enables the live staining of hPSCs just supplemented into cell culture media without visible toxicity. Recently, a noninvasive and quantitative assay of hPSCs with tumorigenic potential using rBC2LCN was also successfully developed. Now rBC2LCN is commercialized as a novel type of detection reagent of hPSCs, which is suitable for industrial application in regenerative medicine. Here, we describe our recent findings about the cellular glycome of hPSCs and the discovery and application of rBC2LCN to regenerative medicine.

Keywords Induced pluripotent stem cells • Embryonic stem cells • Glycan • Lectin • rBC2LCN • Regenerative medicine

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6.1 Introduction

Human pluripotent stem cells (hPSCs) with the ability to self-renew and differentiate into any cell types have attracted attention because they are not only applied to drug discovery screening (Egawa et al. 2012; Yahata et al. 2011) and elucidation of disease mechanism (Brennand and Gage 2011; Imaizumi and Okano 2014; Yoshida and Yamanaka 2010) but also cell sources for regenerative medicine (Kamao et al. 2014; Okano et al. 2013). Two types of human pluripotent stem cells have been generated: human embryonic stem cells (hESCs) derived from blastocysts (Thomson et al. 1998) and induced pluripotent stem cells (hiPSCs) (Takahashi et al. 2007). hiPSCs were first generated in 2007 by introducing a combination of four transcription factors, POU domain, class 5, transcription factor 1 (OCT3/4), sex-determining region Y-box 2 (SOX2), Kruppel-like factor 4 (KLF4), and myelocytomatosis oncogene (c-MYC) into adult human fibroblasts (Takahashi et al. 2007). hiPSCs originating in Japan have great advantage that they have a low ethical road back because of the no use of fertilized embryos and can be established from autologous tissues (Yamanaka 2009). Thus hiPSCs are receiving high expectations from the field of regenerative medicine. In 2010, the world's first clinical trial using hESCs was conducted by a biotechnology company Geron to treat spinal cord injury, although this trial was halted on November 14, 2011, by an economical reason (Strauss 2010). In the USA, there are currently two active clinical trials using hESCs, both being conducted by a biotechnology company called ACT, to treat patients with Stargardt's macular dystrophy and age-related macular degeneration (Schwartz et al. 2012). In Foundation for Biomedical Research and Innovation, Japan, the world's first clinical trial using hiPSCs was recently conducted for age-related macular degeneration patients. Keio University is also planning to start clinical studies for spinal cord injury patients (Okano et al. 2013). Although the clinical application of hPSCs is started as just described above, a system to supply cells by securing quality and safety is not sufficiently developed. One of the most important safety concerns to be addressed to apply hPSCs for regenerative medicine is that residual hPSCs in transplanting cells could form tumors in patients (Andrews 2011; Ben-David and Benvenisty 2011; Goldring et al. 2011; Itskovitz-Eldor 2011; Okano et al. 2013). The residual tumorigenic hPSCs thus have to be detected and removed from transplanting cells prior to introduction into patients, since even 245 undifferentiated hESCs spiked into 10^6 fibroblasts could produce a teratoma (Hentze et al. 2009).

A practical strategy for overcoming the tumorigenic risk of hPSCs is based on the use of cellular biomarkers, which are useful for the detection and targeted elimination of tumorigenic hPSCs (Tang et al. 2011). Cell surface glycans are considered to be ideal targets for cell surface markers, which can be used to detect and isolate each cell type by the following reasons: (1) Glycans are located at the outermost cell surface. (2) Glycan structures are complex and diverse within a cell type or even a single glycosylation site. (3) Glycans are dynamic, which change in

response to intrinsic and extrinsic signals (Gagneux and Varki 1999; Lanctot et al. 2007; Varki 1993). Therefore, cell surface glycans are often referred to as "cell signature," which represent cell properties (Tateno et al. 2010). In fact, all of the cell surface markers, generally used to evaluate hPSCs, are glycans such as stage-specific embryonic antigens (SSEA-3, SSEA-4) and tumor-rejection antigens (Tra-1-60 and Tra-1-81). SSEA-3 and SSEA-4 are carried by globo-series glycolipids, consisting of Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-Cer and its α2-3sialylated form (Siaα2-3Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-Cer) (Kannagi et al. 1983). On the other hand, TRA-1-60 and TRA-1-81 markers have been reported to be expressed on podocalyxin, a heavily glycosylated membrane protein (Schopperle and DeWolf 2007). Their carbohydrate epitopes were first reported to be keratan sulfate (Badcock et al. 1999), but were recently identified by glycan microarray analysis to be type-1 *N*-acetyl-lactosamine linked to type-2 *N*-acetyl-lactosamine (Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAc) (Natunen et al. 2011). More recently, SSEA-5 was proposed as a novel marker for hPSCs that consists of H type-1 glycan (Fucα1-2Galβ1-3GlcNAc) (Tang et al. 2011). However, only little is known why these structures could function as biomarkers of hPSCs, since the overall feature of cell surface glycans of hPSCs has not been well understood.

6.2 Recombinant Shift of Lectins and Development of High-Density Lectin Microarray

To analyze the cellular glycome of hPSCs, we utilized a lectin microarray technology developed by ourselves (Hirabayashi et al. 2013; Kuno et al. 2005). Before challenging this subject, the quality of lectin microarray was improved. In the previous evanescent-type lectin microarray, 43 lectins with diverse glycan-binding specificities prepared from natural sources were selected and used due to various reasons (history, commercial availability, stability, and cost-effectiveness) (Hirabayashi et al. 2013; Kuno et al. 2005). However, lectins from natural sources have many issues with respect to glycan-detection reagents, e.g., in terms of purity, lot-to-lot difference, and instable supply (Tateno et al. 2011). To solve these subjects, we first challenged "recombinant shift" of lectins from naturally prepared ones (Hirabayashi et al. 2013; Tateno et al. 2011). For this purpose, lectins were selected from diverse lectin families intending to cover a wider range of glycan-binding specificities (Tateno et al. 2011). For production, the *E. coli* expression system was chosen to avoid glycosylation of the produced lectins, which might cause nonspecific binding to lectin-like molecules in the objective samples (Tateno et al. 2011). By incorporating 38 recombinant lectins, a high-density lectin microarray containing an almost doubled number of lectins (96 lectins) was developed (Tateno et al. 2011) (Fig. 6.1).

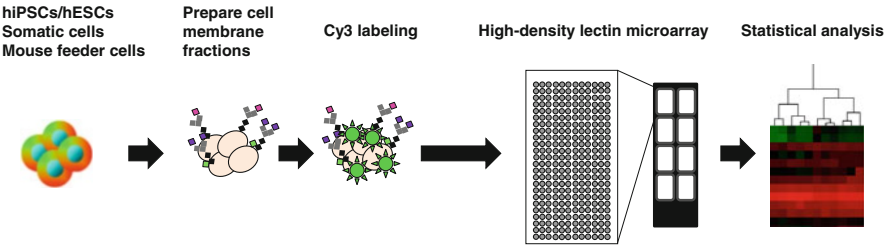

Fig. 6.1 Glycan profiling using high-density lectin microarray

6.3 Comprehensive Glycan Profiling Using High-Density Lectin Microarray

Using the developed high-density lectin microarray, we performed comprehensive glycome analysis of 114 hiPSCs, 9 hESCs, and 11 somatic cells, all of which were of human origins (Tateno et al. 2011). hiPSCs were generated from four different cells: human fetal lung fibroblast cell line (MRC5), amniotic mesoderm (AM), uterine endometrium (UtE), and placental artery endothelial (PL). We also analyzed hiPSCs generated from human dermal fibroblasts with four (201B7) (Takahashi et al. 2007) and three (253G1) transcription factors developed by Dr. Yamanaka's group (Nakagawa et al. 2008). Cell membrane fractions were prepared, labeled with Cy3-*N*-hydroxysuccinimide ester, and 50 ng of protein was analyzed by a high-density lectin microarray as previously described (Tateno et al. 2010) (Fig. 6.1). The obtained data were first analyzed by unsupervised hierarchical clustering. Differentiated somatic cells and undifferentiated hPSCs were separated into two large clusters, indicating that cell surface glycans are clearly different between them (Tateno et al. 2011). All of the hiPSCs of different origins gave glycan profiles similar to hESCs. This indicates that the introduction of reprogramming factors caused uniform convergence into glycan profiles analogous to hESCs. The close analysis of high-density lectin microarray data also revealed drastic change in glycan profiles upon the induction of pluripotency, which can be regarded as "glycome shift" (Tateno et al. 2011). That is, a dozen of lectins were statistically selected, showing significantly different binding signals between differentiated somatic cells and undifferentiated hPSCs, which represent the glycome shift upon the induction of pluripotency. Among them, based on the lectin signals, the expression levels of α2-6Sia, α1-2Fuc, and type-1 LacNAc were presumed to be markedly increased in undifferentiated hPSCs (Fig. 6.2). These results could also be supported by the differential expression of the corresponding glycosyltransferase genes: expression levels of glycosyltransferase genes, *ST6Gal1*, *FUT1/2*, and *B3GalT5*, were apparently elevated in hPSCs compared with somatic cells (Tateno et al. 2011).

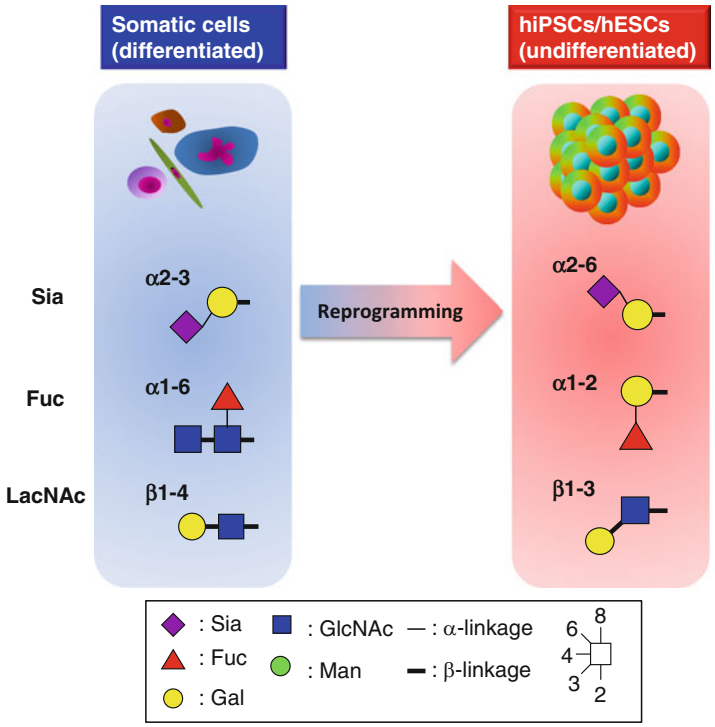

Fig. 6.2 Glycome shift upon induction of pluripotency

6.4 Quantitative Glycan Analysis Using Mass Spectrometry (MS)/High-Performance Liquid Chromatography (HPLC) Mapping

To confirm the results obtained by high-density lectin microarray analysis, quantitative glycan analysis of 201B7 hiPSCs was performed compared to human dermal fibroblasts using glycosidase-assisted MS/HPLC mapping (Hasehira et al. 2012). Forty-seven glycans (*N*-glycans, 37; *O*-glycans, 10) for 201B7 hiPSCs and 27 glycans (*N*-glycans, 20, *O*-glycans, 7) for human dermal fibroblasts were determined. Differences in glycan structures between 201B7 hiPSCs and human dermal fibroblasts were summarized below:

1. The total ratio of high-mannose-type *N*-glycans in 201B7 cells (86.1 % molar fraction) was higher than that in fibroblasts (69.9 %).
2. A significant amount of hybrid-type *N*-glycans was identified in 201B7 cells (1.9 %), but not in fibroblasts (0 %).
3. All of the identified Sia-linkages on *N*-glycans were found to be the α2-6 type in 201B7 cells, while that was in contrast with the α2-3 type in fibroblasts.

4. α1-2Fuc and type-1 LacNAc were specifically identified in 201B7 cells on both *N*-glycans (α1-2Fuc, 1.7 %; type-1 LacNAc, 0.6 %) and *O*-glycans (α1-2Fuc, 8 %; type-1 LacNAc, 9.6 %), but were not detected at all in fibroblasts.

These results fully confirmed the above results obtained by lectin microarray; i.e., the expression of α2-6Sia, α1-2Fuc, and type-1 LacNAc increased upon the induction of pluripotency (Fig. 6.2).

6.5 Discovery of a Novel hPSC-Specific Probe, rBC2LCN

Among the 96 lectins immobilized on the high-density lectin microarray, a lectin, previously known as rBC2LCN (*N*-terminal domain of BC2-L lectin derived from *Burkholderia cenocepacia*), was found to bind all of the undifferentiated cells tested, but not at all to any of the differentiated cells (Fig. 6.3) (Tateno et al. 2011). Moreover, two advanced methods, comprehensive glycan microarray (Tateno et al. 2008) and analytical frontal affinity chromatography (FAC) (Tateno et al. 2007), revealed that rBC2LCN recognizes the unique glycan epitopes, Fucα1-2Galβ1-3GlcNAc/GalNAc-containing glycans such as H type-1 (Fucα1-2Galβ1-3GlcNAc),Htype-3(Fucα1-2Galβ1-3GalNAc),Lewisb(Fucα1-2Galβ1-3(Fucα1-4)GlcNAc), and Globo-H (Fucα1-2Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glc). Notably, all these structures comprise two of the above characteristics of pluripotency, i.e., α1-2Fuc and type-1 LacNAc (Tateno et al. 2011). In fact, rBC2LCN ligands are well related to the previously reported hPSC markers such as SSEA-3/SSEA-4/SSEA-5 and Tra-1-60/81, all of which are synthesized by the key enzymes, *B3GalT5* and *FUT1/2*.

Among the 47 types of glycans derived from hiPSCs described above (Hasehira et al. 2012), one *O*-glycan containing an H type-3 was identified, which was a core2-type *O*-glycan, Fucα1-2Galβ1-3(Galβ1-3GlcNAcβ1-6)GalNAc (Fig. 6.4) (Tateno et al. 2013). In contrast, no glycan constituting H type-1 structure

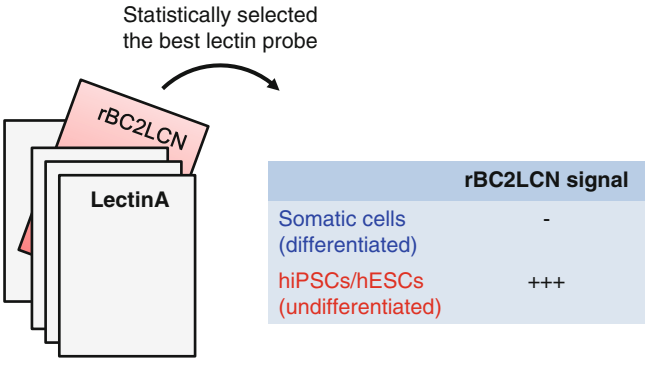

Fig. 6.3 rBC2LCN is a hPSC-specific probe

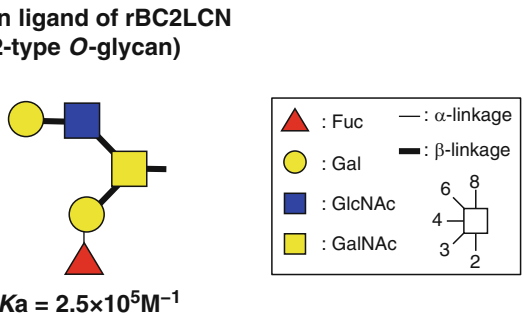

Fig. 6.4 Glycan ligands of rBC2LCN

(Fucα1-2Galβ1-3GlcNAc) was detected in glycoproteins derived from 201B7 hiPSCs. The affinity constant of rBC2LCN to the core2-type *O*-glycan was determined to be 2.5×10^5 M^{-1} by FAC (Fig. 6.4). These results suggest that the core2-type *O*-glycan is an actual target glycan to rBC2LCN in hPSCs (Tateno et al. 2013).

Then, what is a glycoprotein ligand of rBC2LCN? For this search, cell lysates of differentiated somatic cells and undifferentiated hPSCs were run on SDS-PAGE and blotted with peroxidase-labeled rBC2LCN (Tateno et al. 2013). Evident high-molecular-weight protein bands ranging from 150 to >240 kDa were specifically detected for undifferentiated hPSCs, but not for differentiated somatic cells (Tateno et al. 2013). Immunoprecipitation and western blotting analyses revealed that these high-molecular-weight bands found to correspond to heavily glycosylated podocalyxin (Tateno et al. 2013). Human podocalyxin is a type-1 transmembrane protein consisting of 528 amino acids (Kershaw et al. 1997) (Fig. 6.5). The extracellular domain of podocalyxin has a mucin domain, five potential *N*-linked glycosylation sites, and three putative glycosaminoglycan sites (Kershaw et al. 1997) (Fig. 6.5). The reactivity of rBC2LCN to podocalyxin was greatly reduced by the alkaline hydrolysis to remove *O*-glycans by β-elimination, indicating that the carbohydrate antigens of rBC2LCN are expressed on *O*-glycans of podocalyxin (Tateno et al. 2013). Taken together, it is most likely that the cell surface ligands of rBC2LCN are podocalyxin-expressing glycan *a* described above (H3+podocalyxin), although the direct evidence whether glycan *a* is actually displayed on podocalyxin remains to be obtained (Fig. 6.5).

6.6 Live Cell Staining of hPSCs Using rBC2LCN

To investigate whether rBC2LCN could be used as a probe for hiPSCs, various types of hPSCs were stained with fluorescence-labeled rBC2LCN (Onuma et al. 2013). hPSCs were fixed and stained with fluorescence-labeled rBC2LCN at a concentration of 1 μg/mL hiPSCs (253G1, 201B7), and hESCs (KhES-1, KhES-3, and H1) of various strains were strongly stained with fluorescence-labeled rBC2LCN (Onuma et al. 2013). rBC2LCN staining was observed on the cell membrane of

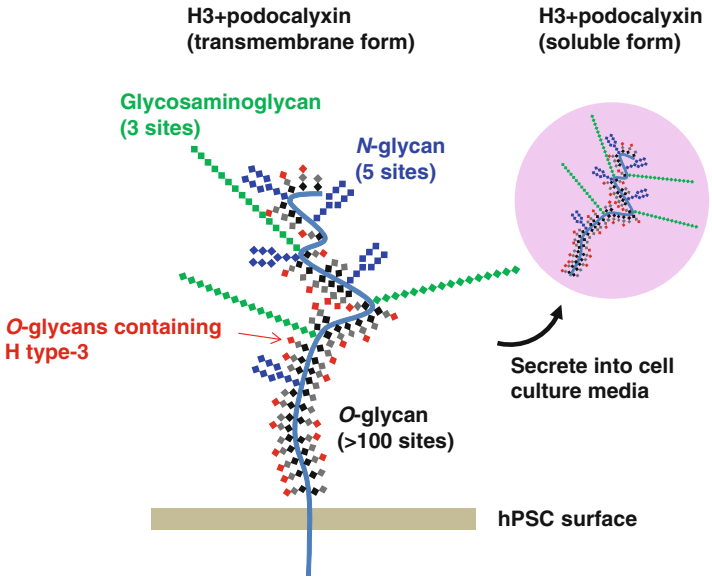

Fig. 6.5 A cell surface glycoprotein ligand of rBC2LCN, podocalyxin, is secreted into cell culture media

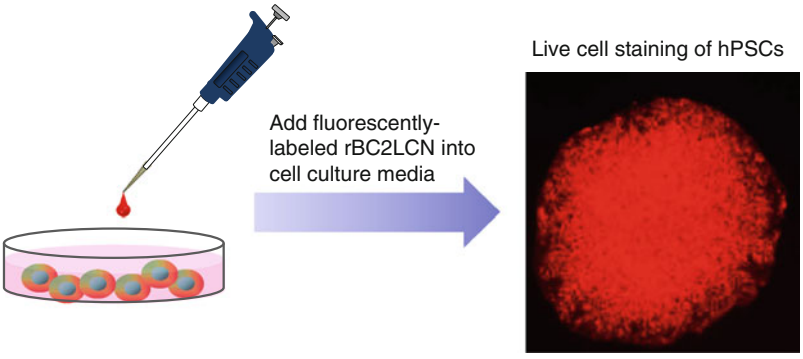

Fig. 6.6 Live cell staining of hPSCs using fluorescently labeled rBC2LCN

hiPSCs by confocal microscopy, which agrees well with the result that the glycoprotein ligand of rBC2LCN is the transmembrane protein, podocalyxin (Onuma et al. 2013) (Fig. 6.5). rBC2LCN was then applied to the live staining of hPSCs (Fig. 6.6). Cy3-labeled rBC2LCN was added into cell culture media at a final concentration of 0.1 μg/mL. After 2-h culture, a clear live cell image could be observed under a conventional fluorescence microscope (Onuma et al. 2013). No significant change of morphology and gene expression of hPSCs was observed in the presence of fluorescence-labeled rBC2LCN (Onuma et al. 2013). The sensitivity and

specificity of rBC2LCN were comparable to those of the established pluripotent marker antibodies, such as anti-TRA-1–60 and anti-SSEA-4 antibodies (Onuma et al. 2013). These results demonstrate the utility of rBC2LCN for monitoring the properties of hPSCs during culture.

6.7 Noninvasive and Quantitative Detection of Tumorigenic hPSCs Using Cell Culture Supernatants

The most obvious concern to apply hPSCs to regenerative medicine is that hPSCs could form tumors in patients. To minimize patient risk, hPSC-based cell products should be assessed for potential safety concerns prior to the introduction of the cells into a patient. However, all of the currently available methods, such as qRT-PCR and flow cytometry, require a significant number of invaluable transplanting tissues for quality assessments (Kuroda et al. 2012). To solve these subjects, a noninvasive technology to detect residual hPSCs contaminated in transplanting cells has been desired. As described above, H3+podocalyxin is a putative cell surface ligand of rBC2LCN (Tateno et al. 2013). We found that H3+podocalyxin is secreted into cell culture supernatants from various types of hPSCs, but not differentiated human somatic cells (Tateno et al. 2014) (Fig. 6.5). Based on this unexpected observation, we have developed a noninvasive and quantitative detection method of hPSCs using cell culture supernatants (Tateno et al. 2014) (Fig. 6.7). Usually, proteins are detected with antibodies, which recognize a particular peptide sequence. However, podocalyxin is a large, heavily *O*-glycosylated sialomucin with a molecular weight of >250 kDa. Such "hyperglycosylated" podocalyxin could not be sufficiently detected with any types of antibodies. We then challenged a novel system of

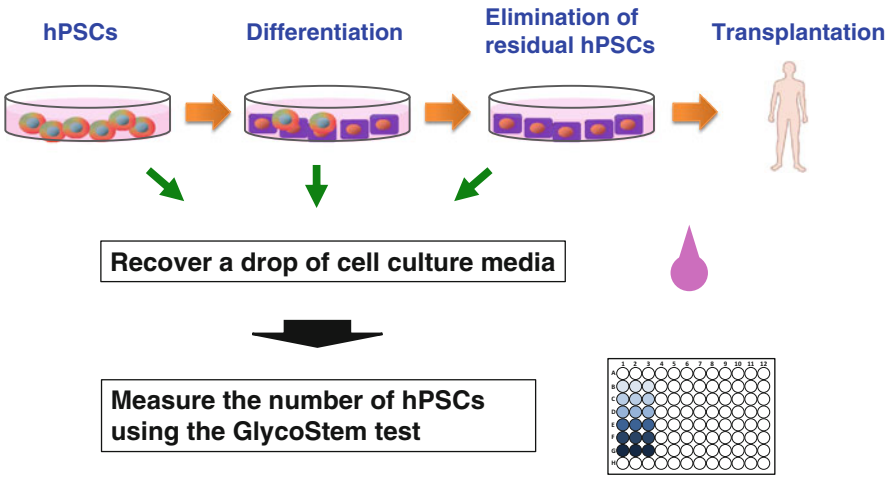

Fig. 6.7 Detection of residual hPSCs contaminated in transplanting cells using cell culture media

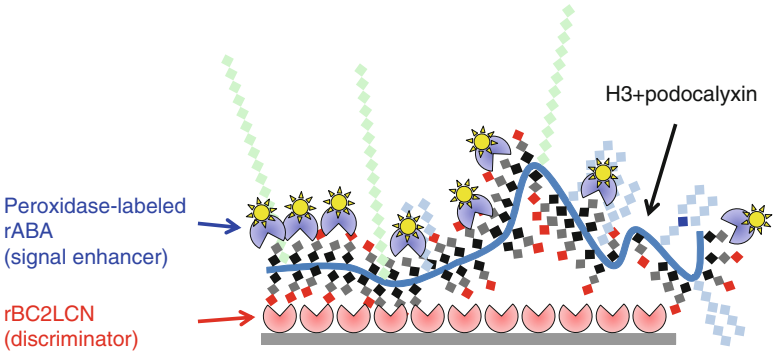

Fig. 6.8 Principle of the Glycostem test

"lectin-lectin" sandwich assay, termed Glycostem test, which utilizes two lectins by focusing on characteristic glycan structures displayed on podocalyxin (Tateno et al. 2014) (Fig. 6.8). The detailed methods are as follows:

1. Immobilize rBC2LCN recognizing soluble H3+podocalyxin on a reaction plate.
2. Incubate a drop (50 μL) of cell culture media for 1 h to capture soluble H3+podocalyxin onto the rBC2LCN-immobilized plate.
3. After washing, incubate peroxidase-labeled recombinant *Agaricus bisporus* lectin (rABA) with the bound H3+podocalyxin on the reaction plate.
4. Add substrate to develop peroxidase-labeled rABA to measure the color strength.
5. Estimate the cell number of hPSCs from the amount of H3+podocalyxin.

There are two important points for the developed system: *First*, rBC2LCN is used as a "discriminator" to capture H3+podocalyxin specifically secreted from hPSCs. *Second*, rABA, an *O*-glycan-binding lectin, is used as a "signal enhancer," which detects *O*-glycans, typically Galβ1-3GalNAc, heavily displayed on podocalyxin (Fig. 6.8). Using the two lectins, a high-selective and high-sensitive detection system could be developed. The developed system allows rapid diagnosis (<3 h) of a large number of samples in a high-throughput ELISA (enzyme-linked immunosorbent assay) manner. Since the rate of contamination of hPSCs in transplanting cells could be quantitatively measured, the developed system is expected as a novel method for safety assessments of hPSC-based cell therapy.

6.8 Practical Applications of Lectins into Industry

rBC2LCN is a new hPSC-specific probe suitable for industrial applications. It has a great advantage over an antibody, because it is small in size (16 kDa), and its produce in *Escherichia coli* (~0.1 g/L) is cost-effective. rBC2LCN is now commercialized as a novel type of hPSC-specific probe from Wako Pure Chemical Industries.

Other 37 recombinant lectins and the Glycostem test described above are also planned to be commercialized from the same company. Although no description has been made in this review, a novel method to efficiently and conveniently remove tumorigenic hPSCs using rBC2LCN has also been successfully developed. Therefore, the practical applications of glycans/lectins into regenerative medicine would be more and more accelerated and fruitful in the near future.

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Chapter 7
Glycan Structure and Neural Plasticity

Tadahisa Mikami and Hiroshi Kitagawa

Abstract Extracellular factors that surround cell surfaces play essential roles in a wide spectrum of neurobiological functions, including neuronal development and neuronal plasticity. Glycans are ubiquitous throughout the extracellular and pericellular spaces, and they may function as microenvironmental cues during neuronal development and remodeling. Recent advances in the field of glyco-neuroscience clearly indicate that distinct glycans, especially sulfated glycosaminoglycan (GAG) chains, are functionally relevant to neuronal plasticity. This chapter reviews current research findings on neuroregulatory glycans and focuses primarily on structural divergence among sulfated GAG chains and their unique and/or partially overlapping contributions to neuronal plasticity during development and during regeneration after central nervous system injury.

Keywords Central nervous system injury • Chondroitin sulfate • Extracellular matrix • Glycosaminoglycan • Heparan sulfate • Hyaluronan • Keratan sulfate • Perineuronal net • Plasticity • Proteoglycan • Sulfotransferase

7.1 Introduction

Neuronal plasticity is the ability of the nervous system to react and change adaptively in response to intrinsic and/or extrinsic inputs. These adaptive changes are typically accompanied by reorganization of neuronal circuits. Early in mammalian development, neuronal circuit formation is highly plastic and therefore can be greatly influenced by the external environment. Such experience-dependent neuronal plasticity is most evident during developmental windows, often called critical periods, after which the established neuronal connections become less plastic (Hensch 2005; Jiang et al. 2005; Tropea et al. 2009). Long-standing evidence indicates that a tightly controlled balance between excitatory and inhibitory circuits is crucial for determining the exquisite timing of critical periods (Fagiolini et al. 2004; Feldman 2000;

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Huang et al. 1999; Sugiyama et al. 2008; Yazaki-Sugiyama et al. 2009). In fact, onset and closure of critical periods can be accelerated by enhanced inhibitory synaptic transmissions (Fagiolini et al. 2004), and conversely reductions in inhibitory transmissions can reactivate experience-dependent plasticity even after the termination of the critical period (Harauzov et al. 2010).

The extracellular matrix (ECM) is essential for proper regulation of neuronal plasticity. Notably, the ECM in the central nervous system (CNS) has a unique composition that is distinct from those in nonneuronal tissues; specifically, the CNS ECM has relatively small amounts of fibrous proteins (e.g., collagens) and high levels of proteoglycans (PGs) (Novak and Kaye 2000). PGs are a class of heavily glycosylated macromolecules that bear sulfated glycosaminoglycan (GAG) chains. Some GAG chains are principal components of perineuronal nets (PNNs), specialized ECMs in CNS, whose emergence during development is tightly linked with the limitation of critical period plasticity (Pizzorusso et al. 2002; Yamaguchi 2000; Yazaki-Sugiyama et al. 2009). Moreover, sulfated glycans play critical roles in synaptic and anatomical plasticity by controlling synaptic properties and rewiring of neuronal network after CNS injury, respectively (Dityatev and Schachner 2003; Galtrey and Fawcett 2007). Therefore, this chapter focuses on the structural characteristics of GAG chains and their functional importance in various aspects of neuronal plasticity.

7.2 Structure and Biosynthesis of GAG Chains

GAG chains are long linear polysaccharides that consist of repetitive disaccharide units; each unit comprises an amino sugar, *N*-acetylgalactosamine (GalNAc) or *N*-acetylglucosamine (GlcNAc), and a galactose (Gal) or a hexuronic acid, either glucuronic acid (GlcA) or iduronic acid (IdoA). Each GAG chain can be classified based on constituent disaccharide building blocks into one of four classes: chondroitin sulfate (CS)/dermatan sulfate (DS), heparin (Hep)/heparan sulfate (HS), keratan sulfate (KS), or hyaluronan (HA) (Fig. 7.1). Three of these classes (CS/DS, Hep/HS, and KS) can also be categorized as sulfated GAG chains that are constituents of proteoglycans (PGs); the respective sulfated polysaccharide chains (CS, HS, or KS) are covalently linked to a panel of core proteins. In contrast, HA is the sole non-sulfated polymer, and HA is never attached to a protein core.

7.2.1 CS and HS Chains

CS and HS chains consist of repetitive disaccharide units [(–4GlcAβ1-3GalNAcβ1–)$_n$] or [(–4GlcAβ1-4GlcNAcα1–)$_n$], respectively. Notably, both CS and HS are synthesized onto a GAG-protein linkage region (GlcAβ1–3Galβ1–3Galβ1–4Xylβ1–*O*-Ser) that is covalently linked to specific serine (Ser) residues embedded in core proteins. The

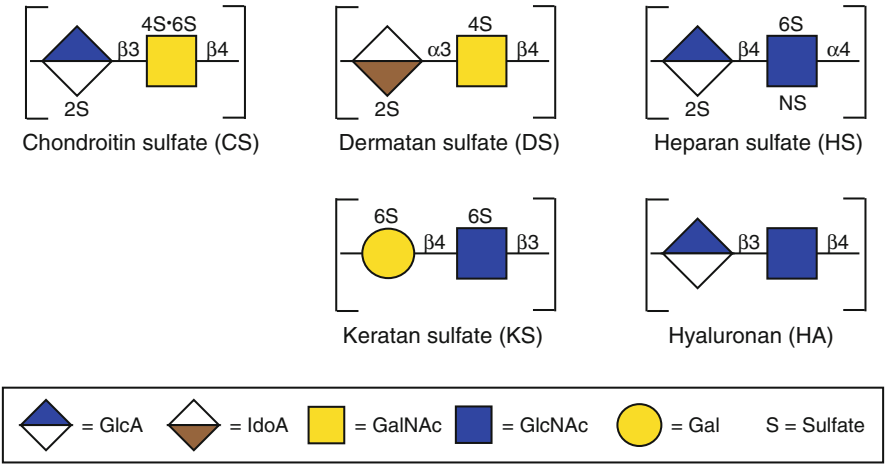

Fig. 7.1 Schematic structures of the repeating disaccharide units found in CS, DS, HS, and HA GAG chains. CS chains comprise GlcA and GalNAc residues. DS is a stereoisomer of CS that contains IdoA instead of or in addition to GlcA. HS chains comprise GlcA and GlcNAc residues. These sugar residues can be sulfated at various positions as indicated by "S." In contrast, HA is a non-sulfated GAG composed of GlcA and GlcNAc residues

tetrasaccharide linkage is assembled through the sequential stepwise addition of individual monosaccharide units, a single Xyl (xylose), two successive Gal residues, and a single GlcA, by the corresponding glycosyltransferases (Fig. 7.2a) (Silbert and Sugumaran 2002; Sugahara and Kitagawa 2000; Uyama et al. 2007). When a GalNAc is the first residue transferred to the nonreducing terminal GlcA residue of the linkage region, a chondroitin (Chn) backbone is synthesized; alternatively, when a GlcNAc instead of a GalNAc is transferred to the linker, HS chains is synthesized. Therefore, the first *N*-acetylhexosamine transfer is critical for the selective assembly of CS versus HS chains (Fig. 7.2a) (Silbert and Sugumaran 2002; Sugahara and Kitagawa 2000; Uyama et al. 2007). Chn polymerization, i.e., the synthesis of a Chn backbone, is catalyzed by a combination of six homologous glycosyltransferases, some of which constitute Chn polymerase complexes (Mikami and Kitagawa 2013). Likewise, the repetitive disaccharide region characteristic of HS chains is synthesized via the action of two HS polymerases, EXT1 and EXT2 (Nadanaka and Kitagawa 2008; Sugahara and Kitagawa 2000; Uyama et al. 2007).

The polysaccharide backbones of CS and HS chains acquire remarkable structural variability via further enzymatic modifications, such as sulfation and uronate epimerization (Fig. 7.3a, b) (Kusche-Gullberg and Kjellén 2003; Uyama et al. 2007). Sulfation of CS can result from the 4-*O*-sulfation or the 6-*O*-sulfation pathways based on the substrate preferences of multiple Chn sulfotransferases (Fig. 7.3a) (Kusche-Gullberg and Kjellén 2003; Mikami and Kitagawa 2013). The non-sulfated O unit [GlcA–GalNAc] serves as a common acceptor substrate for two types of sulfotransferases, chondroitin 4-*O*-sulfotransferases (C4STs) and chondroitin 6-*O*-sulfotransferase-1 (C6ST-1); the former catalyzes 4-*O*-sulfation and the latter

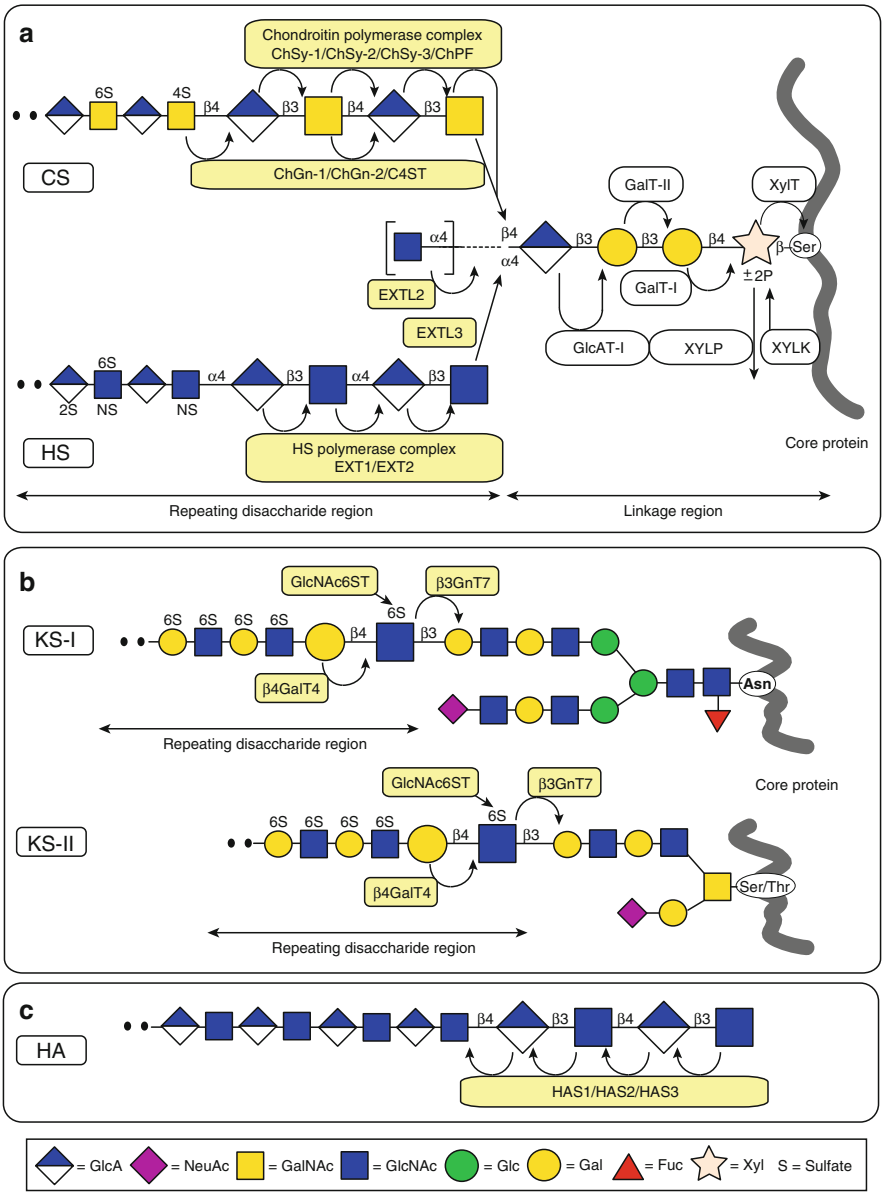

Fig. 7.2 Biosynthetic pathways for GAG synthesis. (**a**) A number of glycosyltransferases and specific kinase/phosphatase(s) participate in the synthesis of the common tetrasaccharide linkage region and repeating disaccharide regions characteristic of CS and HS chains. "±2P" denotes a transient phosphorylation of Xyl residue in the linkage region. *XylT* xylosyltransferase, *XYLK* xylose 2-*O*-kinase, *XYLP* 2-*O*-phosphoxylose phosphatase, *GalT-I* β1,4-galactosyltransferase-I, *GalT-II* β1,3-galactosyltransferase-II, *GlcAT-I* β1,3-glucuronyltransferase-I, *ChSy* chondroitin synthase, *ChPF* chondroitin polymerizing factor, *ChGn* chondroitin GalNAc transferase, *C4ST* chondroitin 4-*O*-sulfotransferase. (**b**) KS can be synthesized as either *N*-linked (KS-I) or *O*-linked (KS-II) glycans. Chain elongation of the repetitive disaccharide region of KS chains is achieved via the action of two glycosyltransferases and a sulfotransferase. *β3GnT7* β1,3-GlcNAc transferase-7, *β4GalT4* β1,4-galactosyltransferase-4, *GlcNAc6ST* GlcNAc 6-*O*-sulfotransferase. (**c**) HA biosynthesis is catalyzed by plasma membrane-bound HA synthases (HASs)

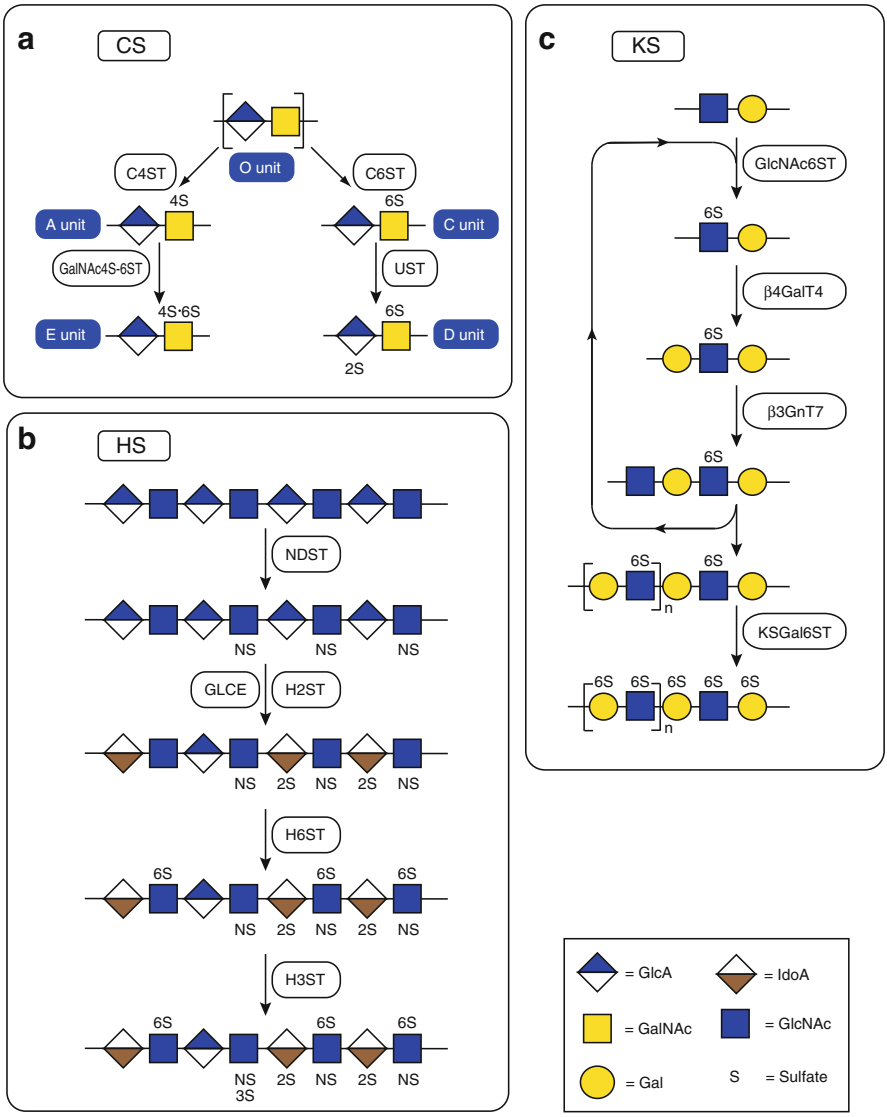

Fig. 7.3 Schematic pathways depicting sulfation of GAG chains. (**a**) The disaccharide units of CS chains are classified into O, A, C, D, or E units based on their sulfation patterns, and their biosynthetic pathway can be classified into either the initial "4-*O*-sulfation" or "6-*O*-sulfation" pathway. *C6ST* chondroitin 6-*O*-sulfotransferase, *UST* uronyl 2-*O*-sulfotransferase, *GalNAc4S-6ST* GalNAc 4-sulfate 6-*O*-sulfotransferase. (**b**) Modification of an HS backbone is initiated by *N*-deacetylation/*N*-sulfation of selected GlcNAc residues. Subsequent modifications occur in the *N*-sulfated regions of the nascent polymers. *NDST N*-deacetylase/*N*-sulfotransferase, *GLCE* uronyl C5 epimerase, *H2ST* uronyl 2-*O*-sulfotransferase, *H6ST* glucosaminyl 6-*O*-sulfotransferase, *H3ST* glucosaminyl 3-*O*-sulfotransferase. (**c**) During KS biosynthesis, GlcNAc-6-*O*-sulfation is tightly coupled with chain elongation. In contrast, Gal-6-*O*-sulfation occurs after completion of chain elongation. KSGal6ST, KS Gal 6-*O*-sulfotransferase

6-*O*-sulfation of GalNAc residues to form monosulfated A [GlcA–GalNAc(4-*O*-sulfate)] or C [GlcA–GalNAc(6-*O*-sulfate)] units, respectively. Subsequent sulfation of the A and C units can also occur, forming disulfated disaccharide E [GlcA–GalNAc(4,6-*O*-disulfate)] or D [GlcA(2-*O*-sulfate)–GalNAc(4-*O*-sulfate)] units, respectively. Recently, production of CS versus HS chains is fine-tuned via alternative mechanisms, including a transient phosphorylation of Xyl residue in the linkage region and an EXTL2-mediated biosynthetic suppression of the polymerization of CS and HS chains (Koike et al. 2009, 2014; Nadanaka et al. 2013). This diversity of modifications creates functional sugar domain structures with diverse biological activities.

7.2.2 KS Chains

KS chains are made up of repetitive disaccharide units; each comprises a Gal and a GlcNAc; therefore, the polymer backbone is identical to poly-*N*-acetyllactosamine structure found on conventional glycoproteins and mucins. Unlike CS and HS GAGs, KS chains are covalently linked to core proteins via either *N*- or *O*-linkages (Fig. 7.2b) (Funderburgh 2000). Sulfation can occur on the C6 position of either or both sugar constituents, and sulfation patterns are strictly regulated during KS biosynthesis (Funderburgh 2000; Kusche-Gullberg and Kjellén 2003). GlcNAc-6-*O*-sulfation mediated by GlcNAc 6-*O*-sulfotransferases (GlcNAc6STs) occurs only on nonreducing terminal GlcNAc residues and not internal GlcNAc residues; moreover, this sulfation is coupled to chain elongation (Figs. 7.2b and 7.3c). In contrast, Gal-6-*O*-sulfation mainly occurs after completion of chain elongation, and this type of sulfation at a nonreducing terminal Gal residue may serve as a signal that terminates chain elongation (Funderburgh 2000; Kusche-Gullberg and Kjellén 2003). Highly sulfated forms of KS chains rich in the disulfated disaccharides that are immunoreactive with a monoclonal antibody, 5D4, are distributed in the brain (Zhang et al. 2006). However, this immunoreactivity is not evident in the brains of mice that lack GlcNAc6ST-1 (Zhang et al. 2006).

7.2.3 HA Chains

HA chains are non-sulfated GAGs that consist of repetitive disaccharide units [(–4GlcAβ1-3GlcNAcβ1–)$_n$]; they are the largest glycans found in vertebrates. Unlike biosynthesis of sulfated GAGs, HA biosynthesis occurs on cell surfaces via the action of a family of plasma membrane-bound enzymes, hyaluronan synthases (HASs) (Fig. 7.2c) (Itano and Kimata 2002). Each member of the HAS family—HAS-1, HAS-2, and HAS-3—can polymerize HA chains on its own, and each has characteristic processivity (Itano and Kimata 2002). HA chains constitute major components of PNNs; therefore, they accumulate massively amid PNNs in CNS

(Carulli et al. 2007; Köppe et al. 1997; Kwok et al. 2010). Notably, HASs that are associated with the surfaces of neurons might act as anchors for HA chains, thus allowing for the formation and stabilization of PNNs (Carulli et al. 2007; Kwok et al. 2010).

7.3 Functions of GAGs in Experience-Dependent Neuronal Plasticity

7.3.1 CS Sulfation Pattern-Dependent Cortical Plasticity

Each PNN is a well-organized pericellular matrix that encapsulates the cell soma, proximal dendrites, and axonal segments of a particular class of neurons; most of these neurons are parvalbumin (PV)-positive inhibitory interneurons (Fig. 7.4a) (Yamaguchi 2000; Yazaki-Sugiyama et al. 2009). The appearance of PNNs coincides with the end of experience-dependent plasticity during postnatal development (Pizzorusso et al. 2002). Because of the abundance of CSPGs in PNNs, the functional involvement of CSPGs in limiting plasticity following closure of the critical periods is well documented (Pizzorusso et al. 2002). Monocular deprivation during the critical period leads to a reduction in the responses by visual cortical neurons that innervate the deprived eye and to an increase in the responses to the nondeprived eye (Fig. 7.4b); however, this capacity for ocular dominance plasticity is absent after the critical period ends (Gordon and Stryker 1996; Hensch 2005; Wiesel and Hubel 1963). Notably, removal of CS moieties via treatment with a bacterial CS-degrading enzyme chondroitinase ABC (ChABC) disrupts PNNs and permits reactivation of ocular dominance plasticity even after closure of the critical period (Fig. 7.4c) (Pizzorusso et al. 2002, 2006). Similar to its effects on ocular dominance plasticity, ChABC treatment of PNNs in the amygdala, which is involved in the formation of erasure-resistant fear memories, can render subsequently acquired fear memories susceptible to erasure even in adult animals (Gogolla et al. 2009). These reports suggest that CS moieties of CSPGs are responsible for PNN formation and restriction of critical period plasticity. However, the importance of sulfation patterns of CS chains in such plasticity has been overlooked in these previous studies because only ChABC has been used and ChABC degrades all CS chains, irrespective of CS sulfation status.

During brain development, sulfation profiles of CS chains change dramatically (Kitagawa et al. 1997; Mitsunaga et al. 2006; Properzi et al. 2005). In general, the proportion of 6-*O*-sulfation gradually decreases, and that of 4-*O*-sulfation progressively increases; consequently, there is a substantial increase in the 4S/6S ratio during development. Consistent with the notion that C4ST and C6ST competitively utilize the acceptor Chn disaccharide structures (see Sect. 2.1), systemic overexpression of human C6ST-1 in mice results in a substantially lower 4S/6S ratio throughout development (Miyata et al. 2012). This reduced 4S/6S ratio in the transgenic mice is due to an increase in the proportion of 6-*O*-sulfation and a decrease in

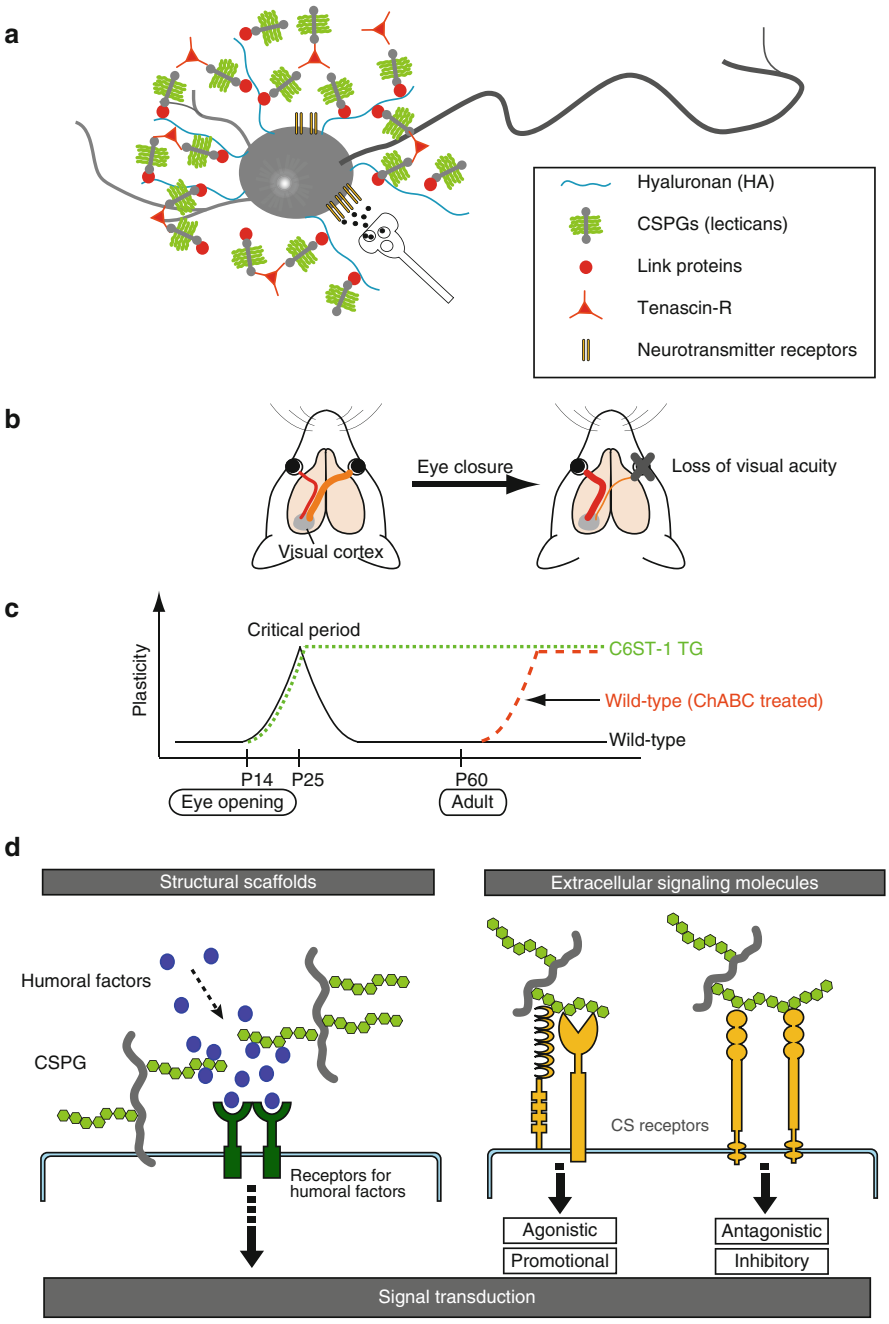

Fig. 7.4 Neuroregulatory functions of GAG chains in the CNS. (**a**) A schematic diagram of PNNs. Each PNN is a ternary complex of HA, CSPGs, and tenascin-R. Link proteins stabilize interactions between HA and CSPGs. PNNs are mainly localized around PV-expressing interneurons and may act as structural scaffolds that support neuronal functions. In addition, PNNs may also restrict

the proportion of 4-*O*-sulfation compared to that in wild-type mice. Interestingly, the transgenic mice that overexpress C6ST-1 retain juvenile level of ocular dominance plasticity even in adulthood (Fig. 7.4c) (Miyata et al. 2012), indicating that sulfation patterns of CS chains regulate the plasticity characteristic of the critical period. Furthermore, reduced PNN formation and decreased accumulation of Otx2 homeoprotein around PV^+ interneurons are also evident in these transgenic mice (Miyata et al. 2012). Otx2 regulates ocular dominance plasticity via its effect on the maturation of PV^+ inhibitory interneurons (Spatazza et al. 2013; Sugiyama et al. 2008). Therefore, the 4S/6S ratio of CSPGs may regulate the Otx2-mediated maturation of PV^+ interneurons that determines the critical period for cortical plasticity. Consistent with this notion, PNNs that bear specific CS chains are reportedly required for effective accumulation of Otx2 in PV-expressing interneurons (Beurdeley et al. 2012); these findings indicate that CS chains function as a structural scaffold (Fig. 7.4d).

7.3.2 CSPGs in PNN Formation

HA chains, link proteins, and tenascin-R are, along with CSPGs, major components of PNNs (Fig. 7.4a) (Galtrey and Fawcett 2007; Kwok et al. 2011a). In a recent model of PNN formation, secreted CSPGs interact with pericellular HA chains; these interactions are stabilized by the presence of link proteins, a group of proteins that bind to both CSPGs and HA chains. Interactions between C-terminal domains in the core protein of CSPGs and tenascin-R are also established, and, consequently, massive macromolecules form in the pericellular spaces (Galtrey and Fawcett 2007; Kwok et al. 2011a). Among CSPGs, lectican family members including aggrecan, versican, neurocan, and brevican are principal constituents of PNNs (Galtrey and Fawcett 2007; Kwok et al. 2011a). However, mice deficient for versican, neurocan, or brevican have largely normal PNNs (Dours-Zimmermann et al. 2009). In contrast, cortical primary neurons derived from aggrecan-deficient mice are abnormal in that they are not stained by lectin *Wisteria floribunda* agglutinin (WFA), a broad PNN marker, indicating an essential role of aggrecan in PNN formation (Brakebusch et al. 2002; Giamanco et al. 2010; Zhou et al. 2001). Despite the lack of WFA

Fig. 7.4 (continued) lateral diffusion of neurotransmitter receptors. (**b**) Ocular dominance plasticity in the visual cortex. In the left visual cortex, cortical neurons are dominated by the right eye (ocular dominance). Following closure of the right eye, responses of visual cortical neurons to stimulation from the right eye are depressed and those to stimulation from left eye are potentiated (ocular dominance plasticity). These altered responses result in permanent loss of visual acuity through the right eye. (**c**) The critical period for ocular dominance plasticity in mice peaks around postnatal day 25 (P25). ChABC treatment reactivates ocular dominance plasticity in adult animals. In addition, systemic overexpression of C6ST-1 in mice results in a juvenile level of ocular dominance plasticity even in adulthood. (**d**) Mechanisms of action of CS chains. CS chains can act as structural scaffolds that regulate humoral factor-mediated signal transduction (*left*); alternatively, CS chains can act as extracellular signaling molecules that trigger CS-receptor-mediated signal transduction (*right*)

staining, pericellular accumulation of other PNN components seems unaffected by the absence of aggrecan. This finding indicates that WFA has a binding preference for CS moieties of aggrecan. Therefore, distinct sugar domain structures on aggrecan that probably depend on the CS sulfation status may be determinants of PNN formation. Furthermore, given that the CS moieties of CSPGs affect their own interaction with other PNN components, identification of PNN components that recognize such functional sugar domains is an important task.

7.4 The Roles of GAGs in Neural Plasticity After CNS Injury

7.4.1 Bifunctional Nature of CS Chains in Neuronal Extension and Regeneration

Traumatic injury to the adult CNS induces glial scar formation. During wound healing following injury, the glial scar prevents further spread of damage and controls inflammatory responses (Rolls et al. 2009). However, these scars can act as major impediments to axonal regeneration (Rhodes and Fawcett 2004; Silver and Miller 2004). When confronted with glial scar tissue, regenerating axons cease extending and display a retracted growth cone with morphologically characteristic dystrophic endings (Raman y Cajal 1928). CSPG deposition is upregulated during glial scar formation, and CSPGs are generally recognized as inhibitors of axon regrowth after CNS injury (Rhodes and Fawcett 2004; Silver and Miller 2004). In fact, the formation of dystrophic growth cone, a hallmark of regeneration failure, is highly reproducible in vitro on a substrate that constitutes a crude gradient of CSPGs (Tom et al. 2004). Conversely, suppression of CSPG synthesis and/or treatment with ChABC promotes axon growth over scar-like surfaces in vitro (McKeon et al. 1995; Smith-Thomas et al. 1995). Moreover, removal of CS moieties from CSPGs via ChABC treatment of lesion sites improves axonal regeneration and functional recovery in vivo. These findings indicate that the CS moieties are a critical component that determines the inhibitory nature of CSPGs (Bradbury et al. 2002; Moon et al. 2001; Silver and Miller 2004).

The inhibitory effect of CS chains on axon growth is amply documented and widely recognized; nevertheless, CS chains do not always impede neurite outgrowth (Sugahara et al. 2003). In fact, several CS preparations such as CS-E, which comprises typical CS chains that are rich in E units, serve as stimulatory substrates for neurite outgrowth of cultured primary neurons in a cell-type-dependent manner (Brown et al. 2012; Gama et al. 2006; Sugahara and Mikami 2007). Such apparently contradictory functions are presumably attributable to the structural diversity of CS chains. The prominent CS subclasses found in mammalian tissues are composed of monosulfated A and C units. CS chains rich in A units (CS-A) negatively regulate axonal guidance and growth of cerebellar granule neurons (Wang et al. 2008). In contrast, CS chains rich in C units (CS-C) do not appear to be inhibitory, but rather to be relatively permissive for axonal regeneration, based on a knockout study of

C6ST-1, which is responsible for the synthesis of C units, in mice (Lin et al. 2011; Sugahara et al. 2003). Thus, the upregulation of C6ST-1 expression under injury-related conditions (Liu et al. 2006; Properzi et al. 2005) may be involved in the protective roles of glial scar.

7.4.2 Molecular Mechanisms for CS-Mediated Regulation of Neuronal Extension

As described above, CS chains promote or inhibit neuronal extension depending on CS structure and the context. Furthermore, accumulating evidence indicates that extracellular CSPGs can affect several intracellular signaling pathways (Kwok et al. 2011b). Such findings may indicate that neuronal cells have distinct CS-recognition mechanisms; therefore, studies to identify functional CS receptor molecules have ensued (Carulli et al. 2005). Contactin-1 (CNTN-1), a glycosylphosphatidylinositol-anchored cell adhesion molecule in the immunoglobulin superfamily, is now recognized as a neuronal cell surface receptor for CS-E; this finding is the first demonstration that distinctive CS chains indeed behave as extracellular signaling molecules that trigger intracellular signaling cascades (Fig. 7.4d) (Mikami et al. 2009). The stimulatory effects of CS chains propagated by CNTN-1 promote neuronal extension; conversely, four other CS receptors—including a protein tyrosine phosphatase PTPσ—mediate the inhibitory effects of CS chains on axonal regeneration (Dickendesher et al. 2012; Fisher et al. 2011; Shen et al. 2009). Notably, the primary role of PTPσ is as a HSPGs receptor (Chien and Ryu 2013); therefore, PTPσ propagates CSPG-mediated inhibition and HSPG-mediated axon outgrowth. In this context, CS chains have been shown to serve as inhibitors of the HS-induced oligomerization of PTPσ that promotes neurite growth (Fig. 7.4d) (Coles et al. 2011). Functional redundancy among the four inhibitory CS receptors may indicate that there are unidentified CS receptors, in addition to CNTN-1, that propagate in CS-induced neurite outgrowth.

The GAG-mediated switch between counteracting PTPσ functions is reminiscent of the bifunctional guidance cue semaphorin 5A (Sema5A). Sema5A interacts with GAG moieties of both CSPGs and HSPGs, and its HSPG-mediated attraction can be converted to inhibitory by extrinsic CSPGs (Kantor et al. 2004). Additionally, Sema3A, which is from another class of semaphorins and acts as a repulsive guidance cue, is reportedly anchored to PNNs via interactions with CS chains (Dick et al. 2013). In view of the fact that Sema3A expression is induced in the scar-associated meningeal cells after adult CNS injury (Pasterkamp et al. 1999), the inhibitory nature of CS chains in glial scars may result, at least in part, from CS-mediated modulation of semaphorin functions.

Alternatively, the CSPG-mediated inhibition may be relevant to the activation status of integrins, which are involved in neuronal cell adhesion and axon growth. Actually, a substrate pre-coated with some CSPGs (e.g., aggrecan) results in neuronal axon growth inhibition that is accompanied by reduced integrin activation;

however, pharmacological or genetic activation of integrins overcomes CSPG-mediated inhibition of axon outgrowth (Afshari et al. 2010; Condic et al. 1999; Tan et al. 2011). In addition, the suppressive effect of CSPGs is reduced by ChABC treatment (Afshari et al. 2010). Collectively, these data indicated that CS chains are important to the control of integrin activation, but currently no direct evidence indicates that there are physical interactions between CS chains and integrins.

7.4.3 KS Chains in Axonal Regeneration/Sprouting

Recent evidence demonstrates that KSPGs also have indispensable roles in axonal regeneration after CNS injury. A genetic deficiency of GlcNAc6ST-1 in mice leads to loss of the 5D4-reactive KS chains in brain samples and a marked reduction of scarring following CNS injury (Zhang et al. 2006). Surprisingly, neuronal regeneration and functional recovery are clearly enhanced in GlcNAc6ST-1-deficient mice, even though the characteristic increase in CS expression around lesion sites is unaltered (Ito et al. 2010). Local administration of keratanase II (K-II), a specific KS-degrading enzyme, to sites of injury also promotes anatomical and functional recovery after spinal cord injury (Imagama et al. 2011). Consistent with these in vivo findings, K-II-catalyzed selective digestion of KS moieties permits neurite outgrowth even on in vitro PG preparations derived from chick brain; such preparations are commonly used as axon growth inhibitory substrata that contain both CSPGs and KSPGs (Imagama et al. 2011). Notably, the promotional effects of K-II in vitro and in vivo are comparable to those of ChABC, but a combined application of both enzymes does not result in additive or synergistic effects (Imagama et al. 2011). These findings indicate that both CS and KS chains are independently required for PG-mediated inhibition of axonal regeneration/sprouting. Identification and characterization of KS receptors that mediate inhibitory effects of KSPGs may further illuminate this phenomenon.

7.4.4 GAG Chains in Plasticity of Spinal Neural Circuitry After Injury

Poor recovery after CNS injury is attributed to the limited capacity of adult CNS for functional rewiring. Upregulation of CSPGs following injury may also restrict plasticity in a recovering CNS. This notion is strongly supported by recent studies on a combination therapy for spinal cord injury (García-Alías and Fawcett 2012). It is generally known that rehabilitation has beneficial effects on the recovery processes (Biernaskie and Corbett 2001), and therefore rehabilitation, as well as environmental stimuli, can enhance the neuronal plasticity, which may be largely restricted by CSPGs (Edgerton et al. 2004). In fact, animals subjected to a specific motor rehabilitation regime after spinal cord injury and acute ChABC treatment reportedly

exhibit better functional recovery than those who received either ChABC treatment or rehabilitation alone (García-Alías et al. 2009). Therefore, ChABC treatment may enhance spinal cord plasticity, and the subsequent training may strengthen precisely functioning connections and also exclude imprecise ones. Taken together, these findings indicate that CSPGs indeed restrict neuronal plasticity within the spinal cord and that such combination therapies are promising strategies that may promote spinal cord repair. In view of the indispensable roles of KSPGs in axonal regeneration (Hilton et al. 2012; Kadomatsu and Sakamoto 2014), an alternative use of K-II in the combination therapy might be useful for validation of KSPG functions.

7.4.5 HS Chains in Axonal Regeneration

In view of the widely accepted roles of HSPGs in axonal growth promotion (Maeda et al. 2011; Nadanaka and Kitagawa 2008; Yamaguchi et al. 2010), HSPGs are thought to have a supportive role in axonal regeneration. Based on several studies, HS chains and their core proteins, including syndecan family members, are upregulated within and/or surrounding the lesion core following CNS injury (Iseki et al. 2002; Moon et al. 2002; Properzi et al. 2008). Notably, mice carrying a gene knockout for CS *N*-acetylgalactosaminyltransferase-1 (ChGn-1), a key enzyme in CS biosynthesis, have significantly better recovery from spinal cord injury than do wild-type or ChABC-treated mice (Takeuchi et al. 2013). This superior recovery is attributed to the expected reduction in CS synthesis and to an unexpected induction of HS biosynthetic machinery (Takeuchi et al. 2013). These findings provide support for notion that HS chains promote axon regeneration, although the underlying mechanisms remain elusive.

7.5 GAGs in Synaptic Plasticity

7.5.1 CS and HA Chains in Synaptic Plasticity

During postnatal development, CSPGs influence synaptic properties. Enzymatic digestion of CS chains impairs synaptic plasticity, as judged by reduced levels of long-term potentiation (LTP) or long-term depression (LTD); these electrophysiological indexes underlie learning and memory (Bukalo et al. 2001). Additionally, ChABC treatment reportedly enhances the motility of dendritic spines and induces the appearance of spine protrusions in a glutamate receptor-independent manner (Orlando et al. 2012); these findings indicate that CS moieties of CSPGs restrict dendritic spine dynamics. Such inhibitory action of CSPGs in cortical neurons operates, at least in part, via targeting of neurotrophin receptors and therefore neurotrophin action (Kurihara and Yamashita 2012).

Lateral diffusion of glutamate receptors is essential for their postsynaptic accumulation, and such accumulation is relevant to the synaptic plasticity of excitatory neurons. Many neurons in mature synapses are wrapped by a net-like ECM that contains CSPG and HA chains. Removal of these CS and HA chains promotes lateral diffusion of glutamate receptors and modulates receptor density and activity in subsynaptic sites (Frischknecht et al. 2009; Pyka et al. 2011). These findings indicate that perisynaptic GAGs (e.g., CS and HA chains) also affect glutamate receptor-dependent short-term synaptic plasticity.

7.5.2 HS Chains in Synaptic Plasticity

Studies involving conditional knockout of EXT1, the rate-limiting enzyme for HS biosynthesis, have demonstrated the various in vivo functions of HS chains in mammalian brain development (Yamaguchi et al. 2010). However, there is little direct evidence that supports the notion that HS chains are functionally relevant to synaptic plasticity. For example, the HSPG agrin is a well-known synaptic stabilizer in neuromuscular junctions (Ngo et al. 2007). Lack of agrin reduces excitatory, but not inhibitory, synaptogenesis (Gingras et al. 2002; Ksiazek et al. 2007). Other HSPGs such as syndecan family members are also reportedly involved in spine formation (Lin et al. 2007; Yamaguchi et al. 2010). However, there is no evidence that the HS moieties of agrin or syndecan HSPGs are required for synaptic functions.

In contrast, a recent study of conditional EXT1-knockout mice provides a significant breakthrough in the understanding of HS functions in synaptic plasticity (Irie et al. 2012). This EXT1-knockout is targeted to postnatal neurons, and the mutant mice recapitulate almost the full range of autistic symptoms. Moreover, electrophysiological analysis of the pyramidal neurons in the mutant amygdala revealed attenuated excitatory synaptic transmission, presumably due to a reduced number of glutamate receptors in synapses. This finding elucidates critical roles of HS chains for normal functioning of glutamatergic synapses.

7.6 Conclusion

This chapter provides a brief overview of functional relevance of glycans in neuronal plasticity. While this review sheds light on sulfated GAG chains only, the roles of the respective GAGs in CNS plasticity can be highly divergent and occasionally redundant. Such functional complexity is attributable to a characteristic feature of the glycans that exhibit structural plasticity. Accumulating evidence indicates that GAG chains may be involved in neural disorders such as Alzheimer's disease, schizophrenia, and neuropathy (Berretta 2012; Cui et al. 2013; Izumikawa et al. 2013; Saigoh et al. 2011; Soleman et al. 2013). Therefore, comprehensive analyses of the relationships between the structure and function of GAGs are needed not only

for a better understanding of the molecular mechanisms underlying GAG-associated neuronal disorders but also for the development of promising new therapeutic approaches to treatments for the related neuronal disorders.

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Chapter 8
The Involvement of Midkine, a Heparin-Binding Growth Factor, in Cancer Development

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Abstract Midkine is a secreted growth factor identified as a retinoic acid-induced gene in embryonal carcinoma cells. In terms of its molecular structure, a midkine consists of two domains, an N-terminal and a C-terminal domain. Previous reports have emphasized the significance of the C-terminal domain, which contains clusters of basic amino acids (clusters I and II). Cluster I is mainly responsible for the strong affinity of midkine to heparin. In addition to heparin, two other glycosaminoglycans, chondroitin sulfate and heparan sulfate, can also bind to midkine. The binding between cluster I in the C-terminal domain of midkine and glycosaminoglycans would mainly mediate the ligand-receptor interaction. Midkine is broadly expressed in various cancers and could have potential as both a tumor marker and prognostic factor. In neuroblastoma, the serum midkine level has been established as a reliable poor prognostic factor. Furthermore, it was recently revealed that midkine is physiologically involved in the tumorigenesis of neuroblastoma. Notch2 is likely to function as a receptor of midkine in neuroblastoma cells. Although anaplastic lymphoma kinase (ALK), another candidate receptor of midkine, was shown to be one of the predisposition genes of neuroblastoma, their ligand-receptor relationship in neuroblastoma has yet to be elucidated. Interestingly, it was reported that both Notch2 and ALK were glycosylated and that these glycosylations were necessary for their functions. Midkine could be an efficient molecular target in cancer therapy. Several molecular tools to target midkine have been developed, such as siRNA, antibodies, and RNA aptamers. Each of them exhibits certain therapeutic activities. Future investigation into the role of sugar chains in these activities would be of benefit. Progress in this and other matters pertaining to the clinical application of these molecular tools is eagerly anticipated.

Keywords Midkine • Glycosaminoglycan • Heparin • Neuroblastoma • Chondroitin sulfate • Heparan sulfate

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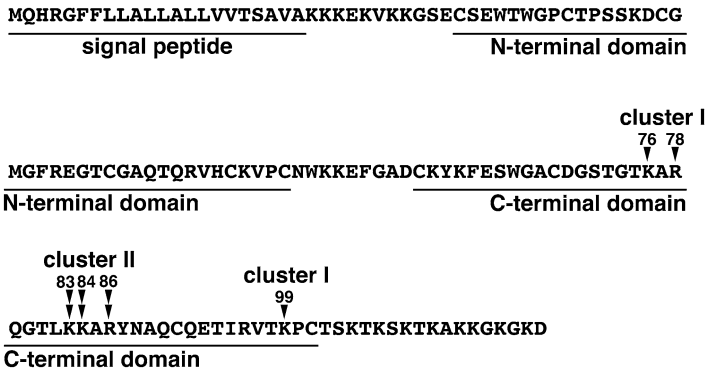

Fig. 8.1 Amino acid sequence of mouse midkine. Mouse midkine consists of 140 amino acids (including 22 amino acids of an unnumbered signal sequence). The N-terminal domain (12–49), C-terminal domain (59–101), cluster I (76, 78, 99), and cluster II (83, 84, 86) are indicated

The growth factor midkine was identified through the search for genes that are differentially expressed during the retinoic acid-induced differentiation of embryonal carcinoma cells (Kadomatsu et al. 1988). The secreted midkine protein, whose molecular weight is 13 kDa, is rich in basic amino acids and cysteines and consists of two domains, an N- and a C-terminal domain (Iwasaki et al. 1997) (Fig. 8.1). Each domain is composed of three antiparallel β-sheets linked by flexible linker regions. It is interesting that the C-terminal domain alone exhibits certain biological activities, such as the induction of neurite outgrowth, fibrinolysis, and nerve cell migration. There are two clusters (clusters I and II) of basic amino acids in the C-terminal domain of midkine (Asai et al. 1997; Akhter et al. 1998; Maeda et al. 1999) (Fig. 8.1). These amino acids, and particularly those in cluster I, are thought to be critical for the biological function of midkine, as described later. Midkine shares around 50 % sequence identity with the only other member of the midkine family, pleiotrophin (Tomomura et al. 1990).

The sugar chains, the title of this book, is one of the key concepts underlying the biological activity and function of midkine. While it has not been reported that the midkine protein itself could be glycosylated, midkine possesses significant and implicative affinity with glycosaminoglycans. At the beginning of this study, it was shown that midkine had heparin-binding ability (Tomomura et al. 1990). Cluster I located in the C-terminal domain of midkine was revealed to be mainly responsible for its binding to heparin (Asai et al. 1997). The mutation at arginine 78 (cluster I in mouse midkine) (Fig. 8.1) resulted in a significant reduction of heparin-binding activity. In contrast, mutation at either arginine 83 or 84 (cluster II) had only slight effects. However, the finding that the mutations at both the 83 and 84 arginines caused even a slight reduction in heparin-binding activity indicates that cluster II is also involved in the binding to heparin. On the other hand, the sulfations in heparin are important for binding to midkine. All three sulfate groups in the heparin disaccharide unit (6-O-sulfate, 2-O-sulfate, and N-sulfate) were involved in the interaction

with midkine. In particular, the N-sulfate group among them was shown to be critically important (Kaneda et al. 1996a). Although the heparin-binding activity and neurite-promoting activity were found to be well correlated among a series of midkine mutants (Asai et al. 1997), the significance of heparin binding to midkine remains to be determined. Interestingly, binding to heparin might affect the dynamics of secreted midkine. Various types of cancer cells express midkine at high levels, which is the main topic of this section. When such cancer cell lines are cultured *in vitro*, they continuously express and secrete midkine. But few midkine proteins can be found in the conditioned media, because most of the secreted midkine is easily captured on the cell surface and/or extracellular matrix. These phenomena should result from the high affinity of midkine to glycosaminoglycans. There are many proteoglycans on the cell surface and extracellular matrix. When an excess amount of heparin is added to the media, the secreted midkine binds to the heparin and stays in the media as a soluble form. Some reports have suggested that the heparin could inhibit the midkine function to induce neurite outgrowth (Raulo et al. 1994; Kinnunen et al. 1996; Kaneda et al. 1996a, b). Nonetheless, it remains unclear whether the soluble midkine and cell surface- or extracellular matrix-bound midkine are biologically active, particularly in cancers. In addition, what about the behavior of midkine *in vivo*? One possible clue to this question was observed in a mouse model of neuroblastoma. Neuroblastoma is a pediatric solid tumor that arises from sympathetic organs, such as the adrenal gland and sympathetic ganglion (Nakagawara and Ohira 2004). Neuroblastoma is one of the cancers in which midkine is closely involved in the tumorigenesis, and the oncogenic transcription factor *MYCN* is thought to be a very important predisposition gene. As an animal model of neuroblastoma, *MYCN* transgenic (Tg) mice have been generated (Weiss et al. 1997). The neuroblastoma tumor tissue developed in *MYCN* Tg mice significantly expresses and secretes midkine. Interestingly, the midkine protein, but not the mRNA, was detected in the normal, metastasis-free liver tissue of these mice. These results indicate that midkine proteins are secreted from tumor cells and circulate through the blood vessels and that some of them are captured on the endothelial surface of the blood vessels in the liver. These bindings might be mediated by the interaction with glycosaminoglycans. On the other hand, the circulating midkine in the bloodstream might also bind to glycosaminoglycans in order to maintain its soluble status. Although the functional significance of and the differences between the captured and released midkine *in vivo* are also not addressed yet, glycosaminoglycans, such as heparin, might regulate these midkine statuses.

It is worth noting that heparin is not the only glycosaminoglycan that binds to midkine. Protein tyrosine phosphatase ζ (PTPζ), syndecans, and glypican-2 are candidate receptors of midkine. PTPζ is a chondroitin sulfate proteoglycan. Syndecans and glypican-2 are heparan sulfate proteoglycans. PTPζ is a single-pass transmembrane protein and possesses protein tyrosine phosphatase activity in its intracellular domain. Midkine binds to PTPζ with significantly high affinity, and this binding can be inhibited by the addition of heparin, heparan sulfate, and chondroitin sulfates (Maeda et al. 1999). In addition, PTPζ treated with chondroitinase ABC shows significantly reduced affinity to midkine. These results indicate that midkine binds to

PTPζ by recognizing chondroitin sulfate on it. Furthermore, the C-terminal domain of midkine is responsible for binding to PTPζ and mutation at arginine 78 (cluster I) (Fig. 8.1) causes a reduction in affinity. Mutations at lysine 83 and lysine 84 have no effect. Taking these results together, cluster I in the C-terminal domain of midkine should be involved in the binding to chondroitin sulfate on PTPζ, just as in the binding to heparin. Midkine-PTPζ interaction mediated by chondroitin sulfate seems to be involved in the haptotaxis of osteoblast-like cells (Qi et al. 2001). Midkine-induced haptotaxis was abrogated by the addition of chondroitin sulfates B and E, treatment with chondroitinases ABC and B, and addition of protein tyrosine phosphatase inhibitor.

In terms of chondroitin sulfate proteoglycans, it has been reported that PG-M/versican, an extracellular matrix protein, is bound to midkine (Zou et al. 2000). By examining midkine-binding proteoglycans in day 13 mouse embryos in which midkine was intensively expressed, PG-M/versican was identified. PG-M/versican is a huge chondroitin sulfate proteoglycan expressed in various developing and differentiated tissues. Digestion of PG-M/versican with chondroitinase ABC, AC-I, or B abolished the binding to midkine. Furthermore, heparin and chondroitin sulfates D and E inhibited the binding between midkine and PG-M/versican. These results indicated that midkine interacts with PG-M/versican by recognizing chondroitin sulfate. The biological significance of this interaction is yet to be elucidated.

Syndecans are heparan sulfate proteoglycans, and syndecan-1, syndecan-3, and syndecan-4 (also known as ryudocan) have been reported to bind to midkine (Mitsiadis et al. 1995; Kojima et al. 1996; Nakanishi et al. 1997). Syndecan-4 binding to midkine was inhibited by the pretreatment of syndecan-4 with heparitinase and the addition of heparin or heparan sulfate (Kojima et al. 1996). These results suggest that the C-terminal domain of midkine (particularly cluster I) recognizes heparan sulfate on syndecans, which is responsible for the interaction between midkine and syndecans. Based on their expression patterns, the midkine-syndecans bindings appear to play roles in the embryogenesis of the central nervous system (Nakanishi et al. 1997). Glypican-2 is also a heparan sulfate proteoglycan. Midkine recognizes heparan sulfate chains of glypican-2, and the interaction participates in neuronal cell migration and neurite outgrowth (Kurosawa et al. 2001).

As described above, PG-M/versican was identified as a midkine-binding glycoprotein expressed in day 13 mouse embryos (Zou et al. 2000). A similar screening also identified LDL receptor-related protein 1 (LRP1) as a midkine-binding glycoprotein expressed in day 13 mouse embryos (Muramatsu et al. 2000). LRP1 is a large transmembrane glycoprotein, and its involvement in midkine signaling has been reported. Upon the binding of midkine to LRP1, the complex is endocytosed and midkine is degraded in lysosomes (Shibata et al. 2002; Suzuki et al. 2004). A portion of the endocytosed midkine can escape from degradation and is released from endosomes into the cytosol. Cytosol midkine binds to nucleolin, a nucleocytoplasmic shuttle protein, and translocates into the nucleus. This nuclear translocation of midkine seems to play a role in promoting cell survival. LRP1 is also glycosylated in a different manner in each tissue (May et al. 2003). The midkine-binding domain in LRP1 has been investigated in detail, and a peptide consisting of 169

Table 8.1 MK-binding proteins and their sugar chains

Protein	Sugar chain	Function
None	Heparin	–
PTPζ	Chondroitin sulfate	Haptotaxis of osteoblast-like cells
PG-M/versican	Chondroitin sulfate	Unknown
Syndecans	Heparan sulfate	Embryogenesis of the central nervous system
Glypican-2	Heparan sulfate	Neuronal cell migration, neurite outgrowth
LRP1	Unknown	Cell survival

amino acids in its extracellular domain was revealed to possess significant affinity to midkine (Chen et al. 2007). Interestingly, when the peptide (named MK-TRAP) was expressed in cells, it appeared as a smear band in SDS-PAGE gels. This result implies that the midkine-binding domain of LRP1 would be glycosylated and that midkine-sugar chains binding would be responsible for the ligand-receptor complex formation. The proteins and their sugar chains that bind to MK are listed in Table 8.1.

One of the major fields in which midkine is involved is cancer. The physiological expression of midkine is especially remarkable during the embryonic period (Kadomatsu et al. 1990). In contrast, its postnatal expression is totally restricted. The expression of midkine in postnatal animals tends to be closely related with various pathogeneses (Kadomatsu et al. 2014a, b; Kishida and Kadomatsu 2014; Muramatsu 2014). In the early stages of the study of midkine, it was reported that midkine was highly expressed in a variety of cancers, including Wilms' tumor (Tsutsui et al. 1993), gastrointestinal cancer (Aridome et al. 1995), astrocytoma (Mishima et al. 1997), colorectal cancer (Ye et al. 1999), prostate cancer (Konishi et al. 1999), and neuroblastoma (Nakagawara et al. 1995). On the other hand, the ectopic expression of midkine has been shown to transform NIH3T3 cells *in vitro* (Kadomatsu et al. 1997). Because midkine is a secreted protein released into the bloodstream, it has been established as a tumor marker, as well as a prognostic factor in several cancers (Ikematsu et al. 2000, 2003, 2008; Obata et al. 2005; Jia et al. 2007; Ota et al. 2008; Ibusuki et al. 2009). Particularly in neuroblastoma, the plasma midkine level was shown to be correlated with existing prognostic factors, such as stages 3 and 4, over 1 year of age, *MYCN* amplification, low TrkA expression, and diploidy/tetraploidy (Ikematsu et al. 2003). Furthermore, the plasma midkine level itself has been shown to be an independent prognostic factor (Ikematsu et al. 2008).

In order to address the physiological role of midkine, it is necessary to analyze the phenotype of *Mdk* (mouse midkine gene) knockout mice (Nakamura et al. 1998). Because midkine is mainly expressed during the embryonic stage, it is likely that there would be some defects in embryogenesis. Unexpectedly, although *Mdk* knockout mice showed some behavioral phenotypes related with postnatal development of the hippocampus, there was no gross abnormality related with embryogenesis. In terms of cancers, there has been no direct and conclusive evidence showing its involvement in tumorigenesis despite many years of investigations. Recently, the phenotype of *MYCN* Tg mice, the neuroblastoma model mice, crossed with *Mdk* knockout mice was reported (Kishida et al. 2013). Neuroblastoma arises from

a sympathetic neuronal lineage derived from the neural crest in early embryogenesis (Nakagawara and Ohira 2004). Despite the vigorous basic and clinical researches, the prognosis of malignant neuroblastoma is still poor. *MYCN* Tg mice were generated in 1997 (Weiss et al. 1997) and have been recognized as an excellent model in which human neuroblastoma is accurately reproduced. The human *MYCN* gene, which is under the control of the rat tyrosine hydroxylase (sympathetic neuron-specific enzyme) promoter, is integrated in *MYCN* Tg mice. These animals spontaneously develop neuroblastoma that originates from the superior mesenteric ganglion (SMG), one of the sympathetic ganglia. The tumors developed in *MYCN* Tg mice are pathologically equivalent to human neuroblastoma in terms of histology and molecular biological characteristics (e.g., gene expressions and chromosomal aberrations) (Weiss et al. 1997, 2000; Hackett et al. 2003). They have been utilized in various basic studies as a reliable model. Around 70 % of *MYCN* Tg mice (hemizygotes) develop tumors derived from SMG. It has been reported that the tumor incidence is partially suppressed, up to 60 %, in *Mdk* knockout mice (Kishida et al. 2013). This is the first physiological result indicating the involvement of midkine in tumorigenesis. But the suppressive effect in the absence of midkine was not striking. Many of the *MYCN* Tg mice still developed neuroblastoma via a midkine-independent mechanism. There are several possible interpretations for these phenotypes. The simplest would be that midkine does not have a particularly dominant effect on the tumorigenesis of neuroblastoma. Second, because neuroblastoma is famous for its intensive and various chromosomal aberrations (gain and loss), a secondary event that compensates for the absence of midkine might occur. In neuroblastoma, a candidate receptor of midkine, Notch2, is thought to function in mediating midkine signaling (Kishida et al. 2013). Notch family receptors, including Notch2, are single transmembrane proteins whose canonical ligands are other transmembrane proteins, such as DLLs and JAGs, on the surface of adjacent cells. Upon the binding of ligand, the intracellular domain (ICD) of Notch is cleaved and directly translocates into the nucleus to activate the transcription of target genes. In *Mdk* $^{-/-}$ *MYCN* Tg mice, the protein level of nuclear Notch2 ICD (activated form) in tumor cells was found to be significantly decreased compared with that in *Mdk* $^{+/+}$ mice (Kishida et al. 2013). These results indicate that Notch2 signaling was attenuated in the absence of midkine. Consistent with this, the expression of a typical Notch target gene, HES1, was also decreased in *Mdk* $^{-/-}$ mice. These physiological data strongly suggest that the midkine-Notch2-HES1 axis is involved in the tumorigenesis of neuroblastoma. It has also been reported that this axis functions in pancreatic cancer (Güngör et al. 2011). The midkine-Notch2-HES1 pathway has been shown to induce epithelial-mesenchymal transition and drug resistance in pancreatic cancer cells. Although direct evidence of a ligand-receptor relationship between midkine and Notch2 is limited so far, it is interesting that Notch proteins undergo essential glycosylation in their extracellular domains (Rana and Haltiwanger 2011).

Glioma is another cancer in which a relationship between midkine and a particular receptor has been suggested (Lorente et al. 2011). In glioma, the suggested functional receptor of midkine is anaplastic lymphoma kinase (ALK). ALK was

originally known for its involvement as a hyperactive fusion gene resulting from chromosomal translocation in leukemia and lung cancer. In glioma cells, midkine signaling through ALK suppresses cannabinoid-induced autophagic cell death. Although a previous study *in vitro* originally suggested that ALK was a putative receptor of midkine (Stoica et al. 2002), this glioma case was the first report showing the midkine-ALK axis in cancer cells. Interestingly, ALK was established as a predisposition gene in neuroblastoma (Mossé et al. 2008; Janoueix-Lerosey et al. 2008; Chen et al. 2008; George et al. 2008). Some hyperactive mutations in ALK were found in both familial and sporadic neuroblastomas. Because the involvement of midkine in neuroblastoma has also been independently reported, it would seem natural to speculate that the midkine-ALK axis also functions in neuroblastoma cells. But so far, there has been no supporting evidence of its presence. For example, the knockdown of midkine in neuroblastoma cell lines harboring ALK mutation resulted in growth suppression (Kishida and Kadomatsu 2014). This result suggests that midkine exerts an ALK-independent (possibly Notch2-dependent) function to promote the proliferation of neuroblastoma cells. Finally, ALK also undergoes glycosylation, which is necessary for the pro-survival signal of neuroblastoma cells (Del Grosso et al. 2011).

The expression pattern of midkine—i.e., high expression in various tumor cells and low expression in normal postnatal tissues—suggests that it has potential not only as a tumor marker but also as a target of cancer therapy. Several tools targeting midkine have been developed, and their effectiveness has also been revealed (Table 8.2). They are mainly classified into two groups: RNAi-based nucleotides to block the midkine protein synthesis and molecules to inhibit secreted midkine function.

Table 8.2 Possible molecular tools to target midkine in cancer therapy

Molecular tools	Note	References
Oligonucleotide	Intratumor injection to rectal carcinoma xenograft	Takei et al. (2001)
		Takei et al. (2002)
	i.v. to in situ hepatocellular carcinoma	Dai et al. (2007a)
		Dai et al. (2007b)
	i.v. of oligonucleotide-loaded nanoparticle to in situ hepatocellular carcinoma	Dai et al. (2009)
Morpholino oligomer	Intratumor injection to prostate and colon carcinoma xenografts	Takei et al. (2005)
siRNA	Intratumor injection to prostate carcinoma xenograft	Takei et al. (2006)
	Intratumor injection to glioma xenograft	Lorente et al. (2011)
Binding peptide	MK-TRAP (LRP1-derived midkine-binding peptide)-expressing rectal carcinoma xenograft	Chen et al. (2007)
Polyclonal antibody	Anchorage-independent colony formation of Wilms' tumor and rectal carcinoma cells	Chen et al. (2007)
Monoclonal antibody	i.p. to osteosarcoma xenograft	Sueyoshi et al. (2012)
RNA aptamer	Intratumor injection to neuroblastoma xenografts	Kishida et al. (2013)

i.v. intravenous injection; *i.p.* intraperitoneal injection

The RNAi-based oligonucleotides have been examined by two groups. Intratumor injection to CMT-93 mouse rectal carcinoma cell-derived xenografts (Takei et al. 2001, 2002) effectively suppressed tumor growth *in vivo*. Intravenous injection to an *in situ* human hepatocellular carcinoma model in combination with antitumor drugs was also indicated to exert moderate suppressive effects (Dai et al. 2007a, b). The same group attempted intravenous injection of oligonucleotide-loaded nanoparticles in the same model (Dai et al. 2009). Intratumor injections of morpholino oligomer to PC-3 prostate carcinoma cell-derived and SW620 colon carcinoma cell-derived xenografts also exerted moderate effects (Takei et al. 2005). More recently, siRNA, rather than oligonucleotide, has become a major tool in the knockdown of specific genes. There are two reports examining the therapeutic effects of siRNA against midkine (Takei et al. 2005; Lorente et al. 2011). Because the stability *in vivo* is the main obstacle of siRNA usage in therapy, the siRNAs were mixed with atelocollagen as a carrier and intratumorally injected in both reports. siRNA in combination with paclitaxel markedly suppressed the growth of PC-3-derived xenografts (Takei et al. 2005). On the other hand, siRNA with cannabinoid inhibited the growth of T98 glioma cell-derived xenografts (Lorente et al. 2011). Because the induction of drug resistance seems to be one of the important functions of midkine in cancer cells, the combination of targeting midkine and an anticancer drug would be synergistically effective.

Another strategy to target midkine is the neutralization of the secreted midkine protein. It has been shown that a polyclonal antibody can suppress anchorage-independent colony formation of G401 Wilms' tumor cells (Chen et al. 2007). Although the results of this *in vitro* assay have frequently been correlated with therapeutic effects *in vivo*, no further experiments have yet been reported. However, the therapeutic effects of a monoclonal antibody were examined in an orthotopic xenograft model of 143B osteosarcoma cells (Sueyoshi et al. 2012). Intraperitoneal injection of monoclonal antibody moderately suppressed the xenograft tumor growth and efficiently inhibited lung metastasis. Thus far, however, the therapeutic results utilizing antibodies are limited. Progress in the near future is expected.

As described, a small peptide named MK-TRAP was developed for the purpose of targeting midkine (Chen et al. 2007). MK-TRAP consists of 169 amino acids derived from a part of the extracellular domain of LRP1 and strongly binds to midkine. Both the transfection of MK-TRAP-expressing plasmid and the addition of secreted MK-TRAP-containing medium to G401 and CMT-93 cells resulted in the suppression of anchorage-independent colony formation. Furthermore, the xenografts derived from CMT-93 cells expressing MK-TRAP showed attenuated growth *in vivo*. MK-TRAP might be as useful as an antibody for the molecular targeting of midkine.

Finally, another candidate tool was recently reported (Kishida et al. 2013). This candidate, an RNA aptamer, consists of 20–80-mer RNA and forms a particular three-dimensional structure. Their molecular structure enables RNA aptamers to directly recognize and bind to particular proteins. This is why RNA aptamers are considered nucleic acid analogues to antibodies. An RNA aptamer against a particular molecule is screened from a library containing 10^{14} different molecules

(Miyakawa et al. 2006, 2008; Ishiguro et al. 2011). Intratumor injection of an RNA aptamer against midkine to TNB1 and YT-nu neuroblastoma cell-derived xenografts significantly attenuated tumor growth *in vivo*. Since a single administration was quite effective, the therapeutic effects of RNA aptamers seem to be superior to those of the other molecular tools listed in Table 8.2. Because RNA aptamers consist of RNA, their stability *in vivo* should be the major subject to be addressed. One of the characteristics of RNA aptamers is their applicability to chemical modification. In fact, certain modifications have been shown to make RNA aptamers stable enough for systemic administration (Ishiguro et al. 2011).

No current molecular tools against midkine are especially based and focused on the interaction between midkine and sugar chains. The involvement of those interactions in cancer has also yet to be addressed. Because its relationship with sugar chains is likely to be one of the most important aspects of midkine, it is quite important that researchers studying midkine and its relation to cancers consider the impact of sugar chains.

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Chapter 9
Tumor-Associated Glycans and Their Functional Roles in the Multistep Process of Human Cancer Progression

Reiji Kannagi, Keiichiro Sakuma, Bi-He Cai, and Shin-Yi Yu

Abstract Cancer develops through a multistep process of carcinogenesis. This process accompanies incremental alterations of expression of biologically functional glycans on the surface of cancer cells. A variety of glycans are expressed in nonmalignant epithelial cells, including several normal glycans serving as ligands for siglecs, the immunosuppressive molecules carried by interstitial immune cells. These normal glycans decrease or disappear and are replaced by cancer-associated glycans at the early stages of carcinogenesis. This glycan transition facilitates production by mucosal immune cells of inflammatory mediators that are known to promote cancer progression. Expression of glycans that regulate growth factor receptor functions is also affected at the early stages of cancers. The major mechanism involved in glycan alteration at the early stages is epigenetic silencing through DNA methylation and/or histone deacetylation/methylation of genes responsible for synthesis of normal glycans, leading to their incomplete synthesis. In the locally advanced stages, multiple glycan-related genes are induced transcriptionally and posttranscriptionally by tumor hypoxia and epithelio-mesenchymal transition, thus further culminating in abnormal expression of cancer-associated glycans. Some such glycans serve as specific ligands for selectins, the cell adhesion molecules carried by vascular endothelial cells, and facilitate tumor vascularization and ultimately hematogenous metastasis. Advanced cancer cells which have undergone epithelio-mesenchymal transition share biological characteristics with so-called cancer stem cells, and glycans associated with such cells are currently known to be frequently expressed in human embryonic stem cells as well.

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Keywords Cancer progression • Sialyl Lewis A • Sialyl Lewis X • Epigenetic silencing • DNA methylation • Histone deacetylation/methylation • Sulfate transporter • Siglec • Selectin • Hypoxia inducible factor • Epithelio-mesenchymal transition (EMT) • Cancer stem cells • Embryonic stem (ES) cells

9.1 Introduction

Cancer develops through a multistep process of carcinogenesis. Tumor progression is caused by genetic and epigenetic alterations of a variety of key regulatory molecules, and this process accompanies an incremental alteration of expression of biologically functional glycans on the surface of cancer cells. Here we will review recent findings on the mechanisms through which genetic and epigenetic alterations affect glycan expression in cancer cells and the pathophysiological roles of altered glycan expression during the course of cancer progression.

9.2 Epigenetic Silencing of Glycan-Related Genes Causing "Incomplete Synthesis" of Normal Glycans at Early Stages of Cancer

It has long been known that glycans undergo drastic changes upon carcinogenesis. We had classified the cancer-associated changes of glycan expression into two categories almost three decades ago; one had been "incomplete synthesis" of normal glycans and the other "*neo*synthesis" of abnormal glycans (Hakomori and Kannagi 1983).

The concept of "incomplete synthesis" had referred to the accumulation of structurally simpler abnormal glycans due to disturbance of synthetic pathways for normal glycans, which mainly occurs during the course of early carcinogenesis. This concept assumed some suppression to occur in the transcription/translation of genes involved in the synthesis of normal glycans during carcinogenesis. At present, the major suppression mechanism is regarded to be epigenetic silencing. The process of "incomplete synthesis" is known to start working at relatively early stages of carcinogenesis.

On the other hand, the concept of "*neo*synthesis" had referred to the appearance of abnormal glycans in cancers, which are not present, or present only in a negligible amount, in normal cells. This had been assumed to be due to transcriptional induction of genes involved in the synthesis of abnormal glycans along with progression of cancers. Nowadays, acceleration of transcription/translation of genes involved in the synthesis of these abnormal glycans is known to occur frequently along with the multistep progression of cancers at advanced stages.

9.3 Examples of Key Glycan-Related Genes Exhibiting Epigenetic Silencing at Early Stages of Cancer

A variety of glycans are expressed in normal epithelial cells, expression of some of which is conventional in that they are also constitutively expressed in cancers. In contrast, some other glycans exhibit preferential expression in nonmalignant epithelial cells and tend to decrease or disappear and be replaced by cancer-associated glycans upon malignant transformation. These glycans are aptly named "normal" glycans, although in a narrow definition of the word (Fig. 9.1).

Such normal glycans include disialyl Lewis A, which was found to be preferentially expressed in nonmalignant epithelial cells of the digestive organs (Kannagi et al. 1988; Itai et al. 1990, 1991), and clinical evaluation of it in patients' sera was proposed to be useful for making differential diagnoses of benign and malignant diseases, especially when routine serum determination of sialyl Lewis A, a well-known cancer-associated glycan, gave false-positive results (Kannagi 2007). Soon it was found that the disialyl Lewis A glycan is a normal counterpart of the cancer-associated glycan, sialyl Lewis A, and that epigenetic silencing of *ST6GalNAc6*, a gene for α2,6sialyltransferase, is the key for diminishing expression of disialyl Lewis A and inducing sialyl Lewis A in cancers (Miyazaki et al. 2004). Downregulation of *ST6GalNAc6* was observed at the early stages of colon carcinogenesis in the normal-adenoma-carcinoma sequence (Bowden et al. 2007). This interconversion of glycans seems to be applicable to a wider range of cancers than initially expected. In addition to the cancers of digestive organs, preferential loss of disialyl Lewis A expression is also noted in prostate cancers (Young et al. 1988), and cancer-associated decrease of the *ST6GalNAc6* mRNA level is noted in breast (Potapenko et al. 2010) and renal (Senda et al. 2007) cancers, as well as glioblastoma (Kroes et al. 2007).

Another example of a "normal" glycan is sialyl 6-sulfo Lewis X, which was found to be preferentially expressed in nonmalignant epithelial cells of the colon and to disappear in colonic cancer cells (Izawa et al. 2000). This finding was in line with the classical histochemical finding on colon cancer glycans that the amount of sulfomucin is decreased in cancers compared to nonmalignant colonic tissues (Shamsuddin et al. 1981). Sialyl 6-sulfo Lewis X glycan is a normal counterpart of the well-known cancer-associated glycan, sialyl Lewis X, and epigenetic silencing of *SLC26A2*, a gene for sulfate transporter DTDST, was found to be a key mechanism responsible for diminished expression of sialyl 6-sulfo Lewis X and appearance of sialyl Lewis X in cancers (Yusa et al. 2010). Downregulation of *SLC26A2* was also observed at the early stages of colon carcinogenesis (Lee et al. 2006). It is notable that not only the genes for glycosyltransferases, which are directly involved in glycan synthesis, but also those for some transporters or enzymes in the intermediate carbohydrate metabolism are capable of playing a key role in the cancer-associated glycan alteration.

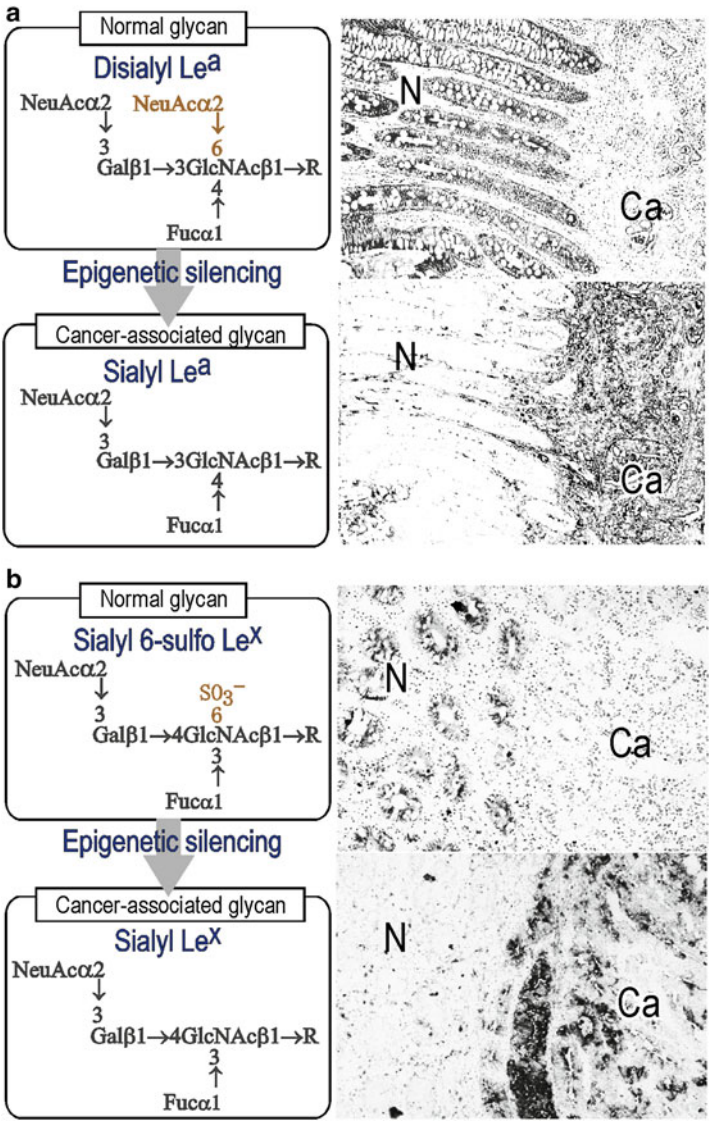

Fig. 9.1 Examples of interconversion of normal glycans into cancer-associated glycans. *Panel a*, transition of normal glycan, disialyl Lewis A, to cancer-associated glycan, sialyl Lewis A, upon malignant transformation. *Panel b*, conversion of normal glycan, sialyl 6-sulfo Lewis X, to a cancer-associated glycan, sialyl Lewis X glycan, upon malignant transformation. Typical distribution patterns shown were obtained by immunohistochemical staining using specific anti-glycan antibodies of consecutive sections prepared from colon cancer tissues. *Ca* cancer cells, *N* nonmalignant epithelial cells (Adopted from references Izawa et al. 2000; Miyazaki et al. 2004; Lim et al. 2008)

9.4 Biological Functions of Normal Glycans

The hallmark of early carcinogenesis is the acquisition of a highly proliferative activity and/or suppression of apoptosis by the transforming cells. Several glycans are known to affect cell proliferation. GM3 and other monosialogangliosides have long been known to suppress EGF receptor signaling by their direct interaction with the receptor molecule (Bremer and Hakomori 1982; Hakomori 2010). Likewise, GM2 is known to suppress c-Met kinase pathway (Todeschini et al. 2008). On the other hand, disialogangliosides, such as GD3 and GD2, are reported to enhance cell proliferation through activation of FAK and Lyn kinases (Furukawa et al. 2012). N-glycans and related genes including *MGAT5* are also known to affect growth factor receptor signaling (Matsumoto et al. 2008; Park et al. 2012; also reviewed in Lau and Dennis 2008). The glycan-related genes involved in O-glycan synthesis such as *GALNT14*, *GALNT2*, and *C1GALT1* on death receptors, and growth factor receptors such as c-Met and EGFR have recently been suggested to affect cancer cell apoptosis and proliferation (Wagner et al. 2007; Wu et al. 2011, 2013). Altered expression of these glycans may well play significant roles during the course of carcinogenesis.

There are other indications for a more indirect association of cell proliferation status with glycan expression. For instance, the decreased transcription of *SLC26A2* in cancers mediates the loss of normal glycan sialyl 6-sulfo Lewis X and induction of cancer-associated glycan sialyl Lewis X and at the same time strongly induces cell proliferation (Yusa et al. 2010). The growth suppressive effect of the *SLC26A2* gene was clearly reproduced in experiments using a Tet-off expression vector for *SLC26A2* (Yusa et al. 2010) (Fig. 9.2). It is not clear whether or not the growth suppression conferred by this gene is due to its effects on glycan sulfation, because *SLC26A2* is a sulfate transporter gene which can affect not only glycan sulfation but also sulfation of other molecules such as proteins and lipids. Still, it can at least be proposed that this is another example indicating the close link between change in glycan expression and cell proliferation status.

Cancer microenvironments also play crucial roles during the course of carcinogenesis. In the mouse model of colon carcinogenesis, mutation in the APC gene induces proliferation of epithelial cells leading to multiple benign polyp formation. Meanwhile, Taketo's group found that the malignant transformation of adenoma cells was observed selectively at the locus where interstitial cells in colonic mucosal membranes produce COX2, which is a pro-inflammatory molecule known to promote cancer progression (Oshima et al. 1996). This finding became the theoretical basis for developing COX2 inhibitors for the chemoprevention of colon carcinogenesis. Although difficulty was encountered during the development of specific COX2 inhibitors because they have intrinsic toxic cardiac effects, it is still true that inflammatory signaling pathways are activated in various cancers including colon cancer linking chronic inflammation to oncogenesis. Alternative modalities are now awaited for the chemoprevention of colon carcinogenesis.

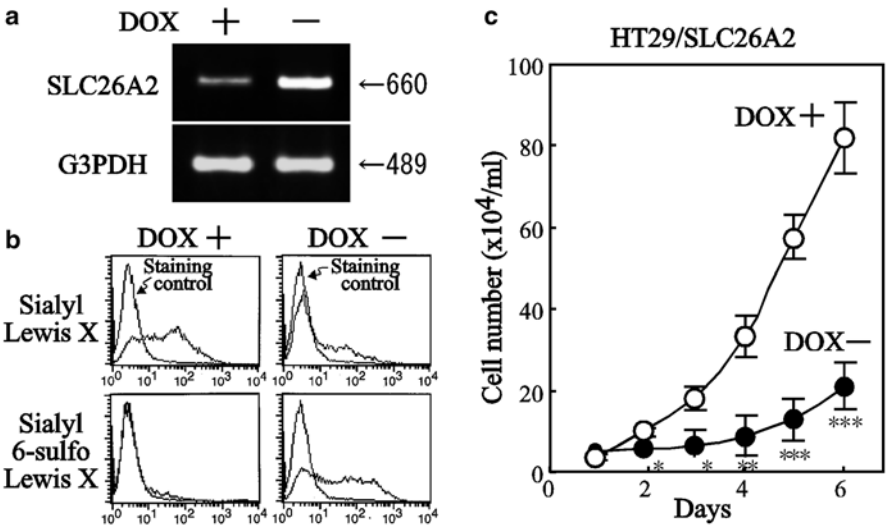

Fig. 9.2 Possible link between glycan expression change and cancer cell growth. Results of RT-PCR (*panel a*), flow-cytometry (*panel b*), and cell proliferation assays (*panel c*) are shown on colon cancer cell line HT29 that was transfected with Tet-off inducible expression vector for *SLA26A2*. When cultured without doxycycline, the *SLA26A2* gene is actively transcribed, resembling nonmalignant epithelial cells, and the normal glycan, sialyl 6-sulfo Lewis X, is strongly expressed on the cell surface. This is associated with suppression of cell proliferation. In contrast, when cultured with doxycycline, transcription of *SLA26A2* is repressed and resembles cancer cells, leading to the extinction of normal glycan expression and induction of the cancer-associated glycan, sialyl Lewis X, which is coupled with enhanced cell proliferation. The difference was statistically significant at *, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$ (Adopted from reference Yusa et al. 2010)

In this context, it is noteworthy that the normal glycans in colonic epithelial cells such as disialyl Lewis A and sialyl 6-sulfo Lewis X were both shown to serve as ligands for siglecs, which are glycan-recognition molecules having immunosuppressive ITIM motifs in their cytoplasmic domains and are expressed by a variety of immune cells. The sialyl 6-sulfo Lewis X glycan was shown to be the ligand for siglec-7, and disialyl Lewis A was found to serve as a ligand for both siglec-7 and siglec-9 (Miyazaki et al. 2004, 2012) (Fig. 9.3). A significant number of tissue macrophage-like cells expressing siglec-7 or siglec-9 were present in normal colonic mucosal membranes, and ligation of either siglec had suppressive effects on the production of COX2 and IL-12 by macrophage-like cells (Miyazaki et al. 2012) (Fig. 9.3). Based on these results, it was proposed that normal glycans may play a role in maintaining immunological homeostasis and preventing cancer progression. The loss of these normal glycans due to epigenetic silencing of the key genes upon malignant transformation is expected to further facilitate carcinogenesis.

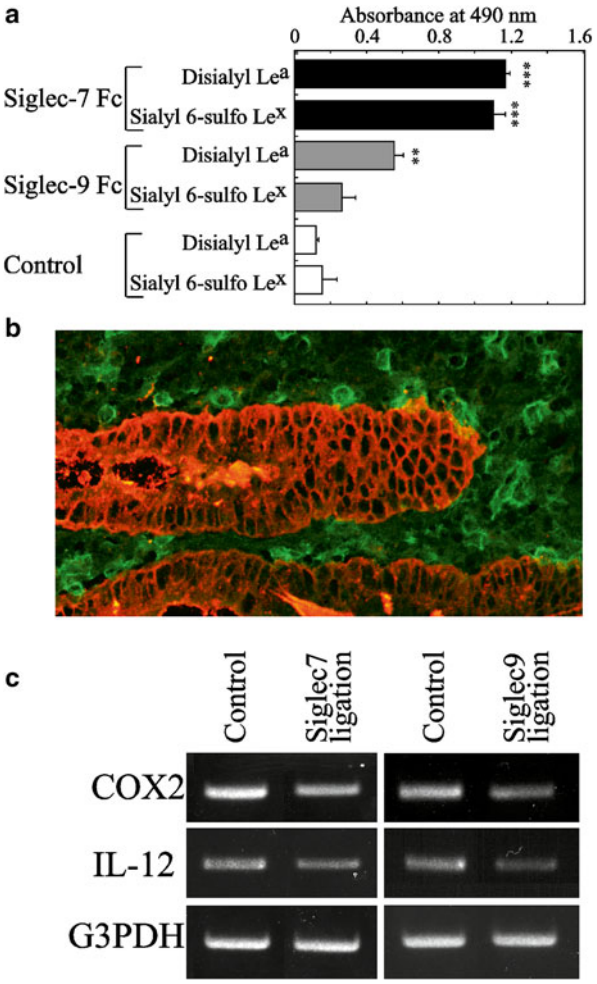

Fig. 9.3 Normal glycans serve as ligands for siglecs. *Panel a*, normal glycans such as disialyl Lewis A and sialyl 6-sulfo Lewis X, but not cancer-associated glycans, serve as specific ligands for siglecs, which are immunosuppressive receptors carried by immune cells in mucosal membranes. *Panel b*, a human colon tissue stained with anti-siglec and anti-glycan MoAbs, suggesting possible interaction between tissue macrophage-like cells expressing siglec-7 (*green*) and colonic epithelial cells expressing its ligand disialyl Lewis A (*red*). *Panel c*, suppression of LPS-induced COX2 and IL-12 in cultured macrophage-like cells by ligation of siglec-7/-9. TPA-treated U937 cells transfected with siglec-7 or siglec-9 were stimulated with LPS with or without a $F(ab')_2$ fragment of an anti-siglec-7 or anti-siglec-9 antibody (Adopted from references Miyazaki et al. 2004, 2012)

9.5 Mechanisms for Epigenetic Silencing of Glycan-Related Genes During the Course of Carcinogenesis

Aberrant promoter CpG island hypermethylation is one of the most common and well-established epigenetic abnormalities in cancer. In earlier studies, DNA methylation of A- and B-enzymes was shown to cause a decrease of normal A- and B-blood type glycans in cancers (Kominato et al. 1999; Chihara et al. 2005). Likewise, loss of the Sd^a blood group substance in colon cancer was shown to be due to DNA methylation of the *B4GALNT2* gene promoter (Kawamura et al. 2008). The decrease in all these glycan epitopes attached to the lactosamine or polylactosamine backbone structures is proposed to facilitate expression of cancer-associated glycan epitopes such as sialyl Lewis A and sialyl Lewis X, synthesized from common precursors, by leaving the surplus substrates for the enzymes responsible for synthesis of the latter two cancer-associated glycans (Kannagi et al. 2008).

Table 9.1 summarizes hitherto known glycan-related genes in cancers which represent epigenetic silencing. There are many examples of genes involved in glycan synthesis which are regulated by DNA methylation. *HS3ST2* (*3OST2*) is a typical example of genes known to be strongly hypermethylated in a variety of cancers (Miyamoto et al. 2003) and is sometimes utilized even as a positive control in DNA methylation analyses. The biological significance of this gene was not known until the heparan sulfate 3-O-sulfotransferase encoded by this gene was recently reported to suppress cell proliferation and migration (Hwang et al. 2013). The exact mechanisms behind these observed phenomena await further investigation, but this is an indication of another link between glycan expression and cancer cell proliferation. The roles of extracellular heparan sulfates as extracellular coreceptors for growth factors have been well documented (Fuster and Esko 2005).

Histone modification is also intimately involved in cancer-associated epigenetic silencing of glycan-related genes. Transcription of *ST6GalNAc6* was initially reported to be recovered either by treatment with histone deacetylation inhibitor (butyrate) or DNA methylation inhibitor (5-aza-2′-deoxycytidine) (Miyazaki et al. 2004), but the effect of the DNA methylation inhibitor was later found to be dependent on the cell lines used in experiments, and histone deacetylation turned out to be the major mechanism for silencing this gene. Histone modification was also proposed to be the major mechanism for epigenetic silencing of the sulfate transporter gene *SLC26A2*, and in the case of this gene, significant participation of histone trimethylation at H3K27 was suspected in addition to histone deacetylation (Yusa et al. 2010) (Fig. 9.4). Accordingly, addition of not only HDAC inhibitors, but histone methyltransferase inhibitor DZNep, was shown to stoichiometrically induce *SLC26A2* transcription (Fig. 9.4). Participation of DNA methylation in cancer-associated suppression of this gene was recently reported for papillary thyroid cancer (Zhang et al. 2012).

Epigenetic drugs are not yet actively utilized for therapy of cancers, but several reports suggest them to be beneficial for chemoprevention of cancers (Ravillah et al. 2014; Timp and Feinberg 2013). Assessment of normal glycan expression may be useful for monitoring therapeutic effects in such regimens.

Table 9.1 Examples of glycan-related genes in cancers which are known to represent epigenetic silencing

Genes	Glycans involved	Mechanisms	References
ABO	ABO(H) blood group substance	DNA methylation	Kominato et al. (1999)
HS3ST2 (*3OST2*)	Heparan sulfate (3S)	DNA methylation	Miyamoto et al. (2003)
GNE	CMP-sialic acid	DNA methylation	Oetke et al. (2003)
ST6GALNAC6	Sialyl Lewis A/disialyl Lewis A	Histone acetylation, DNA methylation	Miyazaki et al. (2004)
EXT1	Heparan sulfate	DNA methylation	Ropero et al. (2004)
EXTL3	Heparan sulfate	DNA methylation	Karibe et al. (2008)
FUT3	Lewis A	DNA methylation	Serpa et al. (2006)
MGAT5	*N*-glycan branching	DNA methylation	Chakraborty et al. (2006)
MGAT4A	*N*-glycan branching	DNA methylation, histone acetylation	Ide et al. (2006)
SULF1	Heparan sulfate (6S)	DNA methylation, histone acetylation	Staub et al. (2007)
B4GALNT2, *ST3GAL6*	Sd^a/sialyl Lewis A	DNA methylation	Kawamura et al. (2008)
B4GALNT2	Sd^a	DNA methylation	Wang et al. (2008)
SLC26A2 (DTDST, sulfate transporter)	Sialyl Lewis X/sialyl 6-sulfo Lewis X	Histone methylation, histone acetylation	Yusa et al. (2010)
FX, *SLC35C1* (GDP-fucose transporter), *FUT4*	Fucose in TRAIL signaling	DNA methylation	Moriwaki et al. (2010)
HS3ST1, *HS3ST2*, *HS3ST3A1* (*3-OST1*, *3-OST2*, *3-OST3A*)	Heparan sulfate (3S)	DNA methylation	Bui et al. (2010)
C4ST1, *DSE*	Chondroitin/dermatan sulfate	DNA methylation	Kalathas et al. (2010)
GMDS, *FX*, *MGAT4A*, *MGAT5*	Core fucose/*N*-glycan branching	DNA methylation	Saldova et al. (2011)
ST3GAL6	Sialyl Lewis X	DNA methylation	Chachadi et al. (2011)
HS3ST3B1 (*3-OST-3B1*)	Heparan sulfate (3S)	DNA methylation, histone acetylation	Song et al. (2011)
B4GALNT1, *ST8SIA1*	Gangliosides	histone acetylation	Suzuki et al. (2011)

(continued)

Table 9.1 (continued)

Genes	Glycans involved	Mechanisms	References
MGAT5B	*N*-glycan branching	DNA methylation, histone acetylation, histone methylation	Kizuka et al. (2011)
B3GALT5	Sialyl Lewis A	DNA methylation, histone acetylation, histone methylation	Caretti et al. (2012)
Cosmc	Mucin core 1	DNA methylation	Mi et al. (2012)
B3GALT1	Sialyl Lewis A	Histone acetylation	Chachadi et al. (2013)
B3GNT7	Sialyl Lewis A/X	DNA methylation	Lu et al. (2014)

Candidate glycans which are expected to be affected by these alterations are also included

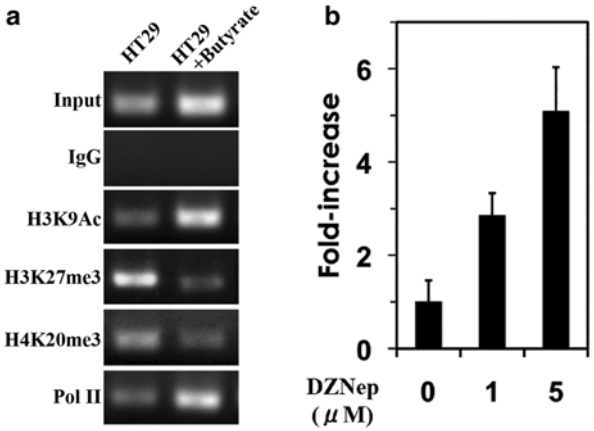

Fig. 9.4 Epigenetic silencing of *SLC26A2* gene in colon cancer cells. *Panel a*, results of ChIP assays in human colon cancer HT29 cells cultured with or without an HDAC inhibitor, butyrate. *Panel b*, effect of histone methyltransferase inhibitor DZNep on the transcription level of the *SLC26A2* gene (Adopted from reference Yusa et al. 2010)

9.6 Acquisition of Resistance to Hypoxia by Cancer Cells in Advanced Stage of Cancers

At the locally advanced stages, cancer cells must cope with hypoxic environments to survive and proliferate, and some cancer cell clones having hypoxia-resistant characteristics appear through accumulation of genetic anomalies. Such hypoxia-resistant cancer cells usually exhibit a higher proliferating rate, enhanced cell

mobility, greater angiogenic activity, and stronger multidrug resistance, thus having multiple advantages over other cancer cell clones, and will eventually occupy all cancer cell nests. The transcription factor HIF-1α plays a central role for cancer cells in acquiring hypoxia-resistant characteristics.

Intense changes of glycan expression are observed in advanced-stage cancers, and this is partly because HIF-1α induces the transcription of a variety of genes involved in the synthesis of glycans (Kannagi 2004, 2010). For instance, tumor hypoxia induces, through the action of HIF-1α, transcription of genes for sialyltransferase, fucosyltransferase, and UDP-galactose transporter, which are involved in the synthesis of sialyl Lewis A and sialyl Lewis X (Koike et al. 2004). Tumor hypoxia thus enhances expression of sialyl Lewis A and sialyl Lewis X in cancer cells, which further help cancer cells in coping with hypoxic environments, since these glycans serve as ligands for vascular E-selectin and mediate adhesion of cancer cells to endothelial cells. Interaction between E-selectins on endothelial cells and its ligands on cancer cells is known to facilitate tumor vascularization (Tei et al. 2002). HIF-1α also induces transcription of genes for several enzymes in the synthetic pathway of the lipid moiety of glycolipids, and this is expected to affect their localization in cell membrane microdomains (Yin et al. 2010).

The gene for sialin, *SLS17A5*, is also induced by HIF-1α (Yin et al. 2006). Normal cells synthesize sialic acid, usually from the de novo synthetic pathway starting from UDP-GlcNAc. Cancer cells seem to enhance the de novo synthetic pathway to some extent to meet the increased demands for sialoconjugate synthesis (Go et al. 2007), but upon progression, cancer cells tend to rely more on the salvage pathway, which reutilizes the sialic acid residues cleaved from exogenous glycoconjugates in lysosomes. Sialin is a lysosomal sialic acid transporter that pumps in free sialic acids released in lysosomes to cytoplasm and is closely involved in the salvage pathway. In humans, sialic acid species provided by the de novo synthetic pathway is limited to NeuAc but not NeuGc, because humans lack the enzyme which converts NeuAc to NeuGc. On the other hand, sialic acids transported by sialin contain NeuGc, the nonhuman sialic acid derived from fetal calf serum under cell culture conditions and from a dietary origin under in vivo conditions. Consequently, the amount of glycans containing NeuGc in cancer cells having enhanced sialin activity is usually higher than that in nonmalignant cells. A glycan containing NeuGc was sometimes known to be antigenic to humans and was termed Hanganatziu-Deicher antigen. This antigen was for a long time counted as a member of cancer-associated glycans, as cancers have a higher amount of NeuGc-containing glycans than normal tissues. The Hanganatziu-Deicher antigen occurs late during cancer progression, mainly in the advanced stages of cancers, because its appearance is driven by HIF-1α, which starts to work in the advanced stages. Recently, cultured ovarian cancer cells having an extremely high NeuGc content were reported, and other mechanisms, in addition to enhanced sialin transcription, were suggested to be involved in this extremely high NeuGc expression (Inoue et al. 2010).

9.7 Glycan Alteration by Epithelio-Mesenchymal Transition of Cancer Cells in Advanced Stages of Cancers

Epithelio-mesenchymal transition (EMT) is a critical event in the advanced stages of cancers which prepares cancer cells for metastasis. EMT is caused by a well-defined set of transcription factors and is found to induce several genes related to glycan expression such as sialyltransferases, fucosyltransferases, and galactosyltransferases, which are also involved in the synthesis of sialyl Lewis A and sialyl Lewis X glycans (Sakuma et al. 2012). Consequently, cancer cells which had undergone EMT have a higher expression of the sialyl Lewis A and sialyl Lewis X glycans and strongly bind to vascular E-selectin (Fig. 9.5). Several decades ago, it had been noticed that cancer cells in the invasion front having a mesenchymal cell-like morphology frequently express these glycans strongly (Ono et al. 1996). Judging from the recent findings mentioned above, this must have been due to the EMT-induced transcription of glycan-related genes.

The best-known function of cancer-associated glycans is that sialyl Lewis A and sialyl Lewis X glycans serve as vascular E-selectin and mediate hematogenous metastasis of cancer cells (Phillips et al. 1990; Takada et al. 1991, 1993). For adhesion to occur, however, these glycans need to be expressed in a density high

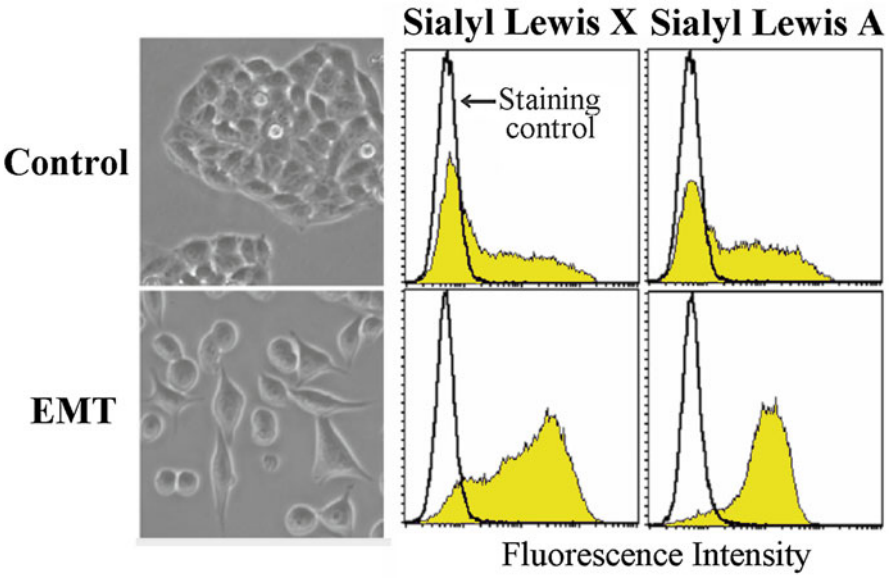

Fig. 9.5 Induction of cancer-associated glycans sialyl Lewis A and sialyl Lewis X in cancer cells underwent epithelio-mesenchymal transition (EMT). Human colon cancer cells DLD-1 were cultured with EGF/bFGF in serum-free medium to induce EMT. Morphological changes (*left panel*) and results of flow-cytometric analyses of sialyl Lewis A and sialyl Lewis X expression (*right panel*) upon EMT are shown (Adopted from reference Sakuma et al. 2012)

enough to be recognized by E-selectin. As disialyl Lewis A and sialyl 6-sulfo Lewis X are only minor components among the glycans in nonmalignant epithelial cells, their interconversion through the "incomplete synthesis" mechanism, i.e., epigenetic silencing of *ST6GalNAc6* or *SLC26A2*, would confer, although significant, only a weak expression of sialyl Lewis A and sialyl Lewis X glycans. The high-density expression of these cancer-associated glycans could be achieved only after further enhancement of their synthesis through transcriptional induction by hypoxia and/or EMT of additional glycan-related genes, the mechanism defined as "*neo*synthesis" in our previous proposition. The two mechanisms, "incomplete synthesis" and "*neo*synthesis," are not mutually exclusive; they sometimes work on the same glycans in a stepwise manner during the multistep progression of cancers. In contrast, appearance of the NeuGc-containing glycans (Hanganatziu-Deicher antigens) in cancer cells can be regarded to have stemmed exclusively from the "*neo*synthesis" mechanism, which specifically occurs in the advanced stages of cancer progression.

9.8 Glycans Associated with Cancer Stem Cells and Embryonic Stem Cells

Characteristics of EMT-induced cancer cells are known to be very similar to those of so-called cancer stem cells (Mani et al. 2008). Expression of sialyl Lewis A and sialyl Lewis X was enhanced in cancer cells after EMT, while a paradoxical decrease was noted in the expression of some other glycans that had been assumed to be cancer associated, such as Lewis Y glycan (Sakuma et al. 2012). This unexpected finding suggested that some hitherto known cancer-associated glycans, exemplified by sialyl Lewis A/X, are linked to a more malignant population of cancer cells such as cancer stem cells, whereas others are not. Examples of glycan-related genes which have been reported to exhibit altered transcription levels in EMT-induced cancer cells and/or cancer stem cells are shown in Table 9.2, together with the candidate glycan species which are expected to be affected by these alterations.

Cell surface glycans are known to be good markers for embryonic stem (ES) cells in the field of stem cell research. Good examples are SSEA-1 for murine ES cells and SSEA-3/-4 for human ES cells. However, the mechanisms of how these glycans appear specifically on the surface of ES cells have not been elucidated yet. It is well known that a combination of several transcription factors such as OCT3/4, Nanog, and Sox-2 plays critical roles in maintaining the stemness in these cells (Takahashi et al. 2007), while transcriptional regulation of ES cell-associated glycan expression still remains largely unknown. There were sporadic publications reporting that SSEA-3/-4 glycans sometimes appear in human cancer cells (Schrump et al. 1988; Suzuki et al. 2013; Gottschling et al. 2013; Lou et al. 2014), and it was recently reported that these glycans are specifically expressed in cancer stem-like cells (Chang et al. 2008; Noto et al. 2013).

Table 9.2 Examples of glycan-related genes reported to exhibit altered transcription levels in EMT-induced cancer cells and/or cancer stem cells

Genes	Glycans involved	Mechanisms	References
B3GALT4 (decrease)	Gg4	EMT induced by hypoxia	Guan et al. (2010)
HAS2 (increase)	Hyaluronan	EMT induced by TGFβ	Craig et al. (2010)
HAS2, *HAS3* (increase)	Hyaluronan	EMT induced by EGF and IL-1β	Chow et al. (2010)
GCNT2 (increase)	I-branching	EMT induced by TGFβ	Zhang et al. (2011)
ST3GAL1/3/4, *FUT3* (increase); *FUT2* (decrease)	Sialyl Lewis A/sialyl Lewis X	EMT induced by EGF/bFGF	Sakuma et al. (2012)
MGAT3 (decrease)	*N*-glycan	EMT induced by TGFβ	Xu et al. (2012)
MGAT3 (decrease)	*N*-glycan	EMT induced by TGFβ	Pinho et al. (2012)
GCNT1 (increase)	*O*-glycan	Breast cancer tumor-initiating cells	Kim et al. (2012)
ST8SIA1 (increase)	GD2	Cancer stem cells induced through EMT	Battula et al. (2012)
UGCG (increase)	Glycolipids, Gb3	Breast cancer stem cells	Gupta et al. (2012)
HAS2 (increase)	Hyaluronan	Breast cancer stem cells	Okuda et al. (2012)
MGAT5, *FUT8*, and *B3GALT5* (increase); *MGAT3* (decrease)	Lectin binding	EMT induced by HGF	Li et al. (2013)
ST6GAL1	*N*-glycan	Colon cancer stem cells	Swindall et al. (2013)
FUT8 (increase)	*N*-glycan	EMT induced by TGFβ	Chen et al. (2013)
ST3GAL5 (increase)	GM3	EMT induced by TGFβ	Kim et al. (2013)
ST3GAL5, *B4GALNT1*, *ST8SIA1*, *ST3GAL2* (increase)	GD2, GD3, GM2, GD1a	Cancer stem cells induced through EMT	Liang et al. (2013)

Candidate glycans expected to be affected by these alterations are also included

SSEA-3/-4 glycans are classified into the globo-series glycolipids (Kannagi et al. 1983), which compose a unique series of glycolipids having glycan structures confined to glycolipids, and are not easily detectable in glycoproteins. Recently, another classical stem cell-specific glycan, TRA-1-60, was reported to be a type 1 chain glycan, having common backbone structures with glycans carried by both glycolipids and glycoproteins (Natunen et al. 2011). This glycan is structurally very similar to that of sialyl Lewis A, sharing the same enzymes in most steps of their synthetic pathways.

The recently introduced fucosylated stem cell-associated glycan, SSEA-5, again had the backbone structure of the type-1 chain glycan (Tang et al. 2011). Finally, sialyl Lewis A itself was shown to be expressed in human ES cells and to disappear upon ES cell differentiation, thus behaving as a stem cell-specific glycan (Tang et al. 2011). These findings strongly suggest that there are some common features between glycans specific to ES cells and those associated with cancer stem cells.

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Chapter 10
Mammalian Sialidase and Tumor Development

Taeko Miyagi, Kohta Takahashi, Kazuhiro Shiozaki, and Kazunori Yamaguchi

Abstract Sialidases are glycosidases responsible for the removal of α-glycosidically linked sialic acid residues from carbohydrate portions of glycoproteins and glycolipids. They are found widely distributed in common in metazoan animals, from echinoderms to mammals, and also in viruses and other microorganisms. In mammals, there are four types of sialidase (NEU1, NEU2, NEU3, and NEU4), encoded by different genes differing in their major subcellular localization and enzymatic properties. They have been implicated to participate in the regulation of various cellular functions, with roles in cell differentiation, cell growth, and cell adhesion and motility, depending on their particular properties, whereas in microorganisms the same enzymes appear to play roles limited to nutrition and pathogenesis. Aberrant expression of mammalian sialidases has been demonstrated in cancer, causing dysregulation of cell homeostasis and contributing to tumor development. The present review aims to provide a brief overview of our recent investigations into the significance of mammalian sialidases and mechanisms underlying their actions relevant to neoplasia.

Keywords Sialidase • Sialic acid • Ganglioside • Glycoprotein • Cancer • Transmembrane signaling • Metastasis

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10.1 Introduction

Sialic acids are acidic monosaccharides generally found in the terminal positions on a variety of glycoproteins and glycolipids. They actually play crucial roles in various biological processes by influencing chemical and biological features of the glycoconjugates, probably due to their negative charge (Schauer 2009). Sialic acids show differences in quantity as well as structure during cell differentiation, proliferation, and carcinogenesis and may contribute as virulence factors in bacterial and viral infection (Varki and Varki 2007; Miyagi and Yamaguchi 2007).

Aberrant sialylation in cancer suggests its association with malignant properties including invasiveness and metastasis, primarily supported by findings of reduced malignancy of cancer cells treated with bacterial sialidase. Structural studies have documented that a general increase in sialylation is often found in cell surface glycoproteins of malignant cells, and altered sialylation of glycolipids is also observed as a ubiquitous phenotype, associated with the appearance of tumor-associated antigens, aberrant adhesion, and blocking of transmembrane signaling (Lau and Dennis 2008; Hakomori 2010). Despite the large number of reports describing the involvement of sialic acids in cancer, the molecular mechanism and significance are not fully understood. Especially, drawing definite conclusions regarding physiological links between sialic acid contents and malignant properties is difficult, due to controversial experimental results.

Sialylation is mainly regulated by sialidases and sialyltransferases which cleave sialic acid residues from and transfer them to glycoconjugates, respectively. As metabolic enzymes, alterations of the levels of ganglioside sialidase activity associated with malignant transformation were reported in 3T3-transformed cells (Yogeeswaran and Hakomori 1975) and in BHK-transformed cells (Schengrund et al. 1973). However, it remained uncertain whether the activities originated from the same or different types of sialidase. Our biochemical isolation and characterization of sialidases from murine tissues then provided evidence for the existence of four types of sialidases differing in their subcellular localization and enzymatic properties (Miyagi and Tsuiki 1984, 1985; Miyagi et al. 1990a). Based on the studies, we previously reported an increase of ganglioside sialidase activity in plasma membrane fractions and a decrease in lysosomal sialidase activity to be associated with the induction of anchorage-independent growth in mouse epidermal JB6 cells exposed to phorbol esters (Miyagi et al. 1990b). To further elucidate the mechanism underlying this aberrant sialylation and with the aim of developing an effective cure for cancer, we have been focusing on mammalian sialidases from their molecular aspects.

Consistent with our biochemical studies, recent progress of the gene cloning studies has validated four types of mammalian sialidases (designated as NEU1, NEU2, NEU3, and NEU4) differing in subcellular localization and enzymatic properties as well as in the chromosomal localization. Sialidase expression levels indeed change in response to various cellular phenomena and especially during tumor development (Miyagi et al. 2012). Among the sialidases, plasma membrane-associated sialidase (NEU3), playing particular roles in the regulation of transmembrane signaling by modulation of gangliosides, is upregulated in various human cancers,

whereas NEU1 and NEU4, controlling largely the desialylation of glycoproteins, show a tendency for downregulation. Our recent studies have shown that aberrant alteration of each sialidase exerts unique influences on cancer initiation and promotion, as well as progression. Altered expression and the pathological significance of sialidases are here introduced mostly focusing on the cases of human colon, renal, and prostate cancers.

10.2 General Properties of Mammalian Sialidases

Since sialidase activity in higher organisms was first detected in preparations of Cohn Fraction VI (Warren and Spearing 1960), numerous papers have demonstrated its presence in a wide variety of mammalian cells and tissues. Molecular cloning has validated four forms of mammalian sialidase and facilitated elucidation of their functional roles (Miyagi and Yamaguchi 2012). General properties of the four human sialidases are compared in Table 10.1. NEU1, NEU2, and NEU3 are now known to be localized mainly in the lysosomes, cytosol, and plasma membranes, respectively, and NEU4 is found in lysosomes or in mitochondria/the endoplasmic reticulum. However, recent observations have also revealed that the subcellular localization can vary with particular cellular stimuli. In fact, NEU1 and NEU4 as well as NEU3 are also observed at cell surface depending on localization of their accessible natural substrates.

Sialidases of mammalian origin have been found to differ from those of microbial origin in their overall primary sequences and enzymatic properties, although they contain RIP (−Phe (Tyr)-Arg-Ileu-Pro-) sequence and Asp boxes (−Ser-X-Asp-X-Gly-X-Thr-Trp-) in the primary sequences, conserved in viral and bacterial sialidases. Distinctive behavioral differences in response to inhibitors between mammalian and microbial sialidases are suggestive of molecular variation. For example, anti-influenza drugs, Tamiflu (oseltamivir) and Relenza (zanamivir), are

Table 10.1 Human sialidases

	Major subcellular location	Good substrate	Frequent change in cancer
NEU1	Lysosome	Oligosaccharides	
		Glycopeptides	
NEU2	Cytosol	Oligosaccharides	Hardly detectable
		Glycoproteins	
		Gangliosides	
NEU3	Plasma membrane	Gangliosides	
NEU4	Lysosomes Mitochondria ER	Oligosaccharides	
		Glycoproteins (including mucin)	
		Gangliosides	

known to be effective in vitro at nanomolar concentrations for the viral sialidase, but Tamiflu hardly affects any of the human sialidases, even at 1 mM, and Relenza inhibits only in the micromolar range (Hata et al. 2008; Chavas et al. 2010). Detailed three-dimensional structural information has been provided for human NEU2 by X-ray crystallography (Chavas et al. 2005). The enzyme possesses the 6-blade β-propeller structural organization typical of bacterial sialidases and viral neuraminidases, with its active site in a shallow crevice. Among human sialidases, the overall amino acid identity of NEU1 to the other forms is relatively low (19–24 %), whereas NEU2, NEU3, and NEU4 show 34–40 % homology to each other. Regarding comparative expression levels, in human, NEU1 generally shows the strongest expression, 10–20 times greater than those of NEU3 and NEU4, while NEU2 expression is extremely low, at only four- to ten-thousandths of the NEU1 value at most in a range of tissues (Yamaguchi et al. 2005; Hata et al. 2008), although profiles differ among the human, rat, and mouse.

10.3 Characteristic Features of the Four Mammalian Sialidases

The human *NEU1* gene was identified by three research groups (Bonten et al. 1996; Milner et al. 1997; Pshezhetsky et al. 1997) as a major histocompatibility complex (MHC)-related sialidase gene on chromosome 6. The human sialidase, NEU1, has been found to be associated with a protective protein (carboxypeptidase A) and β-galactosidase as a complex in lysosomes, dissociation of the complex leading to sialidase inactivation (D'Azzo et al. 1982; Galjart et al. 1988). In sialidase assays in vitro, NEU1 efficiently hydrolyzes oligosaccharides, glycopeptides, and a synthetic substrate, 4-methylumbelliferyl-neuraminic acid (4MU-NeuAc), but hardly acts on gangliosides with optimal pH of 4.5–4.7. Oligosaccharide substrates possessing the α2–3 sialyl linkage can be hydrolyzed faster than those with α2–6 and α2–8 linkages. NEU1 is linked to two neurodegenerative lysosomal storage disorders, sialidosis and galactosialidosis (d'Azzo and Bonten 2010), the former caused by defects in genomic DNA, including frameshift insertions and missense mutations, and the latter featuring a combined deficiency of sialidase and β-galactosidase due to the absence of a functional protective protein. NEU1 has been found to negatively regulate lysosomal exocytosis, a cellular process for the recruitment of lysosomes to the plasma membrane, resulting in an increase in extracellular proteolytic activity (Yogalingam et al. 2008). It is also involved in cellular signaling for immune responses and elastic fiber assembly through transportation to plasma membranes and contributes to the regulation of phagocytosis in macrophages and dendritic cells through the desialylation of surface receptors (Pshezhetsky and Hinek 2011).

NEU2 was the first mammalian sialidase for which cDNA cloning was achieved (Miyagi et al. 1993). The human ortholog was cloned from a genomic library of the human skeletal muscle (Monti et al. 1999), and its structure has been determined by X-ray crystallography (Chavas et al. 2005). NEU2 possesses broad substrate specificity at nearly neutral pH. It participates in muscle cell and neuronal

differentiation in murine cells, and the rat *Neu2* gene contains two E-box pairs in the 5′-flanking enhancer/promoter region (Sato and Miyagi 1995), known to be consensus binding sites for muscle-specific transcription factors, and exhibits transcriptional activity in murine myogenic cells. In PC12 cells the sialidase has been suggested to participate in neuronal differentiation on the basis of nerve growth factor-induced transcriptional activation of the gene (Fanzani et al. 2004). Unlike other human sialidases, NEU2 expression as assessed by quantitative real-time PCR was found to be extremely low or undetectable in many human tissues and cells, even in cancer cells, with notable exceptions like the placenta and testis.

NEU3 was first cloned from a bovine brain library (Miyagi et al. 1999), based on peptide sequence information from purified enzyme protein (Hata et al. 1998), and later from a human cDNA and genomic library (Wada et al. 1999; Monti et al. 2000). The primary sequences covering the entire coding region of the corresponding human, mouse, and rat genes display 83 %, 79 %, and 78 % overall identity with the bovine gene, respectively. The catalyzed hydrolysis is essentially specific for gangliosides other than GM1 and GM2, in the presence of Triton X-100. The bovine and human enzymes specifically hydrolyze gangliosides, and the murine enzyme acts on oligosaccharides; a synthetic substrate, 4MU-NeuAc; and glycoproteins to a certain extent. Desialylation of GM1 and GM2 by murine enzymes is evident in the presence of GM2 activator protein (Li et al. 2001), supporting the existence of an asialo-derivative GA2 pathway for catabolism of GM2 in the mouse. With regard to the glycosidic linkage specificity, NEU3 hydrolyzes gangliosides with α2–3 (GM3) and α2–8 (GD3) linkages almost equally and faster than those with an α2–6 linkage (synthetic GM3). GM3 containing N-glycolylneuraminic acid is hydrolyzed by NEU3 at a lower rate. Unlike the bovine and murine enzymes, the human enzyme shows two peaks at pH 4.5–4.8 and at pH 6.0–6.5 (Wada et al. 1999). The major subcellular localization of the bovine and murine sialidases proved to be plasma membranes on Percoll density gradient centrifugation of cell homogenates and by immunofluorescence staining. The human ortholog NEU3, however, is not always detected on the cell surface but may exist in other cellular membrane components and can mobilize to membrane ruffles together with Rac-1 in response to growth stimuli such as EGF, enhancing cell movement (Yamaguchi et al. 2006). Recent analyses of membrane topology have suggested that the sialidase might be localized partially on the cell surface as a peripheral membrane protein and also in endosomal structures (Zanchetti et al. 2007). NEU3 participates in neurite formation in mice (Hasegawa et al. 2000) and in human neuroblastoma cells (Proshin et al. 2002) and in the regulation and regeneration of rat hippocampal neurons (Rodriguez et al. 2001; Da Silva et al. 2005). It is located in rafts of neuroblastoma cells (Kalka et al. 2001) and in caveolae of HeLa cells (Wang et al. 2002), closely associated with caveolin-1. Interestingly, NEU3 transgenic mice develop impaired insulin signaling and insulin-resistant diabetes mellitus by 18–22 weeks (Sasaki et al. 2003), associated with hyperinsulinemia, islet hyperplasia, and increase in the β-cell mass. All the results so far obtained indicate an essential involvement of NEU3 in the regulation of signal transduction through ganglioside modulation and interaction with signaling molecules (Miyagi et al. 2008).

The fourth human sialidase, NEU4, was identified based on cDNA sequences in public databases (Monti et al. 2004; Seyrantepe et al. 2004; Yamaguchi et al. 2005). With regard to its subcellular localization, two different descriptions have been reported on the basis of gene transfection studies: one featuring targeting to the lysosomal lumen (Seyrantepe et al. 2004) and the other to mitochondria and endoplasmic reticulum (Yamaguchi et al. 2005; Bigi et al. 2010). NEU4 appears to consist of isoforms which differ in their possession of 12 N-terminal amino acid residues for mitochondrial targeting. The isoforms are also differentially expressed in a tissue-specific manner, the brain, muscle, and kidney containing both and the liver and colon possessing predominantly the short form (Yamaguchi et al. 2005). It is a characteristic feature that NEU4 acts on mucin. Unlike other sialidases, NEU4 hydrolyzes sialyl Lewis antigens expressed on *O*-glycans, such as sialyl Le^x and sialyl Le^a (Shiozaki et al. 2011), and polysialic acid on NCAM (polySia-NCAM) (Takahashi et al. 2012). It may be involved in neuronal cell apoptosis, based on the observation that the NEU4 long form probably regulates levels of GD3, known to be an apoptosis-related ganglioside, in mitochondria of neuroblastoma cells (Hasegawa et al. 2007). In contrast to NEU3, NEU4 appears to negatively regulate neurite formation in Neuro2a cells and hippocampal neurons (Shiozaki et al. 2009a; Takahashi et al. 2012).

10.4 Sialidase in Colon Cancer

The upregulation of NEU3 suppresses apoptosis of colon cancer cells and stimulates adhesion to laminins. In human colon cancers, the *NEU3* mRNA level was found to be increased in all tissue specimens ($n=50$) by 3- to 100-fold as compared with that in the adjacent noncancerous mucosa (Kakugawa et al. 2002). In situ hybridization analysis showed NEU3 expression in the epithelial elements of adenocarcinomas, and significant elevation of sialidase activity against gangliosides was also observed in the tumor tissues. To understand the significance of the increased expression, cultured human colon cancer cells were treated with sodium butyrate, and changes in expression during differentiation and apoptosis were examined. NEU3 was downregulated by the treatment, but NEU1 was upregulated. Transfection of the NEU3 gene into cancer cells inhibited apoptosis, accompanied by increased Bcl-2 and decreased caspase-3 levels, whereas NEU3 silencing with siRNA resulted in augmented apoptosis. Colon cancer tissue specimens exhibited marked accumulation of lactosylceramide, a possible NEU3 product, and the addition of the glycolipid to culture reduced the number of apoptotic cells during sodium butyrate treatment. These results indicate that high expression of NEU3 in cancer cells leads to protection against programmed cell death, and in contrast, decreased NEU3 induces apoptosis, implying a critical role of NEU3 in the survival of cancer cells. NEU3 siRNA inhibits and NEU3 overexpression stimulates Ras activation with consequent influence on ERK1/2 and Akt. Ras activation by NEU3 is abrogated by PP2 (Src inhibitor) or AG1478 (EGFR inhibitor), and NEU3 actually enhances EGF-stimulated tyrosine phosphorylation of EGFR, suggesting that the

upstream targets might be tyrosine kinases including Src and EGFR, and subsequent stimulation of the Ras cascade leads to inhibition of cell apoptosis. Glycolipid changes observed seemed to be one of the causes of the cell effects. It is of great interest to note here that NEU3 silencing caused acceleration of apoptosis in various cancer cells, but not to have the same effect in noncancerous cells, including human normal keratinocytes and fibroblasts (Wada et al. 2007). NEU3 may thus be an essential gene for cancer cell survival, and siRNAs targeting this protein could have utility for gene-based therapy of human cancers.

In colon cancer cells, NEU3 differentially regulates cell proliferation through integrin-mediated signaling depending on the extracellular matrix (Kato et al. 2006) and causes increased adhesion to laminins and consequent cell division, but rather decrease in cell adhesion to fibronectin and collagens I and IV. Triggered by laminins, NEU3 enhanced phosphorylation of focal adhesion kinase (FAK) and ERK1/2 but no activation on fibronectin. NEU3 markedly stimulated tyrosine phosphorylation of integrin β4 by its association, along with recruitment of Shc and Grb-2 only on laminin-5 (laminin-332). GM3 depletion, as the result of NEU3 overexpression, appeared to be one of the causes of the increased adhesion on laminins. Among matrix proteins, especially neo-expression of laminin-332 is associated with proliferation of carcinoma cells, and it often accumulates in invading edges of carcinomas. These results indicate that NEU3 differentially regulates cell proliferation through integrin-mediated signaling depending on the extracellular matrix. NEU3 is indeed involved in the activation of signaling molecules, including FAK, Shc, and integrin β4, often upregulated in carcinogenesis, which may cause progression of the malignant phenotype in cancer cells.

NEU3 is involved in colorectal carcinogenesis in vivo. A possible role of NEU3 in promoting tumorigenesis in vivo has been demonstrated by generation of a mouse model of experimental colon carcinogenesis. In human NEU3 transgenic mice treated with a carcinogen, azoxymethane, for induction of precancerous colonic aberrant crypt foci (ACF) (Shiozaki et al. 2009b), ACF were induced significantly more frequently than in their control wild-type counterparts. Enhanced phosphorylation of EGFR, Akt, and ERK and the upregulation of Bcl-xL protein were observed in the transgenic colon mucosa, as described above in the case of NEU3-transfected cells, but no changes were found in cell proliferation, suggesting that the increased ACF formation was due to suppression of apoptosis. Thus, NEU3 upregulation may be essential for the promotion stage of colorectal carcinogenesis in vivo. When *Neu3*-deficient mice were exposed to another carcinogen, dimethylhydrazine, there were no differences in the incidence or growth of tumors from wild-type mice. On the other hand, *Neu3*-deficient mice were less susceptible to colitis-associated colon carcinogenesis induced by azoxymethane and dextran sodium sulfate, indicating an involvement of NEU3 in inflammation-dependent tumor development (Yamaguchi et al. 2012). In addition, we have discovered a close link between NEU3 expression and Wnt/β-catenin signaling in colon cancer cells by analyzing cancer stemlike characteristics and tumor-initiating capability. Activity-loss mutants of NEU3 failed to demonstrate alteration of Wnt and EGFR signaling. However, NEU3 silencing in colon cancer cells resulted in significant decrease in clonogenicity on soft agar and in vivo tumor growth, along with the

downregulation of stemness genes (Miyagi et al. 2015). These data suggest that NEU3 may participate in the maintenance of cancer stem cells and initiation of the process of colon carcinogenesis because of Wnt/β-catenin signaling known to be critical for cell stemness and tumor initiation (Clevers 2006).

The downregulation of NEU1 enhances cancer metastasis. We previously discovered a good inverse relationship between NEU1expression level and metastatic ability in various cell lines. For example, in rat 3Y1 fibroblasts, NEU1 decreased after *Src* transformation, and v-*fos* transfer to these transformed cells induced more severe decrease in the sialidase activity associated with acquisition of greater lung metastatic potential (Miyagi et al. 1994). Introduction of the *Neu1* gene into murine Bl6 melanoma cells resulted in suppression of experimental pulmonary metastasis and tumor progression, with reduction of anchorage-independent growth and increased sensitivity (Kato et al. 2001). This inverse relation of NEU1 expression level with metastatic ability appears also applicable to colon cancers. In mouse colon 26 adenocarcinoma cells, compared to low metastatic NL4 and NL44 cell lines, highly metastatic NL17 and NL22 cells exhibit lower NEU1 expression, accompanied by higher levels of sialyl Le^x and GM3 (Sawada et al. 2002). Furthermore, to examine whether and how NEU1 influences metastatic potential of human colon cancer, the human sialidase gene *NEU1* was overexpressed or silenced in colon cancer HT-29 cells (Uemura et al. 2009). When NEU1-overexpressing cells were injected transsplenically into mice, in vivo liver metastasis was significantly reduced. NEU1 suppressed cell migration, invasion, and adhesion in vitro, whereas the silencing resulted in the totally opposite effects. One of the major molecular changes caused by NEU1 was decreased sialylation of integrin β4, assessed by PNA-lectin blotting of immunoprecipitates with anti-integrin β4 antibody. The desialylation was accompanied by decreased phosphorylation of the integrin followed by attenuation of signaling through the FAK and ERK1/2 pathways. In the cells, NEU1 caused downregulation of matrix metalloproteinase-7, overexpression of which is associated with cancer metastasis. Treatment of the cells with GalNAc-α-O-benzyl, an inhibitor of *O*-glycosylation, showed increased PNA-positive integrin β4 with decreased phosphorylation, indicating that sialic acid removal from the integrin *O*-glycans results in decreased phosphorylation. Biotinylation and immunofluorescence staining showed some NEU1 molecules to be present at the cell surface accessible to the integrin. These results suggest that NEU1 plays important roles in the regulation of integrin β4-mediated signaling, leading to suppression of metastasis. It should be noted here that interestingly, in human colon cancer cells, the NEU1-mediated phenomena are opposite to the case of NEU3 which enhances the phosphorylation of integrin β4 by GM3 depletion and leads to the acceleration of adhesion on laminin-5, as described earlier.

The downregulation of NEU4 enhances cell adhesion to and motility and growth on E-selectin. Sialyl Lewis antigens, sialyl Le^a and sialyl Le^x, are commonly utilized as tumor markers, and their increase in cancer is associated with tumor progression by enhancement of cancer cell adhesion to endothelial E-selectin. However, regulation mechanisms remained not fully understood. In human colon cancer, in contrast to NEU3, NEU4 was found to be downregulated in surgical specimens of cancer compared to noncancerous tissues (Yamanami et al. 2007). To

understand the significance of NEU4 downregulation, we investigated sialyl Lewis antigens as endogenous substrates for the sialidase, since these are known to be associated with tumor progression by enhancement of cancer cell adhesion. NEU4 was found to hydrolyze the antigens in vitro and decrease cell surface levels much more effectively than other sialidases (Shiozaki et al. 2011). On analysis of the desialylation products, NEU4 was found to preferentially catalyze sialyl Lewis antigens expressed on *O*-glycans. Cell adhesion to and motility and growth on E-selectin were significantly reduced by NEU4 overexpression. E-selectin stimulation of colon cancer cells enhanced cell motility through activation of the p38/Hsp27/actin reorganization pathway, whereas NEU4 attenuated the signaling. As some NEU4 molecules were found at cell surfaces as assessed by immunocytochemical analysis, the enzyme might be accessible to sialyl Lewis antigens. Under conditions of hypoxia, NEU4 expression was markedly decreased. NEU4 thus plays an important role in the regulation of sialyl Lewis antigen expression in the colon mucosa and its impairment in colon cancer through enzyme downregulation.

We have recently presented evidence of a capacity of NEU4 for hydrolyzing polysialic acid on NCAM, which has been reported to be expressed in malignant tumors, including gliomas, lung and colon cancers, as well as nervous tissue. A correlation of its existence with tumor development, invasion, and poor prognosis has been suggested (Tanaka et al. 2001; Suzuki et al. 2005). Considering the regulation of polySia by NEU4 (Takahashi et al. 2012), it is feasible that the downregulation of the enzyme in colon cancer might be involved in polysialic acid presence in cancers, probably enhancing tumor development. Figure 10.1 summarizes all the results

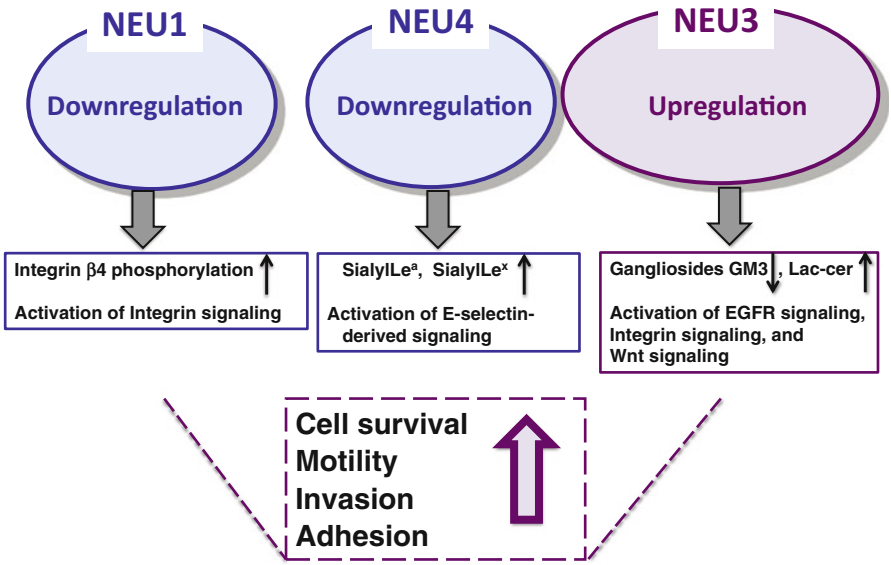

Fig. 10.1 Altered expression of sialidases in human colon cancer. The upregulation of NEU3 and downregulation of NEU1 and NEU4 cause disturbance of cell signaling related to cell growth and apoptosis and cell adhesion and motility, contributing to tumor development

described above that altered expressions of the three major sialidases in human colon cancer that cause tumor progression by disturbing cellular signaling.

10.5 Sialidase in Prostate Cancer

The upregulation of NEU3 has also been reported in human prostate cancer, showing a significant correlation with malignancy as assessed by the Gleason score (Kawamura et al. 2012). Prostate cancers generally become androgen independent and resistant to hormone therapy with progression. To understand the underlying mechanisms and facilitate the development of novel treatments for androgen-independent prostate cancer, we have investigated how upregulation of NEU3 is involved in progression. NEU3 silencing with siRNA in prostate cancer PC-3 and LNCaP cells resulted in increased expression of differentiation markers and in cell apoptosis, but decrease in Bcl-2 as well as a progression-related transcription factor, early growth response gene (EGR-1). In androgen-sensitive LNCaP cells, forced overexpression of NEU3 significantly induced expression of EGR-1, androgen receptor (AR), and PSA both with and without androgen, the cells becoming sensitive to androgen. This NEU3-mediated induction was abrogated by inhibitors for PI 3-kinase and MAP kinase and more specifically by their silencing in the absence of androgen, as confirmed by increased phosphorylation of AKT and ERK1/2 in NEU3-overexpressing cells. To understand further how NEU3 causes elevation of EGR-1, AR, and PSA expression by activation of AKT and ERK, we observed the upstream signaling including EGFR family expression, which has been proposed to escape androgen regulation and switch to androgen-independent cell growth (Traish and Morgentaler 2009). Consistent with other types of cancer, NEU3 was found to activate the PI3K and MAPK pathways, associated with increase in mRNA and protein levels of EGFR and ERBB2 under androgen-deficient conditions. For development of hormone-refractory progression, enhanced AR mRNA and protein expression is known as a critical factor in conversion to a hormone-refractory state in the majority of patients who do not have AR mutations or amplification. The mechanism of increased AR expression includes crosstalk between ARs and growth factor receptors such as EGFR and ERBB2. In this context, NEU3 upregulation may trigger off activation of AR pathway probably via EGFR family followed by ERK and AKT activation, leading to conversion to a hormone-refractory state, as illustrated in Fig. 10.2. In addition, transcriptional activation of NEU3 itself probably occurs by Sp1 transcription factor in positive feedback loop, as Sp1 phosphorylated by ERK and NEU3 is an Sp1 target gene (Yamaguchi et al. 2010). NEU3 may again be a pivotal molecule acting to upregulate growth factor receptors and lead to androgen-independent cell growth. In fact, NEU3 siRNA introduction caused reduction of cell growth of androgen-independent PC-3 cells in culture and of transplanted tumors in nude mice (Kawamura et al. 2012). These data

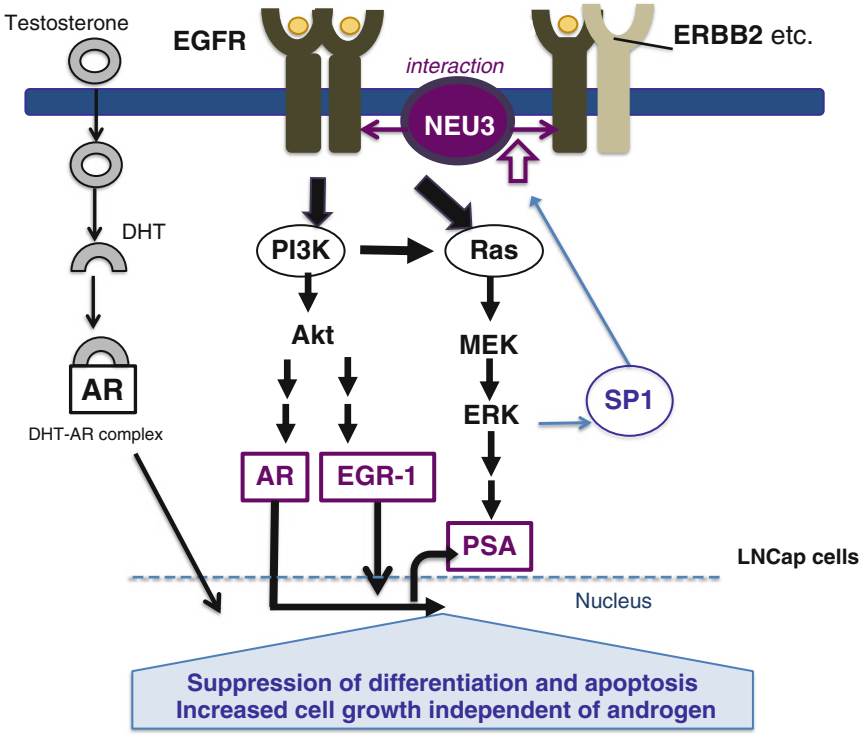

Fig. 10.2 Roles of NEU3 in human prostate cancer. NEU3 enhances expression of AR, EGR-1, and PSA by activating the PI3K and MAPK pathways, associated with increased expression of EGFR and ERBB2 under androgen-deficient conditions. NEU3 upregulation may trigger off activation of AR pathway probably via EGFR family followed by ERK and AKT activation, switching androgen (dihydrotestosterone, DHT) dependent to independent AR activation. Furthermore, transcriptional activation of NEU3 itself probably occurs by Sp1 transcription factor in positive feedback loop, as Sp1 is phosphorylated by ERK and NEU3 is an Sp1 target gene (Kawamura et al. 2012)

altogether suggest that NEU3 regulates tumor progression through AR signaling and thus be a potential tool for diagnosis and therapy of androgen-independent prostate cancer.

10.6 Sialidase in Renal Carcinomas

NEU3 expression was found to be increased at the levels of mRNA and enzyme activity in human renal cell carcinomas (RCCs) compared with adjacent non-tumor tissues, significantly correlating with elevation of interleukin-6 (IL-6), a pleiotropic cytokine that has been implicated in immune responses and pathogenesis of several cancers, including RCCs (Ueno et al. 2006). In human RCC ACHN

cells, IL-6 treatment enhanced *NEU3* promoter luciferase activity 2.5-fold and endogenous sialidase activity significantly. *NEU3* transfection or IL-6 treatment resulted in both suppression of apoptosis and promotion of cell motility, and the two in combination exerted synergistic effects. NEU3 scarcely affected MAPK- or IL-6-induced STAT3 activation but promoted the PI3K/Akt cascade in both IL-6-dependent and IL-6-independent ways. Consistent with these data, NEU3 markedly inhibited staurosporine-induced caspase-3 activity and enhanced IL-6-dependent inhibition, which was abolished by LY294002, a PI3K inhibitor. Furthermore, IL-6 promoted Rho activation, and the effect was potentiated by NEU3, leading to increased cell motility that was again affected by LY294002. NEU3 silencing by siRNA resulted in the opposite: decreased Akt phosphorylation and inhibition of Rho activation. Glycolipid analysis showed a decrease in GM3 and an increase in lactosylceramide after *NEU3* transfection, with these lipids apparently affecting cell apoptosis and motility. The results indicate that NEU3 activated by IL-6 contributes to IL-6-mediated signaling, largely via the PI3K/Akt cascade, in a positive feedback manner and therefore to expression of a malignant phenotype in RCCs (Fig. 10.3).

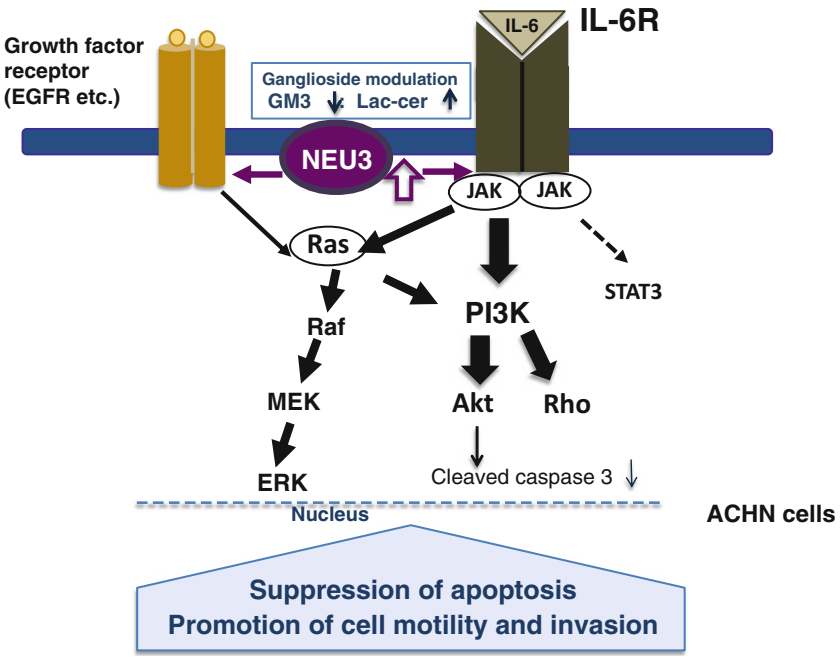

Fig. 10.3 Roles of NEU3 in human renal cancer. Increased expression of NEU3 in human renal cell carcinomas significantly correlates with elevation of interleukin-6 (IL-6). In human renal cell carcinoma ACHN cells, *NEU3* overexpression or IL-6 treatment brings about both suppression of apoptosis and promotion of cell motility, and the combination gives synergistic effects. NEU3 scarcely affects IL-6-induced STAT3 activation but promotes PI3K/Akt pathway in both IL-6-dependent and IL-6-independent ways (Ueno et al. 2006)

10.7 Other Cancers

In head and neck cancer, NEU3 sialidase expression may also be increased both mRNA and ganglioside sialidase activity levels. The levels showed a significant correlation with the histological differentiation grade, lymphatic invasion, and lymph node metastasis. In ovarian clear cell adenocarcinoma, NEU1 and NEU3 expression levels were also elevated in most of the cell lines, but NEU4 expression was hardly changed. A significant correlation of high level of NEU3 expression with T3 factor of pTNM for classification of progression has been observed (Nomura et al. 2006).

The one obvious exception to the general increase of NEU3 in cancer is the downregulation described in acute lymphoblastic leukemia (ALL) in relation to disease progression. Mandal et al. (2010) reported that in ALL, lymphoblasts show the downregulation of NEU3 in the levels of mRNA and ganglioside activity, as compared with cells from healthy controls. Overexpression of NEU3 in the leukemia cell line, MOLT-4, led to apoptosis with decrease in the Bcl2-Bax ratio and increase in ceramide. Therefore, reduced expression could help lymphoblasts to survive. Interestingly, NEU3 activity varied in relation to disease progression, increasing in clinical remission after chemotherapy and decreasing again in patients that relapsed. The downregulation of NEU3 may therefore even be a good clinical marker for the process of the disease.

Neuroblastoma is a frequently lethal tumor occurring in childhood. In the nervous system, gangliosides, which are good substrates for NEU3 and NEU4, are relatively abundantly present. Despite the lack of actual data on relative sialidase levels between neuroblastomas and healthy controls, it is interesting that these two sialidases are possibly involved in the cell differentiation and proliferation of human neuroblastoma cells in a way opposite to the case of carcinomas. We previously observed endogenous NEU3 expression in human neuroblastoma NB-1 cells to be increased during neurite differentiation induced by dibutyryl cAMP (Proshin et al. 2002). Although treatment with dibutyryl cAMP alone enhanced neurite formation, transfection of the NEU3 gave rise to a more prominent outgrowth of neurites with axon-like characteristics, even in the absence of dibutyryl cAMP. On the other hand, Tringali et al. (2012) reported that NEU4 (NEU4L) affected the differentiation of NB SK-N-BE cells. NEU4L overexpression induced activation of Wnt/β-catenin signaling, leading to enhanced proliferation and a more undifferentiated cell phenotype together with an increase of the expression of the pluripotency genes, MYC, NANOG, OCT-4, CD133, and NES (nestin). The cells failed to differentiate after serum withdrawal. The endogenous target of NEU4 causing the malignant phenotype was likely linked to desialylated glycoproteins but not ganglioside changes.

10.8 Perspectives

In this review, we have presented evidence that alteration in sialidase expression may be a defining factor for cancer promotion and progression. In human carcinomas, the upregulation of NEU3 and downregulation of NEU1 and NEU4 are likely

to augment malignant properties underlying cell survival, enhanced cell motility, and invasion. In acute lymphoblastic leukemia and neuroblastomas, in contrast, the upregulation of NEU3 and downregulation of NEU4 seem to lead to reduced malignancy, although more investigation of human subjects is needed for firm conclusions. Further elucidation of the mechanisms underlying the different influences of sialidase expression on tumor progression or regression depending on cell type is clearly of high priority. The fact that sialidase alterations often correlate with cancer progression, however, opens up potential applications in cancer cure and diagnosis. In particular, the downregulation of NEU3 expression by treatment with specific siRNAs, antibodies, or inhibitors may bring about regression in many carcinomas, as proposed in Fig. 10.4. Taking advantage of the limited effects of NEU3 siRNAs on normal cells and of activity-loss mutants affecting EGFR and Wnt signaling, NEU3 siRNAs or inhibitors causing apoptosis in cancer cells could offer a practical tool for cancer therapy.

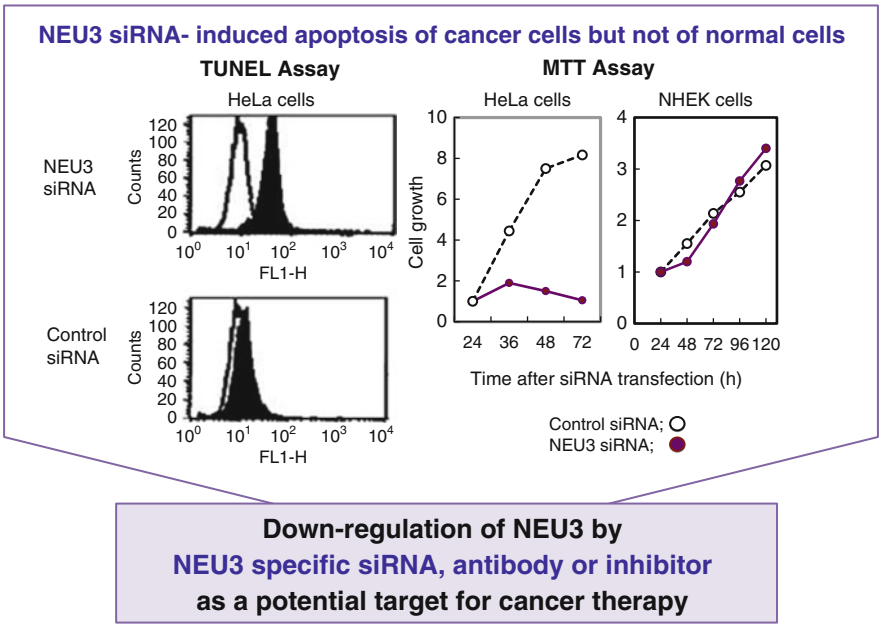

Fig. 10.4 Proposed effects of NEU3 suppression as a potential target for cancer therapy. The downregulation of NEU3 expression with a specific siRNA, antibody, or inhibitor may lead to prevention of cancer progression. In particular, taking advantage of the limited effects on normal cells, NEU3 siRNAs causing apoptosis in various cancer cells including HeLa cells could offer a potential tool for therapy (Wada et al. 2007)

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Chapter 11
Roles of Glycans in Immune Evasion from NK Immunity

Shigeru Tsuboi

Abstract We innately have an ability to reject tumors, thereby limiting cancer progression and metastasis. The major effector lymphocytes in tumor rejection are natural killer (NK) cells. NK cells kill target cancer cells by two different rejection mechanisms, NK receptor-dependent killing and tumor-necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated killing. In spite of these tumor rejection systems, cancer cells make survival in host, facilitating the metastatic spread to other organs. The metastatic spread is still the major cause of cancer deaths. It has been revealed that some cancer cells acquire an ability to evade tumor rejection responses by NK cells to survive longer in host, thereby increasing the chance to metastasize. Several immune evasion strategies have been well documented. Recently, the immune evasion mechanisms from NK immunity using cell-surface glycans have been identified. The cancer cells use the certain types of cell-surface glycans to evade NK immunity in the following three ways: reducing NK activating receptor-mediated signaling, enhancing NK inhibitory receptor-mediated signaling, and modulating TRAIL-mediated killing. In this chapter, we will illustrate those evasion mechanisms in which cell-surface glycans play a central role.

Keywords Cancer • Metastasis: natural killer (NK) cell • Tumor rejection • NK immunity • NK activating receptor • NK inhibitory receptor • NK receptor ligands • Tumor-necrosis factor-related apoptosis-inducing ligand (TRAIL) • Death receptor

11.1 Introduction: Tumor Rejection by NK Immunity

The major cause of cancer deaths is the metastatic spread to other organs. Metastasis occurs when cancer cells acquire invasive phenotypes and the ability to evade tumor rejection in host. Particularly, the evasion of the cancer cells from the host tumor rejection responses greatly increases the chance for metastasis.

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Hematogenous metastasis involves the following multiple steps: Cancer cells (1) invade the tissues surrounding the primary site, (2) enter the blood vessels, (3) disseminate into blood circulation, (4) arrest in the blood vessels at a target organ, (5) extravasate into the tissue of the organ, and (6) proliferate at the new site (Valastyan and Weinberg 2011). Among these steps, dissemination into circulation is the most critical step for cancer cells to establish metastasis, because the circulating cancer cells are exposed to innate immune systems that patrol and kill those cells to limit dissemination. In the tumor rejection responses by innate immune systems, NK cells are the major effector lymphocytes (Vivier et al. 2008; Levy et al. 2011).

NK cells are innate immune cells that have the natural ability to distinguish normal cells from cancer cells and specifically kill cancer cells (Trinchieri 1989). In human, NK cells constitute 10–15 % of peripheral blood lymphocytes. Upon encountering cancer cells in host circulation systems, NK cells eliminate the target cancer cells in mainly two different types of rejection mechanisms. One is the NK receptor-mediated killing of cancer cells. The other one is the tumor-necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated killing of cancer cells. In order to evade these rejection systems using the above two mechanisms, cancer cells take various types of strategies. Of those, the immune evasion strategies in which cancer cell-surface glycans play a central role are illustrated in this chapter.

11.2 Evasion Strategies from NK Receptor-Dependent Killing

11.2.1 Controlling NK Cell Responsiveness Through NK Receptor-Mediated Signaling

Various receptors present on NK cells (NK receptors) are triggered during target cancer cell recognition. NK receptors are divided into two (NK activating receptors and NK inhibitory receptors). Activating receptors and inhibitory receptors induce a positive and negative cell signaling, respectively. NK cell responsiveness is controlled by a balance of these opposite signals generated from NK activating and inhibitory receptors (Moretta et al. 2004; Joncker and Raulet 2008; Purdy and Campbell 2009) (Fig. 11.1). If the activating signaling is dominant over inhibitory signaling, NK cells recognize the target cells as “nonself” and are activated to secrete several apoptosis-inducing substances such as granzyme B, perforin, and cytokines, resulting in target cell killing (Fig. 11.1a). If the inhibitory signaling is dominant, NK cells recognize the target cells as “self,” and the signal blocks the activating signaling to inhibit NK cell activation, resulting in the survival of target cells (Fig. 11.1b) (Lanier 2008).

Recent studies have revealed that some cancer cells develop the strategies to evade NK immunity and survive longer in host circulation system, increasing the chance to metastasize. To evade NK immunity, those cancer cells impair the NK cell responsiveness in two different ways: reducing the NK activating receptor-mediated

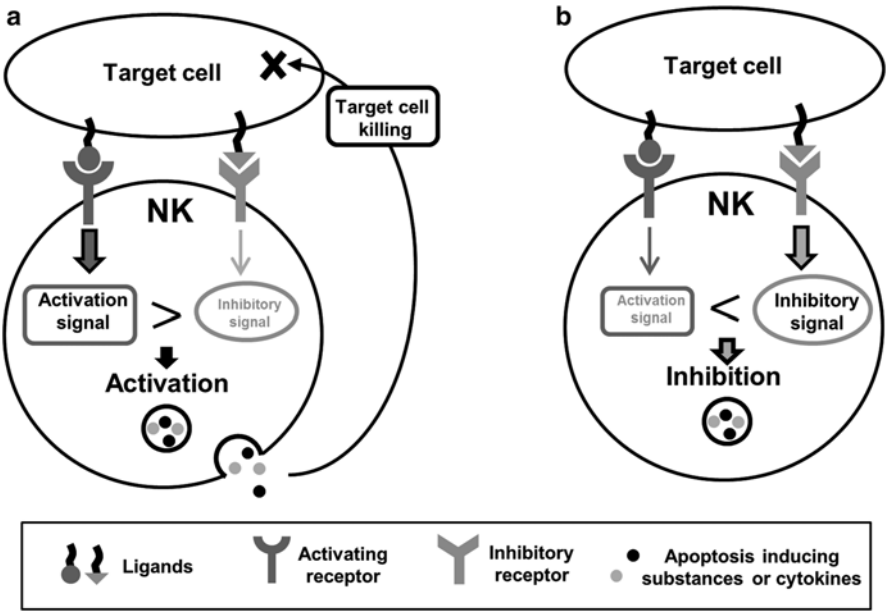

Fig. 11.1 Controlling NK cell responsiveness through NK receptor-mediated signaling. (**a**) If NK activating receptor-mediated signaling is dominant, NK cells are activated to secrete apoptosis-inducing substance, thereby killing target cells. (**b**) If NK inhibitory receptor-mediated signaling is dominant, the signal blocks NK activating receptor-mediated signaling, thereby inhibiting NK cell activation

signaling and enhancing NK inhibitory receptor-mediated signaling. In the following two sections, we will explain the immune evasion mechanisms in which cell-surface glycans play a central role.

11.2.2 Immune Evasion Mechanism Targeting NK Activating Receptor

The major NK cell activating receptors include the natural cytotoxicity receptors (NCRs: NKp30, NKp44, and NKp46), natural killer group 2 member D (NKG2D), Fc receptor CD16, and activating killer cell immunoglobulin-like receptors (KIR) (Moretta et al. 2004; Lanier 2008). Among these receptors, NKG2D is the best studied receptor on its ligands. Known ligands for NKG2D are the major histocompatibility complex (MHC) class I-related chain A/B (MICA/B) and six UL16-binding proteins (ULBP1-6). Their expression was induced upon the transformation of target cells and other cellular stresses (Lanier 2008; Raulet et al. 2013). The analysis of NKG2D knockout mice revealed that the NKG2D-MICA interaction is the most critical in tumor rejection responses (Guerra et al. 2008; Zafirova et al. 2009). In tumor rejection responses, the MICA expression is induced and

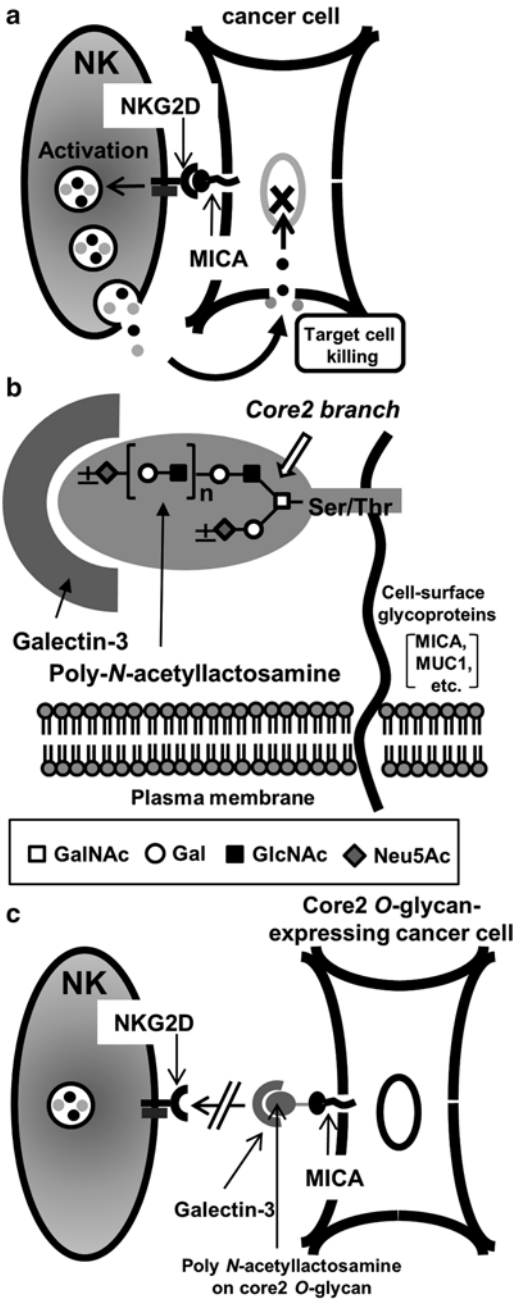

Fig. 11.2 Immune evasion mechanism targeting NK activating receptor (NKG2D) using core2 *O*-glycans. (**a**) NK cells are activated by the interaction of an activating receptor, NKG2D with tumor ligand MICA. Activated NK cells kill target cancer cells. (**b**) Core2 *O*-glycans are a scaffold for the subsequent extension of lactosamine disaccharide unit (Galβ1-4GlcNAc)n called poly-*N*-acetyllactosamine. The number of unit repeats varies depending on the carrier molecules and cell types. A β-galactose-binding protein, galectin-3, binds to this poly-*N*-acetyllactosamine. In core2 *O*-glycan-expressing cancer cells, cell-surface glycoproteins are thus modified with poly-*N*-acetyllactosamine and galectin-3. (**c**) In core2 *O*-glycan-expressing cancer cells, galectin-3 binds to poly-*N*-acetyllactosamine in the NKG2D-binding site of MICA. Modification with poly-*N*-acetyllactosamine and galectin-3 reduces the affinity of MICA for NKG2D, impairing NK cell activation

upregulated upon cellular transformation, and the engagement of NKG2D by MICA triggers the NKG2D-mediated signaling, thereby secreting the apoptosis-inducing substances to kill the target cancer cells (Fig. 11.2a) (Nausch and Cerwenka 2008).

Recently, it has been reported that some cancer cells evade the NKG2D-dependent killing by using cell-surface mucin-type *O*-glycans[1] (hereafter *O*-glycans). *O*-glycans play several important roles in biologically fundamental phenomena including cell differentiation, development, and immunity (Fuster and Esko 2005). *O*-glycans regulate biochemical and functional properties of cell-surface glycoproteins. There are four common *O*-glycan core structures in mammalian tissues, namely, core1, core2, core3, and core4, which depend on the combination of sugar added. Among them, it was reported that the expression of core2 *O*-glycan is associated with metastatic phenotypes of several cancers (Machida et al. 2001; Hagisawa et al. 2005; Hatakeyama et al. 2010). Core2 *O*-glycan contains an *N*-acetylglucosamine branch connected to *N*-acetylgalactosamine (GlcNAcβ1-6GalNAc), which is designated core2 branch (Fig. 11.2b). It has been demonstrated that core2 *O*-glycan-expressing cancer cells evade NK tumor rejection by impairing the NK activating receptor (NKG2D)-mediated signaling in the following sequences. NKG2D-binding site in MICA has several *O*-glycosylation sites, and MICA in core2 *O*-glycan-expressing cancer cells carries core2 *O*-glycan containing the core2 branch. The core2 branch is a scaffold for the subsequent production of lactosamine disaccharide unit, poly-*N*-acetyllactosamine $(Gal\beta1\text{-}4GlcNAc)_n$ on *O*-glycans. This poly-*N*-acetyllactosamine is a ligand for galectins. Galectins are a family of lectins which bind to β-galactoside and have been implicated in numbers of biological processes including immunity and tumor progression (Rabinovich et al. 2002; Yang et al. 2008; Saegusa et al. 2009). In core2 *O*-glycan-expressing cancer cells, *O*-glycans in the NKG2D-binding site in MICA are modified with poly-*N*-acetyllactosamine and that galectin-3 binds to the poly-*N*-acetyllactosamine of MICA *O*-glycans (Fig. 11.2b). The modification of MICA with poly-*N*-acetyllactosamine and galectin-3 masks the NKG2D-binding site in MICA, thereby reducing the affinity of MICA for NKG2D, severely impairing NK cell activation, and finally preventing the NKG2D-mediated killing of core2 *O*-glycan-expressing cancer cells (Fig. 11.2c) (Tsuboi et al. 2011, 2012; Tsuboi 2013). This masking of the tumor-ligand for the NK receptor by core2 *O*-glycans functions as a tumor defense system against NK cell immunity, increasing their survival in host circulation. Core2 *O*-glycan-expressing cancer cells thus evade NK cell attack, resulting in the promotion of metastasis.

It was previously reported that cancer cells evade NK cell tumor immunosurveillance by the following three systems: (1) a large amount of soluble MICA shed by cancer cells downregulate NKG2D expression (Groh et al. 2002; Doubrovina et al. 2003; Clayton et al. 2008); (2) cancer cells sustain the expression of NKG2D ligands. The excess stimulation of NKG2D with the ligands reduces the cell-surface expression of NKG2D, resulting in impaired NK cell activation (Oppenheim et al.

[1] Mucin-type *O*-glycan is a general term for the oligosaccharides which are initially found in cell-surface and secreted mucins. Those oligosaccharides contain the linkage of *N*-acetylgalactosamine (GalNAc) to serine or threonine residues. Mucin-type *O*-glycans are present on other cell-surface glycoproteins such as CD43 and CD34. In this chapter, we focus on the mucin-type *O*-glycans among *O*-glycans.

2005); (3) cancer cells decrease the cell-surface expression of MICA by retaining MICA in the cells (Fuertes et al. 2008). Modifying MICA with poly-*N*-acetyllactosamine plus galectin-3 is the newly identified evasion strategy of NK cell attack (Fig. 11.2c) (Tsuboi et al. 2011).

11.2.3 Immune Evasion Mechanism Targeting NK Inhibitory Receptor

NK cells express numbers of inhibitory receptors that inhibit NK cell activation including killer cell immunoglobulin-like receptors (KIRs), leukocyte immunoglobulin-like receptors (LILRs), Ly49, and CD94-NKG2A. Upon the engagement of these receptors by their ligands, this NK inhibitory receptor-mediated signaling blocks the NK activating receptor-mediated signaling, thereby inhibiting NK cell activation (Fig. 11.3a). It has been reported that some cancer cells evade the NK cell-dependent killing by enhancing NK inhibitory receptor-mediated signaling using cell-surface glycans.

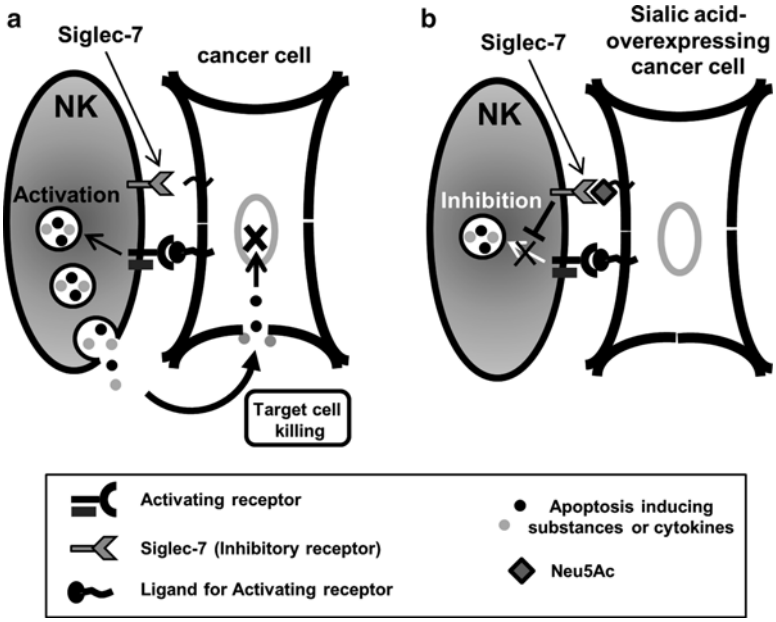

Fig. 11.3 Immune evasion strategy targeting NK inhibitory receptor using sialic acids. (**a**) The interaction of NK activating receptor with tumor ligand mediates NK activating signaling to kill target cancer cells. (**b**) In sialic acid-overexpressing cancer cells, cell-surface sialic acids interact with an NK inhibitory receptor, Siglec-7. Siglec-7-mediated signaling blocks NK activating signaling, thereby inhibiting NK cell activation

The upregulation of cell-surface sialic acids in cancer cells was known to correlate with poor prognosis, and it has been suggested that the hypersialylation of the cancer cell surface may facilitate the cancer cells to evade tumor rejection systems in host (Fukuda 1996; Leivonen et al. 2001; Fuster and Esko 2005). Recent studies have revealed that there is a positive correlation between target cancer cell sialylation state and resistance to NK tumor rejection (Van Rinsum et al. 1986; Ogata et al. 1992), suggesting that sialic acids on cancer cell-surface are involved in immune evasion of sialic acid-overexpressing cancer cells from tumor rejection by NK cells.

Just recently, a novel immune evasion mechanism using sialic acids has been identified. Bertozzi's group demonstrated that increasing sialylated glycans on cancer cells inhibit NK cell activation by enhancing the signaling mediated by an NK inhibitory receptor, sialic acid-binding immunoglobulin-like lectin 7 (Siglec-7) (Hudak et al. 2014). Siglec-7 which NK cells ubiquitously express contains a cytosolic immunoreceptor tyrosine-based inhibitory motif (ITIM) to function as an NK inhibitory receptor. Hudak et al. coated cancer cells with sialylated glycopolymers and incubated the cancer cells with NK cells. Highly sialylated glycans on the cancer cell surface binds to Siglec-7 to engage. The engagement of Siglec-7 induces the recruitment of the Src homology-2 (SH2) phosphatases (SHPs) to inhibit the kinase phosphorylation cascade downstream of NK activating receptors, resulting in the inhibition of NK cell activation (Hudak et al. 2014) (Fig. 11.3b). They revealed that the cell-surface sialic acids play an important role in the evasion mechanism of cancer cells from NK tumor rejection system.

As an immune evasion strategy targeting NK inhibitory receptors, it was previously reported that some cancer cells evade NK tumor rejection system by overexpressing ligands for NK inhibitory receptors to enhance the NK inhibitory receptor-mediated signaling (Mamessier et al. 2011). This is the first example that cancer cells evade NK cell attack by using cell-surface glycans as a ligand for the NK inhibitory receptor.

11.3 Evasion Strategies from TRAIL-Mediated Killing

11.3.1 TRAIL-Mediated Cancer Cell Killing

TRAIL is expressed in various types of cells in the immune system including NK cells. The TRAIL/TRAIL receptor system was shown to have tumor immunosurveillance functions. The TRAIL and its receptors constitute one of the systems which have been shown to regulate intercellular apoptotic responses in the immune system. In tumor immunosurveillance functions by TRAIL expressed in NK cells, the initial step of apoptosis induction by TRAIL is the direct binding of TRAIL to TRAIL receptors called death receptor (DR) 4 or DR5 which are expressed in target cancer cells. Thereby, the receptors are trimerized and transduced the death-inducing signaling to induce apoptosis (Falschlehner et al. 2009) (Fig. 11.4a).

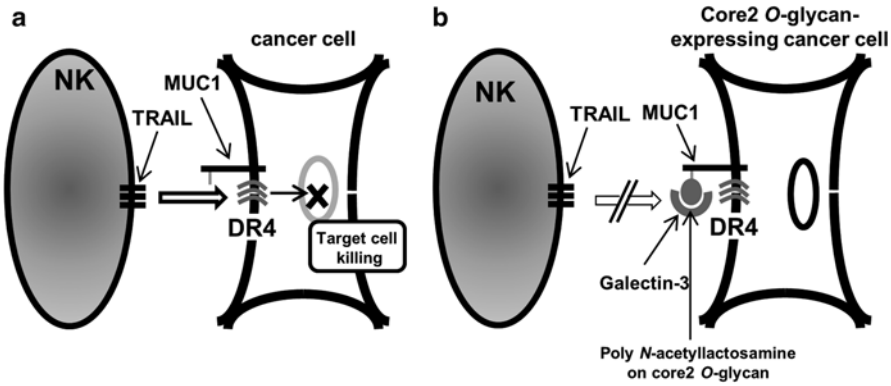

Fig. 11.4 Immune evasion strategy targeting TRAIL-mediated killing. (**a**) TRAIL expressed on the NK cell surface interacts with death receptors (DR4 and DR5) on target cancer cell surface. DR4-mediated signaling induces apoptosis of target cancer cells. (**b**) Modification of a cell-surface mucin, MUC1 with poly-*N*-acetyllactosamine, and galectin-3 shields against the access of TRAIL to DR4, impairing TRAIL-mediated killing of target cancer cells

11.3.2 Immune Evasion Mechanism Targeting TRAIL-Mediated Killing by DR Shielding Effect

An immune evasion mechanism targeting TRAIL-mediated cancer cell killing has been recently identified (Suzuki et al. 2012; Okamoto et al. 2013). In core2 *O*-glycan-expressing cancer cells, cell-surface mucin 1 (MUC1) is heavily core2 *O*-glycosylated. MUC1 core2 *O*-glycans carry poly-*N*-acetyllactosamine, and gaectin-3 binds to MUC1 through this poly-*N*-acetyllactosamine (Fig. 11.2b). Modification of MUC1 with poly-*N*-acetyllactosamine and galectin-3 interferes with the access of the NK cell TRAIL to cancer cell DR4, since MUC1 is one of the molecules that extend farthest from the cell surface. This interfering with the TRAIL-DR4 interaction by MUC1 carrying core2 *O*-glycan impairs TRAIL-mediated killing of the core2 *O*-glycan-expressing cancer cells (Fig. 11.4b) (Suzuki et al. 2012; Okamoto et al. 2013). Thus, MUC1 carrying core2 *O*-glycans functions as a molecular shield against TRAIL to evade TRAIL-mediated killing, resulting in longer survival of the cancer cells in host circulation and the promotion of metastasis.

11.3.3 Immune Evasion from TRAIL-Mediated Killing by Diminishing Glycans

The immune evasion strategy described above is that cancer cells evade TRAIL-mediated killing by increasing the expression of the cell-surface glycan, core2 *O*-glycan. Some cancer cells take an opposite strategy. Those cancer cells evade

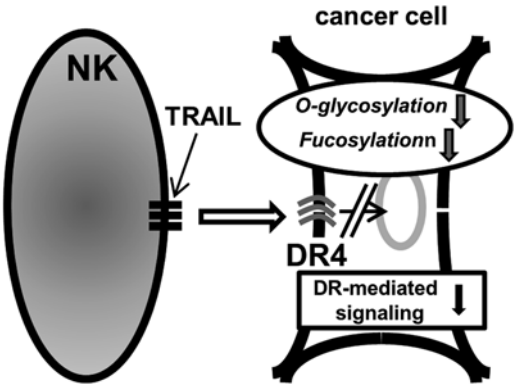

Fig. 11.5 Immune evasion from TRAIL-mediated killing by diminishing glycans. Some cancer cells reduce *O*-glycosylation of DRs, impairing DR-mediated signaling. Some cancer cells deplete cellular fucosylation, also impairing DR-mediated signaling. Those cancer cells evade TRAIL-mediated killing to survive longer in host

TRAIL-mediated killing by diminishing the expression of a certain type of glycan. Two different mechanisms for this type of strategy have been reported (Fig. 11.5).

O-glycosylation of DR. The *O*-glycosylation of cell-surface molecules is initiated by the formation of α-glycosidic linkage of *N*-acetylgalactosamine (GalNAc) to serine or threonine residues. This initial reaction is catalyzed by 24 kinds of peptidyl GalNAc transferase (GALNT) isoform. Wagner et al. described a novel mechanism that regulates DR-mediated signaling in cancer cells through the *O*-glycosylation of DR (Wagner et al. 2007). They discovered that the expression level of *GALNT14*, one of the GALNT isoforms, significantly correlates with TRAIL sensitivity in several types of cancers. They also presented a possible mechanism by which DR *O*-glycosylation increases the susceptibility to TRAIL. *O*-glycosylation of DR4 and DR5 promoted TRAIL-stimulated clustering of these death receptors. The promoted clustering of DRs mediated the recruitment and activation of caspase-8, the apoptosis-initiating protease, thereby inducing cancer cell apoptosis. Interestingly, it has been demonstrated that several types of malignant cancer cell lines including pancreatic cancer, non-small-cell lung carcinoma, and melanoma suppress the *GALNT14* gene expression to reduce DR *O*-glycosylation. Reduced *O*-glycosylation of DR modulates the DR-mediated signaling. Thereby, those cells evade TRAIL attack to survive longer in host by reducing the *O*-glycosylation of DR.

Cellular fucosylation. Another prominent example is cellular fucosylation. It has been reported that fucosylation is one of the most critical modifications in cancer progression and inflammation (Miyoshi et al. 2008). Fucosylation is catalyzed by several fucosyltransferase isoforms which require guanosine diphosphate (GDP)-fucose as a donor substrate. The initial step of the GDP-fucose synthesis is catalyzed by GDP-mannose-4,6-dehydrogenase (GMDS) (Ohyama et al. 1998; Sullivan et al. 1998). Moriwaki et al. discovered that some colon cancer cell lines and tissues which have the mutations of GMDS gene evade NK tumor rejection system (Moriwaki et al. 2009). The mutations result in a virtually complete deficiency of cellular fucosylation. They demonstrated that the fucosylation-deficient colon cancer cells acquire resistance to NK tumor rejection responses in both in vitro and in vivo systems. In addition, they found that DR-mediated signaling was modulated

in the fucosylation-deficient cancer cells and that this modulation caused less susceptibility of those cells to TRAIL-mediated killing (Moriwaki et al. 2009). They also showed that the GMDS-rescued cancer cells restored the sensitivity to TRAIL attack. These results indicate that cellular fucosylation is required for DR-mediated signaling. Although little is known about what role fucosylated glycans play in DR-mediated signaling, those certain types of colon cancer also evade TRAIL-mediated killing to survive longer in host by depleting cellular fucosylation.

11.4 Concluding Remarks

This chapter, with an emphasis on the most recent advances, provides several examples that support the significance of the regulatory roles of cell-surface glycans on NK immunity. Cancer cells develop the immune evasion mechanisms from NK-mediated tumor rejection by inducing or diminishing the certain types of cell-surface glycans.

Our current understanding of the molecular mechanisms described above may lead to the development of new therapeutic methods and agents for preventing the evasion of NK immunity by glycans. For instance, the reduction of the activity of a GlcNAc transferase (C2GnT) which is responsible for the formation of core2 *O*-glycans or the downregulation of C2GnT expression using specific inhibitors or siRNA-based agents in cancer cells could impair the immune evasion mechanism. These agents could restore cancer cell susceptibility to NK immunity.

Immune evasion is a novel function of cell-surface glycans. Recent discoveries of the evasion of NK immunity by glycans implicate that glycans may also play a role in evasion from other aspects in tumor immunity. Further studies on the structures and functions of cell-surface glycans, focusing on the influence on other tumor rejection systems such as cytotoxic T lymphocyte (CTL) tumor immunity, will provide a new insight into the roles of cell-surface glycans in cancer metastasis.

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Chapter 12
Glycomic Analysis of Cancer

Yasuhide Miyamoto

Abstract Remarkable alterations in oligosaccharide structures are associated with many human diseases, including cancers. Numerous clinicopathological and biochemical studies have suggested the involvement of aberrant glycosylation in cancer malignancy, such as metastasis and invasion. Furthermore, altered carbohydrate determinants, including tumor-associated carbohydrate antigens such as SLe^a (CA19-9), have been utilized as useful tumor markers for the diagnosis of cancer. Cancer glycomic analysis (i.e., precise and comprehensive analysis of altered oligosaccharides in cancer tissues and sera) is a widely used tool for (1) investigating the involvement of glycosylation in cancer malignancy and (2) discovering novel carbohydrate tumor marker candidates. Comprehensive clinico-glycomic studies of glycosphingolipids of colorectal cancers have revealed specific alterations related to malignant transformation, as well as characteristic alterations associated with clinical features. Glycomic analyses of colorectal cancers and pancreatic cancers revealed the presence of two kinds of novel fucogangliosides, sialylated type1H (Lewis-negative specific antigen) and sialylated type2H, both of which are isomers of sialyl Le^x and sialyl Le^a. The accumulation of free oligosaccharides in human cancers has been elucidated. Free Neu5Ac-containing complex-type *N*-glycans accumulated in pancreatic cancers. In addition to these free oligosaccharides, free KDN-containing complex-type *N*-glycans accumulated in prostate cancers. *N*-linked and *O*-linked glycans have also been targets for cancer glycomics. In particular, extensive studies of serum glycomic analyses have been performed to find novel glycan cancer biomarker utilizing newly developed high-throughput platform technologies. It is anticipated that these cancer glycomic studies will lead to the discovery of glycan biomarker or therapy targets for cancers.

Keywords Glycosphingolipid • *N*-Glycan • *O*-Glycan • Cancer • Glycomic analysis • Tumor marker • Biomarker

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12.1 Introduction

Glycosylation of the surface of cancer cells is closely associated with malignant transformation and malignancy of cancers (Hakomori 2002). Extensive studies on the oligosaccharide structures of glycosphingolipids (GSLs) and glycoproteins in cancers have revealed that aberrant glycosylation occurs in essentially all types of human cancers, and many altered carbohydrate determinants, including sialyl Le^x (SLe^x) and sialyl Le^a (SLe^a), are classified as tumor-associated carbohydrate antigens (Hakomori 1989, 2002; Lau and Dennis 2008; Brockhausen 1999; Kim and Varki 1997; Fukuda 1996). Subsequent studies have indicated the functional significance of aberrant glycosylation in cancer malignancy, such as metastasis and invasion (Hakomori 1996, 2002; Ono and Hakomori 2004). For example, SLe^a and SLe^x function as ligands for selectins and are thought to be involved in hematogenous metastasis (Kannagi et al. 2004). Furthermore, altered carbohydrate determinants, including tumor-associated carbohydrate antigens such as SLe^a and SLe^x, have been utilized as useful tumor markers for the diagnosis of cancer (Saldova et al. 2008; Kannagi et al. 2004; Peracaula et al. 2008). CA19-9, SLe^a epitope, is one of the most well-known serum tumor markers, which is frequently used for clinical diagnosis of a variety of cancers such as pancreatic, colorectal, and stomach cancers. Thus, cancer glycomic analyses, which precisely and comprehensively determine the altered oligosaccharides in cancer tissues and sera, are now widely used as a valuable tool to investigate the involvement of glycosylation in cancer malignancy as well as identifying novel carbohydrate tumor marker candidates (Alley et al. 2012; Narimatsu et al. 2010). This chapter introduces the achievements of glycomic analyses of glycoconjugates including glycosphingolipids, free glycans, *N*-linked, and *O*-linked glycans derived from human cancers and serum of cancer patients.

12.2 GSLs

Extensive studies have been performed to analyze the structure of GSLs from a variety of tumor tissues, including colon cancer (Siddiqui et al. 1978), melanoma (Portoukalian et al. 1979), gastrointestinal cancers (Magnani et al. 1982), head and neck squamous cell carcinoma (Bolot et al. 1999), metastatic brain tumors (Hamasaki et al. 1999), and renal cell carcinoma (Ito et al. 2001). Furthermore, a series of GSLs that abnormally accumulate in cancerous tissues have been successfully isolated and analyzed (Hakomori et al. 1983, 1984; Fukushi et al. 1984a, b). These studies reveal that each type of tumor is characterized by the accumulation of specific types of GSLs. For example, unusual accumulation of GSLs having type 1 or 2 chain derivatives (i.e., those with Le^a, Le^x, Le^y, or dimeric Le^x, and their sialosyl derivatives, such as sialyl Le^a and sialyl Le^x) is observed in most human adenocarcinoma (Hakomori et al. 1984; Fukushi et al. 1984b; Nudelman et al. 1986a, b), while GD3 is observed in melanoma (Portoukalian et al. 1979).

Structural analyses of these GSLs were performed by conventional techniques. Specifically, GSLs extracted from cancerous tissues were separated by HPLC (high performance liquid chromatography) using organic solvents and traditional thin-

layer chromatography (TLC) methodologies followed by staining with orcinol. However, there are a number of limitations with these analytical techniques in terms of identification and quantification of GSLs.

Ito et al. discovered endoglycoceramidases capable of hydrolyzing the glucosylceramide linkage of most GSLs, leading to the release of their carbohydrate moieties (Ito and Yamagata 1989). Liberated oligosaccharides of GSLs are reductively aminated with fluorescence reagents, such as 2-aminopyridine and 2-aminobenzoic acid, and then subjected to HPLC separation or mass spectrometry. This technique is highly sensitive and capable of analyzing GSLs both quantitatively and qualitatively. Glycomic analyses of GSLs of cancerous tissues using the advanced methods have been performed by several groups. These analyses have verified previous results and discovered novel findings as described below.

12.2.1 Aberrant Glycosylation

Comprehensive clinico-glycomic analyses of GSLs of colorectal cancerous cells and their corresponding normal colorectal epithelial cells were performed from 16 colorectal cancer patients including 5 having liver metastasis and 1 lacking α1-4 fucosyltransferase, Lewis enzyme activity, and 2 showing high serum levels of CA19-9 (Misonou et al. 2009). In order to enhance the accuracy of the analysis, an improved method of isolating epithelial cells was employed using collagenase treatment and magnetic beads labeled with antibody against epithelial cell marker, CD326. As a result, highly purified colorectal cancer cells (CCs) and their corresponding normal colorectal epithelial cells (NCs) were obtained from cancerous and normal tissues, respectively.

The structures of glycans from normal colorectal epithelial cells are characterized by dominant expression of neutral type 1 chain oligosaccharides; lactose, Le^a, and Le^b are dominant and acidic GSLs are almost absent. Three specific alterations were observed in malignant transformation, namely, (1) increased ratios of type 2 oligosaccharides, (2) increased α2-3 and/or α2-6 sialylation, and (3) increased α1-2 fucosylation (Fig. 12.1). These alterations result in increases in the amount of or appearance of Le^x, LST-c, Le^y, Le^b, sialyl Le^x, sialyl Le^a, $IV^6NeuAc\alpha IV^2Fuc\alpha$-$nLc_4$ (ST2H), $V^3Fuc\alpha III^3Fuc\alpha$-$nLc_6$, $VI^3NeuAc\alpha$-nLc_6, and $VI^6NeuAc\alpha III^3Fuc\alpha$-$nLc_6$ (Fig. 12.1). Most of these findings are essentially in agreement with previous results obtained by conventional methods.

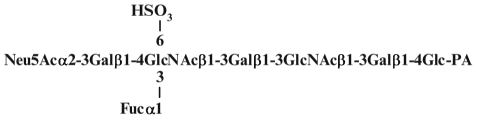
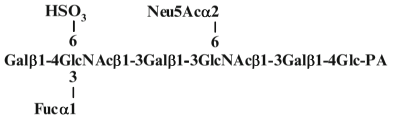
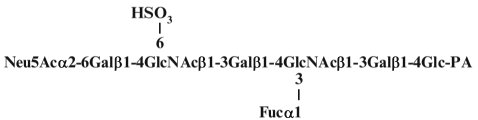
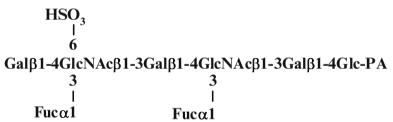
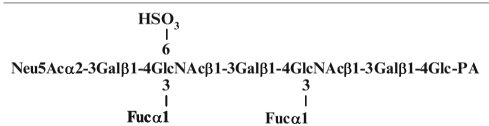
In addition to the general pattern of abnormal accumulation observed in malignant transformation of colorectal cancers, these precise analyses also revealed two further characteristic alterations that are associated with clinical features. One such change was a shift from type 1 dominant NCs to type 2 dominant CCs found in the five cases having hepatic metastasis. Second was a specific elevation of α2-3 sialylation observed in two cases exhibiting high serum levels of CA19-9. Further extensive glycomic analyses of colorectal cancer cells revealed that CCs estimated to have low metastatic potential express a variety of oligosaccharides including very rare sulfated GSLs, such as 6-sulfo Le^x, 6′-sialyl 6-sulfo lactosamine, and 3′-sialyl 6-sulfo Le^x, in addition to sialylated or fucosylated derivatives of type 1 and type 2 hybrid oligosaccharides (Table 12.1) (Shida et al. 2009).

Type-1
GlcNAcβ1-3Galβ1-4Glc
Lc3
Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Lc4
4F
Galβ1-3GlcNAcβ1-3Galβ1-4Glc
4
Fucα1
Lea
3S
NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc
SLex (DU-PAN-2)
4F
NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc
4
Fucα1
SLea (CA19-9)
2F
Galβ1-3GlcNAcβ1-3Galβ1-4Glc
2
Fucα1
Type1H
4F
Galβ1-3GlcNAcβ1-3Galβ1-4Glc
2 4
Fucα1 Fucα1
Leb
6S
NeuAcα2-6Galβ1-3GlcNAcβ1-3Galβ1-4Glc
2
Fucα1
ST1H
Type-2
Galβ1-4GlcNAcβ1-3Galβ1-4Glc
nLc4
3F
Galβ1-4GlcNAcβ1-3Galβ1-4Glc
3
Fucα1
Lex
3S
NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc
SPG
3F
NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc
3
Fucα1
SLex
6S
NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc
LST-c
2F
Galβ1-4GlcNAcβ1-3Galβ1-4Glc
2
Fucα1
Type2H
3F
Galβ1-4GlcNAcβ1-3Galβ1-4Glc
2 3
Fucα1 Fucα1
Ley
6S
NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc
2
Fucα1
ST2H

Fig. 12.1 Proposed synthetic pathways for major groups of GSLs in colorectal cancers and normal colorectal epithelial cells. *Arrows* indicate the pathways predominating in normal colorectal epithelial cells. *Broken arrows* indicate the pathways that are increased in carcinogenesis. Abbreviations: *4F* α1-4 fucosylation of GlcNAc (Lewis enzyme activity), *3F* α1-3 fucosylation of GlcNAc, *2F* α1-2 fucosylation of galactose, *3S* α2-3 sialylation of galactose, *6S* α2-6 sialylation of galactose. The structures of GSLs in normal colorectal epithelial cells from Lewis-positive individuals are composed mainly of Le^a and Le^b (highlighted by a *square* with a *thick line*). By contrast, the structures of GSLs in normal epithelial cells from Lewis-negative individuals are composed mainly of Lc_4 and type1H (highlighted by a *square* with a *thin line*). In malignant transformation, the type 2 ratio, α2-3 and/or α2-6 sialylation, and α1-2 fucosylation are increased. These alterations result in increases in the amounts of, or the appearance of, a variety of oligosaccharides, such as Le^x, Le^y, LST-c, SLe^x, and ST2H as type 2 oligosaccharides and type1H, SLe^a, SLe^c, and ST1H as type 1 oligosaccharides. Note the difference in composition of type 1 oligosaccharides between cancer cells from Lewis-positive and Lewis-negative individuals, i.e., synthesis of SLe^a is increased upon carcinogenesis, and SLe^a becomes one of the major components of cancer cells in Lewis-positive individuals. However, SLe^a is not synthesized in cancer cells and normal epithelial cells from Lewis-negative individuals, but the levels of SLe^c and/or ST1H are increased during carcinogenesis.
SLe^a epitope (NeuAcα2-3Galβ1-3(Fucα1-4)GlcNAcβ1-R) and SLe^c epitope (NeuAcα2-3Galβ1-3GlcNAcβ1-R) are recognized by CA19-9 and DU-PAN-2 antibodies, respectively

Table 12.1 Sulfated GSLs accumulated in colon cancers that are estimated to have low metastatic potential

Structure	Abbreviation
HSO_3-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA	3′-sulfo-Lc_4
HSO_3 (6) GlcNAcβ1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA	agalacto $V^6HSO_3^-{}_{1,2}Lc_6$
HSO_3 (6) Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA; Fucα1 (3)	III^6HSO_3,III^3Fucα-nLc_4
HSO_3 (6) Galβ1-4GlcNAcβ1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA	$V^6HSO_3^-{}_{1,2}Lc_6$
HSO_3 (6) Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA	VI^6NeuAcα,$V^6HSO_3^-{}_{1,2}Lc_6$
HSO_3 (6) Galβ1-4GlcNAcβ1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA; Fucα1 (3)	V^6HSO_3,V^3Fucα-$_{1,2}Lc_6$
HSO_3 (6) Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA; Fucα1 (3)	V^6HSO_3,III^3Fucα-nLc_6

	VI^{3}NeuAcα,V^{6}HSO$_3$,V^{3}Fucα-$_{1,2}$Lc$_6$
	V^{6}HSO$_3$,V^{3}Fucα,III^{6}NeuAcα-$_{1,2}$Lc$_6$
	VI^{6}NeuAcα,V^{6}HSO$_3$,III^{3}Fucα-nLc$_6$
	V^{6}HSO$_3$,V^{3}Fucα,III^{3}Fucα-nLc$_6$
	VI^{3}NeuAcα,V^{6}HSO$_3$,V^{3}Fucα,III^{3}Fucα-nLc$_6$

Type 1 and type 2 hybrid hexasaccharides, Galβ1-4GlcNAcβ1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc is abbreviated as $_{1,2}$Lc$_6$, in order of linkage type of the fourth and sixth galactose

In order to understand the mechanism of aberrant glycosylation, precise analyses of the activities of glycosyltransferases responsible for the aberrant glycosylation in malignant transformation of colorectal cancers (β-galactosyltransferase, sialyltransferase, fucosyltransferase, sulfotransferase) were carried out (Misonou et al. 2009). While some alterations could be accounted for by changes in the activities of related glycosyltransferases, others could not. Increases in the ratio of type 2 oligosaccharides, α2-6 sialylation, and α1-2 fucosylation can be broadly accounted for by changes in the activities of related glycosyltransferases. Thus, in malignant transformation, β1-3 galactosyltransferase activity is markedly decreased, α2-6 sialyltransferase activity toward terminal galactose of nLc4 is increased, and the α1-2 fucosyltransferase activity toward both nLc_4 and Lc_4 is markedly increased with very few exceptions. It is possible that greatly reduced activity of β1-3 galactosyltransferase and a virtually invariant alteration in the activities of β1-4 galactosyltransferase in carcinogenesis result in the increase of type 2 chain oligosaccharides. Similarly, increased type 2 chain oligosaccharides followed by an increase in the activity of α2-6 sialyltransferase toward type 2 lactosamine chains results in the elevation of α2-6 sialylated type 2 oligosaccharides, such as LST-c and $VI^6NeuAc\alpha III^3Fuc\alpha\text{-}nLc_6$. Furthermore, greatly increased activity of α1-2 fucosyltransferase toward both nLc_4 and Lc_4 leads to the elevation of α1-2 fucosylated products, such as Le^y and Le^b. In contrast, elevation of α2-3 sialylation in carcinogenesis does not depend on changes in the related enzyme activities.

Holst et al. also performed comprehensive glycomic analyses of GSLs of colorectal cancer tissues and corresponding control tissues of 13 colorectal cancer patients by methods that include enzymatic release of carbohydrate moieties, fluorescent labeling with 2-aminobeozoic acid, and MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) (Holst et al. 2013). They reported that the alteration of carcinogenesis of colorectal cancers is characterized by (1) increased fucosylation, (2) decreased acetylation, (3) decreased sulfation, (4) reduced expression of globo-type glycans, as well as (5) disialyl gangliosides.

12.2.2 Novel Tumor-Associated Carbohydrate Antigens

Comprehensive glycomic analyses of colorectal cancers (60 patients) and pancreatic cancers (5 patients) revealed the presence of 2 kinds of novel fucogangliosides, as described below (Korekane et al. 2007b; Misonou et al. 2009; Shida et al. 2010) (Fig. 12.1). Both of these are isomers of well-known tumor-associated carbohydrate antigens, sialyl Le^x(SLe^x) and sialyl Le^a (SLe^a):

NeuAcα2-6(Fucα1-2)Galβ1-4GlcNAcβ1-3Galβ1-4Glc (sialyl type2H, ST2H)
NeuAcα2-6(Fucα1-2)Galβ1-3GlcNAcβ1-3Galβ1-4Glc (sialyl type1H, ST1H)

The α2,6-sialylated type2H (ST2H) was found in colorectal cancer cells from about half of the cases (36 cases out of 60 colorectal cancer patients; 3 cases out of 5 pancreatic cancer patients) regardless of Lewis type. Unlike ST2H, the α2,6-sialylated type1H (ST1H) was found specifically in cancer cells from half of the

Lewis-negative patients (3 out of 6 Lewis-negative patients, i.e., 2 cases of colorectal and 1 case of pancreatic cancer). However, the moiety was not found in cancer cells from 59 Lewis-positive patients. Both ST1H and ST2H antigens were absent in normal colorectal and pancreatic cells.

ST1H and ST2H are carbohydrate tumor marker candidates, similar to SLe^x and SLe^a. Specifically, ST1H has a type 1 structure, similar to the SLe^a epitope (CA19-9 epitope), and is a promising candidate tumor marker.

Lewis enzyme (also called FUT3) is the only enzyme responsible for the synthesis of Lewis antigens, such as Le^a, Le^b, and SLe^a in vivo (Kukowska-Latallo et al. 1990). Lewis-negative individuals, who make up approximately 10 % of the population, are homozygotes for the inactive Lewis gene alleles (Mollicone et al. 1994; Nishihara et al. 1994; Elmgren et al. 1997). Such individuals do not possess Lewis enzyme activity and never express Le^a, Le^b, and SLe^a in any tissue (Nishihara et al. 1999; Narimatsu et al. 1996; Yazawa et al. 1995) (Fig. 12.1). Because Lewis-negative individuals cannot produce the SLe^a epitope (CA19-9 epitope), serum levels of CA19-9 in these individuals are either undetectable or very low (i.e., under 1 U/ml). DU-PAN-2 (SLe^c epitope), which is a precursor structure of SLe^a, is another well-known tumor marker (Fig. 12.1). Hence, measurement of CA19-9 and DU-PAN-2 is recommended to apply for Lewis-positive and Lewis-negative individuals, respectively (Narimatsu et al. 1998) (Fig. 12.1). The synthetic flow of DU-PAN-2 and ST1H is different, i.e., DU-PAN-2 and ST1H are synthesized by α2-3 sialylation of Lc_4 and α2-6 sialylation of type1H, respectively (Fig. 12.1). The combination of ST1H and DU-PAN-2 determinants could serve as a highly sensitive tumor marker, especially for Lewis-negative individuals.

12.3 Free Oligosaccharides

The occurrence of free high-mannose-type *N*-glycans is well demonstrated in mammalian cells (Moore 1999; Suzuki and Funakoshi 2006; Winchester 2005). However, with the exception of mouse liver and two kinds of human stomach cancer-derived cell lines (MKN7 and MKN45) (Ohashi et al. 1999; Ishizuka et al. 2008), free complex-type *N*-glycans, especially sialylated species, are not normally observed.

Some valuable information regarding the presence of free sialylated complex-type *N*-glycans were obtained from the glycomic analyses of colorectal, pancreatic, and prostate cancers (Yabu et al. 2013a, b). GSLs and free oligosaccharides, but not glycoproteins, are extracted when organic solvents are used to homogenize tissues or cells. When the organic solvent-extractable fractions were treated with EGCase (endoglycoceramidase), both free oligosaccharides and oligosaccharides released from GLSs were subjected to analysis.

The Neu5Ac-containing complex-type *N*-glycans with a single GlcNAc at each reducing terminus (Gn1 type) were observed as a minor component in colorectal cancer cells (i.e., much lower than GSLs, such as GM3 and LST-c). Structural analyses of oligosaccharides associated with pancreatic cancers revealed, unlike

Table 12.2 Neu5Ac- and KDN-containing free complex-type *N*-glycans accumulated in human cancers

Group		Structure
1	◎	Manβ1-4GlcNAc-PA (3) / Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1
	○	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1 (6) Manβ1-4GlcNAc-PA
		Manβ1-4GlcNAc-PA (3) / Neu5Acα2-6Galβ1-4GlcNAcβ1-4Manα1
		Neu5Acα2-6Galβ1-4GlcNAcβ1-6Manα1 (6) Manβ1-4GlcNAc-PA
		Manα1 (6) Manβ1-4GlcNAc-PA / (3) Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1
		Manβ1-4GlcNAc-PA (3) / Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1
2	◎	Manβ1-4GlcNAc-PA (3) / KDNα2-6Galβ1-4GlcNAcβ1-2Manα1
	○	KDNα2-6Galβ1-4GlcNAcβ1-2Manα1 (6) Manβ1-4GlcNAc-PA
		Manβ1-4GlcNAc-PA (3) / KDNα2-6Galβ1-4GlcNAcβ1-4Manα1
		KDNα2-6Galβ1-4GlcNAcβ1-6Manα1 (6) Manβ1-4GlcNAc-PA
		KDNα2-3Galβ1-4GlcNAcβ1-2Manα1 (6) Manβ1-4GlcNAc-PA

Group 1: Neu5Ac-containing complex-type *N*-glycans accumulated in pancreatic and prostate cancers. Group 2: KDN-containing complex-type *N*-glycans accumulated in prostate cancers
◎ indicates the most abundant species. ○ indicates the second most abundant species. The other species are minor components

colorectal cancer cells, the presence of a variety of free Neu5Ac-containing complex-type *N*-glycans from three out of the five cases (Table 12.2) (Yabu et al. 2013b). The relative amounts of these free Neu5Ac-containing complex-type *N*-glycans were comparable to or much higher than those of GSLs in most, but not all, pancreatic cancers. The Neu5Ac-containing complex-type *N*-glycans derived from human pancreatic cancer cases possess several common characteristic features. Specifically, (1) almost all (>95 %) of the free *N*-glycans are composed of α2,6-Neu5Ac-linked glycans, with α2,3-sialylated glycans making up a very minor part, and (2) the proportion of each free *N*-glycan relative to total free glycans is to some extent dependent on its pentasaccharide backbone. Namely, free

α2,6-Neu5Ac-linked *N*-glycans having a Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAc backbone are the most abundant species (i.e., 48–72 % of total free *N*-glycan content). The second most abundant glycans had a Galβ1-4GlcNAcβ1-2Manα1-6Manβ1-4GlcNAc backbone (8–24 %), followed by glycans with either a Galβ1-4GlcNAcβ1-6Manα1-6Manβ1-4GlcNAc or Galβ1-4GlcNAcβ1-4Manα1-3Manβ1-4GlcNAc backbone. These results indicate that the branch on the α6-Man arm of biantennary *N*-glycans is preferentially removed. Thus, the most abundant glycan is Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAc, followed by Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6Manβ1-4GlcNAc. However, the levels of free *N*-glycans in normal colorectal and pancreatic tissues were barely detectable (Yabu et al. 2013b).

In addition to the free Neu5Ac-containing *N*-glycans accumulated in pancreatic cancers, a relatively large amount of free KDN (deaminoneuraminic acid)-containing *N*-glycans were also found to accumulate in prostate cancer tissues from four out of five patients (Table 12.2) (Yabu et al. 2013a). Indeed, in one of the four cases having bone metastasis, the free KDN-glycans are major components, and the amounts of the free KDN-glycans were much higher than those of GSLs in both primary and bone metastatic prostate cancer tissues. With regard to KDN, refer to the review article by Inoue and Kitajima (2006). KDN is an unusual type of sialic acid that was first discovered in the cortical alveolar polysialoglycoprotein (PSGP) of rainbow trout eggs (Nadano et al. 1986). Subsequent studies revealed that KDN, like the typical type sialic acid Neu5Ac, occurs widely among vertebrates and bacteria, although KDN is only abundant in lower vertebrates and pathogenic bacteria. In mammals, Neu5Ac occurs abundantly in both normal and tumor tissues, whereas KDN is almost undetectable (Inoue et al. 1996).

The characteristic features of free KDN-containing *N*-glycans associated with human prostate cancers are very similar to those of free Neu5Ac-containing *N*-glycans, described above. Specifically, (1) most of the free *N*-glycans are composed of α2,6-KDN-linked glycans, and (2) the branch on the α6-Man arm of biantennary *N*-glycans is preferentially removed. Hence, the most abundant free KDN-containing *N*-glycan is KDNα2-6Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAc followed by KDNα2-6Galβ1-4GlcNAcβ1-2Manα1-6Manβ1-4GlcNAc.

Biochemical analyses have revealed the mechanism responsible for the accumulation of free Neu5Ac-containing *N*-glycans in stomach cancer cell lines. These studies showed impaired lysosomal function leading to inefficient degradation of free *N*-glycans in the lysosomes, as well as the leakage of lysosomal components (including free *N*-glycans) into the cytosol (Ishizuka et al. 2008). A similar mechanism might be responsible for the accumulation of free complex-type *N*-glycans in human pancreatic cancer cells, and the pathological mechanism may be similar to the lysosomal disease, sialidosis. This hypothesis is supported by the finding that the principal Neu5Ac-containing free oligosaccharides which accumulate in cancer cells are also found in excessive urinary excretion from patients with sialidosis (Strecker et al. 1977). A mechanism responsible for the accumulation of KDN-containing free *N*-glycans is also thought to be related to reduced levels of degradation. However, several questions remain to be elucidated. Namely,

(1) KDN-containing *N*-glycans were found in prostate cancers, but not in colorectal and pancreatic cancers. Do free KDN-containing *N*-glycans accumulate specifically in prostate cancers? Do these species accumulate in any other cancer tissue? If not, why do KDN-containing *N*-glycans specifically accumulate in prostate cancers? (2) Four kinds of human neuraminidases, Neu1 (lysosomal), Neu2 (cytosolic), and Neu3 and Neu4 (both membrane bound), are known to mediate the release of a Neu5Ac residue from sialoglycoconjugates. KDN linkages are thought to be resistant to the action of human sialidases. Are sialidases capable of cleaving KDN linkages present in human cells?

12.4 *N*-Glycans and *O*-Glycans

Most of the biomarker discovery studies based on glycomics have focused on mapping changes in serum and cancer cell lines. However, detailed glycomic analyses of *N*-glycans or *O*-glycans using human cancer tissue specimens are somewhat limited. Glycomic analyses of human cancer tissues and serum are introduced below.

12.4.1 Glycomic Studies on Human Cancers

The β1-6 branching of *N*-linked structures resulting from enhanced or induced expression of GlcNAc transferase-V is one of the most common types of aberrant glycosylation observed in experimental and human cancers (Yamashita et al. 1984). Clinicopathological, immunohistological, and biochemical studies show a correlation between the expression of these branching structures and invasion, metastatic potential, and shorter survival rates of the patients (Fernandes et al. 1991; Dennis and Laferte 1989). The formation of bisecting GlcNAc, which is synthesized by GlcNAc transferase-III, competes with the formation of β1-6 branching sites and hence the occurrence of triantennary and tetra-antennary structures. The occurrence of bisecting GlcNAc has been implicated in the suppression of metastasis, leading to higher rates of survival (Yoshimura et al. 1995).

The glycomic analyses of human cancer specimens support these previous findings. Korekane et al. compared the *N*-glycan profiling between those of colon cancer and normal colon epithelia using laser microdissected samples (Korekane et al. 2007a). *N*-Glycans were liberated from the samples by hydrazinolysis, labeled with 2-aminopyridine and then subjected to HPLC separation and mass spectrometry. Korekane et al. reported a marked reduction of *N*-glycans having bisecting GlcNAc in malignant transformation of colon cancers (Korekane et al. 2007a). However, these findings were not evident when using bulk colon cancer tissues and normal colon epithelial tissues. This observation highlights the importance of isolating cancer cells or normal cells with high purity for accurate cancer glycomic studies

(as well as proteomics studies) by using laser microdissection or purification methods with epithelial cell markers.

Balog et al. performed the comprehensive glycomic analyses of *N*-glycosylation of colorectal cancer tissues and corresponding control tissues of 13 colorectal cancer patients by methods that included the release of *N*-glycans by *N*-glycosidase F, fluorescent labeling with 2-aminobenzoic acid, and MALDI-TOF-MS (Balog et al. 2012). As well as verifying a decrease in the occurrence of *N*-glycans containing bisecting GlcNAc, these workers also reported an increase in the levels of sulfated *N*-glycans and paucimannosidic *N*-glycans in colorectal cancer tissues by comparison with surrounding normal colorectal epithelial tissues.

In the above two cancer glycomic analyses, each *N*-glycan was released and then labeled with a fluorescent molecule prior to characterization by HPLC separation and mass spectrometry.

Lectin microarray technology was also applied to cancer glycomic analyses (Kuno et al. 2010; Matsuda et al. 2008, 2010). Tissue-based differential glycan profiling covering both *N*- and *O*-glycans was constructed by 43 kinds of lectins using very small quantities of glycoproteins derived from a very small region (one-dot section comprising about 1,000 cells corresponding to 1.5 mm diameter and 5 μm thickness tissue array) of formalin-fixed paraffin-embedded specimens. Among the 43 lectins, *Wisteria floribunda* agglutinin (WFA) was found to be the best probe to differentiate intrahepatic cholangiocarcinoma lesions from normal bile duct epithelia (Matsuda et al. 2010).

As in GSLs and *N*-glycans, the structures of *O*-glycans (mucin-type glycans) are also altered in malignant transformation (Yang et al. 1994; Vavasseur et al. 1994; Brockhausen 1999, 2006). Compared with *O*-glycans in normal epithelial cells, cancer-associated *O*-glycans can be highly sialylated and less sulfated and are often truncated. Increased levels of Tn (GalNAcα-Ser/Thr) and sialyl Tn (Neu5Acα2-6GalNAcα-Ser/Thr) antigens were observed in a variety of human cancers including colon cancers and ovarian cancers (Orntoft et al. 1990; Inoue et al. 1991). Most of these results were obtained from immunohistochemical analyses using monoclonal antibodies recognizing the individual *O*-glycans. However, very few qualitative and quantitative structural analyses of *O*-glycans from human cancer tissues have been performed. Because serum glycan tumor marker epitopes such as CA19-9 are carried on mucins, detailed glycomic analyses of *O*-glycans in human cancers would provide a new source of tumor markers.

12.4.2 Glycomic Studies on Serum of Cancer Patients

Carbohydrate antigens, such as CA19-9, CA125, DUPAN-II, and AFP-L3, are the most frequently used serum biomarkers for cancer. Serum glycomic studies to discover novel glycan cancer biomarkers have been highlighted (Adamczyk et al. 2012). Numerous glycan tumor marker candidates for various cancers, including liver (Kaji et al. 2013; Kamiyama et al. 2013; Wu et al. 2012; Tang et al. 2010;

Goldman et al. 2009; Comunale et al. 2009; Tanabe et al. 2008; Ressom et al. 2008), ovary (Biskup et al. 2013; Hua et al. 2013; Alley et al. 2012; Abbott et al. 2010; Leiserowitz et al. 2008), pancreas (Li et al. 2009; Okuyama et al. 2006), breast (de Leoz et al. 2011; Alley et al. 2010; Zeng et al. 2010; Storr et al. 2008; Abd Hamid et al. 2008; Kyselova et al. 2008; Kirmiz et al. 2007), prostate (Saldova et al. 2011), lung (Arnold et al. 2011), colorectum (Zhao et al. 2012), bile duct (Silsirivanit et al. 2011), and esophagus (Mechref et al. 2009) cancers, have been reported. Most of these glycomic analyses have been carried out by newly developed high-throughput platform technologies, such as mass spectrometry-based methods and lectin-based methods, which have enabled the efficient analysis of large cohorts of samples.

Among the variety of oligosaccharide tumor marker candidates, most are *N*-linked oligosaccharides. Indeed, there are only a limited number of reported *O*-linked oligosaccharide tumor marker candidates (Leiserowitz et al. 2008; Storr et al. 2008; Kirmiz et al. 2007). In a majority of cancers, the levels of fucosylation and sialylation are found to be significantly altered. Most of these glycomic studies have been carried out to identify changes in serum glycan profiles or through the isolation and identification of glycoproteins that contain these irregular glycan structures. One such study looking for abnormal glycan structures of glycoprotein found that the concentration of fucosylated haptoglobin increased significantly in the serum of pancreatic cancer patients compared to patients with other types of cancer or healthy controls (Okuyama et al. 2006).

However, despite the large number of studies that have been conducted, the successful results of glycomic analyses to identify differences between serum from cancer patients and healthy donors have been somewhat varied. Nonetheless, it is anticipated that improvements in serum glycomics will further enhance the identification of glycan biomarkers for cancer. It is hoped that such biomarkers will be applicable for clinical diagnostic purposes.

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Chapter 13
Glyco-Predisposing Factor of Diabetes

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Abstract Appropriate insulin secretion is an essential process in glucose homeostasis and is initiated by glucose sensing with glucose transporter-2 (GLUT2) in pancreatic β-cells. The disappearance of GLUT2 from the β-cell surface is one of the early markers of the onset of type 2 diabetes, though the molecular mechanism has not been well understood. Recent advance in glycophysiology revealed that specific GLUT2 glycosylation by GnT-IVa is required for the production of carbohydrate epitopes bound to galectin-9 on the β-cell surface. The engagement of galectin-9 with GLUT2 regulates remodeling of GLUT2 clusters among cell surface membrane sub-domains, to control glucose transport activities, and prevents endocytosis to increase cell surface residency of GLUT2 that contributes to sustaining the glucose sensor function of β-cells. The pathway to diet- and obesity-associated diabetes has recently been revealed, in which a high-fat diet leading to diabetes recapitulated the free fatty acid-induced oxidative stress in human and mouse pancreatic β-cells that induced nuclear exclusion of transcription factors regulating GnT-IVa and, subsequently, attenuated GnT-IVa-dependent GLUT2 glycosylation. In β-cells, overexpression of GnT-IVa prevents GLUT2 glycosylation and high-fat diet-induced β-cell dysfunction that ameliorates the onset of type 2 diabetes. These findings indicate that GnT-IV-mediated redistribution of cell surface GLUT2 is a fundamental process to regulate insulin secretion responses to blood glucose levels, a paradigm that can be practically applied to better understand the pathogenesis of type 2 diabetes and provide a clue for the development of drugs.

Keywords Type 2 diabetes • Glucose transporter • Glycosylation • GnT-IVa • Pancreatic β-cells • Oxidative stress

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13.1 Introduction

All organisms require glucose for the production of energy in the form of ATP, as well as a major component in the elaboration of proteins, lipids, and nucleotides. Therefore, maintaining of glucose homeostasis is important for biological activities that are sustained by endocrinological and neurological cooperation in response to dynamic physiological fluctuation of blood glucose levels. In vertebrates, cells specialized in glucose homeostasis commonly express a glucose sensor molecule, glucose transporter (GLUT), on their cell surface, which enables cells to uptake extracellular glucose from interstitial fluid. This uptake is achieved by passive and facilitative transport processes along with the downward gradient of glucose concentration across the cellular plasma membrane, initiating cellular metabolic responses. Therefore, GLUT-dependent cellular glucose uptake was thought to be a physiological mandatory process.

GLUT consists of 13 members, of which 11 are specific for sugar transport without any energy requirements. GLUT family proteins are structurally conserved and share a structure of 12 membrane-spanning regions and a single *N*-glycan in either the first or the fifth extracellular loop domain (Widdas 1988; Baldwin and Lienhard 1981; Joost et al. 2002; Gould and Holman 1993). It has been reported that *N*-glycosylation is indispensable for the stable expression of GLUT on the cell surface (Asano et al. 1991, 1993; Ohtsubo et al. 2005), implying that *N*-glycosylation of GLUT plays an important role in glucose homeostasis, and further suggests that dysglycosylation might be involved in the pathogenesis of glucose homeostasis disorders.

It has been well characterized that the glucose-stimulated insulin secretion of pancreatic β-cells is impaired in the disease process of diabetes. Consistent with this finding, the glucose sensor function of pancreatic β-cells is attenuated in human type 2 diabetes patients and in various diabetic animal models. In addition, a decreased total and cell surface expression of GLUT2 protein is found in pancreatic β-cells in the early stage of diabetes that reflects the failure of glucose-stimulated insulin secretion (Johnson et al. 1990; Orci et al. 1990; Thorens et al. 1990; Unger 1991; Del Guerra et al. 2005). Its molecular mechanism, however, is not well understood.

13.2 Dysglycosylation of GLUT2 in Pancreatic β-Cells in the Disease Process of Type 2 Diabetes

Human chromosomal susceptible regions to type 2 diabetes were analyzed by genetic linkage analyses among type 2 diabetes patients and their relatives, and a chromosomal loci, 2q11.5, was identified (McCarthy 2003; Van Tilburg et al. 2003). The region encompasses a gene of mannosyl (α-1,3-)-glycoprotein β-1,4-N-acetylglucosaminyltransferase (*N*-acetylglucosaminyltransferase-IV) isozyme A,

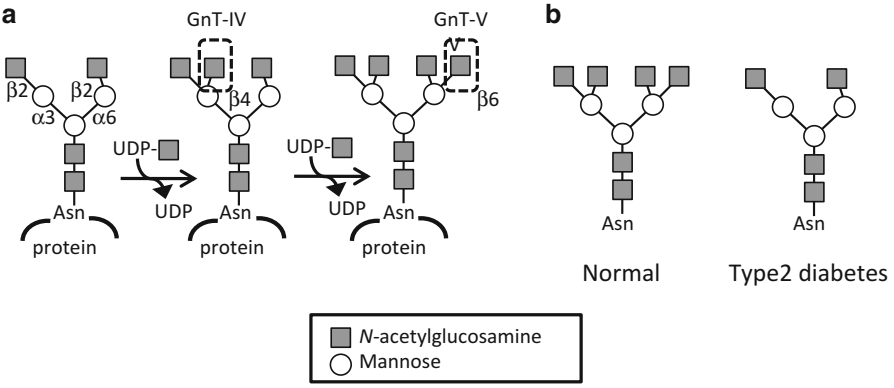

Fig. 13.1 GnT-IVa-dependent formation of multiantennary *N*-glycan structures. (**a**) In mammalian cells, newly synthesized proteins are sequentially *N*-glycosylated by GnT-IV and GnT-V and consequently acquire multiantennary *N*-glycans. (**b**) GLUT2 in normal pancreatic β-cells has a tetra-antennary *N*-glycan, whereas that of type 2 diabetes has a bi-antennary *N*-glycan

abbreviated to GnT-IVa or MGAT4A, which catalyzes the transfer of GlcNAc (*N*-acetylglucosamine) from UDP-GlcNAc to the GlcNAcβ1-2Manα1,3 arm of the core structure of *N*-linked oligosaccharides ($Man_3GlcNAc_2$-Asn) via a β1-4 linkage (Minowa et al. 1998; Yoshida et al. 1999). Moreover, coincident with these findings, DNA microarray analyses of gene expression profile of pancreatic β-cells of type 2 diabetes patients revealed that the expression level of the *MGAT4A* gene is significantly reduced in type 2 diabetes patients (Gunton et al. 2005). These findings suggest that the failure of GnT-IVa function is involved in the pathogenesis of type 2 diabetes.

GnT-IVa is an essential glycosyltransferase to form multiantennary (tri- or tetra-antennary branched) complex-type *N*-glycans (Fig. 13.1a), which is highly expressed in the pancreas (β-cells), small intestine, colon, kidney, and brain, as well as many types of tumor cells (Ohtsubo et al. 2005; Yoshida et al. 1999). The expression of GnT-IVa seems to be regulated by multiple mechanisms. The normal tissue distribution of GnT-IVa largely overlaps with that of GLUT2. Sequence motif analyses among promoter regions of human and mouse *MGAT4A* and GLUT2 genes revealed that they share binding sites of transcription factors, FOXA2 and HNF-1α, which are well characterized to regulate function and development of pancreatic β-cells (Ohtsubo et al. 2011). FOXA2 and HNF-1α are well characterized to transcriptionally regulate GLUT2 expression in pancreatic β-cells (Cerf 2006) that strongly suggest that the expressions of GnT-IVa and GLUT2 are synchronously regulated, consequently enabling GLUT2 to acquire an *N*-glycan formed by GnT-IVa in a specific type of cells. Indeed, the introduction of siRNAs for *FOXA2* and *HNF-1α* into β-cells significantly reduced the expression levels of *Mgat4a* and *GLUT2* (Ohtsubo et al. 2011). Consistent with these findings, in normal human and mice pancreatic β-cells, GLUT2 has multiantennary N-glycans synthesized by

GnT-IVa activity. In contrast, GLUT2 in pancreatic β-cells of human type 2 diabetes and mice receiving a high-fat diet has less branched *N*-glycans (Ohtsubo et al. 2005, 2011) (Fig. 13.1b), which is consistent with reduced expression of *MGAT4A* in pancreatic β-cells in type 2 diabetes patients, described above.

13.3 GnT-IVa-Dependent Glycosylation Regulates In Situ Distribution of GLUT2 and Cellular Glucose Sensor Function

Pathophysiological relevance of GnT-IVa deficiency in the disease process of type 2 diabetes has been well elucidated by the studies of GnT-IVa-deficient mice. GnT-IVa-deficient mice were engineered by genetic disruption of *Mgat4a* gene in embryonic stem cells using Cre-loxP gene targeting system. GnT-IVa-deficient mice were normal in hematology, immunology, behavior, learning, and fecundity, though they showed signs of type 2 diabetes including moderate hyperglycemia, hypoinsulinemia, elevation of free fatty acids, triglycerides, ALT and AST in serum chemistry, impaired glucose tolerance, hepatic steatosis, and diminished insulin action in muscle and adipose tissues (Ohtsubo et al. 2005).

In in vitro insulin secretion assay, primary isolated normal pancreatic β-cells show two peaks of insulin secretion (primary response and secondary response) in response to the elevation of extracellular glucose concentration. Primary isolated GnT-IVa-deficient pancreatic β-cells lost the primary response (Ohtsubo et al. 2005). This insulin secretion pattern resembled that of GLUT2 deficiency that suggested that GnT-IVa deficiency impaired the function of GLUT2 in pancreatic β-cells.

In addition, the analyses of glucose uptake kinetics of primary pancreatic β-cells revealed that the affinity to glucose was not altered and glucose uptake speed was significantly reduced (~10 % of wild type) in GnT-IVa-deficient β-cells (Ohtsubo et al. 2005), implying that the attenuation of glucose uptake in GnT-IVa-deficient β-cells was attributed to the reduced cell surface expression of GLUT2, but not to the impairment of GLUT2 function. This was supported by the analyses of in situ distribution of GLUT2. The reduced β-cell surface residency of GLUT2 in GnT-IVa deficiency was detected by flow cytometry and confirmed by immunohistochemistry of pancreatic β-cells, indicating that the deposition of GLUT2 in plasma membrane was significantly reduced and the greater part intracellularly sequestered in early endosome and lysosome (Ohtsubo et al. 2005).

Pancreatic β-cells have two glucose sensor molecules, GLUT2 and glucokinase (GK), which are rate-limiting molecules in glucose metabolism in β-cells. Under normal conditions, the latter predominantly works as a glucose sensor in the insulin secretion process in β-cells, whereas the former restrains glucose uptake and becomes the limiting step if the cell surface expression level of GLUT2 is lowered by 20 % of normal. Indeed, GLUT2-deficient mice and GLUT2 knockdown mice

exhibit impaired glucose-stimulated insulin secretion (Guillam et al. 1997, 2000; Valera et al. 1994).

GnT-IVa-deficient β-cells showed reduced branch formation of *N*-glycans, the majority of which was altered to a bi-antennary structure (Ohtsubo et al. 2005). These results indicate that GnT-IVa-dependent GLUT2 glycosylation regulates its in situ distribution, and the dysglycosylation of GLUT2 impairs glucose sensor function in β-cells.

13.4 Galectin-Glycan Lattices Regulate Cell Surface Residency of GLUT2

The mechanism of the dysglycosylation-induced intracellular accumulation of GLUT2 has not been well elucidated. For explaining the mechanism, two molecular functions of the GLUT2 *N*-glycan were conjectured: (1) GLUT2 *N*-glycan determines the destination of the intracellular sorting of newly synthesized GLUT2 protein, and (2) GLUT2 *N*-glycan regulates the endocytosis and the cell surface residency of GLUT2 protein. These were tested by pulse-chase labeling experiments of primary isolated pancreatic β-cells that revealed that intracellular transport of newly synthesized GLUT2 protein is normal and they are transported to the cell surface, but cell surface half-life of GLUT2 was significantly shortened in GnT-IVa deficiency (Ohtsubo et al. 2005). These results indicate that the GnT-IVa-dependent *N*-glycan of GLUT2 regulates the stability and residency of GLUT2 protein on the cell surface, but not the intracellular sorting system.

Moreover, the molecular mechanism of *N*-glycan-dependent stabilization of GLUT2 protein on the cell surface was discovered. Lectins are a major protein family, which recognize and bind to specific glycan structures, and play important roles in various biological systems. On the cell surface, glycoproteins bind to lectins via their glycans and form a lattice structure, and thereby, cell surface glycoproteins are organized to exert proper molecular functions (Garner and Baum 2008). Galectin-9 was identified as a lectin associating with GLUT2 in pancreatic β-cells by exploring lectins that selectively bind to the Galβ1-4GlcNAc structure, which is a terminal moiety of GLUT2 *N*-glycan branches. Galectin-9 is a member of the galectin family that selectively binds to β-galactosides with a relatively weak affinity and has two carbohydrate recognition domains that enable it to work as a cross-linker of multiple glycoproteins bearing β-galactosides. Galectin-9 preferentially binds to tri- and tetra-antennary *N*-glycan structures bearing β-galactosides, rather than bi-antennary structures (Sato et al. 2002) that suggest that the glycan-binding specificity of galectin-9 determines the molecular interaction between GLUT2 and galectin-9 and consequently controls the cell surface residency of GLUT2. Indeed, the disruption of the GLUT2-galectin-9 binding on the β-cell surface, by addition of synthetic glycan mimetics (Galβ1-4GlcNAc) to β-cell culture, induced GLUT2 endocytosis and diminished cell surface expression levels of GLUT2 (Ohtsubo et al. 2005).

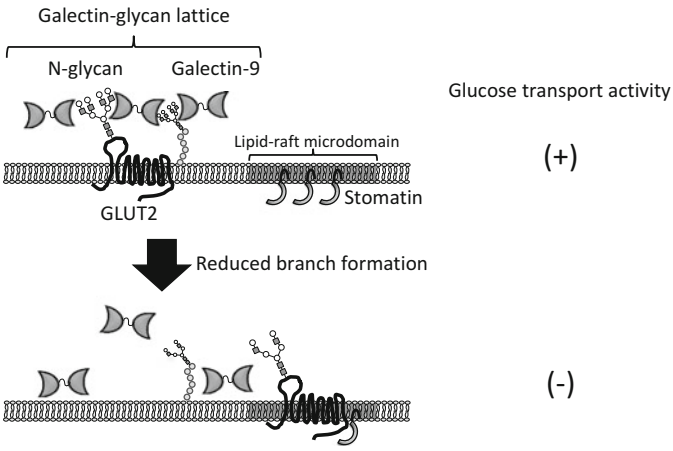

Fig. 13.2 GLUT2 *N*-glycosylation regulates its stability, membrane sub-domain distribution, and glucose sensor function. (*Upper*) GLUT2 binds to galectin-9 by using its *N*-glycan (lattice formation) and stably stays in a non-raft domain of pancreatic β-cell surface that enables cells to uptake glucose efficiently. (*Lower*) Reduced *N*-glycan branch formation attenuates the binding between GLUT2 and galectin-9 that causes the transition of GLUT2 to the lipid raft domain where stomatin resides. The glucose transport activity of GLUT2 is inhibited by binding with stomatin in the lipid raft domain

Collectively, these findings indicate that GnT-IVa produces *N*-glycan epitopes on GLUT2 that bind to endogenous lectins, including galectin-9, leading to a reduction in the rate of GLUT2 endocytosis and thereby maintaining glucose sensor function of glucose-stimulated insulin secretion (Fig. 13.2).

13.5 *N*-Glycosylation Controls Membrane Sub-domain Distribution and Glucose Transport Activity of GLUT2

It has been well established that the redistribution of GLUT1 among membrane subdomains is associated with cellular glucose transport activity (Barnes et al. 2004), since GLUT1 associates with stomatin in lipid rafts. Stomatin erythrocyte membrane protein 7.2b is a 31 kDa integral membrane protein residing in lipid rafts and controls the function of ion channels and transporters (Lapatsina et al. 2012; Rungaldier et al. 2013). GLUT1 and GLUT2 exhibit a high degree of sequence similarity, and their hydropathy plots are virtually superimposable, suggesting these proteins are likely to adopt similar global structures within the membrane (Gould and Holman 1993). These findings imply that the glucose transport activity of GLUT2 is also regulated in the same manner.

Analyses of membrane sub-domain distribution of GLUT2 in primary isolated pancreatic β-cells revealed that GLUT2 almost exclusively resides in the non-lipid raft microdomain in β-cell membrane (Ohtsubo et al. 2013). Furthermore, the disruption of the GLUT2-galectin lattice by genetic inactivation of GnT-IVa, or by treatment of pancreatic beta cells with competitive glycan mimetics, induced the redistribution of GLUT2 into the lipid raft microdomain that was coincident with attenuation of cellular glucose transport activity. Moreover, the disruption of the lipid raft microdomain by methyl-β-cyclodextrin treatment released GLUT2 from lipid rafts and reactivated cellular glucose transport in GnT-IVa-deficient β-cells (Ohtsubo et al. 2013). These results indicate that GLUT2 *N*-glycosylation is involved in membrane sub-domain distribution and glucose transport activity.

Experiments of intracellular protein cross-linking of pancreatic β-cells demonstrated that the disruption of GLUT2-galectin interaction triggered membrane sub-domain redistribution and then allowed GLUT2 to associate with stomatin that was coincident with the attenuation of the glucose transport activity of GLUT2 (Ohtsubo et al. 2013).

These findings indicate that the lipid raft microdomain residency of GLUT2 causes the stomatin interaction and thereby suppresses the transport activity of GLUT2, further suggesting that the glycosylation-mediated membrane sub-domain distribution of GLUT2 is important for the regulation of the glucose sensor function for glucose-stimulated insulin secretion of pancreatic β-cells (Fig. 13.2).

13.6 High-Fat Diet-Induced Oxidative Stress Impairs Transcriptional Regulation of GnT-IVa and GLUT2 in β-Cells

As described above, the expression of GnT-IVa and GLUT2 is transcriptionally co-regulated by HNF-1α and FOXA2, suggesting that the glucose sensor function of pancreatic β-cells should be exclusively controlled by these transcription factors. However, the physiological mechanism of how metabolic fluctuation compromises transcriptional regulation and, subsequently, the glucose sensor function of β-cells are not well understood.

Mouse receiving a high-fat diet is a useful animal model recapitulating the onset of diet- and obesity-associated type 2 diabetes, exhibiting attenuation of glucose-stimulated insulin secretion and the development of insulin resistance (Reimer and Ahrén 2002; Winzell and Ahrén 2004). Pancreatic β-cells of high-fat diet-administrated mice showed diminished GLUT2 glycosylation and intracellular accumulation of GLUT2 associated with reduced expression of GnT-IVa and GLUT2 (Ohtsubo et al. 2005, 2011; Reimer and Ahrén 2002). This was coincident with reduced histone acetylation levels of *MGAT4A* and *GLUT2* gene promoters, reflecting inactivation of these genes (Ohtsubo et al. 2011). These results were consistent with decreased promoter binding of HNF-1α and FOXA2, which are

capable of recruiting histone acetyltransferase, in high-fat diet-administrated mice β-cells. These results indicate that impairment of the glucose sensor function of β-cells is deeply associated with defective HNF-1α- and FOXA2-mediated transcription. In agreement with this, the analyses of in situ distribution of these transcription factors revealed that they localize in the nucleus under normal conditions, whereas they localize in cytoplasm in high-fat diet-administrated mouse β-cells (Ohtsubo et al. 2011).

Furthermore, studies of in vitro β-cell cultures demonstrated that the nuclear exclusion of these transcription factors is induced by treatment with free fatty acid that is ameliorated by treatment with antioxidants (Ohtsubo et al. 2011). These findings indicate that the elevation of the free fatty acid level, associated with high-fat diet administration, induces oxidative stress in β-cell that evokes the nuclear exclusion of HNF-1α and FOXA2 and thereby diminishes *MGAT4A* and *GLUT2* expression and impairs glucose sensor function.

13.7 Replenishment of GnT-IVa in β-Cells Ameliorates High-Fat Diet-Induced Type 2 Diabetes

Based on the above findings, it has been speculated that the supplementation of GnT-IVa activity in pancreatic β-cells improves their glucose sensor function and maintains partial glucose homeostasis under high-fat diet conditions. Engineering and characterizing of transgenic mice overexpressing GnT-IVa in pancreatic β-cells demonstrated that their pancreatic β-cells maintained multiantennary N-glycan synthesis and cell surface residency of GLUT2 that allowed β-cell glucose-stimulated insulin secretion (GSIS) under high-fat diet conditions. This contributed to improving long-term blood glucose levels, glucose tolerance, and peripheral insulin sensitivities (Ohtsubo et al. 2011). These findings indicate that the maintenance of the GnT-IVa-dependent protein glycosylation prevents high-fat diet-induced β-cell dysfunction and ameliorates the onset of type 2 diabetes, further suggesting that glycans and glycosyltransferases can be targeted by antidiabetic drugs in the future.

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Chapter 14
Macrophages Govern Ganglioside GM3 Expression in Adipocytes to Regulate Adipogenesis and Insulin Signaling in Homeostatic and Pathogenic Conditions

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Abstract GM3 ganglioside has been known to participate in insulin signaling by regulating the association of insulin receptor in caveolae microdomains (lipid rafts), which is essential to execute complete insulin metabolic signaling in adipocytes. We propose a working hypothesis: "metabolic disorders, such as type 2 diabetes, are membrane microdomain disorders caused by the aberrant expression of gangliosides." Here, we demonstrate the molecular pathogenesis of type 2 diabetes and insulin resistance focusing on the interaction between insulin receptor and GM3 ganglioside in adipocytes. In addition, GM3 levels of primary adipocytes are tightly maintained by soluble factors secreted from resident macrophages to execute physiological adipogenesis. Thus, GM3 participates not only in the development of the state of insulin resistance through the upregulation of GM3 synthesis by proinflammatory cytokines but also functions as a physiological regulatory factor for adipocytes by keeping proper insulin signaling in lipid rafts. The development of novel therapeutic strategy termed "membrane microdomain ortho-signaling therapy" is expected.

Keywords Adipogenesis • Diabetes • Ganglioside GM3 • Glycosphingolipids (GSLs) • Insulin resistance • Metabolic syndrome • Lipid rafts • Proinflammatory cytokines

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14.1 Introduction

Glycosphingolipids (GSLs) and their sialic acid-containing derivatives, gangliosides, are components of membrane lipids in which the lipid portion is embedded in the outer leaflet of the plasma membrane with the sugar chain extending to the extracellular space; the structural features of GSLs affect membrane fluidity and allow for microdomain formation, contributing to cell–cell interaction and receptor-mediated signal transduction. We have previously shown that in cultured adipocytes in a state of TNFα-induced insulin resistance, removal of GSLs by the inhibition of glucosylceramide synthase, the first step of the biosynthesis of all of GSLs, results in a nearly complete recovery of the insulin receptor signaling (Tagami et al. 2002). Studies in animal models demonstrate that pharmacological inhibition of GSLs ameliorates insulin resistance and prevents some manifestations of metabolic syndrome (Zhao et al. 2007; Aerts et al 2007). Further, we have shown that expression of ganglioside GM3, which is the simplest ganglioside species synthesized by GM3 synthase, is increased in metabolic diseases (Tagami et al. 2002; Sato et al. 2008). SAT-I, ST3GalV, and ST3GAL5 are abbreviations that are frequently used for this enzyme, but ST3GAL5 is recommended for common use (Inokuchi and Uemura 2014). GM3 synthase gene of human and mouse abbreviates *ST3GAL5* and *St3gal5*, respectively. *St3gal5* gene expression and GM3 content are upregulated in the visceral adipose tissue of obese model animals (Tagami et al. 2002) and serum GM3 levels are twofold higher in obese patients with type 2 diabetes and/or dyslipidemia (Sato et al. 2008), suggesting that GM3 is responsible for insulin metabolic signaling.

We have postulated a working hypothesis "insulin resistance as a membrane microdomain disorder" (Inokuchi 2010, 2011, 2014) because of the facts that the abnormal increase of membrane GM3 in adipocytes, induced by proinflammatory cytokine TNFα, resulted in the elimination of insulin receptor (IR) from caveolae (Kabayama et al. 2005, 2007). The association of IR in caveolae is essential to execute the complete insulin metabolic signaling (Couet et al. 1997; Nystrom et al. 1999).

Visceral adipose tissues are composed of not only adipocytes but also immune cells including resident macrophages and T lymphocytes and other types of cells (Suganamani and Ogawa 2011; Samaan 2011; Chalwa et al. 2011; Sun et al. 2012). However, the interplay between adipocytes and the resident macrophages upon regulation of GSL expression is not clear. We demonstrated the expression of GSLs in adipocytes, and their corresponding synthase genes are maintained by soluble factors secreted from resident macrophages under not only inflammatory states but also steady-state physiological conditions. Furthermore, obese *St3gal5*-deficient mice fed with high-fat diets are resistant to developing proinflammatory states in adipose tissues. This review focuses on the connection between the metabolic syndrome and the physiological and pathological implications of GM3 in adipose tissues.

14.2 GM3 Is an Inducer of Insulin Resistance

Insulin elicits a wide variety of biological activities, which can be categorized into metabolic and mitogenic actions. The binding of insulin to IR activates IR internal-tyrosine kinase activity. The activated tyrosine-phosphorylated IR is able to recruit and phosphorylate adaptor proteins such as insulin receptor substrate (IRS). The phosphorylated IRS activates PI3-kinase (PI3K). The activated PI3K translocates to lipid rafts and converts PIP_2 to PIP_3, and then PIP_3 recruits PDK1 to phosphorylate Akt. The full activation of Akt might require phosphorylation of the secondary site by mTORC2 (mTOR complex 2) (Zoncu et al. 2011). This IR–IRS–PI3K–Akt signaling cascade is the representative metabolic pathway triggered by insulin, resulting in the translocation of glucose transporter 4 (GLUT-4) to the plasma membrane to facilitate glucose uptake.

When mouse adipocytes were cultured in low concentrations of TNFα which do not cause generalized suppression of adipocyte gene expression including IRS-1 and GLUT-4, interference of insulin action by TNFα occurred (Guo and Donner 1996). This requires prolonged treatment (at least 72 h), unlike many acute effects of this cytokine. The slowness of the effect suggests that insulin resistance in adipocytes treated with 0.1 nM TNFα was accompanied by progressive increases in cellular GM3 content, ST3Gal5 activity, and its mRNA content, indicating that TNFα upregulates GM3 synthesis at the transcriptional level in cultured adipocytes (Tagami et al. 2002; Kabayama et al. 2005). On the other hand, ceramide levels were transiently increased up to 6 h upon TNFα treatment and returned to normal by 24 h. This observation suggests the distinct and independent roles of GM3 and ceramides in the development of insulin resistance in adipocytes (Inokuchi 2014). To elucidate whether the increased GM3 in 3T3-L1 adipocytes treated with TNFα is involved in insulin resistance, we used an inhibitor of glucosylceramide synthase, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) (Inokuchi and Radin 1987), to deplete cellular glycosphingolipids derived from glucosylceramide. D-PDMP proved able to counteract the TNFα-induced increase of GM3 content in adipocytes and completely normalize the TNFα-induced defect in tyrosine phosphorylation of IRS-1 in response to insulin stimulation (Fig. 14.1) (Tagami et al. 2002). These findings are supported by the observation that knockout mice lacking *St3gal5* exhibit enhanced insulin signaling (Yamashita et al. 2003). It has been reported that treatment of adipocytes with TNFα induces an increase in the serine phosphorylation of IRS-1 (Hotamisligil et al. 1993). This phosphorylation is an important event since immunoprecipitated IRS-1, which has been serine phosphorylated in response to TNFα, is a direct inhibitor of insulin receptor tyrosine kinase activity. We have shown that TNFα- induced serine phosphorylation of IRS-1 in adipocytes is completely suppressed by inhibition of GM3 biosynthesis with D-PDMP treatment, suggesting that the elevated GM3 synthesis induced by

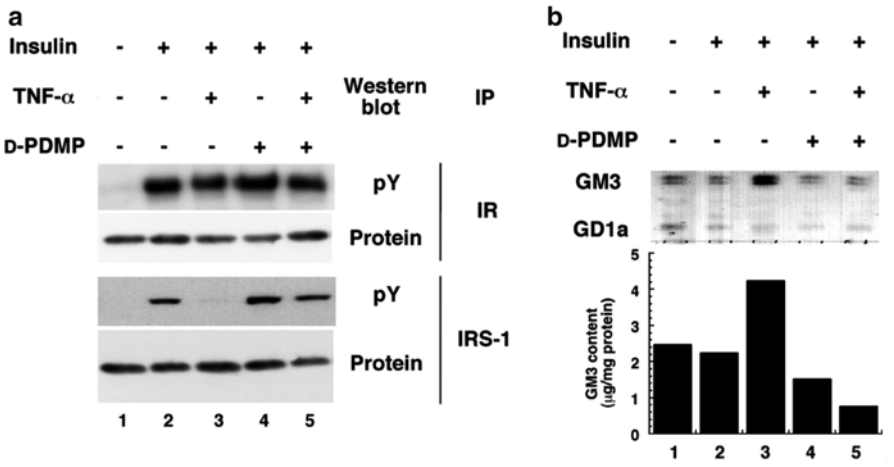

Fig. 14.1 TNFα increases the expression of GM3, and prevention of GM3 synthesis reverses TNFα-induced suppression of insulin signaling in adipocytes (Tagami et al. 2002). (**a**) 3T3-L1 adipocytes were cultured in maintenance medium without (lanes 1, 2, and 4) or with (lanes 3 and 5) 0.1 nM TNFα for 96 h, and in order to deplete GM3, 20 μM D-PDMP was also included (lanes 4 and 5). Before insulin stimulation (100 nM for 3 min), cells were starved in serum-free media containing 0.5 % bovine serum albumin in the absence or presence of TNFα and D-PDMP as above for 8 h. Proteins in cell lysates were immunoprecipitated with antiserum to IR and IRS-1, fractionated by SDS-PAGE, and transferred to Immobilon-P. Western blot was then proved with anti-phosphotyrosine monoclonal antibody, stripped, and reproved with antiserum to IR and IRS-1. (**b**) 3T3-L1 adipocytes were incubated in the absence or presence of TNFα and D-PDMP as in A, and the ganglioside fraction was visualized by resorcinol staining on high performance thin layer chromatography (HPTLC)

TNFα caused the upregulation of serine phosphorylation of IRS-1 (Tagami et al. 2002). An improved D-PDMP analog (Zhao et al. 2007) and an iminosugar derivative glucosylceramide synthase inhibitor (Aerts et al. 2007) were proven to have therapeutic value by oral administration in diabetic rodent models.

Diabetic rodent models, Zucker *fa/fa* rat and *ob/ob* mice, produce significant levels of TNFα in adipose tissues, and much less expression was seen in the lean control animals (Hotamisligil et al. 1993). Thus, we were interested in measuring the expression of *St3gal5* mRNA in the epididymal fat of these obese diabetic models. Northern blot analysis of *ST3Gal5* mRNA contents in the adipose tissues from these two typical models of insulin resistance exhibited significantly high levels compared to their lean counterparts (Fig. 14.2a upper panel) (Tagami et al. 2002). Comparison of the mobility of GM3 bands on thin layer chromatography (TLC) between the lean animals and *ob/ob* mice and Zucker fatty rats indicates the appearance of GM3 species showing low mobility (more hydrophilic) in both obese and diabetic animals (Fig. 14.2a lower panel).

In addition to the analyses of GSLs in *ob/ob* mice and Zucker fatty rats, we further asked whether the expression of GM3 is also affected in diet-induced obese mice (ref new paper). After feeding for 10 weeks, mean BW of standard diet (SD) and high- fat diet (HFD) groups were 31.0 ± 0.6 g and 46.0 ± 0.8 g, respectively. Fasted blood glucose levels of SD and HFD groups were 137 mg/dl and 203 mg/dl,

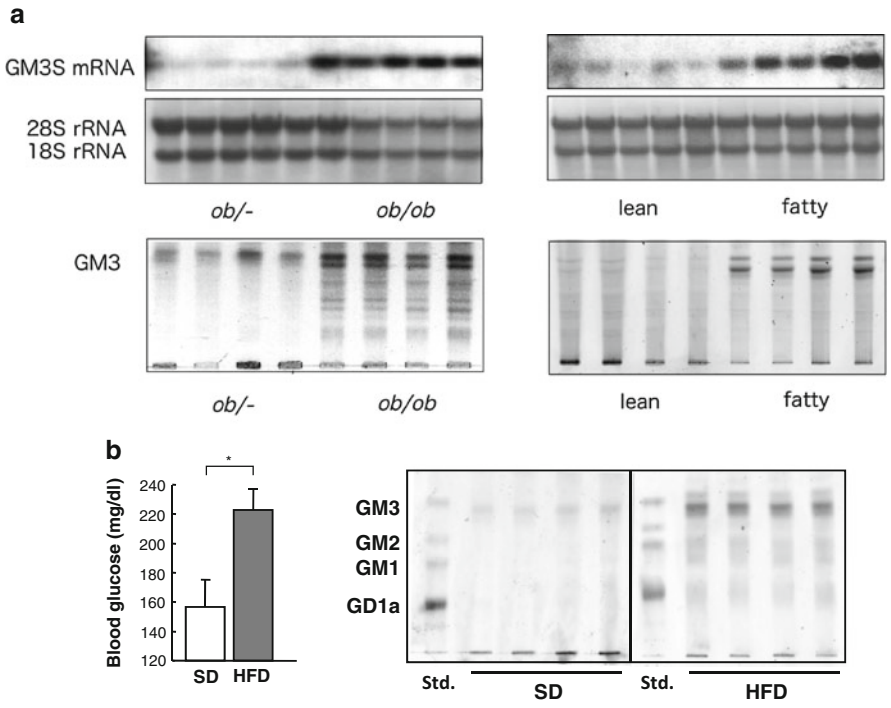

Fig. 14.2 Elevation of GM3 synthesis in visceral adipose tissues of diabetic model animals. (**a**) Significant increase of GM3 biosynthesis in the epididymal fats in *ob/ob* mice and Zucker fatty rats (Tagami et al 2002). Northern blot analysis of *ST3Gal5* mRNA contents in the adipose tissues from *ob/ob* mice and Zucker fatty rats of insulin resistance (*upper panel*). Comparison of the mobility of GM3 bands on TLC between the lean animals and these two typical models (*lower panel*). (**b**) 6-week-old C57BL6 mice were fed HFD for 10 weeks, and their fasted blood glucose (*left panel*) and GM3 on TLC (*right panel*) were measured (Nagafuku et al. 2015)

respectively (Nagafuku et al. 2015) (Fig. 14.2b). As shown in Fig. 14.2b, the GM3 levels in the epididymal fat of the HFD group were threefold higher than that of the SD group. In addition, the mRNA levels of *St3gal5* in the HFD group were also increased threefold (Nagafuku et al. 2015). These results strongly suggest the possibility that the increased expression of GM3 in abdominal adipose tissues might contribute to the induction of malfunction of adipose tissue such as chronic low-grade inflammatory states in obesity.

14.3 Insulin Resistance as a Membrane Microdomain Disorder

In a state of insulin resistance induced in adipocytes by TNFα, we presented evidence that the transformation to a resistant state may depend on increased ganglioside GM3 biosynthesis following upregulated GM3 synthase gene expression.

Thus, increased GM3 during chronic exposure to TNFα functions as a suppressor of insulin signaling (Tagami et al. 2002). Since GSLs, including GM3, are important components of lipid rafts, we pursued the possibility that increased GM3 levels in lipid rafts confer insulin resistance upon TNFα-treated adipocytes. We examined GM3–protein interactions occurring within the plasma membrane of living cells by performing a cross-linking assay using a photoactivatable radioactive derivative of GM3. Adipocytes were preincubated with [^{3}H]GM3(N3) and then irradiated to induce cross-linking of GM3. Target proteins were then separated by sodium docyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography. A specific radioactive band corresponding to the 90-kDa IRβ-subunit was immunoprecipitated with anti-IRβ antibodies, confirming the direct association of GM3 and IR. Therefore, we found that IR forms complexes with caveolin-1 and GM3 independently in 3T3-L1 adipocytes (Kabayama et al. 2007). Lipids are asymmetrically distributed in the outer and inner leaflets of plasma membranes. In typical mammalian cells, most acidic phospholipids are located in the inner leaflet, and only acidic glycosphingolipids such as sulfatides and gangliosides are in the outer. The binding of proteins to lipid membranes is often mediated by electrostatic interactions between the proteins' basic domains and acidic lipids. Gangliosides, which bear sialic acid residues, exist ubiquitously in the outer leaflet of the vertebrate plasma membrane. GM3 is the most abundant ganglioside and the primary ganglioside found in adipocytes (Ohashi 1979). GSLs, including gangliosides, are oriented at a defined angle to the axis of the ceramide (Hakomori 2002). In addition, GM3 spontaneously forms clusters with its own saturated fatty acyl chains, regardless of any repulsion between the negatively charged units in the sugar chains (Sonnino et al. 2006). Thus, GM3 clusters with other cell surface gangliosides such as glycosphingolipid-enriched microdomains (GEM) generate a negatively charged environment just above the plasma membrane. Conversely, IR has a sequence in its transmembrane domain, homologous among mammals, that allows presentation of the basic amino acid lysine (IR944) just above the transmembrane domain. Therefore, during lateral diffusion, an electrostatic interaction between the lysine residue at IR944 and the GM3 cluster could occur due to their proximity on the plasma membrane. Our live cell studies using fluoresence activated photobleaching (FRAP) techniques proved a mechanism in which the dissociation of the IR-caveolin-1 complex is caused by the interaction of a lysine residue, located just above the transmembrane domain in IRβ-subunit and the increased GM3 clustered at the cell surface (Kabayama et al. 2007). Based on this evidence, we propose a mechanism behind the shift of IR from the caveolae to the GEM in adipocytes during a state of insulin resistance (Fig. 14.3).

Reportedly, insulin signaling in the skeletal muscle of *ST3Gal5* KO mice was enhanced compared to wild-type B6 mice (Yamashita et al. 2003). However, it has been recently reported that the inhibition of insulin signaling of C2C12 myotubes exposed to saturated fatty acid was not reversed by treatment with a glucosylceramide synthase inhibitor (D-PDMP analog) (Chavez et al. 2014). Thus, the involvement of GM3 in the pathophysiology of insulin resistance in the skeletal muscle requires further study.

The role of ceramide acyl chain length in insulin signaling was explored by using a ceramide synthase 2 (CerS2) null mouse, which is unable to synthesize very long

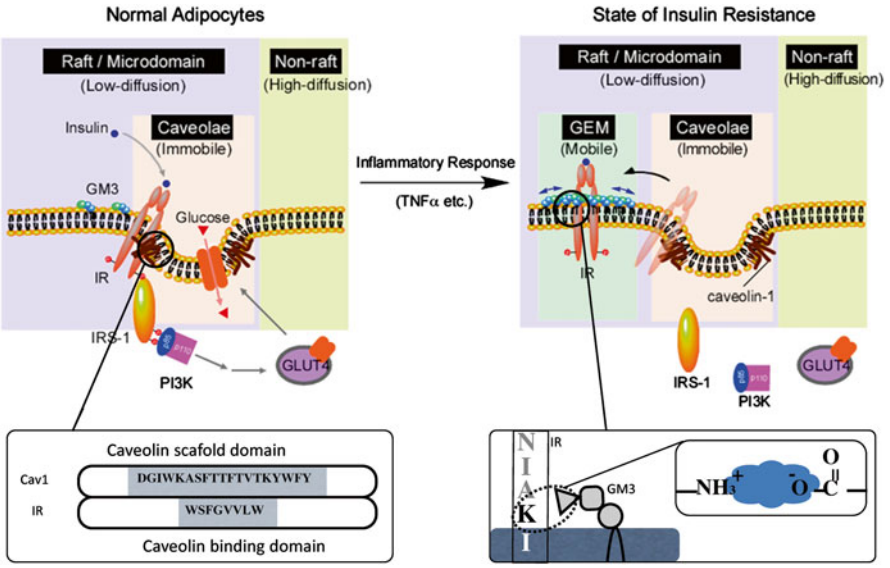

Fig. 14.3 Proposed mechanism behind the shift of insulin receptors from the caveolae to the glycosphingolipid-enriched microdomains (GEM) in adipocytes during a state of insulin resistance. A schematic representation of raft/microdomains comprising caveolae and non-caveolae rafts such as GEM. Caveolae and GEM reportedly can be separated by an anti-CAV1 antibody. IR may be constitutively resident in caveolae via its binding to the scaffolding domain of CAV1 through the caveolin-binding domain in its cytoplasmic region. Binding of IR and CAV1 is necessary for successful insulin metabolic signaling. In adipocytes, the localization of IR in the caveolae is interrupted by elevated levels of the endogenous ganglioside GM3 during a state of insulin resistance induced by TNFα (Kabayama et al. 2005, 2007). This study has proven a mechanism, at least in part, in which the dissociation of the IR/CAV1 complex is caused by the interaction of a lysine residue at IR944, located just above the transmembrane domain, and the increased GM3 clustered at the cell surface

acyl chain (C22–C24) ceramides (Park et al. 2013). In CerS2 null mice, IR and Akt phosphorylation in response to insulin was abrogated in the liver. The lack of insulin receptor phosphorylation in the liver correlated with its inability to translocate into detergent-resistant membranes (DRMs). Moreover, DRMs in CerS2 null mice displayed properties significantly different from those in wild-type mice, suggesting that the altered sphingolipid acyl chain length directly affects IR translocation to lipid rafts and subsequent signaling.

14.4 GM3 Functions as a Physiological Regulator for Insulin Signaling and Adipogenesis

Visceral adipose tissue, particularly mesenteric adipose tissue, is important in the pathogenesis of metabolic syndrome (Chawla et al. 2011; Bays et al. 2008; de Ferranti and Mozaffarian 2008; Xu et al. 2013; Matsuzawa 1997; Saltiel 2012;

Samaan 2011; Sorisky et al. 2013; Suganamani and Ogawa 2011). To investigate the fundamental characteristics of mesenteric adipocytes, we established a physiologically relevant differentiation system of rat mesenteric-stromal vascular cells (mSVCs) to mesenteric-visceral adipocytes (mVACs) (Shimizu et al. 2006; Sato et al. 2008). We optimized the insulin concentration at levels comparable to those in vivo (0.85 ng/ml) by including physiological concentrations of insulin-like growth factor (IGF-1, 200 ng/ml). We found that IGF-1 and insulin worked synergistically, because IGF-1 alone could enhance CCAAT/enhancer binding protein alpha (C/EBPα) and adipocyte lipid binding protein (aP2) mRNA expression; however, IGF-1 could not induce lipid droplet accumulation associated with adipocyte maturation without the physiological concentration of insulin.

Using this culture system, we have explored the commitment of the resident macrophages in mSVCs on physiological adipogenesis. Adipogenesis of SVCs in mesenteric adipose tissues was increased following the removal of resident macrophages, which was accompanied with enhanced insulin signaling and concomitant decrease of GSLs including glucosylceramide, lactosylceramide, and GM3. Phosphorylation levels of both IR and IRS-1 after insulin stimulation were increased by depleting macrophages and protein level of IR per se was increased in the mSVCs (Nagafuku et al. 2015) (Fig. 14.4a).

Thus, GSL levels especially GM3 of adipocytes are tightly maintained by soluble factors secreted from resident macrophages to execute physiological adipogenesis. In addition, adipogenesis of mouse embryonic fibroblasts (MEFs) prepared from *St3gal5* null mice was accelerated with enhanced insulin signaling (Nagafuku et al. 2015). Thus, there is evidence for the direct involvement of GM3 in adipogenesis and insulin signaling in adipose tissues (Fig. 14.4b). These results demonstrate that GM3 levels of preadipocytes and mature adipocytes are tightly maintained by soluble factors secreted from resident macrophages to execute physiological adipogenesis with proper insulin signaling.

Fig. 14.4 (continued) without macrophages. Adequate adipogenesis with proper insulin signaling in the presence of resident macrophages is shown in the middle, while acceleration of adipogenesis with enhanced insulin signaling in the absence of resident macrophages is depicted on the right. Phosphorylation levels of both insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) after insulin stimulation were dramatically increased, and protein levels of IR per se were increased by depleting macrophages (Nagafuku et al. 2015). On the other hand, the activation of macrophages by proinflammatory cytokines suppresses adipogenesis and enhances *St3gal5* gene expression which results in the development of the state of insulin resistance (Figs. 14.1, 14.2, and 14.3; Tagami et al. 2002; Inokuchi 2014; Nagafuku et al. 2015). (**b**) Evidence for the direct involvement of GM3 in adipogenesis. MEFs were prepared from E14 embryos of wild-type C57/BL6 mice and *St3gal5-deficient* mice and subjected to adipogenesis. Enhanced adipogenesis of *St3gal5*-deficient MEF which is accompanied with the increased insulin signaling was observed. Phosphorylation levels of both IR and IRS-1 after insulin stimulation were increased (Nagafuku et al. 2015)

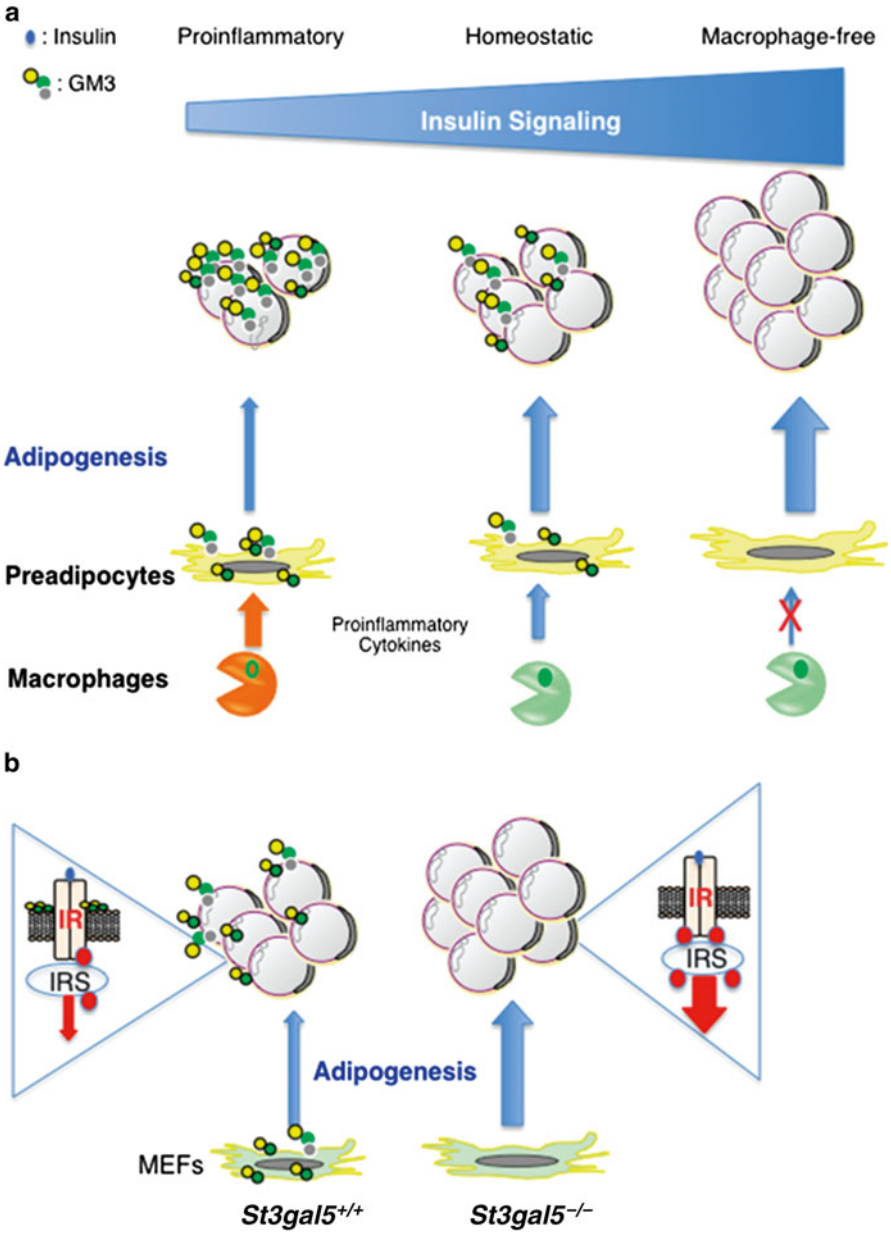

Fig. 14.4 Control of homeostatic and pathogenic balance in adipose tissue by ganglioside GM3. (**a**) Adipogenesis of mesenteric preadipocytes is increased following depletion of resident macrophages. The expression of GSLs including GM3 in mesenteric preadipocytes and adipocytes could be maintained by soluble factors secreted from resident macrophages to execute physiological adipogenesis. Thus, surprisingly primary adipocytes themselves are not capable of making GSLs

14.5 Serum GM3 as a New Biomarker of Metabolic Syndrome

GM3 is the major ganglioside present in serum and is known to be associated with serum lipoproteins (Senn et al. 1989). We examined a relationship between serum GM3 levels and adiposity indices, as well as between serum GM3 levels and metabolic risk variables (Sato et al. 2008). Serum GM3 levels were significantly increased in type 2 diabetic patients with severe obesity (visceral fat area >200 cm^2, BMI >30). The GM3 level was positively correlated with LDL-c (0.403, $P=0.012$) in type 2 diabetes mellitus, but not affected by blood pressure. In addition, high levels of small dense LDL (>10 mg/dL) were associated with the elevation of GM3. Serum GM3 levels were affected by glucose and lipid metabolism abnormalities and by visceral obesity. Small dense LDL is reportedly associated with the development of atherosclerosis (Austin et al. 1998; Tribble et al. 1995), and GM3 has been detected in atherosclerotic lesions (Bobryshev et al. 1997, 2001, 2006). Thus, our findings provide evidence that GM3 may be a useful marker for the management of metabolic syndrome including insulin resistance, as well as for the early diagnosis of atherosclerosis. The structural diversity of ceramide species is generated by various factors in the N-acyl chains, 1) the length (C16–C24), 2) alpha hydroxylation and (3) desaturation, and in the sphingoid bases, (1) d18:1, d18:0 and (2) hydroxylation at C4, resulting in a substantial number of potential combinations. We are currently performing liquid chromatograhy-mass spectrometry (LC-MS/MS) analyses to identify the GM3 species which are specifically involved in metabolic syndrome.

14.6 Pathogenic Control of Adipocytes by the Increased Expression of GM3

Adipose tissue macrophages are present in two main subtypes, M1 and M2. A concept of M1/M2 polarization has been developed for macrophages according to patterns of expression of cytokines, surface markers, and metabolic enzymes (Konner and Brunning 2011). M1 macrophages are potent effector cells that produce inflammatory cytokines such as TNFα, IL1-β, and IL-6. In contrast, M2 macrophages are present in almost all organs in the body as resident cells under physiological conditions, where they serve to maintain tissue homeostasis (Martinez et al. 2009; Zeyda and Stulnig 2007). These cells exert anti-inflammatory functions by producing IL-10 and arginase I enzyme (Arg1). IL-10 potentiates insulin signaling in adipocytes (Odegaard and Chawla 2011; Lumeng et al. 2007), and Arg1 reduces nitric oxide synthesis and inflammation via metabolizing arginine to ornithine (Martinez et al. 2009). Consumption of HFD shifts cytokine expression of murine adipose tissue macrophages from M2- to M1-like patterns by decreasing the expression of IL-10 and Arg1 and increasing TNFα and iNOS (Lumeng et al. 2007).

We found several interesting gene expression profiles in the genes in the epididymal adipose tissue of *ST3Gal5*$^{-/-}$ mice under the HFD condition but not in the standard diet condition as follows (Nagafuku et al. 2015); proinflammatory cytokine

TNFα was significantly decreased, and on the other hand, anti-inflammatory cytokine IL-10 was significantly increased compared to the *St3gal5*$^{+/-}$ mice. The expression level of adiponectin was significantly increased but that of atherogenic genes such as *PAI-1* and *iNOS* was suppressed. M2 signature genes such as *MGL1* and *Arg1* tend to increase in *St3gal5*-deficient mice. In addition, both glucose tolerance test (GTT) and insulin tolerance test (ITT) showed significant improvements of insulin resistance in the HFD condition. These results indicate the critical involvement of GM3 in the development of obesity-induced chronic low-grade inflammatory states and insulin resistance (Fig. 14.5) (Nagafuku et al. 2015).

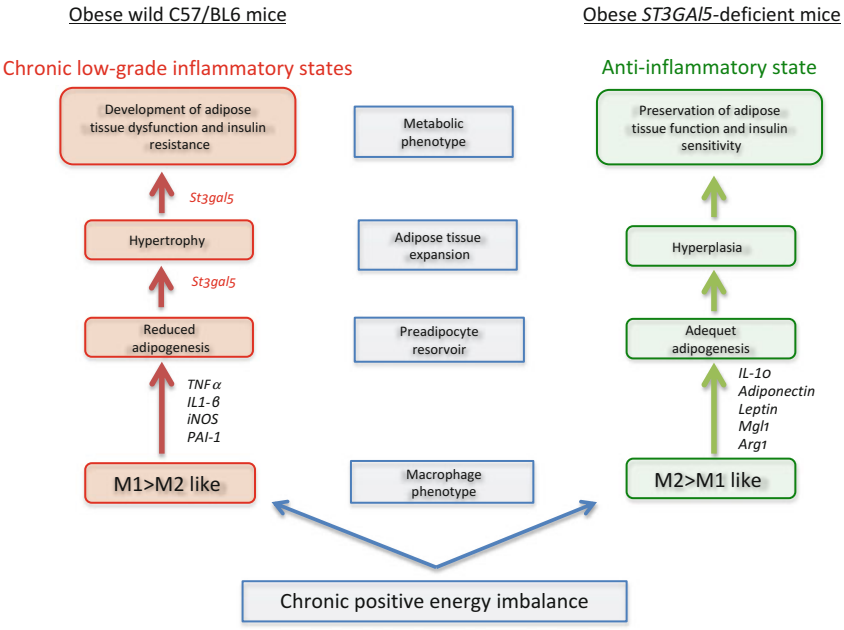

Fig. 14.5 A model of how GM3 regulates adipose tissue remodeling during chronic positive energy imbalance. Chronic positive energy imbalance (high-fat diet: HFD) leads to obesity, and macrophage phenotype may influence the mechanism by which adipose tissue expands. During the obesity, the mass of adipose tissue increases by hyperplasia and hypertrophy, and the latter is associated with the activation of stress signaling. When proinflammatory M1 macrophages dominate, an inadequate preadipocyte reservoir may exist due to reduced preadipocyte survival, proliferation, and/or adipogenic capacity. Energy storage will occur via exaggerated adipose hypertrophy, resulting in dysfunctional adipose tissue and contributing to an inflamed, insulin-resistant state. Chronic increase of GM3 through the upregulation of *St3gal5* gene by proinflammatory cytokines such as TNFα and IL-β could participate in the development of insulin resistance (Tagami et al. 2002; Kabayama et al. 2007). In contrast, *St3gal5*-deficient mice improve insulin action without showing a significant impact on diet-induced obesity (Nagafuku et al. 2015). Obese *St3gal5*-deficient mice showed anti-inflammatory M2-like phenotypes in visceral adipose tissue (epididymal fat) (Nagafuku et al. 2015). Significant increases of *adiponectin* and *interleukin-10* (*IL-10)* compared to obese wild-type mice were observed. IL-10 and adiponectin have a crucial role in maintaining the insulin sensitivity of adipocytes (Odegaard and Chawla 2011; Lumeng et al. 2007)

14.7 Perspective

Adipocytes are more than inert energy depots, and adipose tissue is a biologically active organ that carries out important physiological processes including energy homeostasis and whole-body insulin sensitivity. Dynamic remodeling of the adipose tissue architecture occurs with its expansion. During positive caloric balance, the development of metabolic disease is more closely related to how the fat is stored through adipocyte hypertrophy versus hyperplasia than simply the amount of fat that is stored (Fig. 14.5). In the past it was widely hypothesized that inhibitors of adipogenesis were potential anti-obesity therapeutics. However, evidence from a variety of experiments in mice and humans suggests that inhibitors of adipogenesis are a poor choice for amelioration of metabolic disease states because limiting fat cell expansion is associated with insulin resistance. As proposed more than a decade ago, a failure in adipocyte differentiation can cause type 2 diabetes (Danforth 2000), and this hypothesis is generally recognized and supported by independent lines of investigation in adipocyte biology.

Interactions between macrophages and adipose progenitor cells are important to consider because they may influence the number of preadipocytes and/or their differentiation capacity and induce adipose tissue dysfunction by inhibiting overall adipogenic capacity (Fig. 14.5). The presence of resident and infiltrating macrophages is well documented, and studies in the last decade suggest that these macrophages are modulated in conditions of obesity and type 2 diabetes. It is well established that the proinflammatory TNFα (Torti et al. 1985) and IL1-β (Suzawa et al. 2003) are potent inhibitors of adipocyte differentiation. Of note, it is also known that both of these cytokines induce insulin resistance in adipocytes (Stephens et al. 1992; Jager et al. 2007). Moreover, TNFα expression is induced in the adipose tissue of obese diabetic rodents (Hotamisligil et al. 1993) and humans (Hotamisligil et al. 1995). It is now largely accepted that TNFα expression in adipose tissue comes from macrophages (Weisberg et al. 2003). Together, these studies suggest that macrophages in adipose tissue produce TNFα and IL1-β, which can inhibit differentiation of preadipocytes and induce insulin resistance in mature adipocytes. However, the ability of these cytokines to induce insulin resistance by inhibiting adipogenesis has not been considered. Nonetheless, there are new model systems that clearly suggest that limitations in adipose tissue expansion are associated with insulin resistance. Mice that are very obese but have unlimited adipose tissue expansion are metabolically healthy and insulin sensitive (Kim et al. 2007). Overall, these studies largely support the idea that adequate numbers of preadipocytes that are differentiation competent allow for hyperplastic growth with the effect being to preserve metabolic function in the face of obesity.

As described in this review, our research employing GM3 synthase (*St3gal5*) gene and its knockout mice has proved the critical involvement of GM3 in both homeostatic adipogenesis by controlling insulin signaling and the development of obesity-induced chronic low-grade inflammatory states and insulin resistance in adipose tissue (Figs. 14.3, 14.4), and 14.5). We demonstrated that the expression

of GM3 in adipocytes is governed by soluble factors secreted from resident macrophages to execute physiological adipogenesis. GM3 expression in adipose tissue is further increased under obesity-induced proinflammatory conditions, and GM3 synthase-deficient mice fed with a high-fat diet are resistant to developing insulin resistance and chronic low-grade inflammatory states. Thus, GM3 functions as a novel homeostatic and pathogenic mediator in adipose tissue.

Our data substantiate a rationale for designing novel therapies against metabolic syndrome including type 2 diabetes on inhibition of GM3 biosynthesis for maintaining homeostatic insulin signaling (Fig. 14.6). The extensive reduction of all gangliosides by inhibiting GM3 biosynthesis would carry physical and chemical modifications of the all-cellular plasma membrane and in particular of lipid

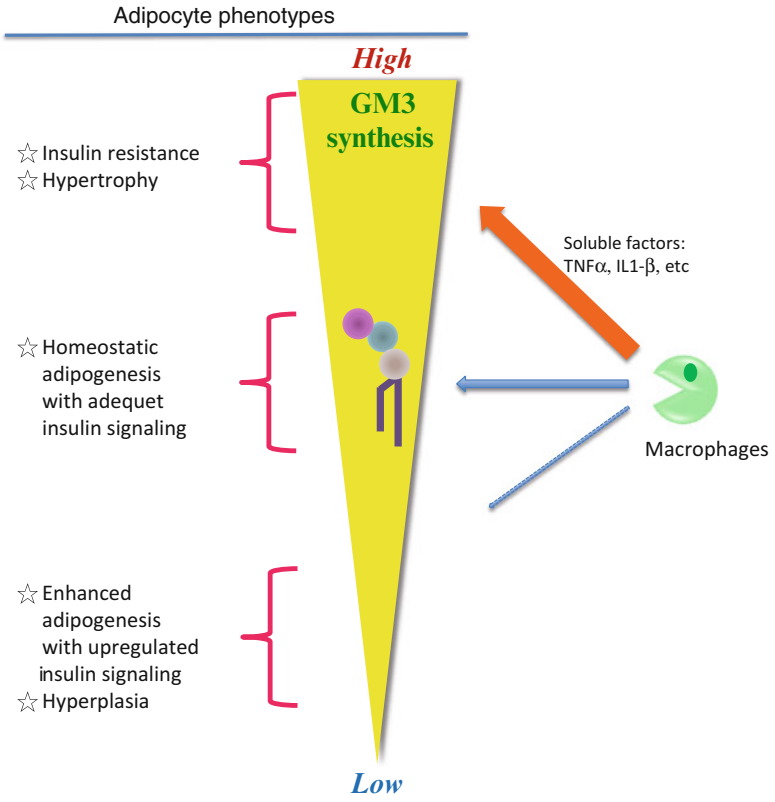

Fig. 14.6 GM3 could be a homeostatic and pathogenic mediator for adipogenesis and insulin signaling. Interactions between macrophages and adipose progenitor cells are important to consider because they may influence the number of preadipocytes and/or their differentiation capacity and induce adipose tissue dysfunction by inhibiting overall adipogenic capacity, depicted on Figs. 14.4 and 14.5. Controlling GM3 levels could be considered as a potential therapeutic intervention for restoring healthy adipose tissue function in obese individuals, distinct from weight-reduction strategies

microdomains too dramatic to be of therapeutic value. However, we expect that such extensive depletion of gangliosides will not be necessarily for the treatment of metabolic disorders. When we demonstrated the effectiveness of D-PDMP on the impaired insulin signaling in TNFα-treated 3T3-L1 adipocytes, the normalization of elevated levels of GM3 was enough to ameliorate the state of insulin resistance (Tagami et al. 2002).

GM3 is dominantly expressed in insulin-responsive organs such as the skeletal muscle, liver, and adipose tissue as well as lymphocytes in humans. Thus, the presence of GM3-dependent membrane microdomains (lipid rafts) is reflecting characteristics of individual cells. In order to accumulate gangliosides in lipid rafts, hydrogen donor and acceptor and saturated and relatively long acyl chains, compared to those of phospholipids, should exist in their ceramide backbone to accelerate their self-aggregation. The structural diversity of the sphingoid base and the N-acyl chain of ceramide moiety is key to defining the behavior of gangliosides in living cell membranes and the localization of lipid rafts. It is essential to employ "sphingolipidomics" to precisely characterize ceramide structures present. A comprehensive study to elucidate the functional supra-biomolecular complex consisting of gangliosides and functional proteins in microdomains should generate a novel concept and strategy of "membrane microdomain ortho-signaling therapy."

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Chapter 15
O-Mannosyl Glycan and Muscular Dystrophy

Hiroshi Manya and Tamao Endo

Abstract Glycosylation is an important posttranslational modification in mammals. The major glycans of glycoproteins can be classified into two groups, *N*-glycans and *O*-glycans, according to their glycan-peptide linkage regions. The development of sensitive methods for analyses of glycan structures has identified *O*-mannosyl glycans in mammals; these structures used to be considered specific to yeast. Originally, *O*-mannosyl glycan was considered to be present on a limited number of glycoproteins of the brain, nerves, and skeletal muscles, especially on α-dystroglycan (α-DG). However, since a clear relationship between *O*-mannosyl glycan and the pathomechanisms of some congenital muscular dystrophies in humans was established, this glyco field has been expanding both biochemically and pathologically. Because the glycosylation of α-DG is defective in congenital muscular dystrophies that show muscular dystrophy with abnormal neuronal migration, these disorders are collectively called α-dystroglycanopathy. Although it is known that *O*-mannosyl glycans have various structures, the biosynthetic pathway responsible remains only partially understood. In addition, many new causative genes of α-dystroglycanopathies are continuously being found. In this article, we discuss the structure, biosynthesis, and pathology of *O*-mannosyl glycans.

Keywords *O*-Glycosylation • *O*-Mannosyl glycan • Glycosyltransferase • Glycan biosynthesis • Muscular dystrophy • α-Dystroglycanopathy • Dystrophin-glycoprotein complex

15.1 Introduction

O-Mannosyl glycan is a type of *O*-glycan for which the reducing terminal mannose is attached to the hydroxyl group of serine (Ser) and threonine (Thr) residues of proteins. In 1997, we reported that the major glycans of peripheral nerve

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α-dystroglycan (α-DG) include *O*-mannosyl glycan, which mainly consists of the four sugars Siaα2-3Galβ1-4GlcNAcβ1-2Man-Ser/Thr (Chiba et al. 1997). We also identified the same *O*-mannosyl glycans in skeletal muscle α-DG (Sasaki et al. 1998). These data suggested that the presence of *O*-mannosyl glycans on α-DG may be important for the functions of α-DG. We attempted to elucidate the mammalian biosynthetic pathway of *O*-mannosyl glycans and found that three glycosyltransferases are responsible for the biosynthesis of *O*-mannosyl glycan: protein *O*-mannosyltransferase 1 (POMT 1), POMT2, and protein *O*-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGNT1) (Takahashi et al. 2001; Yoshida et al. 2001; Manya et al. 2004). POMT1 and POMT2 transfer Man to Ser/Thr residues, whereas POMGNT1 sequentially catalyzes the synthesis of GlcNAcβ1-2Man linkage. During our studies, we found that mutations in the *POMGNT1* gene are causative in muscle-eye-brain disease (MEB) (Yoshida et al. 2001). Furthermore, it has been reported that the *POMT1* and *POMT2* genes are responsible for Walker-Warburg syndrome (WWS) (Beltran-Valero De Bernabe et al. 2002; van Reeuwijk et al. 2005; Manya et al. 2008). MEB and WWS are autosomal recessive disorders characterized by congenital muscular dystrophies with neuronal migration disorders. A defect in *O*-mannosyl glycan of α-DG was also observed in the brain and skeletal muscle of MEB and WWS patients (Yoshida et al. 2001; Muntoni et al. 2002; Endo 2005). Based on these pioneer findings, many researchers entered this field, and it is now known that the aberrant glycosylation of α-DG is the primary cause of some forms of congenital muscular dystrophy, so-called α-dystroglycanopathy (Table 15.1 and Fig. 15.1) (Endo 2005).

After we reported the presence of *O*-mannosyl glycan Siaα2-3Galβ1-4GlcNAcβ1-2Man-Ser/Thr on α-DG (Chiba et al. 1997), ensuing studies revealed various structures in *O*-mannosyl glycans, such as mannose branching and peripheral structures (Fig. 15.2). GlcNAcβ1-2Man, GlcNAcβ1-6Man, GlcNAcβ1-4Man, and Man-6-phosphodiester have been found as branching linkage on mannose residues (Chiba et al. 1997; Inamori et al. 2003, 2004; Kaneko et al. 2003; Yoshida-Moriguchi et al. 2010), and HNK-1 (Dwyer et al. 2012; Morise et al. 2014), Le^x (Smalheiser et al. 1998), and the repeat of -3GlcAβ1-3Xylα1- have been reported as peripheral structures (Inamori et al. 2012). In particular, the repetitive GlcA-Xyl structure and Man-6-phosphodiester linkage are unique in *O*-mannosyl glycans. Furthermore, it has been suggested that both the GlcA-Xyl repeat and phosphodiester linkage are required for the laminin-binding activity of α-DG (Yoshida-Moriguchi et al. 2010; Inamori et al. 2012). Recent reports have also demonstrated that several new proteins, which were previously identified as causative gene products for α-dystroglycanopathies, are involved in *O*-mannosyl glycan biosynthesis. Therefore, it is possible that the biosynthetic mechanisms of diverse *O*-mannosyl glycan structures are strictly regulated by an elaborate system.

In this review, we describe the biosynthetic mechanism of *O*-mannosyl glycans in mammals and also discuss the relationship between α-dystroglycanopathies and the glycobiology of protein *O*-mannosylation.

Table 15.1 Summary of genes responsible for α-dystroglycanopathies

Gene	Protein function	Clinical phenotype (former)[a]	Database	Section
POMT1	*O*-Man transferase	MDDGA1, B1, C1 (WWS)	MIM607423	15.3.2
POMT2	*O*-Man transferase	MDDGA2, B2, C2 (WWS)	MIM607439	15.3.2
POMGNT1	β1,2-GlcNAc transferase	MDDGA3, B3, C3 (MEB)	MIM606822	15.3.3
LARGE	β1,3-GlcA, α1,3-Xyl transferase	MDDGA6, B6 (MDC1D)	MIM603590	15.3.4
B3GNT1	β1,3-GlcNAc transferase	MDDGA13 (WWS)	MIM605517	15.3.4
DAG1	α, β-dystroglycan	MDDGC9 (LGMD2P)	MIM128239	15.3.4
GTDC2	β1,4-GlcNAc transferase	MDDGA8 (WWS)	MIM614828	15.3.5
B3GALNT2	β1,3-GalNAc transferase	MDDGA11 (WWS)	MIM610194	15.3.5
SGK196	*O*-Man kinase (POMK)	MDDGA12 (WWS)	MIM615247	15.3.5
FKTN	Unknown	MDDGA4, B4, C4 (FCMD)	MIM607440	15.3.7
FKRP	Unknown	MDDGA5, B5, C5 (LGMD2I, MDC1C)	MIM606596	15.3.7
TMEM5	Unknown	MDDGA10	MIM605862	15.3.7
DPM1	Dol-P-Man synthase	CDG-Ie	MIM603503	15.3.8
DPM2	Dol-P-Man synthase	CDG-Iu	MIM603564	15.3.8
DPM3	Dol-P-Man synthase	CDG-Io	MIM605951	15.3.8
DOLK	Dol-kinase	CDG-Im	MIM610746	15.3.8
GMPPB	GDP-Man pyrophosphorylase	MDDGA14, B14, C14 (LGMD)	MIM615320	15.3.8
ISPD	Unknown	MDDGA7 (WWS)	MIM614631	15.3.8

[a]These syndromes with cerebral ocular and muscular dystrophy are attributed to the abnormal glycosylation of α-dystroglycan and are now designated as muscular dystrophy-dystroglycanopathy (MDDG) types A (congenital with brain and eye anomalies), B (congenital with mental retardation), and C (autosomal recessive limb-girdle muscular dystrophies). The former names were *WWS* Walker-Warburg syndrome, *MEB* muscle-eye-brain disease, *MDC* congenital muscular dystrophy type, and *LGMD* limb-girdle muscular dystrophy type. *CDG* is a congenital disorder of glycosylation

15.2 The Dystrophin-Glycoprotein Complex and α-Dystroglycanopathy

15.2.1 α-Dystroglycanopathy

Muscular dystrophies are genetic diseases that cause progressive muscle weakness and wasting, and α-dystroglycanopathies are autosomal recessive disorders characterized by congenital muscular dystrophies with neuronal migration disorders (Endo 2005). In particular, an *O*-mannosyl glycan defect of α-DG was observed in the brain and skeletal muscle of α-dystroglycanopathy patients (Muntoni et al. 2002; Endo 2005). To date, approximately 18 genes have been reported as causative for

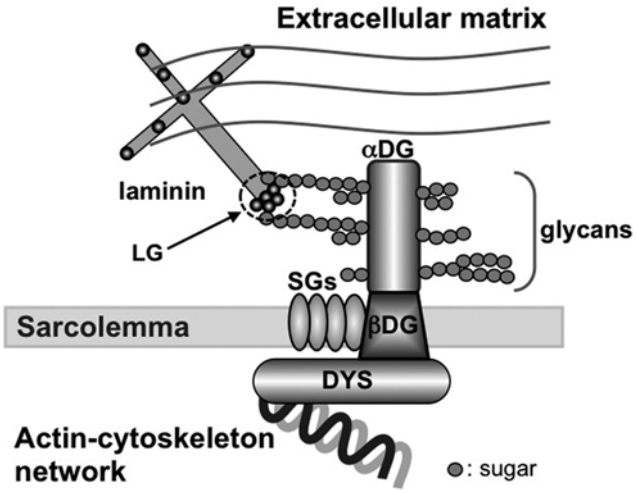

Fig. 15.1 Molecular organization of the dystrophin-glycoprotein complex in sarcolemma. *α-DG* α-dystroglycan, *β-DG* β-dystroglycan, *DYS* dystrophin, *SGs* sarcoglycans, *LG* laminin globular domain. α-DG is an extracellular peripheral membrane glycoprotein that is anchored to the cell membrane by binding to a transmembrane glycoprotein, β-DG. The α-DG-β-DG complex is thought to stabilize the plasma membrane by acting as an axis through which the extracellular matrix is tightly linked to the cytoskeleton because α-DG strongly binds to extracellular matrix proteins containing laminin globular domains, such as laminin, and because the cytoplasmic domain of β-DG interacts with DYS, which in turn binds to the actin cytoskeleton. α-DG is heavily glycosylated, and its sugars play a role in binding to laminin. The α-DG-β-DG complex also associates with the SGs that are the gene products of different muscular dystrophies. Defects in laminin or DYS also cause other muscular dystrophies

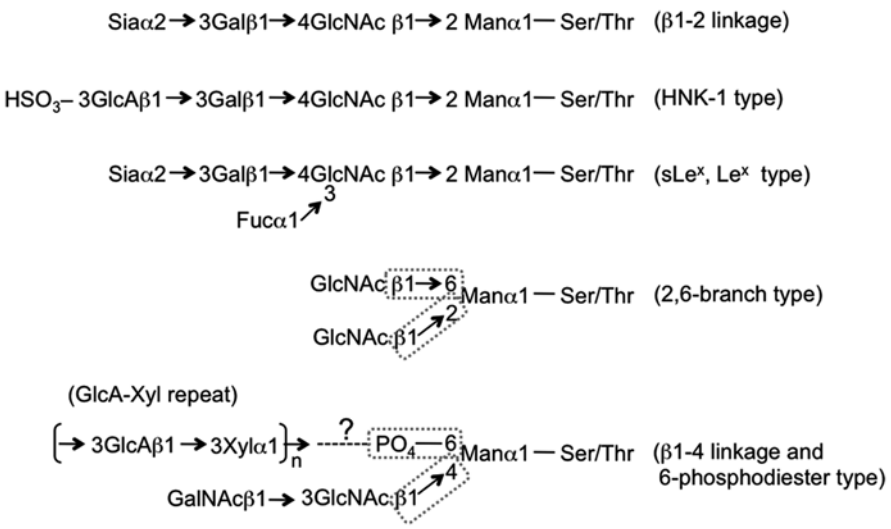

Fig. 15.2 Proposed *O*-mannosyl glycans in mammals. *Fuc* fucose, *Gal* galactose, *GalNAc* *N*-acetylgalactosamine, *GlcA* glucuronic acid, *GlcNAc* *N*-acetylglucosamine, *Man* mannose, *Sia* sialic acid, and *Xyl* xylose. *Dotted boxes* indicate the branched linkages of *O*-mannosylglycans

the abnormal *O*-mannosylation of α-DG (Table 15.1). The severity of the clinical manifestations of α-dystroglycanopathy is partly correlated with the genotype of the responsible gene. However, even when the causative gene is the same, it is known that the clinical manifestations have a broad spectrum, from the severe type that results in fatality with brain malformation in early childhood to the milder form that presents as adult-onset limb-girdle muscular dystrophy without brain malformation (Godfrey et al. 2007; Stalnaker et al. 2011). It should be emphasized that there are many α-dystroglycanopathies for which the causative genes have not yet been identified. It is clear that other causative genes are present in uncharacterized patients and that a molecular diagnosis of each patient is necessary to gain an improved understanding of the pathology of α-dystroglycanopathy.

15.2.2 O-*Mannosyl Glycan on α-DG*

In skeletal muscle, α-DG is a component of the dystrophin-glycoprotein complex, which acts as a transmembrane linker between the extracellular matrix and intracellular cytoskeleton (Fig. 15.1) (Michele and Campbell 2003). This linker is thought to stabilize the sarcolemma, which is subjected to the strenuous activity of skeletal muscle. *O*-Mannosyl glycans of α-DG play a role in binding to extracellular matrix components and synaptic molecules such as laminin, agrin, perlecan, neurexin, pikachurin, and slit (Ervasti and Campbell 1993; Bowe et al. 1994; Gee et al. 1994; Chiba et al. 1997; Peng et al. 1998; Talts et al. 1999; Sugita et al. 2001; Sato et al. 2008; Wright et al. 2012). The failure of α-DG to bind to these ligands because of a defect in α-DG glycosylation is thought to interrupt the normal muscular function and migration of neurons in the developing brain (Michele and Campbell 2003; Endo 2005). These ligand proteins have common laminin globular (LG) domains, which are important for binding to α-DG (Talts et al. 1999). LG domains are known to function in cell adherence, cell migration, intracellular signaling, and cell differentiation by binding to various glycoproteins, glycolipids, and proteoglycans (Tisi et al. 2000).

15.2.3 Ligand Proteins for α-DG

Laminin is a trimeric protein consisting of α, β, and γ subunits. Because several isoforms exist as trimers of the α, β, and γ subunits, various combinations of these subunits could be formed; indeed, more than 15 trimeric isoforms have been identified thus far (Golbert et al. 2011). The laminin α2 chain contains a LG domain and is a major subunit of muscle laminin; it is a causative gene product for congenital muscular dystrophy 1A (MDC1A) (Yamada et al. 1996; Gawlik and Durbeej 2011). It has been reported that the presence of the GlcA-Xyl repeating structure on α-DG is required for interaction with laminin, as shown in Figs. 15.1 and 15.2 (Inamori et al. 2012).

Agrin is a heparan sulfate proteoglycan that induces the formation of postsynaptic specializations at the neuromuscular junction (NMJ) (Gautam et al. 1996). α-DG is known to serve as an agrin receptor, thereby potentially regulating agrin-induced acetylcholine receptor clustering at the NMJ (Gee et al. 1994). Perlecan is a heparan sulfate proteoglycan in the basement membrane surrounding skeletal muscle fibers that is known to be a multifunctional extracellular matrix molecule; it interacts with other molecules, such as laminin and α-DG, to form the basement membrane and the NMJ (Peng et al. 1998; Henry et al. 2001). Furthermore, perlecan interacts with various growth factors, such as fibroblast growth factor (FGF), and receptors related to the regulation of intracellular signaling (Arikawa-Hirasawa et al. 1999).

Neurexins are cell-surface proteins specific to neurons and are mostly located on the presynaptic membrane (Sudhof 2008). Three genes encode neurexins (neurexin-1, neurexin-2, and neurexin-3) and are transcribed into two isoforms: α-neurexin (1α, 2α, and 3α) with a long chain and β-neurexin (1β, 2β, and 3β) with a short chain. Furthermore, there are more than 1,000 splicing variants (Tabuchi and Sudhof 2002). Although neurexins are involved in synapse construction and the release of neurotransmitters (Sudhof 2008), the significance of the relationship between neurexin and DG is still unclear.

Pikachurin co-localizes with α-DG at ribbon synapses and plays a crucial role in the formation of the photoreceptor ribbon synapse (Sudhof 2008; Kanagawa et al. 2010). Slit and its receptor ROBO were identified as axon-guidance molecules and function as a repulsive cue in preventing axons from migrating to inappropriate locations during the assembly of the nervous system. It has been reported that the binding of α-DG and slit is required for the proper localization of slit, suggesting an important role for α-DG as a determination factor for axon-guidance cues in the mammalian nervous system (Wright et al. 2012).

Further studies are necessary to elucidate the role of α-DG glycans in the binding and recognition between α-DG and these ligands. It is noteworthy that the apparent molecular weight of α-DG (e.g., 156 kDa in skeletal muscle, 120 kDa in brain, and 140 kDa in cardiac muscle) varies among different tissues, presumably according to glycosylation status. Many common structural features and tissue-specific differences in glycosylation have been elucidated through the characterization of α-DG purified from different tissues, as described below (Ervasti et al. 1997; Kuga et al. 2012). Different glycoforms of α-DG may have unique or variable functions in different tissues.

15.3 Biosynthesis of *O*-Mannosyl Glycan

15.3.1 Structure and Biosynthetic Pathway

The biosynthesis of *O*-mannosyl glycan is initiated by the transfer of Man to Ser/Thr residues by POMT1/2 (Fig. 15.3) (Manya et al. 2004). As shown in Figs. 15.2 and 15.3, three mannose-linkage structures (GlcNAcβ1-2Man, GlcNAcβ1-4Man,

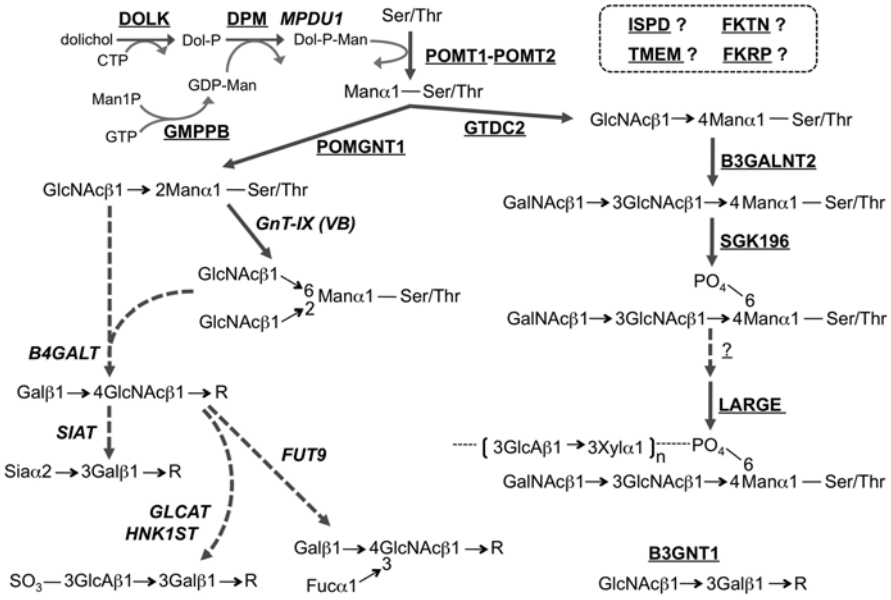

Fig. 15.3 Possible biosynthetic pathway of *O*-mannosylglycan in mammals. *Fuc* fucose, *Gal* galactose, *GalNAc N*-acetylgalactosamine, *GlcA* glucuronic acid, *GlcNAc N*-acetylglucosamine, *Man* mannose, *Sia* sialic acid, *Xyl* xylose, *POMT* protein *O*-mannosyltransferase, *POMGNT1* protein *O*-linked mannose β-1,2-*N*-acetylglucosaminyltransferase 1, *LARGE* acetylglucosaminyltransferase-like, *BGNT1* β1,3-*N*-acetylglucosaminyltransferase 1, *GTDC2* glycosyltransferase-like domain-containing protein 2, *B3GALNT2* β1,3-*N*-acetylgalactosaminyltransferase 2, *SGK196* protein kinase-like protein SGK196, *GnT-IX (VB)* α-1,6-mannosyl-glycoprotein β-1,6-*N*-acetylglucosaminyltransferase IX (VB), *FKTN* fukutin, *FKRP* fukutin-related protein, *TMEM5* transmembrane protein 5, *DPM* dolichol-phosphate-mannose synthase, *DOLK* dolichol kinase, *GMPPB* GDP-mannose pyrophosphorylase B, *MPDU1* mannose-phosphate-dolichol utilization defect 1, *ISPD* isoprenoid synthase domain-containing protein, *B4GALT* β-1,4-galactosyltransferase, *SIAT* β-2,3-sialyltransferase, *GLCAT* β-1,3-glucuronyltransferase, *HNK1ST* HNK-1 sulfotransferase, and *FUT9* α-1,3-fucosyltransferase. The causative gene products for α-dystroglycanopathies are underlined. Non-causative gene products for α-dystroglycanopathies are shown in *italics*

and GlcNAcβ1-6Man) exist in *O*-mannosyl glycans (Chiba et al. 1997; Inamori et al. 2003, 2004; Kaneko et al. 2003; Yoshida-Moriguchi et al. 2013). The GlcNAcβ1-2Man linkage was first identified in skeletal muscle, peripheral nerve, and brain α-DG as part of the main structure Siaα2-3Galβ1-4GlcNAcβ1-2Man-Ser/Thr and is formed by the action of POMGNT1 (Takahashi et al. 2001; Yoshida et al. 2001). The GlcNAcβ1-6Man linkage was identified in brain tissue only as a GlcNAcβ1-2(GlcNAcβ1-6)Man branching structure formed by β1,6-*N*-acetylglucosaminyltransferase-IX (GnT-IX), which is specifically expressed in the brain (Inamori et al. 2003; Kaneko et al. 2003). In addition, the formation of GlcNAcβ1-6Man by GnT-IX requires the presence of the GlcNAcβ1-2Man

structure (Inamori et al. 2004). It has been reported that GnT-IX is associated with the HNK-1 structure on the 2,6-branched *O*-mannosyl glycan of receptor protein tyrosine phosphatase β/tyrosine phosphatase receptor type Z1 (RPTPβ/PTPRZ1), which is an important regulator of remyelination in the brain (Kanekiyo et al. 2013; Morise et al. 2014). Peripheral structures are synthesized by galactosyltransferases (GALTs), sialyltransferases (SIATs), glucuronyltransferases (GLCATs), sulfotransferase (HNK1ST), and α1,3-fucosyltransferase-9 (FUT9), which comprise a series of Golgi-resident glycosyltransferases (Kaneko et al. 1999; Sasaki et al. 2004; Nakagawa et al. 2012; Morise et al. 2014; Terayama et al. 1997; Seiki et al. 1999; Bakker et al. 1997; Kudo et al. 2007).

The GlcNAcβ1-4Man and Man-6-phosphodiester linkages were identified from recombinant α-DG expressed in HEK293T cells (Yoshida-Moriguchi et al. 2010), and the GlcNAcβ1-4Man linkage was found to be formed by glycosyltransferase-like domain-containing 2 (GTDC2) (Manzini et al. 2012; Jae et al. 2013; Ogawa et al. 2013; Yoshida-Moriguchi et al. 2013). GalNAc is transferred to GlcNAcβ1-4Man by β1,3-*N*-acetylgalactosaminyltransferase 2 (B3GALNT2), forming the GalNAcβ1-3GlcNAc linkage (Jae et al. 2013; Stevens et al. 2013; Yoshida-Moriguchi et al. 2013). Furthermore, glycosylation-specific kinase 196 (SGK196, protein *O*-mannose kinase; POMK) transfers a phosphate group to the C6 position of the mannose of GalNAcβ1-3GlcNAcβ1-4Man to form the GalNAcβ1-3GlcNAcβ1-4(phosphate-6)Man structure (Jae et al. 2013; Yoshida-Moriguchi et al. 2013). Although the GlcA-Xyl repeating structure attached to the phosphodiester linkage on the Man-6 position is considered to be required for laminin binding, unidentified molecules may also be present between the GlcA-Xyl repeat and Man-6-phosphate, and little is known about the actual structure of the post-phosphoryl modification (Yoshida-Moriguchi et al. 2010). The GlcA-Xyl repeat is elongated by like-acetylglucosaminyltransferase (LARGE) (Inamori et al. 2012). LARGE2, a paralog of LARGE, catalyzes the same glycosylation reactions as LARGE and exhibits xylosyltransferase and glucuronyltransferase activities to form the GlcA-Xyl repeat (Ashikov et al. 2013; Inamori et al. 2013).

It is currently unknown whether the 2,4-substituted *O*-mannose structure GlcNAcβ1-2(GlcNAcβ1-4)Man can be formed (Yoshida-Moriguchi et al. 2013), although it is clear that a defect of POMGNT1 causes an insufficiency of laminin binding (Yoshida et al. 2001; Kanagawa et al. 2009; Miyagoe-Suzuki et al. 2009), likely because of defective post-phosphoryl modification, which suggests that the 2-substituted *O*-mannose structure is required for post-phosphoryl modification. However, the role of the GlcNAcβ1-2Man linkage in the post-phosphoryl modification remains unclear. Future studies are necessary to clarify the regulatory mechanism for the formation of the *O*-mannosyl branching structure.

Details regarding the functions of the causative gene products for the α-dystroglycanopathies listed in Table 15.1 and their roles in the biosynthetic pathway of *O*-mannosyl glycans are described below.

15.3.2 Protein O*-Mannosyl Transferase (POMT)*

POMT1 and POMT2 catalyze the initial step of *O*-mannosyl glycan biosynthesis, that is, the transfer of a mannosyl residue from dolichol-phosphate-mannose (Dol-P-Man) to Ser/Thr residues of certain proteins (Manya et al. 2004). *O*-Mannosylation is an essential protein modification that is evolutionarily conserved from eukaryotes to mycobacteria (VanderVen et al. 2005; Lommel and Strahl 2009), and it is essential for maintaining cell shape and integrity (Gentzsch and Tanner 1996) and contributes to the quality control of proteins in the yeast endoplasmic reticulum (ER) (Harty et al. 2001; Nakatsukasa et al. 2004; Xu et al. 2013). Reduced levels of protein *O*-mannosyltransferases in *Drosophila melanogaster* were reported to result in defects in embryonic muscle development (Ichimiya et al. 2004; Lyalin et al. 2006; Haines et al. 2007; Ueyama et al. 2010), and *pomt1* deletion in mice resulted in embryonic lethality (Willer et al. 2004). Recently, we also demonstrated that *O*-mannosyltransferase is required for muscle development in zebrafish (Avsar-Ban et al. 2010).

The genes encoding protein *O*-mannosyltransferases have been characterized in the yeast *Saccharomyces cerevisiae* and constitute the *pmt* family; *S. cerevisiae* possesses six *pmt* homologues (*Scpmt1–6*) (Strahl-Bolsinger et al. 1999). In *D. melanogaster*, zebrafish, and mammals (human, mouse, and rat), two homologues (*POMT1* and *POMT2*) have been identified (Willer et al. 2002, 2004; Ichimiya et al. 2004; Manya et al. 2004, 2006; Avsar-Ban et al. 2010). *POMT1* and *POMT2* are classified as *pmt4* and *pmt2*, respectively, in *D. melanogaster*, zebrafish, and mammals (Girrbach and Strahl 2003).

POMT1 and POMT2 are located in the ER membrane (Akasaka-Manya et al. 2006; Manya et al. 2010). In humans, the transferase activity requires the formation of a POMT1 and POMT2 heterocomplex; cotransfection of POMT1 and POMT2 upregulates POMT activity in cultured cells, whereas the expression of only one of these proteins does not (Manya et al. 2004; Akasaka-Manya et al. 2006). Because the POMT1-POMT2 complex does not form in a mixture of membrane fractions from POMT1-transfected cells and POMT2-transfected cells, the complex may form during the synthesis of POMT1 and POMT2 in the ER (Manya et al. 2004; Akasaka-Manya et al. 2006).

Scpmt1 has been proposed to consist of seven transmembrane helices (Strahl-Bolsinger and Scheinost 1999). The Scpmt1 *N*-terminus and loops 2, 4, and 6 are located in the cytoplasm, and the *C*-terminus and loops 1, 3, and 5 are located in the ER lumen. Two hydrophilic regions (loops 5 and 1) are important for enzymatic activity (Strahl-Bolsinger and Scheinost 1999; Girrbach et al. 2000; Girrbach and Strahl 2003). We recently constructed models in which human hPOMT1 and hPOMT2 have seven- and nine-transmembrane helices, respectively, and in which the *C*-termini and loops 1, 3, and 5 are located in the ER lumen (Manya et al. 2010). The loop 5 regions of both hPOMT1 and hPOMT2 have amino acid sequences similar to those of the catalytic domains of Scpmt1 (Manya et al. 2010), suggesting that

loop 5 may be the catalytic domain of hPOMT1 and hPOMT2. Because the *N*-glycosylation sites are found in loop 5, it is possible that these *N*-glycans contribute to the hydrophilicity and affect the folding of the catalytic domain. The finding that the removal of *N*-glycans from either hPOMT1 or hPOMT2 inhibits POMT activity suggests that loop 5 is required for the correct folding of the catalytic center in the POMT1-POMT2 complex (Manya et al. 2010). Further studies are necessary to elucidate the exact catalytic domains and the significance of complex formation for enzymatic activity.

It has been reported that individual Scpmts have different specificities for protein substrates (Lehle et al. 2006), suggesting the presence of certain sequences required for recognition by Scpmts. However, such sequence specificity has not yet been identified (Hutzler et al. 2007). Nonetheless, because *O*-mannosyl glycans were detected in a limited number of proteins and because the mucin-like domain of α-DG is highly glycosylated (Brancaccio et al. 1995; Yamada et al. 1996; Brancaccio et al. 1997; Chiba et al. 1997; Kanagawa et al. 2004), *O*-mannosylation in mammals may require a specific sequence. To elucidate the regulation of *O*-mannosylation, it is important to determine whether there is a preferential amino acid sequence for this modification. To address this question, we synthesized a series of peptides that fully covered the mucin-like domain of α-DG and then examined whether these peptides acted as acceptors for protein *O*-mannosylation. Two similar peptide sequences, corresponding to residues 401–420 and 336–355, were strongly mannosylated by POMT (Manya et al. 2007). Because the positions of the Pro and Thr residues in the two peptides were very similar, we proposed that IxPT(P/x) TxPxxxxPTx(T/x)xx is the preferential amino acid sequence for mammalian *O*-mannosylation. A BLAST search for proteins with this sequence revealed only α-DG, suggesting that the primary *O*-mannosylated protein is α-DG. In the mannosylated peptide corresponding to residues 401–420, Thr414 was found to be most prominently modified by *O*-mannosylation, with *O*-mannosylation occurring sequentially rather than at random. Furthermore, pre-mannosylated (T414Man)-peptide 401–420 was found to be a more effective acceptor than peptide 401–420 (Manya et al. 2007), indicating that the mannosylation of Thr414 leads to effective subsequent *O*-mannosylation. Therefore, α-DG appears to be a prominent acceptor of *O*-mannosylation in vitro. In addition, another group suggested that the upstream region of the α-DG mucin domain is required for the regulation of *O*-mannosylation in vivo (Breloy et al. 2008).

15.3.3 Protein O-*Mannose β1,2*-N-*Acetylglucosaminyltransferase 1 (POMGNT1)*

POMGNT1 catalyzes the formation of GlcNAcβ1-2Man by transferring GlcNAc from a uridine diphosphate GlcNAc (UDP-GlcNAc) to an *O*-mannose of glycoproteins (Takahashi et al. 2001). The human *POMGNT1* gene was cloned from a cDNA sequence homologous to human β1,2-*N*-acetylglucosaminyltransferase I (GnT-I).

Although the overall amino acid sequence identity of POMGNT1 and GnT-I was only 23.2 %, the predicted catalytic domains of the two proteins are highly homologous. POMGNT1 and GnT-I are typical type II membrane proteins that are localized in the Golgi apparatus, and both of them form GlcNAcβ1-2Man linkages. A difference is that POMGNT1 catalyzes GlcNAcβ1-2Man linkage in *O*-mannosyl glycans but not in *N*-glycans, whereas GnT-I catalyzes the same linkage in *N*-glycans but not in *O*-mannosyl glycans (Takahashi et al. 2001).

Human POMGNT1 is composed of 660 amino acids and four domains: an N-terminal cytoplasmic tail, a transmembrane domain, a stem domain, and a catalytic domain (Akasaka-Manya et al. 2004). Because the stem domain of POMGNT1 has low homology with that of GnT-I and because the deletion of 298 amino acids from the *N*-terminus, including the stem domain of POMGNT1, did not affect the activity of POMGNT1, the function of the stem domain is still unknown.

We previously established assay methods for POMGNT1 activity using a synthetic mannosyl peptide (Man-peptide) derived from the α-DG sequence as an acceptor substrate. POMGNT1 recognizes Man-peptide and benzyl-α-D-mannose as an acceptor (Takahashi et al. 2001; Manya et al. 2008; Akasaka-Manya et al. 2011) but not free mannose, *p*-nitrophenyl-α-D-mannose, or mannose-2-aminobenzamide (Takahashi et al. 2001). For POMGNT1 to recognize a mannosyl peptide, three or more amino acid residues are required (Akasaka-Manya et al. 2011). Because the amino acid sequence of mannosyl peptides affects the activity of POMGNT1, the amino acid sequence may be a determinant of efficient GlcNAc elongation.

*15.3.4 Acetylglucosaminyltransferase-Like (LARGE) and β1,3-*N*-Acetylglucosaminyltransferase 1 (B3GNT1)*

The novel glycosaminoglycan-like disaccharide repetitive structure [-3GlcAβ1-3Xylα1-]$_n$ attached to α-DG is synthesized by LARGE and is required for laminin binding to α-DG (Figs. 15.1 and 15.3) (Inamori et al. 2012). Human LARGE is composed of 756 amino acids and four domains: an N-terminal transmembrane domain, a coiled-coil domain, an α1,3-Xyl transferase domain, and an β1,3-GlcA transferase domain (Inamori et al. 2012). LARGE transfers Xyl and GlcA from UDP-Xyl and UDP-GlcA via two catalytic domains and elongates the Xyl-GlcA repeating structure. It has been reported that overexpression of LARGE leads to the hyperglycosylation of α-DG in cultured cells (Barresi et al. 2004). Although it is reasonable that overexpression of LARGE can recover the glycosylation and laminin-binding activity of α-DG in LARGE-deficient cells, it has been demonstrated that LARGE overexpression is also effective in FKTN- and POMGNT1-deficient cells derived from FCMD and MEB patients, respectively (Barresi et al. 2004). Furthermore, the adeno-associated virus-mediated overexpression of LARGE was shown to rescue α-DG function in FKRP-mutant mice (Vannoy et al. 2014). By contrast, the transgenic expression of LARGE in another dystrophic mouse model with an FKRP mutation resulted in a worsening of muscle pathology

(Whitmore et al. 2014). The reason for the discrepancy between these studies is unclear at present. Because the GlcA-Xyl repeat of α-DG is a tunable scaffold for extracellular matrix proteins (Goddeeris et al. 2013), optimal levels of LARGE expression may be required to achieve the expected functions of LARGE.

LARGE is known to be involved in the processing of the N-terminal domain of α-DG by furin, and this processing is necessary for α-DG maturation (Kanagawa et al. 2004). Physical interaction between LARGE and α-DG is required for α-DG glycosylation by LARGE, and the processing of α-DG occurs after glycosylation (Kanagawa et al. 2004). It is notable that the α-DG protein itself is important for interacting with LARGE. A *DAG1* mutation, the 575C-T transition resulting in a Thr192-to-Met substitution (T192M), was identified in a Turkish woman with limb-girdle muscular dystrophy-dystroglycanopathy (MDDGC9, Table 15.1) (Hara et al. 2011). This mutation (T192M) in *DAG1* reduces the interaction of α-DG with LARGE and leads to the defective glycosylation (GlcA-Xyl repeat formation) of α-DG. Therefore, although DAG1 is not a glycosylation-related molecule, it is included in the list of causative genes for α-dystroglycanopathies (Table 15.1).

B3GNT1 is known to synthesize poly-*N*-acetyllactosamine (LacNAc) [-4GlcNAcβ1-3Galβ1-]$_n$ repeats in conjunction with B4GALT1 (Yu et al. 2001). In α-DG glycosylation, B3GNT1 was shown to form a complex with LARGE and synthesize laminin-binding glycans (Bao et al. 2009). However, the glycan structures synthesized by the B3GNT1/LARGE complex were not identified accurately. This study also demonstrated that the defect in B3GNT1 reduced levels of laminin-binding glycans and increased cell migration and tumor formation by cancer cell lines. Recently, mutations in *B3GNT1* were identified in WWS patients (Shaheen et al. 2013).

15.3.5 Glycosyltransferase-Like Domain-Containing Protein 2/Protein O*-Mannose β1,4-*N*-Acetylglucosaminyltransferase 2 (GTDC2/POMGNT2), β1,3-*N*-Acetylgalactosaminyltransferase 2 (B3GALNT2), and Protein Kinase-Like Protein SGK196/Protein* O*-Mannose Kinase (SGK196/POMK)*

As described above, α-DG (recombinant protein expressed in HEK293T cells and Cos7 cells) has a 4-substituted *O*-mannose and 6-*O*-phosphorylated *O*-mannose structure, GalNAcβ1-3GlcNAcβ1-4(P-6)Man (Fig. 15.2) (Yoshida-Moriguchi et al. 2010; Yagi et al. 2013), and it was assumed that an unknown modification, including a phosphodiester bond formation on 6-*O*-phosphoryl mannose, is required for laminin binding (Yoshida-Moriguchi et al. 2010). This phosphodiester linkage formation on α-DG is decreased in skeletal muscle from α-dystroglycanopathy patients (Yoshida-Moriguchi et al. 2010); however, little is known about the phosphodiester-linked glycan structure and the mechanism of post-phosphorylation modification.

As shown in Fig. 15.3, GTDC2 catalyzes the formation of GlcNAcβ1-4Man by transferring GlcNAc from a UDP-GlcNAc to an *O*-mannose residue

(Yoshida-Moriguchi et al. 2013). Next, B3GALNT2 catalyzes the formation of GalNAcβ1-3GlcNAcβ1-4Man by transferring GalNAc from UDP-GalNAc to GlcNAcβ1-4Man (Yoshida-Moriguchi et al. 2013). Notably, *GTDC2*-knock-out mice die within the first day of birth and exhibit abnormal neuronal migration (Yagi et al. 2013). SGK196 then transfers a phosphate group from adenosine 5′-triphosphate (ATP) to the C6 position of the mannose of GalNAcβ1-3GlcNAcβ1-4Man and forms the GalNAcβ1-3GlcNAcβ1-4(P-6)Man structure (Yoshida-Moriguchi et al. 2013). These three enzymes were reported to be localized in the ER, and the GalNAcβ1-3GlcNAcβ1-4Man structure was demonstrated to be required for phosphorylation by SGK196 (Yoshida-Moriguchi et al. 2013). Thus, a series of reactions from *O*-mannosylation by POMT1/2 to phosphorylation by SGK196 may occur in the ER. Therefore, the 2,4-branched structure, GlcNAcβ1-2(GlcNAcβ1-4)Man, may not be formed because POMGNT1 is localized in the Golgi apparatus (Xiong et al. 2006). However, as *POMGNT1*-knock-out mice show defects of the phosphodiester linkage (Kuga et al. 2012) and laminin-binding activity (Miyagoe-Suzuki et al. 2009), further examination is required to elucidate the regulatory mechanism for the branching of *O*-mannose residues.

*15.3.6 β1,6-*N*-Acetylglucosaminyltransferase-IX/VB (GnT-IX/GnT-VB)*

The 2,6-substituted *O*-mannose structure GlcNAcβ1-2(GlcNAcβ1-6)Man has been identified from brain α-DG in mammals (Fig. 15.2). The GlcNAcβ1-6Man linkage is formed by GnT-IX (GnT-VB), which is specifically expressed in the brain (Inamori et al. 2003; Kaneko et al. 2003). Because the transfer of GlcNAc to the *O*-mannose C6 position by GnT-IX requires the GlcNAcβ1-2Man structure (Inamori et al. 2004), POMGNT1 must function before GnT-IX in *O*-mannosyl glycan biosynthesis (Fig. 15.3). GnT-IX is involved in forming the HNK-1 structure of the 2,6-branched *O*-mannosyl glycan on RPTPβ and is also involved in oligodendrocyte survival and in the recovery from demyelinating disease (Kanekiyo et al. 2013). Recently, we demonstrated that *O*-mannose-linked HNK-1 in the brain is mainly carried by phosphacan, a secreted splicing variant of RPTPβ (Morise et al. 2014). However, there is no report regarding the relationship between *GnT-IX* mutation and α-dystroglycanopathy in the brain.

15.3.7 Fukutin (FKTN), Fukutin-Related Protein (FKRP), and Transmembrane Protein 5 (TMEM5)

FKTN, FKRP, and TMEM5 are type II membrane proteins that have some similarities to glycosyltransferases and may be involved in post-phosphoryl modifications. However, their exact functions have yet to be determined (Table 15.1).

FKTN is responsible for Fukuyama congenital muscular dystrophy (FCMD) (Kobayashi et al. 1998). *FKRP* was identified by its homology to *FKTN* and is responsible for MDC1C or limb-girdle muscular dystrophy 2I (LGMD2I) (Brockington et al. 2001a, b). FKTN and FKRP are localized in the Golgi apparatus, and FKTN and FKRP are assumed to belong to the nucleotidyltransferase fold protein superfamily based on their sequence information. FKTN and FKRP also have similarities to enzymes involved in phosphorylcholine modification or the mannosylphosphorylation of polysaccharides in bacteria and yeast. FKTN interacts with POMGNT1 and co-localizes with POMGNT1 in the Golgi apparatus (Xiong et al. 2006). The transmembrane region of FKTN mediates its localization to the Golgi apparatus and participates in the interaction with POMGNT1. This interaction is supported by the observation that a missense Y371C mutation found in FCMD patients causes FKTN to remain in the ER and also causes the mislocalization of POMGNT1 to the ER (Xiong et al. 2006). Most FCMD patients have a 3-kb retrotransposal insertion in the 3′ noncoding region of *FKTN* (Kobayashi et al. 1998). In transgenic *FKTN* knock-in mouse brains containing this retrotransposal insertion, POMGNT1 activity was reduced, and hypoglycosylated α-DG was detected (Xiong et al. 2006). These results suggest that FKTN regulates the cellular localization and enzymatic activity of POMGNT1. In addition to FKTN, FKRP is also involved in the post-phosphoryl modification of *O*-mannose residues on α-DG (Kuga et al. 2012). However, it remains unclear how defects in FKTN or FKRP result in the loss of post-phosphoryl modification.

Mutations in *TMEM5* cause severe cobblestone lissencephaly, consistent with α-dystroglycanopathy (Vuillaumier-Barrot et al. 2012; Jae et al. 2013). TMEM5 is similar to exostosin (EXT1), which is a glycosyltransferase for heparan sulfate proteoglycan synthesis (Vuillaumier-Barrot et al. 2012), but the function of TMEM5 is not clear.

15.3.8 Synthases of Sugar Donors

Dol-P-Man is used as a sugar donor substrate in the Man transfer catalyzed by POMT1/2 (Fig. 15.3). Dol-P-Man is synthesized by Dol-P-Man synthase (DPM), which consists of three subunits, DPM1, DPM2, and DPM3, in the ER (Maeda et al. 2000). DPM1 is a catalytic subunit located at the cytoplasmic face and binds to the C-terminal region of DPM3. DPM2 and DPM3 have two transmembrane domains, and both the N- and C-terminal tails are oriented toward the cytoplasmic face. Thus, DPM2 and DPM3 tether DPM1 to the ER membrane. Because DPM is involved in *N*-glycosylation, *O*-mannosylation, *C*-mannosylation, and the glycosylphosphatidylinositol (GPI)-anchor, it is presumed that defects in DPM would affect a wide range of glycosylations and cause severe disorders. In fact, the genes encoding the three DPM subunits are known to be causative genes for the congenital disorders of glycosylation type I (CDG-I) (Lefeber et al. 2009; Barone et al. 2012; Yang et al. 2013), and CDGs caused by *DPM* mutations are associated with a high incidence of α-dystroglycanopathy. *O*-Mannosylation is selectively inhibited by a mutation in

DPM3 (Lefeber et al. 2009), suggesting that the amount of Dol-P-Man may not be the only regulatory factor for *O*-mannosylation in the ER.

In addition to DPM, several enzymes involved in the synthesis of Dol-P-Man have been found to be causative for α-dystroglycanopathy (Table 15.1 and Fig. 15.3). Dolichol kinase (DOLK) catalyzes the CTP-mediated phosphorylation of dolichol at the cytosolic leaflet of the ER membrane. Mutations in *DOLK* cause CDG-Im (DOLK-CDG) in association with α-dystroglycanopathy (Lefeber et al. 2011). Patients show a combined *N*-glycosylation and *O*-mannosylation deficiency with dilated cardiomyopathy. GDP-Man pyrophosphorylase B (GMPPB) catalyzes the conversion of mannose-1-phosphate and GTP to GDP-Man, which is required for the synthesis Dol-P-Man, as described above. Mutations in *GMPPB* cause LGMD-type α-dystroglycanopathy (Carss et al. 2013), and a defect in *O*-mannosylation and not *N*-glycosylation was observed in cases of mutant *GMPPB*. Finally, the complicated relationships between the amount of available Dol-P-Man and *N*-glycosylation and *O*-mannosylation are briefly discussed. Man-P-dolichol utilization defect 1 (MPDU1) was identified to be related to Dol-P-Man synthesis, and mutations in *MPDU1* cause CDG-If because of abnormal *N*-glycosylation (Kranz et al. 2001; Schenk et al. 2001). The clinical phenotype of CDG-If does not include muscular dystrophy or dilated cardiomyopathy, suggesting that *O*-mannosylation deficiency may not occur in this disease despite the reduced availability of Dol-P-Man in the ER. These data suggest that the amount of available Dol-P-Man affects the *O*-mannosylation of α-DG, but an unknown mechanism may regulate the usage of Dol-P-Man for protein glycosylation in the ER.

Isoprenoid synthase domain-containing protein (ISPD) belongs to the family of 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) synthases (Table 15.1) in the 2-*C*-methyl-D-erythritol 4-phosphate pathway (the MEP pathway, the biosynthetic pathway producing isoprenoid precursors from pyruvic acid and glyceraldehyde 3 phosphoric acid). It was reported that mutations in *ISPD* cause WWS (Roscioli et al. 2012; Willer et al. 2012) and decrease in POMT activity (Willer et al. 2012). However, the mechanism of POMT activity reduction by *ISPD* mutations and the function of ISPD are still not known. Although isoprenoid serves as a precursor to cholesterol and dolichol, the MEP pathway is not essential in mammals. Conversely, the mevalonate pathway, which incorporates isoprenoid from acetyl CoA, is essential in mammals. Based on homology information, ISPD may be involved in the synthesis of sugar donor substrates, such as CDP-sugar or dolichol-sugar. Nonetheless, further studies are necessary to elucidate the function of ISPD.

15.3.9 O-*Mannosylated Proteins*

Although *O*-mannosylation is a major protein modification in yeast, as described in Section 15.3.2, it remains unclear how many proteins, in addition to α-DG, are actually *O*-mannosylated in mammals. Recent studies have demonstrated that various proteins are modified by *O*-mannosyl glycans, including CD24, RPTPβ (see Section

15.3.6), neurofascin 186, lecticans, cadherins, and plexins (Bleckmann et al. 2009; Dwyer et al. 2012; Pacharra et al. 2012, 2013; Vester-Christensen et al. 2013; Lommel et al. 2013). The *O*-mannosyl modifications of these proteins were mainly detected by glycoproteomic analyses using mass spectrometry. However, the detailed structures and roles of *O*-mannosyl glycans in each core protein are not well understood.

CD24 is a GPI-anchored protein that is mainly expressed in B cells and is known as a cell adhesion molecule (Bleckmann et al. 2009). Neurofascin is a type I membrane protein and has two isoforms, neurofascin 155 and 186; it belongs to the L1 family of the immunoglobulin superfamily and is involved in axon subcellular targeting and synapse formation during neural development by interaction with neuron-glia cell adhesion molecule (NgCAM). Neurofascin 155 is known to promote neurite outgrowth, whereas neurofascin 186 inhibits neuronal adhesion (Pacharra et al. 2012). Lecticans constitute a family of chondroitin sulfate proteoglycans, including aggrecan, brevican, neurocan, and versican and are a major component of the neuronal extracellular matrix in the mammalian brain (Pacharra et al. 2013). The cadherin superfamily is a large group including 80 or more proteins with an extracellular cadherin (EC) domain. Vester-Christensen et al. suggested that cadherins are the major carrier proteins of *O*-mannosyl glycans (Vester-Christensen et al. 2013). Cadherins are type I transmembrane proteins and play important roles in calcium-dependent cell adhesion. Classic cadherins are involved in the formation and maintenance of cellular binding within tissues through the formation of adherens junctions. Lommel et al. reported that the *O*-mannosylation of epithelial (E)-cadherin is crucial for the formation of adherens junctions (Lommel et al. 2013). Plexins are type I membrane proteins that are receptor proteins for semaphorins, serving as axon-guidance cues for neural development (Vester-Christensen et al. 2013).

These *O*-mannosylated proteins appear to be highly important for the development of the nervous system through cell adherence at the cell surface or the extracellular matrix. The roles of *O*-mannosyl glycans for these adhesion and recognition molecules will be elucidated in the future.

15.4 Conclusion

The glycan analysis of α-DG revealed the presence of *O*-mannosyl glycans in mammals, and several glycosyltransferases catalyzing the processing of these glycans were found to cause some forms of congenital muscular dystrophy. These muscular dystrophies are termed α-dystroglycanopathies. The common hypoglycosylation of α-DG has been shown to greatly reduce its affinity for extracellular matrix components, such as laminin, thereby disrupting the α-DG-extracellular matrix linkage and leading to membrane fragility. Recently, this glyco field has greatly expanded because of the combined effects of improvements in glycan analysis technology and

gene sequencing technology. Glycan analysis has revealed various new *O*-mannosyl glycan structures, whereas gene sequencing has identified many new causative genes for uncharacterized α-dystroglycanopathies. Both technologies are cooperatively enabling great advances in this research field. Because the hypoglycosylation of α-DG is a common feature in α-dystroglycanopathies, α-DG must be a potential target for new glycotherapeutic strategies for these diseases in the future.

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Chapter 16
Glycans and Chronic Obstructive Pulmonary Disease (COPD)

Congxiao Gao and Naoyuki Taniguchi

Abstract Chronic obstructive pulmonary disease (COPD) is a progressive lung disease that is not fully reversible. It is a highly complex disease in which both environmental exposures and host susceptibility factors should be taken into account in its management. Although cigarette smoke is the major risk factor, quitting smoking does not fully repress the airway inflammation and restore the lost lung function.

Our group has been interested in changes of glycosylation that are related to the onset, biomarker discovery, and therapeutics of various diseases including cancer, neurological disease, and lifestyle-related diseases such as diabetes and COPD. Changes in glycan structures are currently being used as cancer biomarkers, prognosis, and treatment of the disease. Cell membrane receptors are mostly glycosylated proteins, and changes in the glycosylation of them have been shown to lead to dysfunctions in downstream signal pathways.

In COPD, alterations in the structures of glycans lead to dysfunction of receptor molecules, such as the TGF-beta receptor related to the upregulation of protease, or result in alterations in mucus viscosity and impaired mucociliary transport. Although the mechanism responsible for this is still uncertain, it appears that environmental factors such as exposure to cigarette smoke, pollutants, and bacteria/virus infections can alter the activities of some glycosyltransferases, and eventually this may affect the synthesis of glycans. Here, we show some glycan structures and relationships with the pathogenesis of COPD. Our recent findings regarding the role of glycan changes in COPD may open a new avenue toward the development of glycotherapeutics for COPD.

Keywords Chronic obstructive pulmonary disease (COPD) • Core fucose • Sialyl-Lewis^x structure • Glycosaminoglycans

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16.1 Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by two main changes, namely, emphysema with damaged alveolar walls and irregularly shaped air sacs and chronic bronchitis, in which a thick mucus secretion occurs in the airways. Long-term cigarette smoking is the leading cause of COPD, but air pollutants, chemicals, or dust can also contribute to this disease. Aging, poor nutrition, or, rarely, a genetic condition called alpha-1 antitrypsin (AAT) deficiency may play roles in the development and progression of COPD. The prevalence of COPD is increasing drastically, and 210 million people have now been diagnosed with COPD worldwide, and many people may have the disease but are not even aware of it. According to a WHO report, COPD will be the world's third leading cause of mortality by the year 2030. Subjects with COPD often show a progressive limitation in airflow that is progressive and not fully reversible, and viral and bacterial infections including endemic influenza can easily evoke "exacerbation," in which lung function deteriorates and is life-threatening. Currently, through smoking cessation, the application of bronchodilators and steroids can, to some extent, make patients feel better and remain more active; there are no suitable drugs available that are capable of reducing the progression of the disease or the mortality associated with it or the incidence of exacerbations.

Many attempts are being made to explore the nature of COPD from different perspectives, such as early lung development, the balance between protease and antiprotease, and oxidative stress. In this review, we discuss the pathology associated with COPD from the viewpoint of glycan changes.

16.2 Core Fucose Structure

Core fucose is a glycan structure in which a fucose unit is bound to the innermost GlcNAc residue of *N*-linked oligosaccharides on a glycoprotein via an α1,6 linkage (Fig. 16.1). The biosynthesis of core fucose is catalyzed by GDP-L-Fuc:*N*-acetyl-β-d-glucosaminide α1,6 fucosyltransferase (FUT8), which is distinct from other members of the fucosyltransferase family of enzymes. The *Fut8* gene can be found in most mammalian tissues, and a relatively high expression was confirmed in the rat intestine, brain, and lung. Furthermore, the prevalence of core fucose structures can change, depending on the physiological and pathological conditions. Accumulating evidence indicates that the core fucose structure can affect protein function. For example, the deletion of this glycan structure has been reported to improve the binding of IgG1 to FcγRIIIA, resulting in an enhanced antibody-dependent cellular cytotoxicity activity (Shinkawa et al. 2003).

A study using alpha 1,6-fucosyltransferase knockout mice (*Fut8*$^{-/-}$) (Wang et al. 2005) has suggested that core fucose structures play important roles in maintaining physiological homeostasis. Up to 70 % of these mice died within the first 3 postnatal

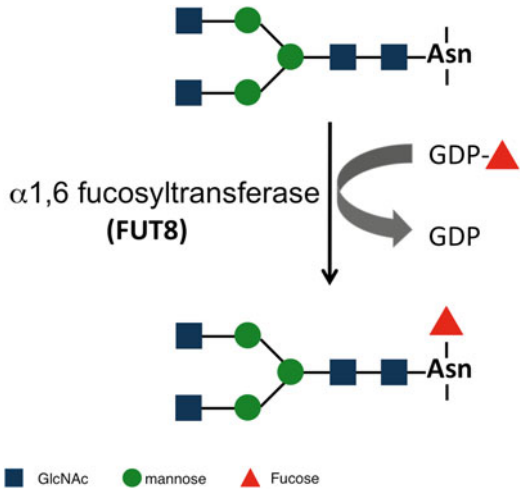

Fig. 16.1 The biosynthesis of core fucose is catalyzed by α1,6 fucosyltransferase (FUT8)

days, and the growth of the survivors was severely retarded. Moreover, core fucose deletion results in changes in both pulmonary structures and respiratory function. From postnatal day 7, the diameters of the pulmonary alveoli of *Fut8*$^{-/-}$ mice were increased significantly, compared with those in wild-type ones. Lung compliance, respiratory volume, and rate in the resting condition increased, while ventilator responses to systemic hypoxia (12 % O_2) or hypercapnia (5 % CO_2/21 % O_2) were significantly attenuated in *Fut8*$^{-/-}$ mice. These data suggest that the core fucose structure is one of the essential components for regulating lung proliferation and differentiation after birth. In addition, a real-time PCR analysis, focusing on genes related to the integrity of the lung extracellular matrix, showed that the expression levels of matrix metalloproteinase (MMP)-9, MMP-12, and MMP-13 and McolB (a mouse orthologue of human MMP-1) were increased. As MMPs are proteinases that have important role in the normal turnover of ECM components, their overexpression is consistent with the fragmentation and significantly reduced the number of elastic fibers observed from *Fut8*$^{-/-}$ mice. These changes, interestingly, can be partly attributed to impaired TGF-β1 signaling. When the TGF-β1 receptor lacks α1,6 fucosylation, its activation and signaling are perturbed. Along with the TGF-β1 receptor, several receptor-mediated signaling pathways, including epidermal growth factor (EGF) receptors (Wang et al. 2006) and integrins (Zhao et al. 2006), have been reported to be downregulated as a result of a loss of core fucosylation. On the other hand, although the precise mechanism for this is unclear, the expression level of the vascular endothelial cell growth factor receptor (VEGFR)-2, which is involved in the production of ceramide and alveolar cell apoptosis, was significantly suppressed. Thus the reduction of VEGFR-2 can be considered to be another cause of the alveolar destruction in *Fut8*$^{-/-}$ mice (Wang et al. 2009).

Although cigarette smoking (CS) is considered to be the primary risk factor for COPD, there is abundant evidence to suggest that the disease state results from

interactions between environment exposure and host factors, such as genetic predisposition, age, nutrients, lung growth, etc. A unique study using *Fut8* heterozygous knockout mice (*Fut8*$^{+/-}$) indicates that an abnormal level of core fucose is involved in the pathogenesis of CS-induced COPD (Gao et al. 2012). From the early days of CS exposure, a marked decrease in FUT8 activity was found in both *Fut8*$^{+/-}$ and wild-type mice, and the lowered enzyme activity level persisted throughout the whole CS exposure period. Consistent with the large number of inflammatory cells that accumulated in the lung, both the transcription level and activity level of MMP-9 were increased. As a result, rapid ECM destruction of the *Fut8*$^{+/-}$ mice lungs occurred and emphysematous lesions developed in the alveolar wall after only a 3-month exposure to CS. On the other hand, 6 months are generally required in the case of wild-type mice. Coincident with the *Fut8*$^{-/-}$ mice, the increase in proteinase levels can be partly attributed to a lack of control of the TGF-β1 receptor-Smad2-MMP signal pathway. In addition, the gene expression profile of the Smad family by RT-PCR analyses revealed that the expression of the Smad7 gene was increased to a greater extent in CS-exposed *Fut8*$^{+/-}$ mice. Smad7 is considered to be an inhibitor of the phosphorylation of Smad2 and therefore would have the ability to indirectly enhance the CS-evoked production of MMP. Meanwhile, core fucosylation has been reported to be decreased in smokers, as evidenced by an analysis of plasma *N*-glycans obtained from 1914 individuals (Knezevic et al. 2010), which is consistent with the results obtained from *Fut8*$^{+/-}$ mice.

It was found that the lung function designated as a faster annual decline of forced expiratory volume % in one second (FEV1) was significantly related to a lower Fut8 activity. Moreover, patients with a lower Fut8 activity experienced exacerbations more frequently (Kamio et al. 2012). A polymorphism within *Fut8* gene is recently reported to be associated with emphysema (Yamada et al. 2011). These data strongly indicate the existence of an association between reduced Fut8 activity and the progression of COPD.

16.3 Sialyl-Lewis^{X} Structure

In addition to the destruction of the lung parenchyma, the hypersecretion of mucus, which results in the obstruction of the small airways, is another typical pathological change associated with COPD. The amount and structure of oligosaccharides in secretory mucins appear to be different, depending on the type and stage of the lung disease, whereas such changes can lead to alterations in the viscosity of the mucus, resulting in mucociliary transport being perturbed. Previous studies have shown that glycan structures containing fucose and sialic acid, such as sialyl-Lewis^{X} [NeuAcα2,3Galβ1,4(Fucα2,3)GlcNAc] on a mucin, exhibited a characteristic rise in bronchoalveolar lavage in patients with bronchitis, and the viscosity of MUC5Ac, a major type of secretory mucin in the airway, was increased significantly. As most of these oligosaccharides are important components of receptors that recognize

viruses and bacteria, an increase in the levels of mucins that contain such glycans would result in the capture pathogens being facilitated. High levels of expressions of glycosyltransferases related to the synthesis of sialyl-Lewis^X are one of the potential mechanisms. To address this issue, an approach using NCI-H292 cells, a tracheal epithelial cell line, was undertaken. TNF-α stimulation appeared to upregulate the level of expression of fucosyltransferase 3 (FUT3), ST3 β-galactoside α-2,3-sialyltransferase 4 (hST3GALIX), and C2/4GnT(β1,6 GlcNAc-transferase), which partly contributes to the phosphatidylinositol-specific phospholipase C signaling pathways (Ishibashi et al. 2005). In addition, several elegant studies have demonstrated that mucins that contain a large number of O-glycans and in which the degree in sulfation, sialylation, and fucosylation changed were related to airway inflammation. Furthermore, it was also reported that a high density of the sialyl-Lewis^X epitope on the mucin was favorable for the pathogenesis of *Pseudomonas aeruginosa* (Xia et al. 2005). It is well known that the selectins are a family of cell-adhesion proteins that mediate the rolling of leukocytes on activated endothelial cells through the recognition of the sialyl-Lewis^x. A novel glycobiology-based therapy designated as GMI-1070 is E-selectin antagonist and targets vaso-occlusive crisis of the patients (Chang et al. 2010). This type of drug is a very promising one as a glycan-based therapeutics in the future.

16.4 Glycosaminoglycans

Glycosaminoglycans (GAG) are linear unbranched polysaccharides, consisting of repeating disaccharide units in which the sugar composition, linkage, and modification are varied. GAG can be classified into four groups: hyaluronan (HA), chondroitin sulfate (CS)/dermatan sulfate, keratan sulfate (KS), and heparan sulfate/heparin. In the lung, GAGs are important components of the extracellular matrix, distributed in the interstice, subepithelial tissues, bronchial walls, and airway secretions. They are considered to be essential for normal lung function and in responding to an injury by regulating signal transduction events. KS is reported to be the most abundant GAG in bronchoalveolar lavage and is produced by bronchial epithelial cells. It is composed of *N*-acetylglucosamine and galactose units, and the sulfation of KS may affect its function. The KS polymer has been suggested to inhibit the expression and activation of MMP-2 in corneal and skin explant cultures, and its disaccharide repeating unit [SO_3^--6]Galβ1–4[SO_3^--6]GlcNAc suppresses IL-12 production in macrophages stimulated with LPS. On the other hand, bacterial/virus infections in the lung evoke inflammatory responses, which can be actively promoted by various inflammatory mediators that are released by immune cells and activated epithelial cells. While, in some cases, the reason is not clear, exacerbations are often the result of bacterial/virus infections in the lung. COPD exacerbation refers to a worsening or a “flare-up” of COPD. People with advanced COPD seem to be prone to such life-threatening disorders. Highly significant levels of bacteria

are observed in the airways of subjects with exacerbated COPD as compared to those in healthy adults. The most commonly isolated bacteria are *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis*, while there is growing evidence to show that *Pseudomonas aeruginosa* is particularly important and is involved in advanced COPD. Flagellin from *P. aeruginosa* is a potent activator of the Toll-like receptor (TLR)-5 for producing interleukin 8 (IL-8), and this interaction can be attenuated by the presence of KS disaccharides. Interestingly, the mechanism by which this disaccharide unit blocks the interaction of flagellin with the TLR-5, in part, involves the downregulation of EGFR phosphorylation (Shirato et al. 2013). Since such suppression is observed in the case of other analogues, for example, *N*-acetyllactosamine or disaccharides derived from chondroitin-6-sulfate, this suggests that oligosaccharide structure information including sugar composition and the extent of modification by sulfate will likely be important in designing chemical inhibitors.

In addition to KS, HA, the most abundant non-sulfated GAG in the lung, has its own way to protect the respiratory system, such as regulating water balance and osmotic pressure and enhancing the cellular host defense mechanism by stimulating the activity of blood neutrophils. Furthermore, the intratracheal administration of HA has been reported to prevent experimental emphysema induced by porcine pancreatic elastase. Several studies have demonstrated that the biological effects of HA appear to vary depending on its average molecular mass. Very recently a synthetic monosaccharide, 2,4-*O*-di-sulfated iduronic acid (Di-S-IdoA), was found to attenuate leukocyte recruitment into inflammatory sites and BALF in the model mice and would be a potential drug for asthma treatment (Nonaka et al. 2014).

With their diverse oligosaccharide structures, GAGs play important and multiple roles in the lung, functioning not only to maintain structure homeostasis but also to modulate inflammatory responses. Elucidation of the composition and substitution pattern of GAGs in the airway may help to understand the pathology and could contribute to the development of a therapeutic strategy for the treatment of COPD.

16.5 Perspectives

The most important posttranslational modification in mammals is glycosylation. A large body of evidence exists to show that glycan structures can be changed drastically, as a function of the status of a disease. The molecular nature of glycosylation, such as the glycan structures introduced above, may provide further clues as to how glycans may contribute to the recognition of pathogens and disease pathogenesis (Fig. 16.2). Further studies designed to create a history of changes in oligosaccharide structures, glycoprotein-binding properties, and the concentrations of serum glycoproteins in different stages of COPD might address the issue of which specific agents interfere with the glycosylation and can be useful in providing effective glycobiology-based therapy and possibly altering the status of COPD.

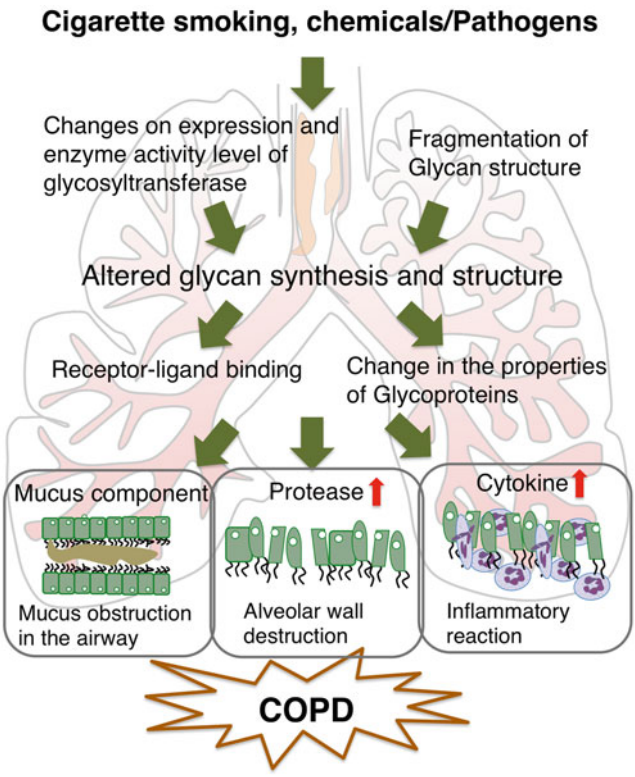

Fig. 16.2 Changes in the contents of sugar chains are now considered to be fundamental features of various diseases, including COPD. Environmental toxins or pathogens can alter the properties of glycosyltransferase and, as a result, alter the synthesis of oligosaccharides. Theses glycan changes have been shown to lead to the dysfunction of signal pathways that control the production of proteases or result in alterations in the viscosity of mucus molecules, thus perturbing mucociliary transport

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Chapter 17
α1,6-Fucosyltransferase Knockout Mice and Schizophrenia-Like Phenotype

Wei Gu, Tomohiko Fukuda, and Jianguo Gu

Abstract The glycan core fucose is catalyzed by α1,6-fucosyltransferase (Fut8), which transfers a fucose residue to the innermost GlcNAc residue via α1,6-linkage on N-linked glycans (N-glycans) in mammals. N-glycan is always attached to the nitrogen atom of an asparagine (Asn) side chain that is present in the Asn-X-Ser/Thr motif on a protein, where X is any amino acid except proline. The α1,6-fucosylated (core-fucosylated) N-glycan is ubiquitously distributed in all tissues. Interestingly, the unique structure of the core-fucosylated hybrid, one of three major types of N-glycans, is highly expressed in brain tissues, and the expression pattern of N-glycans is altered during brain development. The Fut8-deficient ($Fut8^{-/-}$) mice exhibit emphysema-like changes in the lungs and severe growth retardation due to dysregulation of the TGF-β1 receptor and the EGF receptor, respectively. To understand the role of core fucosylation in brain tissue, a combination of neurological and behavioral tests for $Fut8^{-/-}$ mice was examined. $Fut8^{-/-}$ mice displayed multiple behavioral abnormalities, such as increased locomotion, decrease in working memory, strenuous hopping behavior, and prepulse inhibition deficiency, which were consistent with a schizophrenia-like phenotype. Here, we summarized the knowledge of the biological functions of core fucosylation, especially its role in brain and neural cells, and discussed possible underlying molecular mechanisms.

Keywords α1,6-Fucosyltransferase • Fut8 • Core fucose • Core fucosylation • Schizophrenia • Cellular signaling • Knockout mice

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17.1 Introduction

Schizophrenia is a common, chronic, and severe brain disorder that is characterized by episodic positive symptoms such as delusions, hallucinations, and thought disorders and/or persistent negative symptoms such as flattened affect, impaired attention, social withdrawal, and selective cognitive deficits in attention, learning, and memory (Ross et al. 2006). It ranks as one of the leading causes of disability worldwide, as it affects 1 % of the world's population (Freedman 2003; Stanta et al. 2010). Schizophrenia seems to have strong genetic component. Indeed, more than 130 genes reportedly predispose to schizophrenia, but few of these results have been replicated, and fewer still have biological support (Ross et al. 2006). Although evidence has accumulated for plausible candidate genes for schizophrenia, e.g., neuregulin1, disrupted in schizophrenia 1, and dystrobrevin binding protein 1, substantial controversy remains regarding both the meaning of the positive genetic findings and the implications for the therapeutic strategies (Lewis and Sweet 2009). Some studies suggest that interactions between genetics and environmental factors increase the risk that an individual will develop schizophrenia. For example, a functional polymorphism in the gene encoding catechol-O-methyltransferase (COMT), which is thought to affect the availability of dopamine (DA) in the cortex, increases the risk for the development of psychosis associated with cannabis use during adolescence (Caspi et al. 2005). Similarly, serious obstetric complications might interact with variants of genes that are regulated by hypoxia and/or genes that are involved in vascular function to influence the risk of developing schizophrenia (Nicodemus et al. 2008).

Until now, research has long focused on identifying gene or protein expression-level changes in schizophrenia and ascertaining the functional effects of those alterations. Recently, the posttranslational modification, N-glycosylation, becomes a new target of investigation for schizophrenia (Khoury et al. 2011). For example, abnormal N-glycans on AMPA, NMDA, and $GABA_A$ receptors were found in patients with schizophrenia (Tucholski et al. 2013a, b; Mueller et al. 2014).

Complex N-glycans are required for the development of the embryo, and the complete lack of complex N-glycans in N-acetylglucosaminyltransferase I (GnT-I)-deficient mice is lethal with defects in neural tube formation (Ioffe and Stanley 1994). These observations suggest that high-mannose N-glycans are insufficient for normal ontogeny. On the other hand, restriction of N-glycan branching to the formation of only hybrid structures by N-acetylglucosaminyltransferase II (GnT-II) inactivation in mice results in a very low rate of survival to adulthood and a postnatal phenotype that is similar to human "congenital disorder of glycosylation IIa" (CDG-IIa), which manifests as severe multisystem defects, psychomotor abnormalities, and mental retardation (Jaeken et al. 1994; Wang et al. 2001). Furthermore, the specific elimination of GnT-I and GnT-II genes in neuronal cell types reveals that hybrid N-glycans are essential for neuronal and postnatal viability in mice, whereas complex N-glycans appear dispensable in this cell lineage (Ye and Marth 2004). On the other hand, the importance of fucosylation has recently

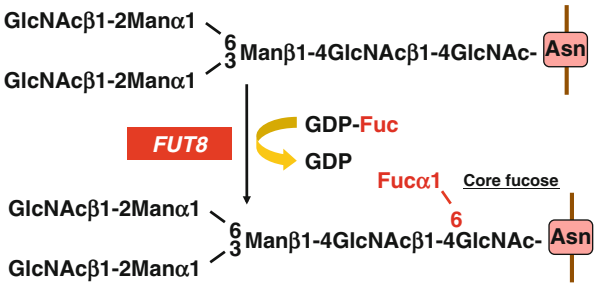

Fig. 17.1 Reaction pathway for the synthesis of core fucose (*GlcNAc* N-acetylglucosamine, *Man* mannose, *Fuc* fucose, *GDP-Fuc* guanosinediphospho-fucopyranoside, *Asn* asparagine)

been underlined by identification of the monogenetic inherited human disease CDG-llc, also termed "leukocyte adhesion deficiency II," which is caused by defective Golgi GDP-fucose transporter (SLC35C1) activity (Lubke et al. 1999, 2001). CDG-llc patients show hypofucosylation of glycoproteins and present clinically with mental and growth retardation, persistent leukocytosis, and severe infections (Marquardt et al. 1999a). These symptoms can be partially corrected by oral L-fucose (Marquardt et al. 1999b).

Core α1,6-fucosylation of N-glycans is ubiquitously observed in eukaryotes except plant and fungi. This type of fucosylation is catalyzed by eukaryotic α1,6-fucosyltransferase (Fut8). Fut8 transfers fucose moiety from GDP-β-L-fucose to the innermost GlcNAc residue to form α1,6-fucose, the so-called core fucose, in hybrid and complex N-linked oligosaccharides of glycoproteins as shown in Fig. 17.1. Many glycoproteins were regulated by core fucosylation. For example, α-fetoprotein (AFP) is well known as a core-fucosylated glycoprotein. This glycoprotein is core fucosylated specifically in hepatocellular carcinoma present as *Lens culinaris* agglutinin-A-reactive AFP (AFP-L3), but in chronic liver disease (Sato et al. 1993). In addition, it is known that the depletion of the core fucose in human IgG1 enhances the antibody-dependent cellular cytotoxicity (ADCC) activity by ~100-fold (Shields et al. 2002; Shinkawa et al. 2003), which is an approach that has been applied in clinical trials (Suzuki et al. 2007; Niwa et al. 2004). Recently, two research groups unraveled the molecular mechanism for the enhanced interaction between sFcγRIIIa and a fucosylated Fc, since fucosylation of the Fc N-glycans inhibited this interaction, because of steric hindrance (Ferrara et al. 2011; Mizushima et al. 2011), which is directly demonstrating that core fucosylation affects the conformation of a target glycoprotein.

The core-fucosylated glycoproteins are widely distributed in mammalian tissues, especially in the brain (Uozumi et al. 1996). In fact, the majority of N-glycans present in mouse brain tissue are core fucosylated (Shimizu et al. 1993); the expression pattern of N-glycans is altered during brain development (Nakakita et al. 2005). In this review, we described some biological functions of Fut8, especially in the central nervous system, and discussed possible underlying molecular mechanisms.

17.2 Fut8-Deficient (Fut8$^{-/-}$) Mice and Phenotypes

To understand the physiological role of core fucosylation, the Fut8 gene was deleted by knockout. It has been reported that disruption of the Fut8 gene in mice results in phenotypes of growth retardation, death during postnatal development, and lung emphysema. The molecular mechanisms for the Fut8$^{-/-}$ mice suffering from emphysema-like changes in their lungs could be ascribed to a lack of core fucosylation of the transforming growth factor-β1 (TGF-β1) receptor, which consequently resulted in dysregulation of TGF-β1 receptor activation and signaling (Wang et al. 2005) and downregulation of the expression of the vascular endothelial cell growth factor receptor-2 (VEGFR-2) (Wang et al. 2009). Moreover, the loss of core fucosylation downregulates both EGF receptor-mediated cell-signaling pathways (Wang et al. 2006) and integrin α3β1-mediated cell adhesion (Zhao et al. 2006). The loss of core fucose in both α4β1 integrin and vascular cell adhesion molecule 1 (VCAM-1) (Li et al. 2008), or mu heavy chains (Li et al. 2012), led to a decreased binding between pre-B cells and stromal cells, which impaired early B cell development and functions and intracellular signaling of precursor B cell receptors, respectively. Taken together, these results suggest that core fucose plays a key role in regulating important physiological functions via modification of functional proteins (Fig. 17.2).

17.3 The Fut8$^{-/-}$ Mice Exhibited Multiple Behavioral Abnormalities with a Schizophrenia-Like Phenotype

To examine the effects of core fucosylation on the central nervous system, we characterized Fut8$^{-/-}$ mice using neurological and behavioral assays that included open-field and hopping tests, a social interaction assessment, the Y-maze test, and the

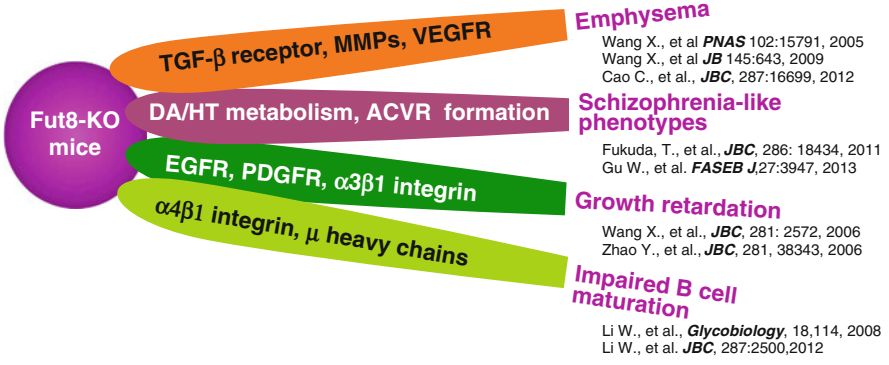

Fig. 17.2 Importance of Fut8 expression for physiological functions. The disruption of the Fut8 gene in mice results in phenotypes of growth retardation, lung emphysema, schizophrenia-like changes, and delayed B cell maturation

prepulse inhibition (PPI) of startle test. The Fut8$^{-/-}$ mice exhibited multiple behavioral abnormalities with a schizophrenia-like phenotype (Fukuda et al. 2011).

17.3.1 Impaired Prepulse Inhibition (PPI) in the Fut8$^{-/-}$ Mice

The PPI of startle is a cross-species measure that measures the ability of a non-startling "pre-stimulus" to inhibit the response to a startling stimulus. There have been numerous reports of PPI deficits in schizophrenia patients (Braff et al. 2001; Kumari et al. 2008; Ludewig et al. 2003). Thus, the PPI deficits associated with schizophrenia are the most thoroughly characterized and the most widely replicated. Although startle amplitudes were not significantly different, between Fut8$^{-/-}$ mice and wild-type (Fut8$^{+/+}$) littermates, Fut8$^{-/-}$ mice showed complete deficits in PPI compared with the Fut8$^{+/+}$ and the hetero (Fut8$^{+/-}$) mice. Of particular interest, Fut8$^{+/-}$ mice showed a significant PPI deficiency compared with Fut8$^{+/+}$ littermates tested under the restraint stress in a cylindrical mouse restrainer for 3 h (Fig. 17.3). These results strongly suggest that Fut8 might play a causal role in the disorder, and the environment is also one of the very important factors for such disorders. In fact, exposure to various mild stressors has been shown to activate dopamine-containing neuronal systems (Carlson et al. 1991).

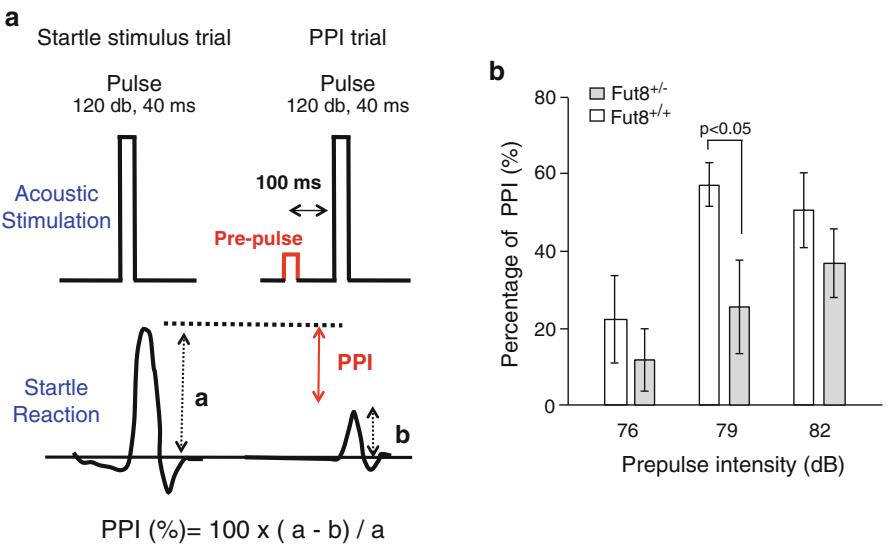

Fig. 17.3 Restraint stress-induced PPI deficiency in Fut8$^{+/-}$ mice. (**a**) Illustration of how PPI is tested. (**b**) The effects of restraint stress (3 h) on PPI of the startle reflex were assayed under different prepulse intensities. Of particular interest, Fut8$^{+/-}$ mice showed a significant PPI deficiency when presented with a 79-db prepulse and a decrease in PPI following prepulses at all of other intensities compared with Fut8$^{+/+}$ littermates tested under the same conditions

17.3.2 Alterations in Social Interaction and Short-Term Memory in the Fut8$^{-/-}$ Mice

Deficits in social interaction are hallmarks of schizophrenia (Kopelowicz et al. 2006; Piskulic et al. 2007). As compared with Fut8$^{+/+}$, Fut8$^{-/-}$ mice spent significantly less time engaging in active contact, such as sniffing or following each other, when the pairs of mice with the same genotype and same sex were allowed to explore a novel environment for 10 min (Fig. 17.4a). The Y-maze task experiment showed that spontaneous alterations were reduced in Fut8$^{-/-}$ mice compared with Fut8$^{+/+}$ mice (Fig. 17.4b), suggesting that short-term memory was decreased in Fut8$^{-/-}$ mice.

17.3.3 Novelty-Induced Hyperactivity in the Fut8$^{-/-}$ Mice

Novelty-induced hyperactivity has been viewed as a preclinical model of the positive symptoms of schizophrenia and of psychomotor agitation in particular (Hashimoto et al. 2001; Pletnikov et al. 2008; van den Buuse et al. 2009). When Fut8$^{-/-}$ mice were placed into a novel environment, the locomotor activities and hopping movements were greatly increased, as compared with those in Fut8$^{+/+}$ mice (Fig. 17.5).

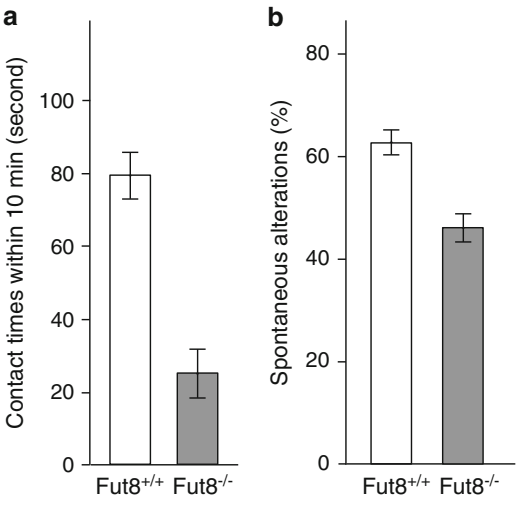

Fig. 17.4 Alterations in social interaction (**a**) and short-term memory (**b**) in the Fut8$^{-/-}$ mice, compared with those in Fut8$^{+/+}$ mice

17.4 Unbalance Between the Dopamine and Serotonin Systems in the Fut8$^{-/-}$ Mice

Because locomotor hyperactivity is commonly associated with increased dopaminergic tone (Gainetdinov et al. 1999), the effect of Fut8 deficiency on monoamine turnover was determined. In Fut8$^{-/-}$ mice, DA turnover was unchanged, while serotonin (5-HT) turnover was decreased. In fact, the importance of 5-HT in controlling locomotor activity has been demonstrated in a study with 5-HT$_{1B}$-receptor knockout mice, which show hyperlocomotor activity and aggressive behavior (Saudou et al. 1994). A similar phenomenon has also been observed in PACAP-knockout mice, which show slightly decreased 5-HIAA levels in the cortex and striatum with abnormal jumping behavior and other psychomotor behavioral abnormalities (Hashimoto et al. 2001). Furthermore, the enhanced locomotor activity of Fut8$^{-/-}$ mice was significantly reduced to the normal levels of Fut8$^{+/+}$ mice by treatment with the typical antipsychotic drug haloperidol (Fig. 17.5), which is a dopamine D2 receptor antagonist (Kapur et al. 2000). The treatment with haloperidol might normalize the balance between the dopamine and serotonin systems in the Fut8$^{-/-}$ mice. On the other hand, treatment with atomoxetine, which is a non-stimulant approved for the treatment of attention-deficit hyperactivity disorder (Garnock-Jones and Keating 2009), did not significantly inhibit the hyperactivity of Fut8$^{-/-}$ mice, also suggesting that Fut8 might play a role in schizophrenia-like disorders.

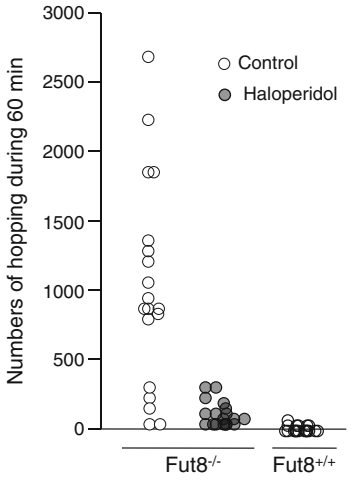

Fig. 17.5 Novelty-induced hyperactivity in the Fut8$^{-/-}$ mice. The novelty-induced hopping of Fut8$^{-/-}$ mice was significantly reduced to the normal levels of Fut8$^{+/+}$ mice by treatment with the typical antipsychotic drug haloperidol, which is a dopamine D2 receptor antagonist

17.5 The Core Fucosylation Is Important for Early-Form Long-Term Potentiation (E-LTP)

The etiology of schizophrenia is only partially understood, but multi-episode patients show significantly disturbed neuronal plasticity, suggesting that synaptic activity and connectivity are altered during the progression of the disease. Recently, cognitive impairments such as deficits in learning and memory have also been shown to be a fundamental feature of the disorder (Savanthrapadian et al. 2013). Long-term potentiation (LTP), a well-established model based on the neurophysiological study of learning and memory, was found to be an important mechanism that underlies synaptic changes and plasticity in schizophrenia (Frantseva et al. 2008; Sanderson et al. 2012). Preliminary data showed that the HFS-induced LTP was dramatically decreased in Fut8$^{-/-}$ mice (unpublished data). It is well known that the induction of LTP began with the activation of AMPA receptors, which induced the opening of NMDA receptor and led to calcium entry that initiated a biochemical cascade through the activation of CaMKII, and the end product of this biological reaction was the long-lasting potentiation of AMPA receptor-mediated excitatory postsynaptic current (Lisman et al. 2012; Kullmann and Lamsa 2007). Thus, it is important to analyze the core fucosylation on these receptors for their biological functions.

17.6 The Core Fucosylation-Regulated TGF-β/Activin-Mediated Signaling

To explore the underlying mechanisms in the nervous system, PC12 cell that is often used for cell differentiation study was used since it highly expresses core-fucosylated N-glycans. Knockdown of the Fut8 gene resulted in an upregulation of neurite formation and phospho-Smad2 levels. In agreement with these results, the restoration of the Fut8 gene in the knockdown cells downregulated neurite formation and phospho-Smad2 levels (Gu et al. 2013). Surprisingly, core fucosylation specifically downregulated the complex formation of activin receptors and their downstream, which contradicted previous observation, in which core fucosylation is required for TGF-β-mediated signaling in lung tissues and fibroblast cells (Wang et al. 2005). Taken together, core fucosylation may play reciprocal double effects in the TGF-β/activin-mediated signaling, i.e., a positive effect for TGF-β and a negative effect for activin (Fig. 17.6).

It is worth noting that the relationship between TGF-β signaling and homeostatic regulation of excitatory and inhibitory synapses in the central nervous system has also been studied in animal models or cell cultures (Krieglstein et al. 2011). As described above, Fut8$^{-/-}$ mice showed strenuous hopping behavior and increased locomotor activity in a novel environment, decreased social interaction,

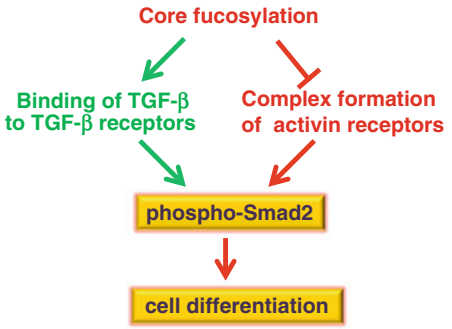

Fig. 17.6 A working model for the effects of core fucosylation on TGF-β/activin-mediated cellular signaling and cell differentiation

and impaired alertness, which might be related to schizophrenia-like behaviors. It is reasonable to speculate that downregulation of TGF-β signaling in the Fut8$^{-/-}$ mice may also have contributed to these phenomena. However, this is not a simple case, since core fucosylation negatively regulates activin signaling as described above. The TGF-β and activin isoforms commonly recruit adaptors Smad2 and Smad3. The phosphorylated Smads interact with a "common Smad," Smad4, which together translocates as a heteromeric complex into the nucleus (ten Dijke and Hill 2004; Shi and Massague 2003). Therefore, this raises the question of why core fucosylation differentially regulates similar signaling pathways via two different receptors. At the present time, this remains unclear, but we could speculate as to the following: (1) The expression patterns of TGF-βs and activins or their receptors are different. TGF-β2 and TGF-β3 are reportedly found in neural progenitor cells, differentiating neurons and radial glial cells as well as astrocytes and numerous neuron populations (Unsicker et al. 1991; Flanders et al. 1991), whereas activin βA is expressed in neocortical and neostriatal regions of rat embryos (Andreasson and Worley 1995). (2) The roles of TGF-β and activin signaling are complicated. Activin is known to have functional roles at excitatory and inhibitory synapses (Dow et al. 2005). However, blocking activin signaling enhanced GABA release and strengthened functions of GABA receptors (Zheng et al. 2009), which caused the low-anxiety phenotype or depression-like behavior of the mice. The functional roles of TGF-β members are also known to modulate synaptic transmission. The loss of TGF-β2 impaired the presynaptic GABAergic inhibitory and excitatory synaptic transmission (Heupel et al. 2008). In addition, activin-A has reportedly induced tyrosine hydroxylase expression, which is required in the tyrosine metabolic pathway for the synthesis of dopamine (Bao et al. 2005). Coincidentally, the enhanced locomotor activity and strenuous hopping behavior of Fut8$^{-/-}$ mice were restored by treatment with a dopamine D2 receptor antagonist as shown in Fig. 17.5. (3) TGF-β and activin have overlapping intracellular signaling cascades. Therefore, the dual effects of core fucosylation might be important for spatiotemporal regulation in the central nervous system.

17.7 Perspective

Core fucose affects biological functions via modification of functional proteins, such as α3β1, α4β1 integrin, EGF receptor, activin, and TGF-β receptors. For example, the core fucosylation is required for α3β1 integrin-mediated cell adhesion and cell signaling. In brain tissue, reelin may arrest neuronal migration and promote normal cortical lamination by binding α3β1 integrin and modulating integrin-mediated cellular adhesion (Dulabon et al. 2000). As described above, most complex-type N-glycans contain core fucose in mouse brain tissues. It is well accepted that multiple genes of each with a small effect, rather than a single causative gene, act in concert with nongenetic factors to increase the risk of mental disorders (Hyman 2000). Therefore, we believe that there may be many functional molecules, such as dopamine and serotonin transporters or receptors, AMPA and NMDA receptors, activin and TGF-β receptors, etc., which depend on the core fucosylation for their biological functions. Thus, the lack of core fucosylation of each target molecule has a small effect, but collectively, the absence of core fucosylation has a significant effect on behavior. A detailed characterization of the functions of core fucosylation on those receptors is required in further studies, which may provide clues for the discovery of novel therapies for schizophrenia and related disorders.

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Chapter 5
Role of Glycans in Viral Infection

Tadanobu Takahashi and Takashi Suzuki

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In chapter titled "Role of Glycans in Viral Infection", the affiliation of the authors T. Suzuki and T. Takahashi was wrong. The correct affiliation has been updated inside the chapter.

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