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Review
ANDOH-TAJIMA Award

Various biological functions of carbohydrate chains learned from glycosyltransferase-deficient mice

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Abstract: Carbohydrate chains are attached to various proteins and lipids and modify their functions. The complex structures of carbohydrate chains, which have various biological functions, are involved not only in regulating protein conformation, transport, and stability but also in cell–cell and cell–matrix interactions. These functional carbohydrate structures are designated as “glyco-codes.” Carbohydrate chains are constructed through complex reactions of glycosyltransferases, glycosidases, nucleotide sugars, and protein and lipid substrates in a cell. To elucidate the functions of carbohydrate chains, I and my colleagues generated and characterized knockout (KO) mice of galactosyltransferase family genes. In this review, I introduce our studies about galactosyltransferase family genes together with related studies performed by other researchers, which I presented in my award lecture for the Ando-Tajima Prize of the Japanese Association for Laboratory Animal Science (JALAS) in 2019.
Key words: carbohydrate chains, galactosyltransferase, glycobiology, glyco-code, knockout mice

Introduction of Myself and My Research

In 1982, when I was a fourth-year student in the Faculty of Science, Kyoto University, I was very impressed with the giant mouse reported in the top pages of newspapers. Mice that were twice their regular size were generated by microinjecting the gene for rat growth hormone into fertilized mouse eggs. These findings were reported by Drs. Palmitter and Brinster in *Nature* [28]. At that moment I ambiguously thought that I would like to undertake that kind of research in future.

I went on to the Graduate School of Science, Kyoto University, and fortunately had a chance to learn about transgenic technology under the supervision of Dr. Iwakura and Prof. Kawade at the Virus Research institute Kyoto University. We started to develop transgenic technology through trial and error and could finally generate transgenic (Tg) mice carrying the interferon-β (IFN-β) gene under the control of the metallothionein promoter and enhancer. The male IFN-β Tg mice were sterile, without germ cells after the pachytene stage, probably because the metallothionein promoter and enhancer is constitutively active in the testis and IFN-β has a harmful effect on cell growth, especially on germ cells [17]. Furthermore, I unexpectedly found that IFN-α as well as IFN-β were expressed in the IFN-β Tg mice. Several experiments revealed that IFN-α was induced by IFN-β in the mouse body [5]. I obtained my Ph.D. from Kyoto University in 1989.

After obtaining my Ph.D., I moved to Osaka Bioscience Institute and joined Dr. Nagata’s laboratory as a postdoctoral fellow. Here, I was engaged in working on G-CSF projects [6, 7] and learned a lot about molecular biology. Then, I relocated abroad to Prof. Gruss’s laboratory at the Max Planck Institute for Biophysical Chemistry in Goettingen, Germany. Here, I studied *Pax* genes during mouse development [4] and tried to make gene

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knockout (KO) mice using ES cells. In 1993, I returned to Japan and began work in Prof. Iwakura's laboratory at the Institute of Medical Science, the University of Tokyo, as a research associate. Here, I studied interleukin (IL)-1 function by generating IL-1α/β double KO mice [13] and IL-1 receptor antagonist KO mice [14]. So far, my research remained focused on studying the biology of cytokines such as IFN, G-CSF, and IL-1. Before I turned 40, I decided to change my research interests and started novel research in the field of glycobiology.

Glycobiology Research by Generating KO Mice of Various Glycosyltransferases

Carbohydrate chains are attached to various proteins and lipids and modify their functions. Although amino acid sequences of proteins are strictly encoded by DNA sequences, carbohydrate chain sequences are not encoded by the genetic code. The only attachment rule is that Asn-linked *N*-glycans and Ser/Thr-linked *O*-glycans are attached to Asn in the Asn-X-Ser/Thr motif and Ser/Thr without any motif, respectively. Three dimensional complex structures of carbohydrate chains are constructed by complex reactions of glycosyltransferases, glycosidases, nucleotide sugars, and protein and lipid substrates in a cell. Complex structures of carbohydrate chains have various biological functions, not only in regulating protein conformation, transport, and stability but also for cell–cell and cell–matrix interactions. The functions of many proteins are modified by these glycosylation patterns. Therefore, functional carbohydrate structures are designated as glyco-codes. My ultimate goal is to elucidate the glyco-code. The importance of carbohydrate chains in the body has been revealed by generating KO mice of glycosyltransferases. Notably, KO mice of GlcNAcT-I, which transfers *N*-acetylglucosamine (GlcNAc) to high mannose-type *N*-glycans to synthesize complex- and hybrid-type *N*-glycans, showed embryonic lethality [15, 23]. This indicated that complex- and hybrid-type *N*-glycans are essential for mouse development.

My colleagues and I are interested in galactose residues that are suggested to have various biological functions. There are four kinds of galactosyltransferase families: α-1,3, α-1,4, β-1,3, and β-1,4-galactosyltransferases, containing 3, 1, 7, and 7 genes, respectively, according to the linkage of sugar chains [12]. Among them we focused on β-1,4-galactosyltransferases (β4GalTs; gene name, *B4galts*) containing *B4galt-1* to *B4galt-7* genes (Fig. 1). Using

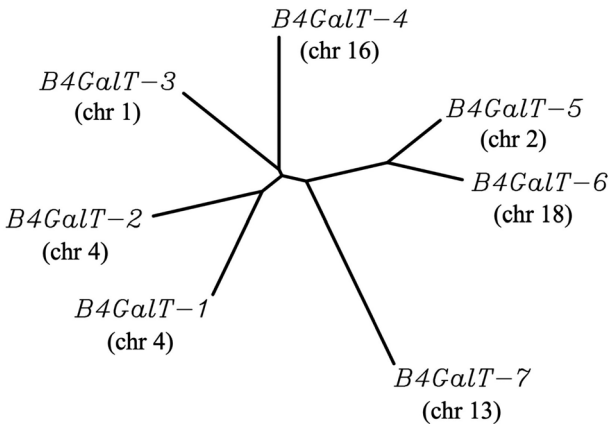

Fig. 1. Phylogenetic tree of the β-1,4-galactosyltransferase gene family. Phylogenetic tree of *B4galt-1* to *-7* genes. The mouse chromosome number in which each *β4GalT* gene is located is indicated in parentheses.

gene targeting methods in ES cells, we first generated KO mice of β4GalT-1, which transfers galactose to the terminal GlcNAc of complex-type *N*-glycans in the Golgi apparatus. While *B4galt-1* KO mice were fertile and born normally, they showed growth retardation after birth and had short life spans. Epithelial cell proliferation in the skin and small intestine was enhanced, and cell differentiation in intestinal villi was abnormal. These observations suggest that β4GalT-1 plays critical roles in the regulation of proliferation and differentiation of epithelial cells [3]. At around the same time, Dr. Shur reported *B4galt-1* KO mice which showed similar growth retardation with hypoplasia of the posterior pituitary [21].

Carbohydrate Chains in Inflammation and Hematopoietic Stem Cells

One of the well-known functions of carbohydrate chains is the interaction of selectins and their carbohydrate ligands, which are involved in adhesion of leukocytes to endothelial cells during inflammation and lymphocyte homing to peripheral lymph nodes (PLNs) under physiological conditions (Fig. 2) [18, 20]. One of these selectin ligands is sialyl Lewis x (sLe^x), which is mainly presented at the terminus of *N*-acetyl lactosamine repeats on core 2 *O*-glycans (Fig. 3). Another selectin ligand is sialyl 6-sulfo Lewis x (Le^x), which is mainly presented at the terminus on core 1 *O*-glycans (Fig. 3). The former is involved in leukocyte adhesion to endothelial cells during inflammation, while the latter is involved in lymphocyte homing to PLNs. Most of the core 2 *O*-glycans on the leukocyte membrane glycoproteins of *B4galt-1* KO mice lacked galactose residues, and soluble P-selectin binding to their neutrophils and mono-

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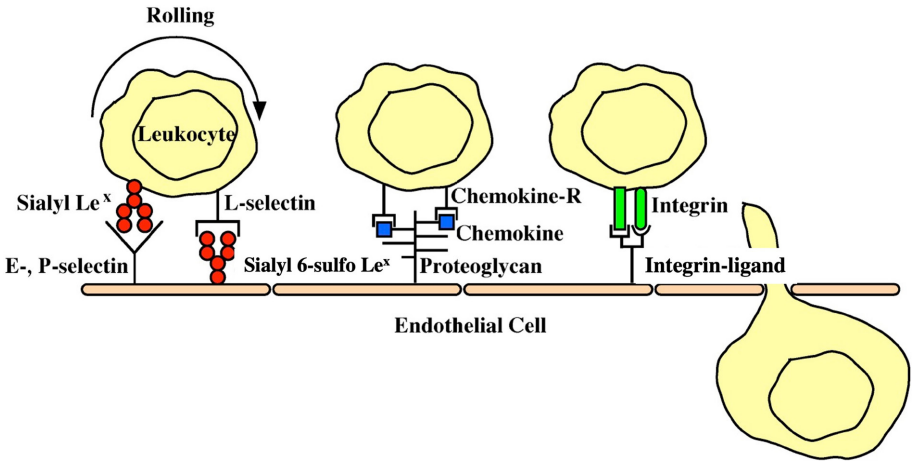

Fig. 2. A model of stepwise adhesion of leukocytes and endothelial cells during inflammation and lymphocyte homing to peripheral lymph nodes (PLNs). In the first step, leukocytes in the bloodstream start to roll by the interaction of sialyl Lewis x (sLe^x) on leukocytes and E/P-selectins on endothelial cells near inflammatory sites. During lymphocyte homing to PLNs, the interaction of L-selectin on lymphocytes and sialyl 6-sulfo Le^x on the high endothelial venule (HEV) in PLNs is important. Then, chemokines expressed on proteoglycans of endothelial cells bind to chemokine receptors induced on leukocytes. The chemokine signals induce integrins and integrin ligands on leukocytes and endothelial cells, respectively. Finally, activated leukocytes infiltrate into inflammatory sites.

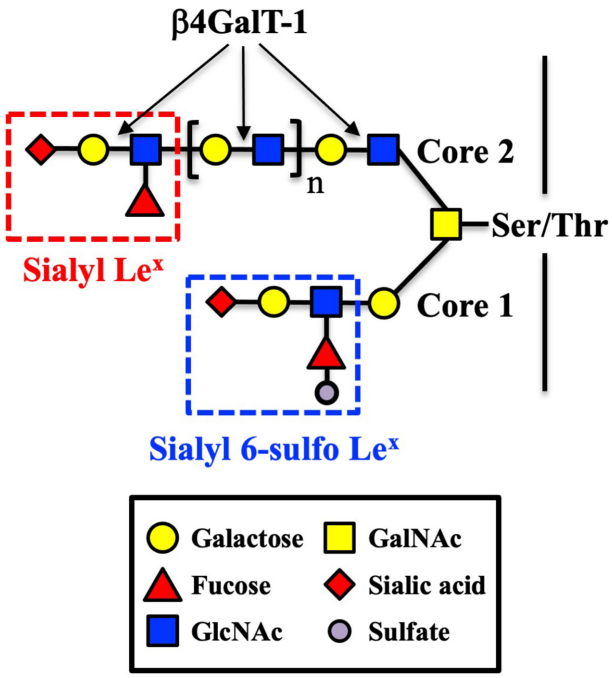

Fig. 3. Sialyl Lewis x (sLe^x) on core 2 *O*-glycans and sialyl 6-sulfo Lewis x (Le^x) on core 1 *O*-glycans. Composed of sialic acid, galactose, GlcNAc, and fucose, sLe^x is mostly expressed at the terminus of *N*-acetyl lactosamine repeats on core 2 *O*-glycans. Sialyl 6-sulfo Le^x, in which fucose of sLe^x is sulfated, is on core 1 *O*-glycans.

cytes was significantly reduced, indicating an impairment of selectin-ligand biosynthesis. *B4galt-1* KO mice exhibited blood leukocytosis but normal lymphocyte homing to PLNs because core 1 *O*-glycans were normally expressed. Acute and chronic inflammatory responses, including the contact hypersensitivity (CHS) and delayed-type hypersensitivity (DTH) responses, were suppressed in these mice. Our results demonstrate that β4GalT-1 is a major galactosyltransferase responsible for the selectin-ligand biosynthesis and that inflammatory responses of *B4galt-1* KO mice are impaired because of defects in selectin-ligand biosynthesis [3]. We also examined the effect of β4GalT-1-deficiency in skin wound healing. *B4galt-1* KO mice showed significantly delayed wound healing with reduced re-epithelialization, collagen synthesis, and angiogenesis compared with control mice. Neutrophil and macrophage recruitment at wound sites was also impaired because of selectin-ligand deficiency in *B4galt-1* KO mice [24].

Very recently, we reported about the role of carbohydrate chains in the homing and engraftment of hematopoietic stem/progenitor cells (HSPCs) to the bone marrow (BM). We found that transplanted BM cells deficient in β4GalT-1 could not support survival in mice exposed to a lethal dose of irradiation. BM cells obtained from *B4galt-1* KO mice showed normal colony-forming activity and hematopoietic stem cell (HSC) numbers. However, colony-forming cells were markedly reduced in the BM of recipient mice 24 h after transplantation of β4GalT-1-deficient BM cells, suggesting that β4GalT-1-deficiency severely impairs HSPC homing [29]. Although E/P-selectin double KO mice also show partially impaired HSPC homing as recipient mice [10], the phenotype of *B4galt-1* KO mice is much more severe

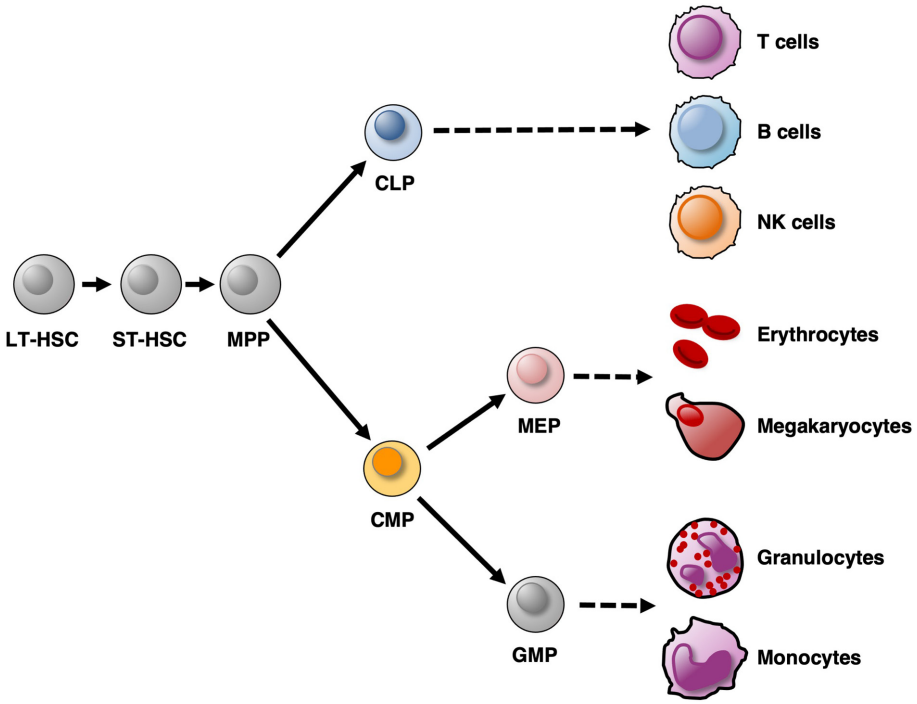

Fig. 4. Hematopoietic stem/progenitor cell (HSPC) differentiation. Illustration of HSPC differentiation to terminal blood cells. LT-HSC, long-term hematopoietic stem cell (HSC); ST-HSC, short-term HSC; MPP, multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; MEP, megakaryocyte-erythroid progenitor.

than that of E/P-selectin double KO mice, suggesting that carbohydrate chains other than sLe^x play an important role in HSPC homing.

In addition, HSC differentiation is also disturbed in *B4galt-1* KO mice (Fig. 4). Although the HSC number was normal, the number of multipotent progenitors (MPPs) increased 3-fold, while the number of common myeloid progenitors (MEPs) and common lymphoid progenitors (CLPs) decreased 0.5-fold compared with control in the BM [29]. In particular, erythrocytes and platelets were markedly reduced in the peripheral blood (unpublished data). Further analysis of HSC differentiation is in progress.

Carbohydrate Chains in Human Diseases

Recent studies indicate that aberrant glycosylation causes various human diseases, such as metastasis of tumor cells [11], muscular dystrophy [32], and dyserythropoietic anemia [8]. The congenital disorders of glycosylation (CDG) are also known to be inherited in multisystemic disorders characterized by the defective glycosylation of glycoproteins [22]. *B4galt-1* KO mice spontaneously developed human immunoglobulin A nephropathy (IgAN)-like glomerular lesions with IgA deposition and expanded mesangial matrix. The mice also showed high serum IgA levels with increased polymeric forms as in human IgAN [26]. IgAN is the most common form of glomerulonephritis, and a significant proportion of patients progress to renal failure. However, pathological molecular mechanisms of IgAN are poorly understood. In humans, serum IgA1 showed aberrant galactosylation and sialylation of *O*-glycans in its hinge region that is thought to contribute to the pathogenesis of IgAN (Fig. 5) [1]. Mouse IgA has *N*-glycans but not *O*-glycans, and β4-galactosylation and sialylation of the *N*-glycans on the serum IgA from *B4galt-1* KO mice was completely absent [26]. We propose that carbohydrate chains of serum IgA are involved in the development of IgAN, regardless of whether the carbohydrates are *O*-glycans or *N*-glycans.

Mutations in the key enzyme of sialic acid biosynthesis, UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE), result in distal myopathy with rimmed vacuoles (DMRV)/hereditary inclusion body myopathy (HIBM) in humans [9]. Among the various GNE mutations, one GNE founder mutation (V572L) has been reported in Japanese families affected by DMRV [2]. We generated mice with a V572L point mutation in the GNE kinase domain. Unexpectedly, these mutant mice had no apparent myopathies or motor dysfunctions. However, they had a short life span and exhibited renal impairment with massive albuminuria.

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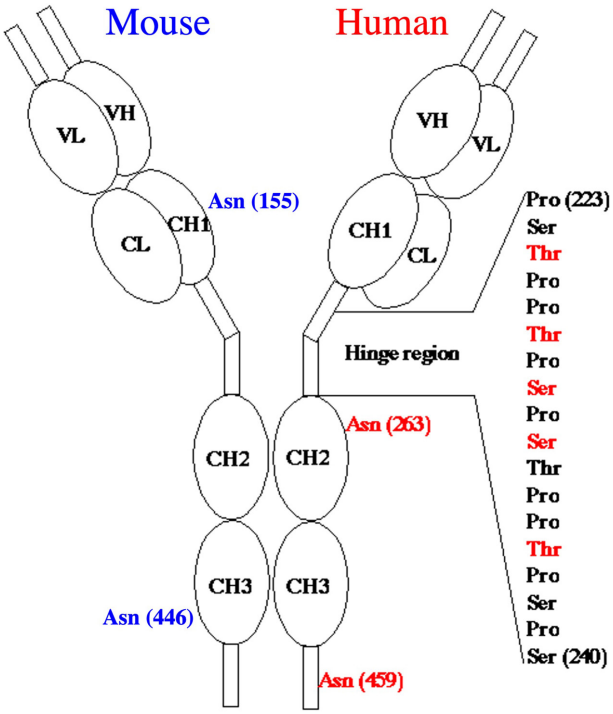

Fig. 5. Illustration of human IgA1 and mouse IgA with *O*- and *N*-glycosylation sites. Human IgA1 (right) has several *O*-glycosylation sites in the hinge region and two *N*-glycosylation sites in the CH2 region and C-terminus. Mouse IgA (left) has two *N*-glycosylation sites in the CH1 and CH3 regions but no *O*-glycosylation sites in the hinge region.

Histological analysis showed enlarged glomeruli with mesangial matrix deposition, leading to glomerulosclerosis and abnormal podocyte foot process morphologies in the kidneys. Glycan analysis using several lectins revealed glomerular epithelial cell hyposialylation, particularly the hyposialylation of podocalyxin, which is an important molecule for the glomerular filtration barrier. Furthermore, administering sialic acid, Neu5Ac, to the mutant mice from embryonic stages significantly suppressed the albuminuria and renal pathology and partially restored the glomerular glycoprotein sialylation. These findings suggest that the nephrotic-like syndrome observed in these mutant mice resulted from impaired glomerular filtration due to the hyposialylation of podocyte glycoproteins, including podocalyxin [16].

Carbohydrate Chains in Development

Next, we generated *B4galt-5* KO mice. Although most widely and strongly expressed β4GalT-1 is dispensable for mouse embryogenesis, *B4galt-5* KO mice unexpectedly showed embryonic lethality. While β4GalT-1 is responsible for *N*- and *O*-galactosylation of glycoproteins, we found that β4GalT-5 is responsible for glycosphingolipids (GSLs) synthesis. β4GalT-5 is lactosylceramide (LacCer) synthetase, which transfers galactose to glucosylceramide (GlcCer) to synthesize LacCer, a

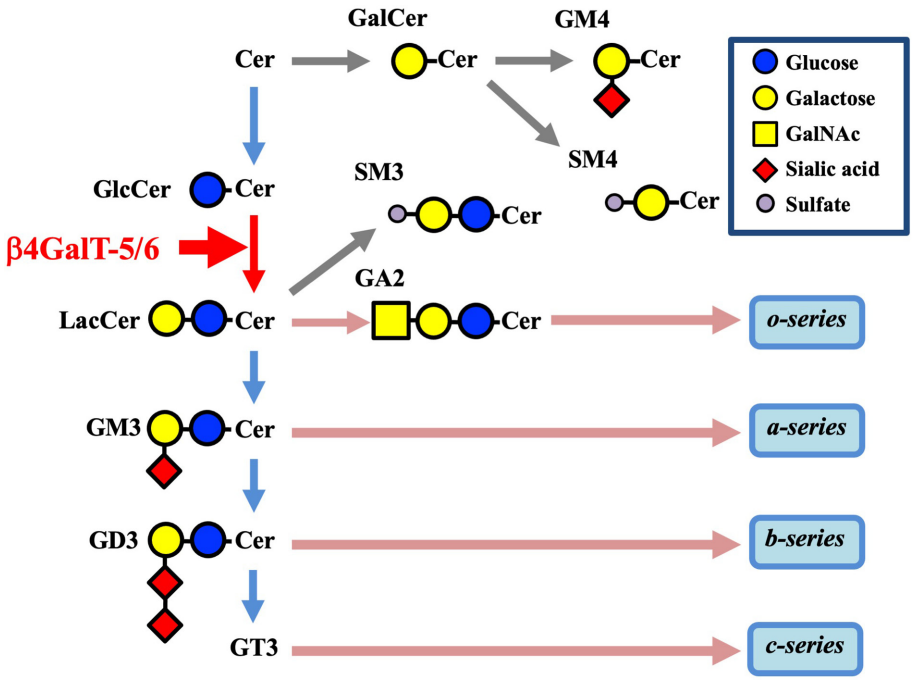

Fig. 6. Biosynthetic pathway of glycosphingolipids (GSLs). Ceramide (Cer) is the biosynthetic origin of GSLs. Glucosylceramide (GlcCer) is synthesized from Cer by GlcCer synthetase, and subsequently, lactosylceramide (LacCer) is synthesized from GlcCer by LacCer synthetase. Our results indicate that LacCer synthetase is encoded by *B4galt-5* and *B4galt-6* genes in mice. LacCer is the starting point of various gangliosides, including *o*-, *a*-, *b*-, and *c*-series gangliosides.

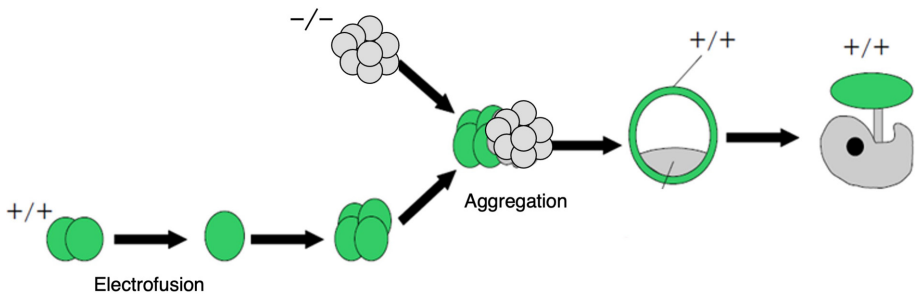

Fig. 7. Tetraploid chimeric analysis. A wild-type (wt, +/+) two-cell stage egg marked by green fluorescent protein (GFP) is electrofused to make a one-cell tetraploid egg. The 4-cell stage wt tetraploid embryo is aggregated with a diploid mutant (mt, −/−) 8-cell stage embryo to make a chimeric embryo. When the chimeric embryo differentiates into a blastocyst and further, the trophectoderm layer and placental tissues are mostly derived from the wt tetraploid egg, respectively, although the embryo is derived from the mt egg. This is because the tetraploid egg cannot differentiate toward epiblast lineages. If the embryonic lethal phenotype is caused by a defect in extraembryonic tissues, these chimeric embryos could be rescued and develop further.

core structure of GSLs, including gangliosides [25] (Fig. 6). LacCer synthetase activity and the amounts of LacCer and GM3 ganglioside in *B4galt-5* KO embryos were markedly reduced. *B4galt-5* KO embryos showed developmental retardation from E7.5 and died by E10.5 [25], as reported by Furukawa's group [19]. Hematoma, hemorrhage, and abnormal localization of trophoblast giant cells were observed in extraembryonic tissues, in contrast to normal formation of three embryonic layers. *B4galt-5* KO embryos developed until E12.5 as chimeras with wild-type tetraploid cells, which could form the extraembryonic membranes (Fig. 7), indicating that extraembryonic defects caused the early embryonic lethality [25]. Our results suggest that β4GalT-5 is essential for extraembryonic development during early mouse embryogenesis.

Carbohydrate Chains in the Nervous System

B4galt-5 is strongly expressed in the central nervous system (CNS), and various gangliosides are abundantly accumulated in the brain. We generated *B4galt-5* conditional KO (cKO) mice using Nestin-Cre mice. Unexpectedly, *B4galt-5* cKO mice developed normally and exhibited normal behavior, although *B4galt-5* expression was markedly reduced in the brain. *B4galt-6*, most homologous to *B4galt-5* (Fig. 1), was reported to encode LacCer synthetase in the rat brain [27]. However, *B4galt-6* KO mice were reported to appear normal [30]. To elucidate whether β4GalT-5 and/or β4GalT-6 are responsible for LacCer synthetase in the brain, we generated double KO (DKO) mice by crossing *B4galt-5* cKO mice and *B4galt-6* KO mice. LacCer synthase activity and major brain gangliosides were completely absent in brain homogenates from the DKO mice, although LacCer synthase activity was about half its normal level in *B4galt-5* cKO mice and *B4galt-6* KO mice. The DKO mice were born normally but they showed growth retardation and motor deficits at 2 weeks and died by 4 weeks of age. Histological analyses showed that myelin-associated proteins were rarely found localized in axons in the cerebral cortex, and axonal and myelin formation were remarkably impaired in the spinal cords of the DKO mice. Neuronal cells, differentiated from neurospheres (neural stem cells) that were prepared from the DKO embryos, showed impairments in neurite outgrowth and branch formation, which can be explained by the fact that neurospheres from DKO mice could not strongly interact with laminin due to a lack of gangliosides, such as GM1a. Furthermore, the neurons were immature, and perineuronal nets (PNNs) were poorly formed in DKO cerebral cortices. Our results indicate that LacCer synthase is encoded by *B4galt-5* and *-6* genes in the mouse CNS (Fig. 6) and that gangliosides are indispensable for neuronal maturation, PNN formation, and axonal and myelin formation [33]. Since β4GalT-6 was reported to be responsible for LacCer synthetase in rat brains [27], we are currently examining rat LacCer synthetases to reveal the difference between rats and mice.

While *B4galt-1* is widely and strongly expressed in various tissues except the CNS, *B4galt-2*, most homologous to *B4galt-1* (Fig. 1), is strongly expressed in the CNS. To elucidate the role of carbohydrate chains on proteins in the CNS, we generated *B4galt-2* KO mice. *B4galt-2* KO mice were born and grown normally and were fertile. In a behavioral test battery, the *B4galt-2* KO mice showed normal spontaneous activity in a novel environment but impaired spatial learning/memory and motor coordination/learning. Immunohistochem-

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istry showed that the amount of HNK-1 carbohydrate was markedly decreased in the brain of *B4galt-2* KO mice, whereas the expression of polysialic acid was not affected [34]. Glucuronyltransferase (GlcAT-P) KO mice lacking the HNK-1 carbohydrate also showed impaired spatial learning/memory, although their motor coordination/learning was normal [31]. Histological examination showed an abnormal alignment and reduced number of Purkinje cells in the cerebellum of *B4galt-2* KO mice. These results suggest that the Galβ1–4GlcNAc structure in the HNK-1 carbohydrate is mainly synthesized by β4GalT-2 and that the glycans synthesized by β4GalT-2 have essential roles in higher brain functions, including some that are HNK-1 dependent and some that are not [34].

Conclusions

For the last twenty years, our group has studied carbohydrate chain functions in an animal body by generating and examining KO mice of various galactosyltransferases. To our surprise, each galactosyltransferase has an individual function, and carbohydrate chains synthesized by it have essential biological functions in inflammation, hematopoiesis, development, and the nervous system. In some cases, aberrant glycosylation causes human diseases. In the present situation, although we cannot draw a whole picture of the glyco-code, I believe accumulation of these kinds of studies by many researchers will elucidate the glyco-code in the near future.

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