

## Physiological roles of *N*-acetylglucosaminyltransferase V (GnT-V) in mice

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Oligosaccharide modification by *N*-acetylglucosaminyltransferase-V (GnT-V), a glycosyltransferase encoded by the *Mgat5* gene that catalyzes the formation of  $\beta$ 1,6GlcNAc (*N*-acetylglucosamine) branches on *N*-glycans, is thought to be associated with cancer growth and metastasis. Overexpression of GnT-V in cancer cells enhances the signaling of growth factors such as epidermal growth factor by increasing galectin-3 binding to poly-lactosamine structures on receptor *N*-glycans. In contrast, GnT-V deficient mice are born healthy and lack  $\beta$ 1,6GlcNAc branches on *N*-glycans, but develop immunological disorders due to T-cell dysfunction at 12-20 months of age. We have developed *Mgat5* transgenic (Tg) mice (GnT-V Tg mice) using a  $\beta$ -actin promoter and found characteristic phenotypes in skin, liver, and T cells in the mice. Although the GnT-V Tg mice do not develop spontaneous cancers in any organs, there are differences in the response to external stimuli between wild-type and GnT-V Tg mice. These changes are similar to those seen in cancer progression but are unexpected in some aspects. In this review, we summarize what is known about GnT-V functions in skin and liver cells as a means to understand the physiological roles of GnT-V in mice. [BMB Reports 2012; 45(10): 554-559]

### INTRODUCTION

Changes in glycosylation are observed during cellular differentiation and carcinogenesis and are regulated by proteins known as glycosyltransferases. Every oligosaccharide is synthesized by a characteristic glycosyltransferase encoded by a specific glyco-gene. These genes make up approximately 1% of the human genome. Recent advances in glycotecology and molecular biology have clarified the biological functions of oligosaccharides (1) and shown that these functions are often the result of complicated molecular interactions. Even if a single

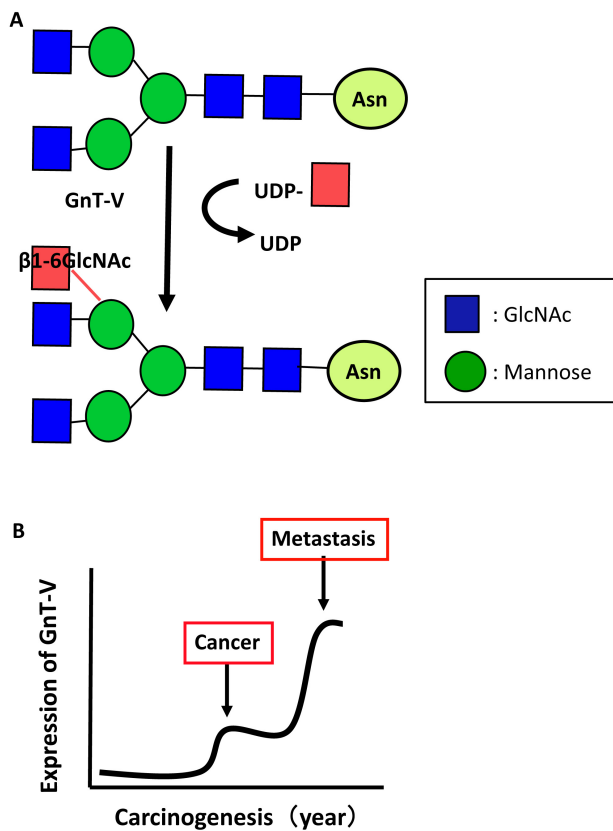
glyco-gene is knocked out or over-expressed in mice, a variety of different glycoproteins will undergo changes to their oligosaccharide structures. It is impossible to only change the oligosaccharide structure on one single glycoprotein with current molecular biology techniques. Therefore, comprehensive and focused analyses are required to understand the specific biological functions of each oligosaccharide, and researchers have focused on the important target glycoproteins that play pivotal roles in a specific phenomenon or organ. For example, epidermal growth factor (EGF) is studied for its role in skin proliferation, and transforming growth factor- $\beta$  (TGF- $\beta$ ) receptor and its signaling pathways are the focus of research into liver fibrosis.

*N*-Acetylglucosaminyltransferase-V (GnT-V), a glycosyltransferase encoded by the *Mgat5* gene that catalyzes the formation of  $\beta$ 1,6GlcNAc (*N*-acetylglucosamine) branches on *N*-glycans (Fig. 1A), is believed to be associated with cancer growth and metastasis (2, 3). It was first reported that GnT-V was directly associated with cancer metastasis in 1987 (4), and the *Mgat5* gene was subsequently cloned from rats (5) and humans (6) in 1993. Three distinct pathways are believed to be involved in the promotion of cancer metastasis by GnT-V. The first pathway involves the upregulation of EGF receptor (EGF-R) signaling through lattice formation between poly-lactosamine on  $\beta$ 1,6GlcNAc branches and galectin-3, resulting in the inhibition of receptor endocytosis (7). In the second pathway, GnT-V promotes metastasis by inhibiting protease degradation through the addition of  $\beta$ 1,6GlcNAc branches (8). The third pathway involves the inhibition of E-cadherin/ $\beta$ -catenin complex function, which leads to the release of cancer cells from the original tissue (9). Induction of mammary tumor growth and metastasis by the polyomavirus middle T oncogene is considerably reduced in GnT-V knockout mice (*Mgat5*<sup>-/-</sup> mice) compared to littermate controls, suggesting that GnT-V is essential for cancer growth as well as metastasis (10). However, clinical studies of GnT-V immunohistochemistry have demonstrated that GnT-V is not always a marker for poor prognosis. Although the prognosis is poor in colon, mammary, and esophageal cancers that exhibit high expression of GnT-V, the opposite has been observed in lung, thyroid, and liver cancers. This discrepancy might depend on the different target proteins of GnT-V in these various cancers. In contrast to its varying role as a prognosis marker, GnT-V expression is upre-

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**Fig. 1.** GnT-V reaction and biological significance of GnT-V in cancer. (A) Reaction pathway catalyzed by GnT-V. (B) The expression of GnT-V is enhanced in the early stages of carcinogenesis as well as at the metastatic end stage.

gulated in the early stages of almost all cancers (Fig. 1B). Thus, the upregulation of EGF-R signaling and the activation of proteases could represent early events in carcinogenesis. Therefore, we have established GnT-V transgenic mice (GnT-V Tg mice) using a  $\beta$ -actin promoter to determine which organs would develop cancer in these mice. Unexpectedly, however, no spontaneous cancers were observed in any of the organs in the GnT-V Tg mice. We did, however, uncover physiological roles for GnT-V in skin and liver cells in response to certain kinds of stimuli in these tissues.

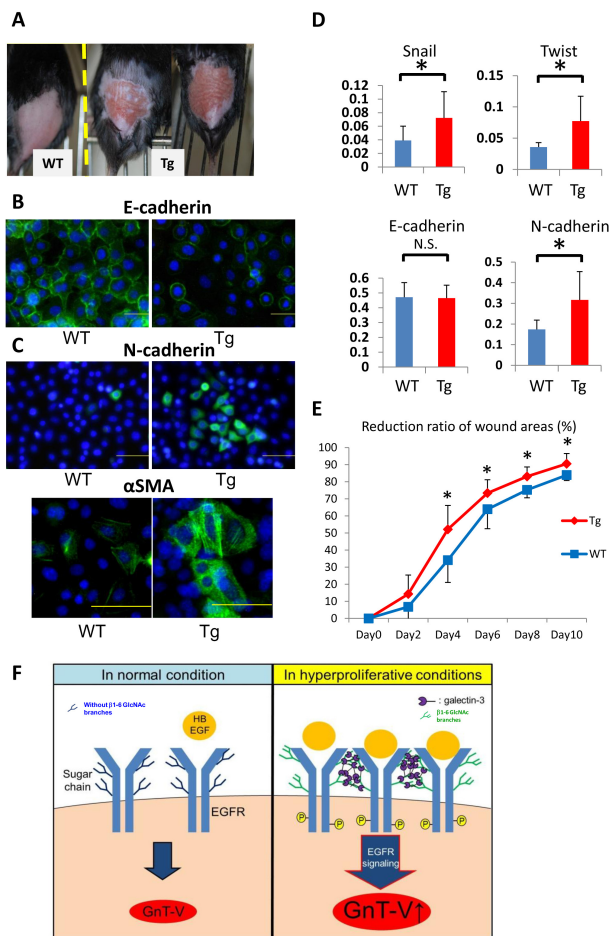
### AN EPITHELIAL-MESENCHYMAL TRANSITION (EMT)-LIKE PHENOTYPE WAS OBSERVED IN GnT-V Tg MICE

EMT, which is characterized by the loss of epithelial adhesion and gain of mesenchymal features, plays a role in fundamental biological processes such as wound healing and embryonic development as well as in cancer invasion and metastasis (11). In adults, EMT, which is driven by the release of cytokines in response to tissue injury, mediates the production of fibro-

blasts during inflammation and wound healing (12-14). Re-epithelialization in wound healing involves the motility or migration of epithelial cells, and the migrating epithelial cells at the wound margins acquire mesenchymal features and go through the early stages of EMT (12). Previous *in vitro* studies by Demetriou *et al.* demonstrated that overexpression of GnT-V in Mv1Lu cells enhanced the migration of these cells to scratch wounds suggesting an effect of GnT-V on EMT induction in cells (15). Recently, we have reported the enhancement of an EMT-like phenotype in the skin of GnT-V Tg mice (Fig. 2A) (16). The skin of the GnT-V Tg mice was more easily damaged by repeated removal of the corneum with cellotape, suggesting that cell-cell adhesion was extremely impaired in these mice. As expected, the expression of E-cadherin, one of the most important cell-adhesion molecules, was dramatically decreased in cultured keratinocytes derived from the GnT-V Tg mice (Fig. 2B). In contrast, the expression of mesenchymal proteins such as N-cadherin and  $\alpha$ -smooth muscle actin in these cells was increased (Fig. 2C). Additionally, the migration of keratinocytes derived from GnT-V Tg mice was significantly enhanced. These changes were dependent on the increased expression of EMT-regulatory transcription factors such as twist and snail (Fig. 2D) that are generally induced through the activation of the EGF or TGF $\beta$  receptor-mediated signaling pathways. It has been reported that the addition of  $\beta$ 1-6GlcNAc branches onto these receptors inhibits endocytosis and prolongs the signaling of these molecules in cancer cells (7). EGF-R signaling was enhanced in GnT-V Tg mouse keratinocytes, suggesting that similar glyco-signaling occurs in normal keratinocytes. To evaluate the EMT-like phenotype and the enhanced migration of GnT-V Tg mouse keratinocytes *in vivo*, we performed a cutaneous wound-healing assay by creating 8-mm round wounds on the backs of GnT-V Tg and control mice. The wound areas in GnT-V Tg mice were significantly smaller than controls from 4 days onward (Fig. 2E). Notably, re-epithelialization was faster in the wounds on the GnT-V Tg mice, suggesting enhanced keratinocyte motility at the wound edge. Taken together, the EMT-like features observed in the skin of the GnT-V Tg mice contributed to keratinocyte motility and cutaneous wound healing, which was mediated in part by upregulated EGF receptor signaling.

### GnT-V MAINTAINS SKIN HOMEOSTASIS BY REGULATING THE PROLIFERATION OF KERATINOCYTES THROUGH EGF-R SIGNALING

We previously reported that the expression of GnT-V was enhanced in inflammation and during cell proliferation (17, 18). Upregulation of GnT-V during these processes can enhance the signaling of growth factors and/or cytokines via the addition of  $\beta$ 1-6GlcNAc branches onto their receptors. EGF-R-mediated signaling also plays a crucial role in maintaining homeostasis in the skin. The proliferation of mature, healthy epidermal cells is maintained by balancing signals from various



**Fig. 2.** EMT-like phenotypes are observed in GnT-V Tg mice. (A) Macro view of the skin on the backs of wild-type and GnT-V Tg mice 30 minutes after tape stripping. (B) Primary cultured keratinocytes derived from wild-type and GnT-V Tg mice were stained with anti-E-cadherin (green) and Hoechst 33342 (blue). Scale bar: 100  $\mu$ m. (C) Primary cultured keratinocytes derived from wild-type and GnT-V Tg mice were stained with anti-N-cadherin (green), anti- $\alpha$ SMA (green), and Hoechst 33342 (blue). Scale bar: 100  $\mu$ m. (D) The EMT-associated transcription factors (snail and twist), E-cadherin, and N-cadherin were evaluated by quantitative RT-PCR, and target gene expression was normalized to GAPDH. Results are expressed as means  $\pm$  S.D. (n = 6). \* $P$  < 0.05. (E) Reduction of the wound area on days 2, 4, 6, 8, and 10. \* $P$  < 0.05. Photographs of re-epithelialization in wild-type and GnT-V Tg mice on day 6 are shown. Dotted yellow lines show the re-epithelialized edge of the epidermis. Bars indicate means  $\pm$  S.D. (F) GnT-V cycle in the skin. GnT-V and HB-EGF co-operate in the proliferation of keratinocytes through the enhancement of each other's expression and function. All of the data in this figure are derived from references 22 and 26.

EGF family members such as heparin-binding epidermal growth factor-like growth factor (HB-EGF), transforming growth factor- $\alpha$ , and amphiregulin, all of which bind to EGF-R in the

epidermis (19-21).

In spite of the fact that expression of GnT-V protein was extremely high in the skin compared to other organs in GnT-V Tg mice, no histological changes were observed in the GnT-V Tg mice or in the GnT-V skin under normal conditions (16). However, GnT-V deficient mice showed a decrease in epidermal hyperproliferation after treatment with phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) (22). TPA induces the production of HB-EGF, which plays important roles in keratinocyte proliferation. HB-EGF is produced in a membrane-anchored form that is then cleaved by proteases on the cell surface. TPA stimulates the activation of these proteases and this leads to increased levels of the soluble form of HB-EGF. The results of TPA-induced epidermal hyperproliferation in GnT-V-deficient mice suggested down-regulation of EGF-R signaling via HB-EGF, and EGF-R signaling in response to HB-EGF was shown to be downregulated in cultured GnT-V-deficient mouse keratinocytes. Furthermore, the addition of HB-EGF or TPA to conditioned medium induced up-regulation of GnT-V expression in cultured keratinocytes (22). Increased GnT-V activity contributes to the addition of  $\beta$ 1-6GlcNAc branches onto the *N*-glycans of EGF, which leads to the lattice formation. This lattice formation inhibits the endocytosis of EGF-R and enhances EGF-R signaling. Thus, the induction of GnT-V by HB-EGF plays an important role in maintaining skin homeostasis during hyperproliferation. The effect of GnT-V in the skin under normal and hyperproliferative conditions is outlined in Fig. 2F and could be referred to as the "GnT-V cycle in the skin". GnT-V and HB-EGF co-operate in the proliferation of keratinocytes through the enhancement of each other's expression and function.

## THE ROLE OF GnT-V IN THE DEVELOPMENT OF STEATO-HEPATITIS

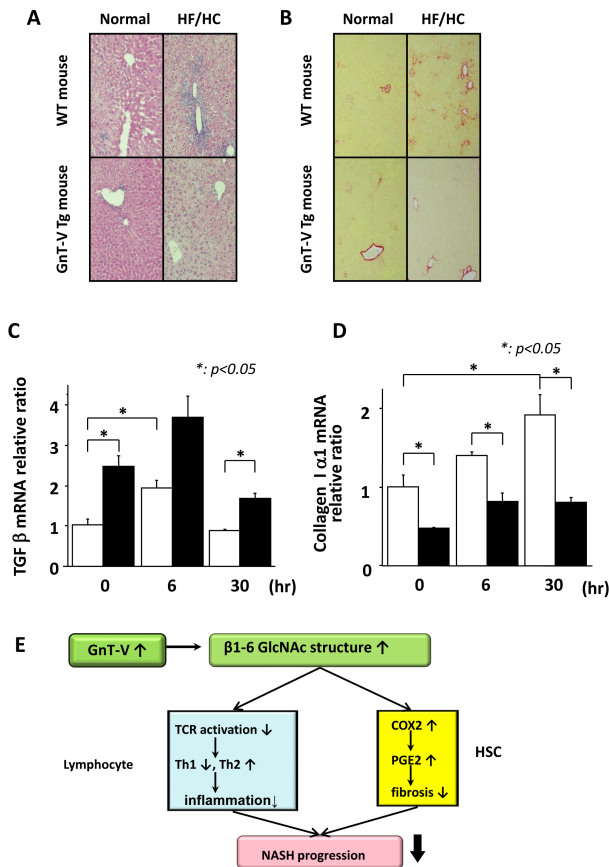
Although the expression of GnT-V is quite low in normal liver tissue, it is increased under conditions of chronic hepatitis and liver regeneration (17, 18). Previous studies have shown that glycosyltransferase transgenic mice develop fatty liver due to the accumulation of lipoproteins in their hepatocytes (23, 24). However, GnT-V Tg mice did not show fatty liver phenotypes under normal conditions. One of the reasons for the lack of fatty changes in the GnT-V Tg mouse liver might be that the expression of GnT-V is lower in the liver than in the other organs in GnT-V Tg mice. Nonalcoholic fatty liver disease (NAFLD) is among the most common chronic liver diseases in the world and is a growing medical problem in industrialized countries (25). A wide spectrum of histological changes have been observed in NAFLD, ranging from simple steatosis, which is generally non-progressive, to nonalcoholic steatohepatitis (NASH), and a proportion of patients with NASH go on to develop cirrhosis and hepatocellular carcinoma (HCC) (26). Recent studies indicate that dietary cholesterol is an important risk factor for the progression of NASH in both humans and rodents.

When we treated GnT-V Tg and wild-type mice with a high fat and high cholesterol (HFHC) diet, lymphocyte infiltration was dramatically suppressed in the GnT-V Tg mice compared to wild-type mice (Fig. 3A). HFHC treatment is one of the best mouse models for NASH because it induces both inflammation and fibrosis in the mouse liver (27). Because the expression of GnT-V was shown to be elevated in chronic hepatitis (17), we gave the GnT-V Tg mice an HFHC diet as a means of understanding the biological effect of up-regulation of GnT-V in the liver (28). When mice were fed a normal chow (NC) diet, the total body weight, liver weight, and liver to body weight ratio were all significantly higher in GnT-V Tg

mice compared to wild-type mice. In contrast, the liver weight and liver to body weight ratio were significantly lower in GnT-V Tg mice compared to wild-type mice when fed the HFHC diet. There were no differences in the serum triglyceride, liver triglyceride, or cholesterol levels between GnT-V Tg mice compared to wild-type mice with either the NC diet or the HFHC diet. There were no differences in the liver cholesterol contents between GnT-V Tg mice and wild-type mice fed the NC diet, but liver cholesterol levels were significantly lower in GnT-V Tg mice compared to wild-type mice when they were fed the HFHC diet. The suppression of lymphocyte infiltration in the GnT-V Tg mouse liver is thought to be due to a shift towards the Th2 response in their T cells. Accordingly, the levels of Th1-cytokines such as interleukin-6, interferon- $\gamma$ , and tumor necrosis factor  $\alpha$  were dramatically suppressed in GnT-V Tg mouse livers (28). Serum alanine aminotransferase levels are also lower in GnT-V Tg mice compared to wild-type mice.

### MOLECULAR MECHANISMS OF INHIBITION OF LIVER FIBROSIS IN GnT-V Tg MOUSE LIVERS

Feeding GnT-V Tg mice an HFHC diet for 4 weeks inhibited fibrosis in their livers compared to wild-type mice (Fig. 3B). Although liver fibrosis is related to hepatic inflammation, the accumulation of extracellular matrix proteins is dependent on the functions of non-parenchymal cells in the liver. Hepatic stellate cells (HSCs) play the most important roles among non-parenchymal cells during liver fibrosis (29). HSCs produce TGF- $\beta$ , which is a very important cytokine in fibrosis, and TGF- $\beta$  stimulates HSCs to produce more TGF- $\beta$  in a positive feedback cycle. When HSCs in primary culture were treated with exogenous TGF- $\beta$ , the HSCs from GnT-V Tg mice showed higher TGF- $\beta$  production than HSCs derived from wild-type mice (Fig. 3C). Even in the absence of exogenous TGF- $\beta$ , RT-PCR showed that TGF- $\beta$  mRNA levels are increased in GnT-V Tg HSCs. These results suggest that GnT-V enhances TGF- $\beta$  signaling, which is dependent on similar mechanisms as EGF-R signaling (7). Determination of Smad3 localization is a useful indicator of whether or not TGF- $\beta$  signaling is enhanced in a cell. Smad3 protein expression in cytoplasmic extracts of HSCs was decreased after TGF- $\beta$  stimulation, and TGF- $\beta$  stimulation significantly increased nuclear Smad3 protein levels in both wild-type and GnT-V Tg HSCs. Interestingly, nuclear Smad3 protein levels were significantly higher in the GnT-V Tg HSCs than in wild-type HSCs, both before and after TGF- $\beta$  stimulation. These results indicated that TGF- $\beta$  signaling in HSCs was enhanced in GnT-V Tg mice. However, the expression of collagen I was dramatically decreased in GnT-V Tg HSCs (Fig. 3D). To determine why collagen expression was decreased in GnT-V Tg HSCs in spite of the enhanced TGF- $\beta$  signaling, we performed microarray analysis comparing GnT-V Tg HSCs and wild-type HSCs. One of the genes found to be elevated in GnT-V Tg HSCs compared to wild-type HSCs was



**Fig. 3.** Role of GnT-V in the development of steato-hepatitis in mouse models. (A) Representative photomicrographs of livers from mice fed a normal chow (NC) diet or high-fat and high-cholesterol (HFHC) diet. (B) Representative photomicrographs of livers from mice fed a NC diet or HFHC diet, stained with picrosirius red. (C) Expression levels of TGF- $\beta$  mRNA in HSCs before and after TGF- $\beta$  stimulation were measured with RT-PCR. HSCs derived from GnT-V Tg mice and wild-type mice were stimulated with 1 ng/ml of TGF- $\beta$  for the indicated times. (D) Expression levels of collagen I  $\alpha$ 1 mRNA in HSCs before and after TGF- $\beta$  stimulation were measured by RT-PCR. (E) Summary of the mechanism by which GnT-V suppresses NASH progression in mice.

cyclooxygenase-2 (COX-2), the rate limiting enzyme in prostaglandin E2 (PGE2) production. It has been reported that TGF- $\beta$  stimulates COX-2 expression and that COX-2-derived PGE2 inhibited TGF- $\beta$ 1-induced collagen production in HSCs via negative feedback (30). Our hypothesis for how GnT-V is related to this process is shown in Fig. 3E. Celecoxib, a selective COX-2 inhibitor, decreased PGE2 production and upregulated collagen I gene expression in GnT-V Tg HSCs. Continuous induction of TGF- $\beta$  stimulation by GnT-V should enhance COX2 expression, and this negative feedback signal could reduce collagen expression in the livers and HSCs of GnT-V Tg mice. The precise mechanism of how this might occur is under investigation.

## CLOSING

Knockout mice of glyco-genes are sometimes lethal if they are involved in the initial step of oligosaccharide synthesis (31). In the cases of other glyco-genes, however, the knockout mice often show no phenotypes or only small differences in a limited number of organs compared to wild-type mice. In such cases, stimulation experiments should be performed. In human diseases, the complete deficiency of a particular gene is relatively rare. On the other hand, partial deficiencies in multiple genes and abnormal inductions of some genes involved in inflammation and regeneration are common. The phenotypes that we observed in the skin and livers of GnT-V Tg mice could not be detected under normal conditions. It is believed that certain changes in the micro- and macro-environment can lead to the onset of disease, and we and other groups have performed these kinds of experiments using transgenic glyco-gene knockout mice (10, 22, 28, 32, 33). In next era of glycobiology research, such experiments will produce mouse models of diseases that involve oligosaccharide remodeling, and tumor induction in GnT-V Tg mice is one of the most promising studies. It is also important to take into consideration which kinds of carcinogens and carcinogenic mice are used in these experiments.

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