

# Chapter 7

## Function of Unique *O*-Glycan Structures in Protecting Gastric Mucosa Against *Helicobacter pylori* Infection and Gastric Cancer Development

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**Abstract** Gastric gland mucin secreted from gland mucous cells located in lower portions of the gastric mucosa contains unique *O*-linked oligosaccharides displaying terminal  $\alpha$ 1,4-linked *N*-acetylglucosamine ( $\alpha$ GlcNAc).  $\alpha$ GlcNAc inhibits growth and motility of *Helicobacter pylori*, a microbe causing gastric cancer, by inhibiting biosynthesis of its cell wall component cholesteryl- $\alpha$ -D-glucopyranoside. In addition,  $\alpha$ GlcNAc serves as a tumor suppressor for gastric differentiated-type adenocarcinoma, and its loss in gastric cancer cells is associated with progression and poor prognosis of patients with this subtype of gastric cancer. This chapter summarizes protective functions of  $\alpha$ GlcNAc against gastric cancer development.

**Keywords**  $\alpha$ 1,4-*N*-acetylglucosaminyltransferase • Gastric cancer • *Helicobacter pylori* • Knockout mouse • Mucin • Patient prognosis • Terminal  $\alpha$ 1,4-linked *N*-acetylglucosamine residue

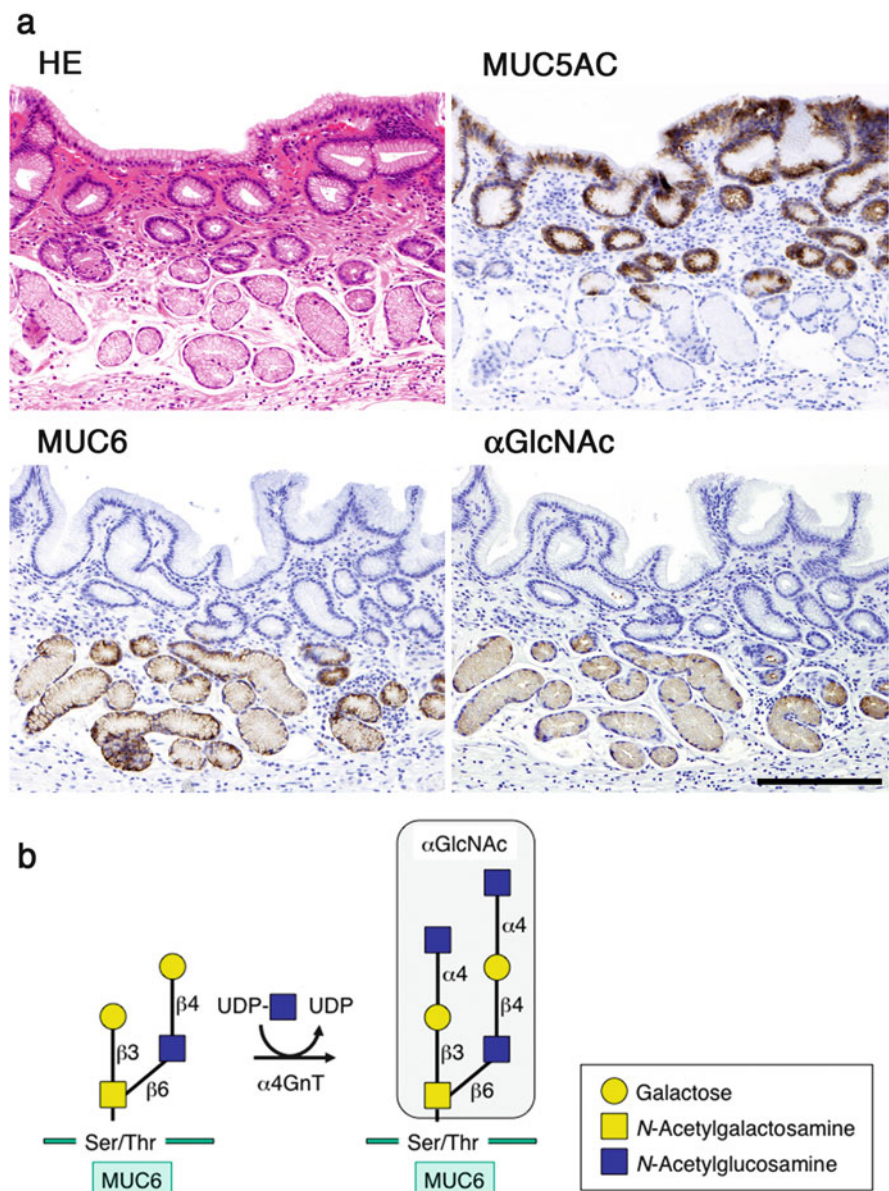
### 7.1 Introduction

Gastric cancer ranks fourth in the most commonly diagnosed cancers and second in the most common causes of cancer-related death worldwide and thus remains one of the most common malignancies (Ferlay et al. 2010). On the other hand, gastric mucins play important roles in forming the surface mucous gel layer, which protects tissues from the external environment (Ota and Katsuyama 1992). However, how gastric mucins alter gastric cancer pathogenesis remains unknown. Gastric mucins are divided into surface and gland mucins (Ota et al. 1991). The first are secreted from surface mucous cells lining the gastric mucosa and contain surface mucin-specific glycans such as Lewis-related blood group carbohydrates attached

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**Fig. 7.1** Expression of MUC5AC, MUC6, and  $\alpha$ GlcNAc in gastric mucosa and the  $\alpha$ GlcNAc biosynthetic pathway. (a) MUC5AC is expressed in surface mucous cells, while MUC6 is detected in pyloric gland cells of human gastric mucosa. Note that  $\alpha$ GlcNAc is coexpressed with MUC6 in pyloric gland cells. Hematoxylin and eosin (HE) staining (*upper left*) and immunohistochemistry using CLH2 antibody for MUC5AC (*upper right*), CLH5 antibody for MUC6 (*lower left*), and HIK1083 antibody for  $\alpha$ GlcNAc (*lower right*). Bar indicates 200  $\mu$ m. (b)  $\alpha$ 1,4-N-Acetylglucosaminyltransferase ( $\alpha$ 4GnT) forms  $\alpha$ GlcNAc primarily attached to MUC6

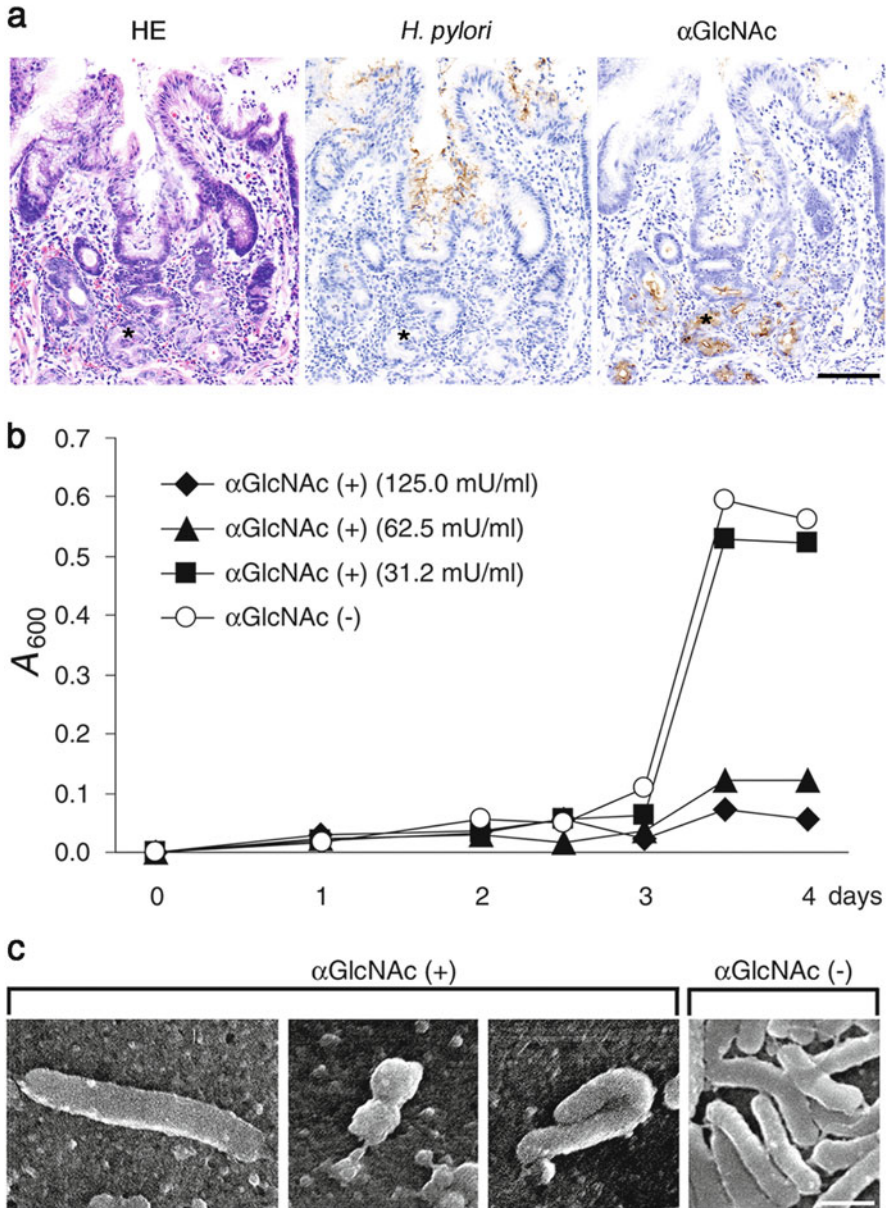
to the mucin core protein MUC5AC (Nordman et al. 2002) (Fig. 7.1a). By contrast, the latter are secreted from gland mucous cells such as pyloric gland cells and mucous neck cells located in the lower layer of the gastric mucosa and contain gland mucin-specific *O*-glycans exhibiting terminal  $\alpha$ 1,4-linked *N*-acetylglucosamine residues (hereafter termed  $\alpha$ GlcNAc) attached to MUC6 (Ishihara et al. 1996; Zhang et al. 2001; Ferreira et al. 2006).  $\alpha$ GlcNAc is a unique *O*-glycan, as its distribution is limited to gastric gland mucous cells and Brunner's glands of the duodenal mucosa (Nakamura et al. 1998).

$\alpha$ GlcNAc biosynthesis is catalyzed by  $\alpha$ 1,4-*N*-acetylglucosaminyltransferase ( $\alpha$ 4GnT), which transfers *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to terminal  $\beta$ -linked galactose (Gal) residues present in *O*-glycans with an  $\alpha$ 1,4-linkage (Fig. 7.1b). Previously, we used an expression cloning to isolate  $\alpha$ 4GnT cDNA from a human stomach cDNA library (Nakayama et al. 1999). Then, using  $\alpha$ 4GnT cDNA as a molecular tool, we investigated  $\alpha$ GlcNAc function in the pathogenesis of gastric cancer. In this chapter, I describe the roles of  $\alpha$ GlcNAc in the gastric mucosa and focus in particular on its protective function against *Helicobacter pylori* (*H. pylori*) infection and gastric cancer development.

## 7.2 Role of $\alpha$ GlcNAc in *H. pylori* Infection

### 7.2.1 $\alpha$ GlcNAc Acts as a Natural Antibiotic in Antagonizing *H. pylori*

*H. pylori* is a gram-negative bacteria first isolated from gastric mucosa by Marshall and Warren (1984). This microbe is known to cause various gastric diseases, including chronic active gastritis, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma (Peek and Blaser 2002). Although *H. pylori* infects over half the world's human population, only a fraction of infected patients develop severe gastric disease. Interestingly, *H. pylori* largely colonizes surface mucins, while it is rarely found in gland mucins (Hidaka et al. 2001) (Fig. 7.2a), suggesting that  $\alpha$ GlcNAc protects the gastric mucosa against *H. pylori* infection. To test the hypothesis, we cultured *H. pylori* in the presence of various levels of recombinant soluble CD43 (sCD43) carrying  $\alpha$ GlcNAc (hereafter termed  $\alpha$ GlcNAc (+)) (Kawakubo et al. 2004), which were secreted from Lec2 cells, a mutant CHO cell line defective in a sialic acid transporter (Deutscher et al. 1984). That line had been cotransfected with three expression vectors encoding  $\alpha$ 4GnT, core2  $\beta$ 1,6-*N*-acetylglucosaminyltransferase (C2GnT) (Bierhuizen and Fukuda 1992), and sCD43, respectively. In this assay, sCD43 serves as a glycan scaffold, as it contains 80 *O*-glycosylation sites in its extracellular domain (Fukuda 1992). Surprisingly, *H. pylori* growth was suppressed in a dose-dependent manner in the presence of  $\alpha$ GlcNAc (+) (Fig. 7.2b). In addition, we observed significantly reduced motility and abnormal morphology, such as elongation and bending in *H. pylori*



**Fig. 7.2** Antimicrobial activity of  $\alpha$ GlcNAc against *H. pylori* infection. **(a)** Histopathology of chronic active gastritis of human gastric mucosa caused by *H. pylori* infection. The microbe is rarely found in gland mucin expressing  $\alpha$ GlcNAc (\*). Hematoxylin and eosin (HE) staining (*left*), immunohistochemistry using anti-*H. pylori* antibody (*middle*), and HIK1083 antibody for  $\alpha$ GlcNAc (*right*). Bar indicates 100  $\mu$ m. **(b)** Growth curves of *H. pylori* cultured in the presence of sCD43 carrying  $\alpha$ GlcNAc ( $\alpha$ GlcNAc (+)) or sCD43 lacking  $\alpha$ GlcNAc ( $\alpha$ GlcNAc (-)). One milliunit of  $\alpha$ GlcNAc (+) corresponds to 1  $\mu$ g GlcNAc $\alpha$ -pNP. A600: absorbance at 600 nm. **(c)** Scanning electron micrographs showing *H. pylori* incubated with 31.2 mU/ml  $\alpha$ GlcNAc (+) or the same concentration of  $\alpha$ GlcNAc (-) protein for 3 days. Bar indicates 1  $\mu$ m (Panels **b** and **c** are

cultured with  $\alpha$ GlcNAc (+). By contrast, when the microbe was incubated with control sCD43 lacking  $\alpha$ GlcNAc (hereafter termed  $\alpha$ GlcNAc (-)) secreted from cotransfected Lec2 cells with C2GnT and sCD43 expression vectors, we did not observe these effects, indicating that  $\alpha$ GlcNAc antagonizes *H. pylori* growth like a natural antibiotic. Similar antibiotic effects were also obtained when *H. pylori* was incubated with *p*-nitrophenyl- $\alpha$ -*N*-acetylglucosamine (GlcNAc $\alpha$ -*p*NP), recombinant soluble CD34 carrying  $\alpha$ GlcNAc, or  $\alpha$ GlcNAc-containing gland mucin prepared from human gastric mucosa (Kawakubo et al. 2004).

### 7.2.2 $\alpha$ GlcNAc Inhibits Cholesterol $\alpha$ -Glucosyltransferase Activity

To define the molecular mechanism underlying  $\alpha$ GlcNAc antimicrobial activity, we focused in particular on morphological changes seen in *H. pylori* cultured in the presence of  $\alpha$ GlcNAc (Fig. 7.2c) (Kawakubo et al. 2004). We noted that those changes were similar to those seen in *H. pylori* cultured in the presence of  $\beta$ -lactamase inhibitors (Enroth et al. 1999). Thus, we speculated that treatment with  $\alpha$ GlcNAc had an effect on the *H. pylori* cell wall. Hirai et al. (1995) previously demonstrated that the *H. pylori* cell wall contains a unique glycolipid, cholesteryl- $\alpha$ -D-glucoopyranoside (CGL), as well as its derivatives. CGL biosynthesis is catalyzed by cholesterol  $\alpha$ -glucosyltransferase ( $\alpha$ CgT), which transfers glucose (Glc) from UDP-Glc to cholesterol with an  $\alpha$ 1,3-linkage. Molecular mimicking between  $\alpha$ 1,4-linked GlcNAc in gland mucin and  $\alpha$ 1,3-linked Glc in CGL suggested that  $\alpha$ GlcNAc suppressed  $\alpha$ CgT enzymatic activity by an end-product inhibitory mechanism. Thus, we analyzed glycolipid fractions isolated from *H. pylori* cultured in the presence of  $\alpha$ GlcNAc (+) or  $\alpha$ GlcNAc (-) using mass spectrometry (Kawakubo et al. 2004). We found that CGL levels in *H. pylori* cultured with  $\alpha$ GlcNAc (+) were significantly lower than those seen in *H. pylori* cultured with  $\alpha$ GlcNAc (-), suggesting that  $\alpha$ GlcNAc directly inhibits CGL biosynthesis by *H. pylori* in vivo. Subsequently, we used expression cloning to isolate  $\alpha$ CgT gene from *H. pylori* (Lee et al. 2006) and proved that  $\alpha$ CgT enzymatic activity is inhibited by core2-branched *O*-glycans displaying  $\alpha$ GlcNAc in vitro (Lee et al. 2008). We also showed that an active form of  $\alpha$ CgT is present in the *H. pylori* membrane fraction, suggesting that bacterial  $\alpha$ CgT is likely accessible to  $\alpha$ GlcNAc in gland mucin (Hoshino et al. 2011).



**Fig. 7.2** (continued) from Kawakubo et al. 2004; Copyright 2004 American Association for the Advancement of Science)

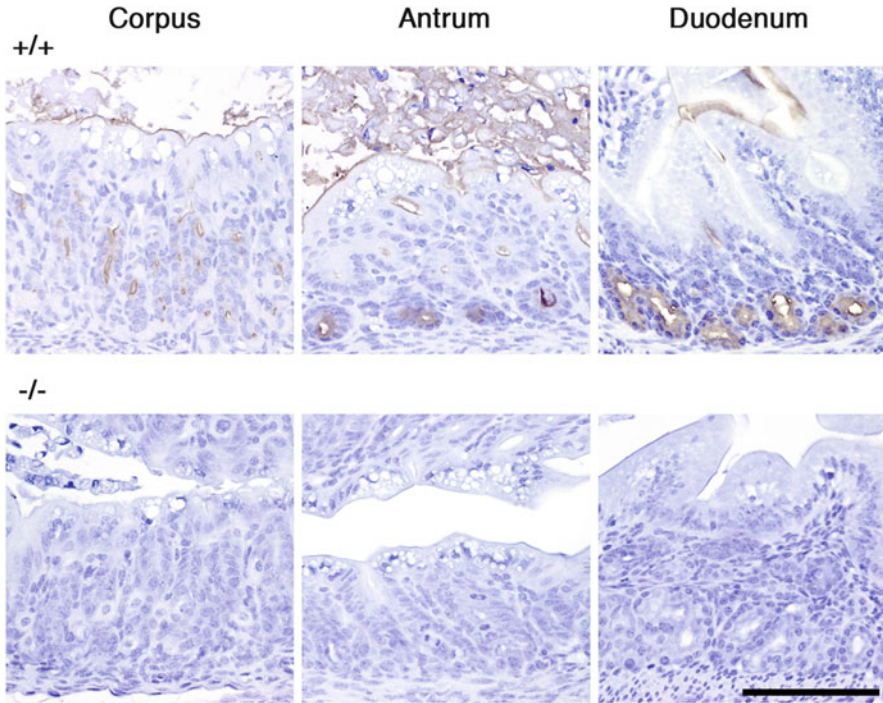
### 7.2.3 CGL Is Indispensable for *H. pylori* Survival

*H. pylori* requires exogenous cholesterol for CGL biosynthesis. Thus to further define CGL function in *H. pylori*, we created *H. pylori* lacking CGL by culturing the microbe in the absence of cholesterol (Kawakubo et al. 2004). Resultant *H. pylori* exhibited reduced growth and motility, and all microbes died following prolonged (21-day) incubation in cholesterol-free media, indicating that CGL is indispensable for *H. pylori* survival. Overall, these results show that  $\alpha$ GlcNAc inhibits CGL biosynthesis by *H. pylori* by suppressing  $\alpha$ CgT, thus protecting the gastric mucosa from infection. In fact,  $\alpha$ GlcNAc does not exhibit antimicrobial activity against bacteria lacking CGL such as *E. coli* and *S. aureus* (Kawakubo et al. 2004). Most recently, we reported that  $\alpha$ CgT enzymatic activity in *H. pylori* was highly correlated with the degree of glandular atrophy in gastric mucosa infected by the bacteria and that the monoacyled form of cholesteryl-6-*O*-phosphatidyl- $\alpha$ -D-glucopyranoside (CPG), a minor constituent of CGL derivatives in the *H. pylori* cell wall, is the most potent antigen for invariant natural killer T cells, thus eliciting an immune response in gastric mucosa (Ito et al. 2013).

## 7.3 $\alpha$ GlcNAc Serves as a Tumor Suppressor for Gastric Cancer

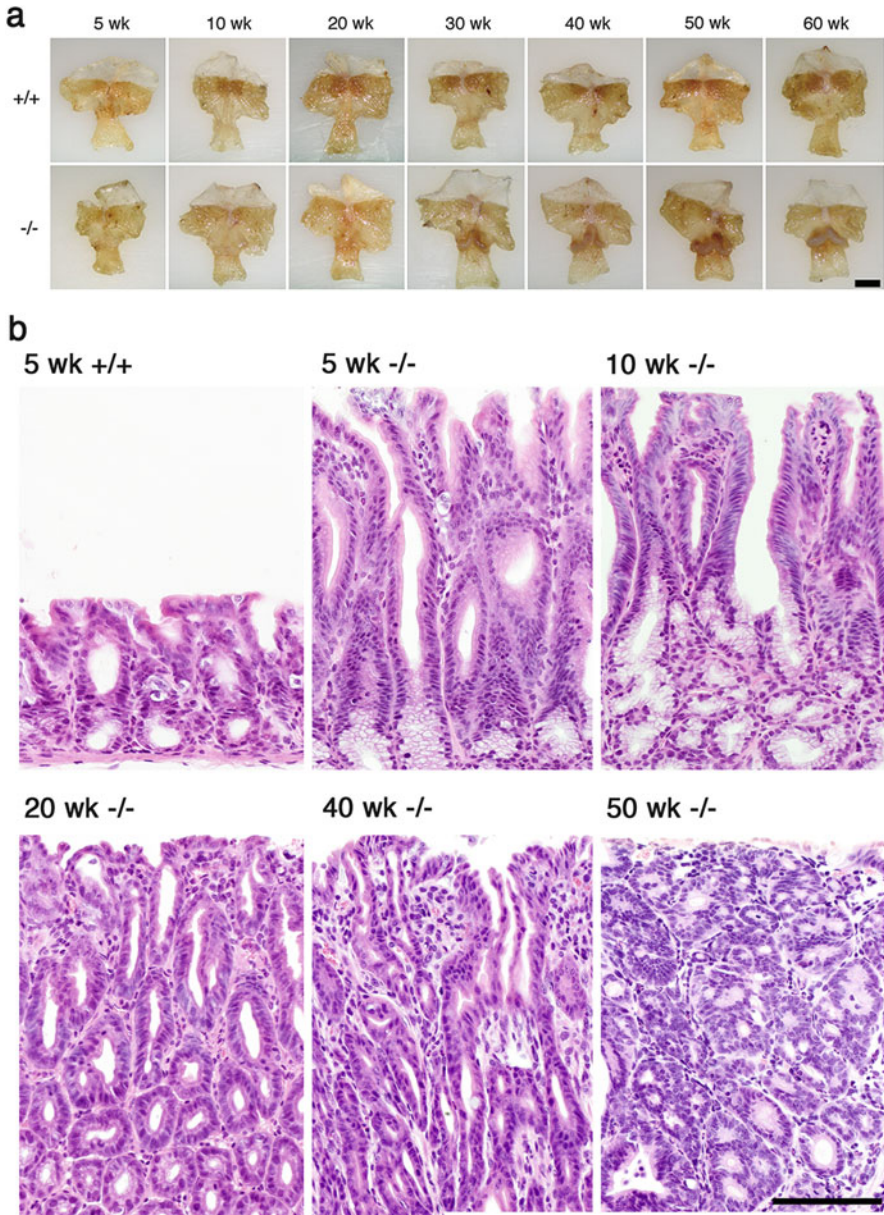
### 7.3.1 $\alpha$ GlcNAc Suppresses Tumorigenesis of Gastric Differentiated-Type Adenocarcinoma

We then asked whether  $\alpha$ GlcNAc had a more general or broader protective function in the gastric mucosa. To address to this question, we generated mice deficient in  $\alpha$ 4GnT by disrupting the *A4gnt* gene and analyzed  $\alpha$ GlcNAc function in vivo (Karasawa et al. 2012). Immunohistochemistry using the  $\alpha$ GlcNAc-specific antibody HIK1083 and MALDI-TOF-MS analyses revealed that *A4gnt*-deficient mice showed a complete lack of  $\alpha$ GlcNAc expression in gastric gland mucin and duodenal Brunner's gland, formally establishing that  $\alpha$ 4GnT is the sole enzyme catalyzing addition of  $\alpha$ GlcNAc to *O*-glycans in vivo (Fig. 7.3). Surprisingly, *A4gnt*-deficient mice, even in the absence of *H. pylori* infection, spontaneously developed tumor in the antrum as early as 5 weeks of age, and tumor size gradually increased as mice aged (Fig. 7.4a). Histopathology of tumors revealed that the mutant mice exhibited hyperplasia by 5 weeks of age, low-grade dysplasia by 10 weeks, and high-grade dysplasia by 20 weeks in the glandular stomach (Fig. 7.4b). In 30-week-old mice, gastric adenocarcinoma developed in 2 of 6 *A4gnt*-deficient mice, and adenocarcinoma incidence increased by 50 weeks of age. Furthermore, all 50- and 60-week-old mice exhibited gastric adenocarcinoma. These pathologies were consistently seen in the antrum of the glandular stomach, and cancer cells were mostly restricted to the mucosa. No sign of distant metastasis



**Fig. 7.3**  $\alpha$ GlcNAc loss in *A4gnt*-deficient mice.  $\alpha$ GlcNAc is expressed in mucous neck cells of the gastric corpus, pyloric gland cells of the gastric antrum, and Brunner's glands of the duodenum of wild-type mouse (+/+), while it is completely absent in these mucous cells of *A4gnt*-deficient mouse (-/-). Shown is immunohistochemistry of gastroduodenal mucosa from 1-week-old mice with  $\alpha$ GlcNAc-specific HIK1083 antibody. Bar indicates 100  $\mu$ m

was noted up to 60 weeks of age. Gastric adenocarcinoma is largely classified into differentiated (or intestinal) or undifferentiated (or diffuse) types, based on tumor cell morphology and histogenesis background (Lauren 1965; Nakamura et al. 1968). Interestingly, the gastric adenocarcinoma seen in *A4gnt*-deficient mice was only of the differentiated type, while undifferentiated-type adenocarcinoma, such as signet ring cell carcinoma, never arose. This indicates that mutant mice develop gastric differentiated-type adenocarcinoma through a hyperplasia-dysplasia-carcinoma sequence in the absence of *H. pylori* infection. Intestinal metaplasia was rarely detected in gastric mucosa of either *A4gnt*-deficient or wild-type mice during the 60-week observation period, indicating that metaplasia is not associated with gastric tumorigenesis in this model. These results establish that  $\alpha$ GlcNAc serves as a tumor suppressor for gastric differentiated-type adenocarcinoma.

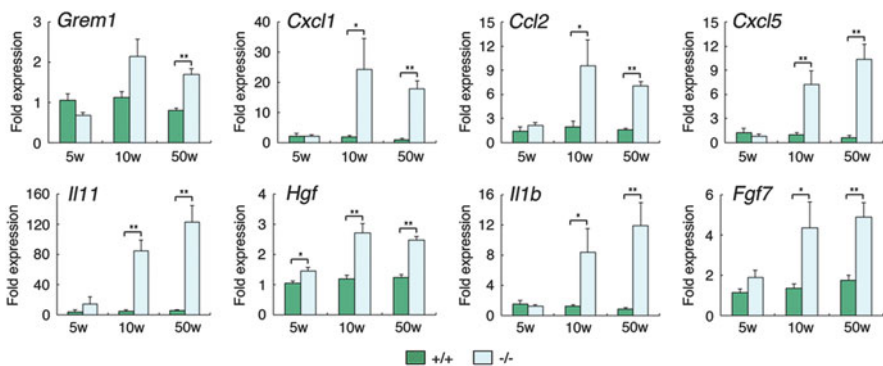


**Fig. 7.4** Gastric pathology of *A4gnt*-deficient mice. **(a)** Macroscopic appearance of the stomach removed from wild-type mice (+/+) and *A4gnt*-deficient mice (-/-). Bar indicates 5 mm. **(b)** Representative histopathology showing hyperplasia at 5 weeks, low-grade dysplasia at 10 weeks, high-grade dysplasia at 20 weeks, and differentiated-type adenocarcinoma at 40 and 50 weeks in the antral mucosa of *A4gnt*-deficient mice. For comparison, pyloric mucosa from a 5-week-old wild-type mouse is shown (*upper left*). Bar indicates 100  $\mu$ m (Panel **a** is from Karasawa et al. 2012; Copyright 2012 The American Society for Clinical Investigation)



### 7.3.2 $\alpha$ GlcNAc Suppresses Tumor-Promoting Inflammation in *A4gnt*-Deficient Mice

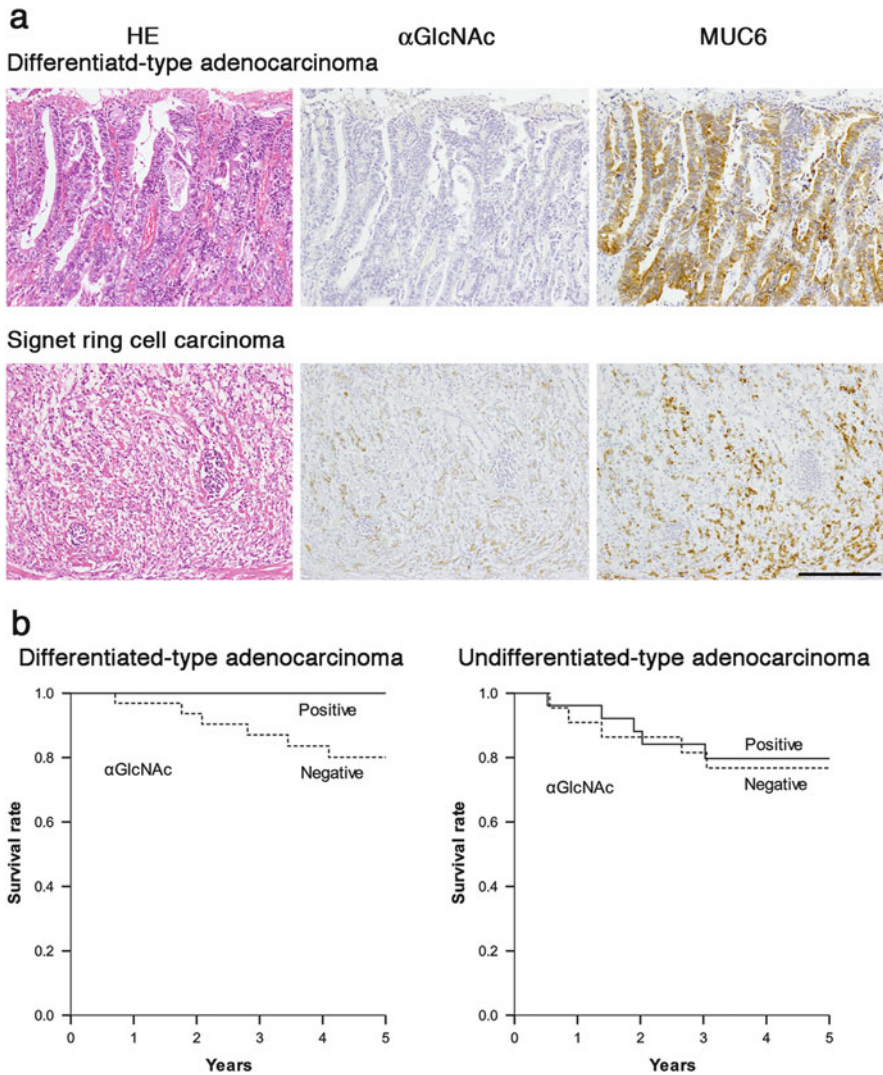
It remained unclear why *A4gnt*-deficient mice develop only differentiated-type adenocarcinoma in the gastric mucosa. To clarify molecular mechanisms underlying such a specific tumor suppression function by  $\alpha$ GlcNAc, we carried out microarray analysis followed by quantitative RT-PCR using mRNA derived from gastric mucosa of *A4gnt*-deficient and wild-type mice at 5, 10, and 50 weeks of age (Karasawa et al. 2012). Our analysis identified eight genes upregulated in *A4gnt*-deficient mice compared with wild-type mice (Fig. 7.5). Among these genes significantly upregulated in the gastric mucosa of mutant mice older than 10 weeks were those encoding inflammatory chemokine ligands such as *Ccl2*, *Cxcl1*, and *Cxcl5*; proinflammatory cytokines such as IL-11 and IL-1 $\beta$ ; and growth factors such as Hgf and Fgf7. In addition, Hgf was upregulated even in 5-week-old mutant mice, indicating that altered gene expression patterns are apparent even at low-grade dysplasia stages, prior to gastric cancer development. Of the altered factors, *Ccl2* is of particular interest as it attracts tumor-associated macrophages, which exert pro-tumorigenic immune responses and promote tumor angiogenesis (Grivennikov et al. 2010; Mantovani et al. 2010). In fact, both infiltration of inflammatory cells such as mononuclear cells and neutrophils and angiogenesis increased progressively in the gastric mucosa as *A4gnt*-deficient mice aged. IL-11 is also noteworthy because it functions in progression of inflammation to gastric tumorigenesis via gp130 signaling, followed by STAT3 phosphorylation (Ernst et al. 2008; Howlett et al. 2009). Taken together, our results indicate that  $\alpha$ GlcNAc loss triggers gastric carcinogenesis through inflammation-associated pathways in vivo.



**Fig. 7.5** Genes upregulated in the gastric mucosa of *A4gnt*-deficient mice compared with those in age-matched wild-type mice, as determined by quantitative RT-PCR analysis. *Grem1*, Gremlin 1; *Cxcl1*, Chemokine (C-X-C motif) ligand 1; *Ccl2*, Chemokine (C-C motif) ligand 2; *Cxcl5*, Chemokine (C-X-C motif) ligand 5; *Il11*, IL-11; *Hgf*, HGF; *Il1b*, IL-1 $\beta$ ; *Fgf7*, FGF7. \* $P < 0.05$ ; \*\* $P < 0.01$  (From Karasawa et al. 2012; Copyright 2012 American Society for Clinical Investigation)

### 7.3.3 *$\alpha$ GlcNAc Loss in Gland Mucin Is Associated with Progression of Human Gastric Differentiated-Type Adenocarcinoma*

Lastly, we asked whether  $\alpha$ GlcNAc loss occurred in human gastric adenocarcinoma and whether such loss was associated with tumor progression. To do so, we used immunohistochemistry to assess expression of  $\alpha$ GlcNAc and its scaffold MUC6 in 214 surgically resected gastric adenocarcinomas and then compared those expression patterns with clinicopathological parameters such as vessel invasion and stage and cancer-specific survival (Shiratsu et al. 2014). MUC6 was detected in gastric cancer cells in 102 (47.6 %) of 214 patients. In differentiated-type adenocarcinoma, 33 (58.9 %) of 54 MUC6-positive cancer lacked  $\alpha$ GlcNAc expression, while in undifferentiated-type adenocarcinoma, 22 (45.8 %) of 48 MUC6-positive cancer lacked  $\alpha$ GlcNAc expression, indicating that there was no significant difference between absence of  $\alpha$ GlcNAc expression in differentiated and undifferentiated tumor types. However, when the comparison was made between a subtype of undifferentiated-type adenocarcinoma, signet ring cell carcinoma, and differentiated-type adenocarcinoma (Fig. 7.6a), only 6 (26.1 %) of 23 signet ring cell carcinoma patients lacked  $\alpha$ GlcNAc expression, significantly at lower frequency compared with differentiated-type adenocarcinoma ( $P = 0.0049$ ). Notably,  $\alpha$ GlcNAc loss was significantly correlated with depth of invasion, stage, venous invasion, and more importantly, poorer patient prognosis in MUC6-positive differentiated-type adenocarcinoma (Fig. 7.6b). On the other hand, no significant correlation between  $\alpha$ GlcNAc loss in tumor cells and any clinicopathological variable or cancer-specific survival of patients with undifferentiated-type adenocarcinoma was observed. Thus,  $\alpha$ GlcNAc loss in MUC6-positive cancer cells is significantly associated with progression and poor prognosis in differentiated-type but not undifferentiated-type adenocarcinomas of the stomach, consistent with phenotypes seen in *A4gnt*-deficient mice (Karasawa et al. 2012). As described in Sect. 7.3.2, inflammatory chemokine ligands, proinflammatory cytokines, and growth factors were upregulated in mutant mice, and these molecules are also thought to function in human gastric cancer development. For example, Ohta et al. (2003) reported that CCL2 expression by human gastric carcinoma cells increases with tumor cell invasiveness, and its expression level is positively correlated with angiogenesis and macrophage recruitment. Verbeke et al. (2012) demonstrated that CXC chemokines, including CXCL1/CXCL5, facilitate progression of gastric cancer tumors. Nakayama et al. (2007) observed that IL-11 expression is significantly higher in differentiated compared to undifferentiated types of adenocarcinoma and that IL-11 functions in gastric carcinoma progression. HGF and FGF7 play important roles in gastric epithelial proliferation. Mohri et al. (2012) suggest that HGF expression is an important prognostic factor in gastric cancer. FGF7 is upregulated



**Fig. 7.6**  $\alpha$ GlcNAc expression in human gastric adenocarcinoma and correlation with cancer-specific survival of gastric cancer patients. **(a)** Hematoxylin and eosin (HE) staining (*left*) and  $\alpha$ GlcNAc (*middle*) and MUC6 (*left*) expression in differentiated-type adenocarcinoma (*upper panels*) and signet ring cell carcinoma (*lower panels*). Bar indicates 200  $\mu$ m. **(b)** Cancer-specific survival in patients with MUC6-positive gastric cancer. In differentiated-type adenocarcinoma, patients with  $\alpha$ GlcNAc-negative tumors had a significant poorer outcome than did patients with  $\alpha$ GlcNAc-positive tumors ( $P=0.048$ ). By contrast, in undifferentiated-type adenocarcinoma, there was no significant difference in survival rate of patients harboring  $\alpha$ GlcNAc-positive or  $\alpha$ GlcNAc-negative tumors ( $P=0.549$ ) (Modified from Shiratsu et al. 2014)

by IL-1 $\beta$  (Palmieri et al. 2003). Kai et al. (2005) demonstrated that tumor IL-1 $\beta$  expression levels are elevated more than 50-fold over those seen in normal gastric mucosa and significantly higher in nonscirrhous compared with scirrhous carcinomas. Thus, all of these factors likely promote tumor-promoting inflammation. Accordingly, our results suggest that  $\alpha$ GlcNAc loss is correlated with gastric cancer progression in inflammation-related pathways in humans. It remains to be determined how  $\alpha$ GlcNAc loss in gastric cancer enhances tumor-promoting inflammation in the stomach. Recently, we demonstrated that reduced  $\alpha$ GlcNAc in Barrett's esophagus could also predict its potential to develop into Barrett's adenocarcinoma (Iwaya et al. 2014).

## 7.4 Conclusion

We conclude that gastric gland mucin-specific  $\alpha$ GlcNAc has a protective function against gastric cancer development in two ways: first, as a natural antibiotic against *H. pylori* and second, as a tumor suppressor for gastric differentiated-type adenocarcinoma. Based on these findings, we anticipate future development of new strategies to detect, diagnose, treat, and prevent gastric cancer.

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