Cigarette smoking alters sialylation in the Fallopian tube of women with implications for the pathogenesis of ectopic pregnancy

Short title: Sialylation and ectopic pregnancy in women

Summary sentence: The expression of sialyltransferases decreases during tubal ectopic pregnancy in women and cigarette smoking alters the expression of ST6GAL1 and ST3GAL5, potentially resulting in a decreased tubal transport and an increased receptivity for blastocysts.

Key words: sialic acid, sialyltransferases, tubal ectopic pregnancy, cigarette smoking, cilia, galectin

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Abbreviations: $\alpha_{2,3}$-galactoside sialyltransferases, ST3GAL; $\alpha_{2,3}$-galactoside sialyltransferases, ST6GAL; galactose, Gal; *Maackia amurensis*, MAM; *Mucin 1*, MUC1; N-acetylglucosamine, GlcNAc; *Sambucus sieboldiana*, SNA

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Abstract

We recently reported potential involvement of galectin-1 and galectin-3, \( \beta \)-galactoside-binding lectins, in pathogenesis of tubal ectopic pregnancy. However, the precise role of galectins and their ligand glycoconjugates remain unclear. Sialylation serves surface negativity to block blastocyst implantation, and \( \alpha \)2,6-sialylation on terminal galactose catalyzed by a sialyltransferase, ST6GAL1, inhibits the binding of galectin-1, which is increased in expression level during tubal ectopic implantation. We here investigated the expression of \( \alpha \)2,3- and \( \alpha \)2,6-galactoside sialyltransferases (\( ST3GAL1−6 \) and \( ST6GAL1−2 \)) and the localization of sialic acids in the Fallopian tube from women with or without ectopic implantation. The expression level of \( ST6GAL1 \) was higher in the mid-secretory phase than the proliferative phase \((P<0.0001)\) of non-pregnant women. In the Fallopian tube with ectopic implantation, \( ST6GAL1 \) was lower \((P<0.0001)\) as were \( ST3GAL3 (P=0.0029), ST3GAL5 (P=0.0089), \) and \( ST3GAL6 (P=0.0018) \). Cigarette smoking, a major risk factor for tubal ectopic pregnancy, was associated with a reduced mid-secretory phase expression of \( ST6GAL1 (P=0.0298) \) and elevated expression of \( ST3GAL5 (P=0.0006) \), an enzyme known to be involved in ciliogenesis. Both \( \alpha \)2,3- and \( \alpha \)2,6-sialic acids were localized to the surface of the Fallopian tube epithelium. Sialic acid-containing ciliated inclusion cysts, which are
known to be associated with abnormal ciliogenesis, were observed within the epithelium, and the number of cysts increased ($P=0.0177$) in women who smoked, suggesting that abnormal ciliogenesis is associated with smoking. These results suggest that cigarette smoking alters sialylation in the Fallopian tube epithelium and potentially involved in a decreased tubal transport and an increased receptivity for blastocyst in human Fallopian tube.
Introduction

Ectopic pregnancy is defined as a pregnancy implanted outside the uterus, and over 95% of the ectopic implantation occurs in the Fallopian tube (Walker 2007; Varma and Gupta 2012). It occurs in up to 2% of pregnancies and remains one of the leading causes of maternal death in the first trimester (Farquhar, 2005; Varma and Gupta, 2012). Although the pathogenesis of tubal ectopic pregnancy is still unclear, it seems to be due to the failure of tubal transport activity and/or increase in tubal receptivity for blastocyst implantation. Major risk factors for the tubal ectopic pregnancy are tubal damage as a result of surgery or infection as well as cigarette smoking and in vitro fertilization (reviewed by Shaw et al, 2010).

Sialic acids are a group of derivatives of a negatively charged acidic sugar with a common nine-carbon carboxylated backbone. Sialic acids often occupy the terminal positions of cell surface glycoproteins and glycolipids. Because of their negative charge and consistent occurrence in exposed positions of cell-surface molecules, they often function as key determinants of oligosaccharide structures that mediate a variety of biological phenomena, such as cell-cell interaction, cell migration, adhesion, and regulate pathological events such as inflammation and cancer metastasis. The apposition of the blastocyst to the epithelium during implantation involves the interaction of
glycosylated proteins, lipids, and mucins. In rabbits, the uterine luminal epithelium undergoes extensive differentiation accompanied by a loss of surface negativity prior to the time of blastocyst implantation (Anderson and Hoffman, 1984). Similarly, the loss of surface negativity and the alterations in glycocalyx have been noted prior to implantation in rats and mice (Hewitt et al, 1979; Chávez and Anderson, 1985). Sialic acids seem to be a major component of the negative charge since a treatment with neuraminidase, which hydrolyses sialic acid residues, replicates the effect (Jenkinson and Searle, 1977). Previous studies have reported decreased sialic acids in the endometrium at the time of implantation in rats and rabbits (Rajalakshmi et al, 1972; Anderson et al, 1986) and there is reduced sialylation in the decidua of early pregnancy in women (Jones et al, 2010). An increase of sialic acids seems to cause delayed implantation in rats (Sankaran and Prasad, 1973), suggesting an involvement of sialic acids in the normal blastocyst implantation.

Little is known about the role of glycoconjugates including sialic acids in the ectopic implantation of blastocysts in the Fallopian tube. We recently reported the altered expression and localization of galectin-1 and galectin-3 in the Fallopian tube from women with ectopic implantation (Nio-Kobayashi et al, 2015). Galectins are endogenous lectins that bind to β-galactoside sugar residues on cell surface, and
galectin-1 increases but galectin-3 decreases during ectopic implantation in women (Nio-Kobayashi et al., 2015). This suggests the potential involvement of lectin-glycoconjugate interaction, especially galectin-1-glycoconjugate binding, in the pathogenesis of ectopic implantation although the glycoconjugate targets for galectins and their functions during normal and pathological implantation are still unclear. Galectins possesses high affinity to galactose $\beta$-linked to $N$-acetylgalactosamine ($N$-acetyllactosamine) and this binding can be modified by sialylation. While $\alpha$2,6-sialylation on the terminal galactose inhibits the binding of galectin-1, it has no effect on the binding of galectin-3. Thus, sialylation is an important modification for the ligand recognition of galectins, and it may alter during ectopic implantation in association with the change in galectin expression. A family of glycosyltransferases, called sialyltransferases, catalyzes the transfer of a sialic acid to an acceptor carbohydrate. The enzymes which transfer sialic acid to the terminal galactose residues of glycochains are classified into two groups according to the linkage of the catalyzed sialic acid: $\beta$-galactoside $\alpha$2,3-sialyltransferases (ST3GAL) and $\beta$-galactoside $\alpha$2,6-sialyltransferases (ST6GAL). There are six members of the ST3GAL family and two members of the ST6GAL family in mice and humans (Harduin-Lepers et al., 2001).

We hypothesized that glycoconjugates are altered in tubal ectopic pregnancy and
there are antecedents in women at increased risk of tubal ectopic pregnancy. To elucidate the function of sialic acids during normal tubal function and ectopic implantation, we investigated 1) the expression of \( ST3GAL1−6 \) and \( ST6GAL1−2 \) in the Fallopian tubes collected from non-pregnant women across the menstrual cycle and from women with tubal ectopic pregnancy; 2) the localization of \( \alpha2,3- \) or \( \alpha2,6 \)-sialic acids in Fallopian tubes collected from non-pregnant women across the menstrual cycle and from women with tubal ectopic pregnancy. To examine Fallopian tube from women with increased risk for ectopic pregnancy, we studied 3) the effects of cigarette smoking on the expression of sialyltransferases and the localization of sialic acids.
Results

Expression of enzymes involved in sialylation in the Fallopian tube

Transcripts encoding both $\beta$-galactoside $\alpha_2,3$-sialyltransferases ($ST3GAL1$−$6$) and $\beta$-galactoside $\alpha_2,6$-sialyltransferases ($ST6GAL1$−$2$) are expressed in the Fallopian tubes of non-pregnant women. These enzymes are expressed both in the proliferative and secretory phase of the menstrual cycle (Table 2). Transcript abundance of $ST6GAL1$ was increased during the mid-secretory phase when compared to the proliferative phase ($P<0.0001$). However in the Fallopian tube from women with ectopic implantation, there was significant decrease in the expression of $ST6GAL1$ ($P<0.0001$). In addition women with tubal ectopic pregnancy had reduced expression of $ST3GAL3$ ($P=0.0029$), $ST3GAL5$ ($P=0.0089$) and $ST3GAL6$ ($P=0.0018$) in the Fallopian tube when compared to that in non-pregnant women during the mid-secretory phase (Table 2).

Influence of cigarette smoking on the expression of $\beta$-galactoside sialyltransferases in the Fallopian tube

We next examined whether cigarette smoking, a key risk factor for tubal ectopic pregnancy, could affect the expression of sialyltransferases in the human Fallopian tube. When relative gene expression was compared using all samples with mixed menstrual
cycle (n=14 for confirmed non-smokers, n=6 for confirmed smokers), the expression of
**ST3GAL5** was significantly high in smokers ($P=0.0169$; Table 3). Because the
expression of **ST6GAL1** altered during menstrual phase, we separately examined the
change in the expression of **ST6GAL1** on either proliferative phase or mid-secretory
phase. Although the sample number of confirmed smokers was small (n=3 for
proliferative or mid-secretory phases), the expression of **ST6GAL1** in the mid-secretory
phase was significantly decreased in the Fallopian tube from confirmed smokers
($P=0.0298$; Fig.1A). An increased expression of **ST3GAL5** was also significant during
the mid-secretory phase ($P=0.0006$; Fig. 1B).

*Localization of sialic acids in the Fallopian tube*

To examine sialylation within the Fallopian tube, we performed lectin histochemistry
using $\alpha_{2,3}$- or $\alpha_{2,6}$-sialic acid-specific plant lectins. Examination of both MAM
(binding to $\alpha_{2,3}$-sialic acids) and SNA (binding to $\alpha_{2,6}$-sialic acids) in the human
Fallopian tube revealed abundant and selective localization of both sialic acids, which
are catalyzed by sialyltransferases including **ST3GAL** and **ST6GAL** families (Fig. 2).
The intense reactivity for both MAM and SNA was found diffusely in the stroma of the
Fallopian tubes (Fig 2A, B). In the epithelium, both $\alpha_{2,3}$- and $\alpha_{2,6}$-sialic acids were
localized to the apical region (Fig. 2C, D). Cilia of ciliated cells abundantly possessed both $\alpha_{2,3}$- and $\alpha_{2,6}$-sialic acids (Fig. 2E, F). Non-ciliated secretory cells were labeled with various intensities and patterns for both MAM and SNA (arrows in Fig. 2E, F); $\alpha_{2,3}$-sialic acids were largely restricted to the apical surface of epithelium (Fig. 2E) while vesicular structures containing $\alpha_{2,6}$-sialic acids were found in the cytoplasm of both types of cells (Fig. 2F). This localization was consistent in the proliferative and secretory phases of the menstrual cycle and in the Fallopian tube of ectopic pregnancy, although the expression of some sialyltransferases altered. Sialylation is therefore marked in the cilia of the luminal side of epithelial cells in the Fallopian tube.

*Formation of sialic acid-containing ciliated cysts in the Fallopian tube epithelium*

Although both $\alpha_{2,3}$- and $\alpha_{2,6}$-sialic acids were mainly restricted to the apical surface in the epithelium, strongly positive spherical structures were also sporadically observed within the epithelium (arrows in Fig. 3A, B). These structures were identified as “ciliated cysts”, which are known to appear in association with abnormal ciliogenesis. Careful observations of the MAM- or SNA-stained sections (Fig. 3C–F) demonstrated that $\alpha_{2,3}$- and $\alpha_{2,6}$-sialic acids concentrated at the concaving bottom of the cilia (arrows in Fig. 3C) and stained ciliary structures with a jug-like shape below the
luminal surface of the epithelium (arrow in Fig. 3D). In other cells, stained cilia became round cysts in the cytoplasm and were completely buried into the epithelium (arrow in Fig. 3E). Finally, ciliated cysts were localized at the bottom of the epithelium (arrow in Fig. 3F) with neighboring elongated nuclei (asterisk in Fig. 3F).

*The effect of smoking on sialic acid localization in the Fallopian tube epithelium*

The same localization of sialic acids to the apical epithelium and stromal regions was seen in both women who smoked and in non-smokers. Although ciliated cysts containing $\alpha_2,3$- and $\alpha_2,6$-sialic acids were observed in both non-smoking and smoking women, their frequency was increased in women with a current smoking history ($P=0.0177$ for $\alpha_2,3$-sialic acid-positive cysts; $P=0.0802$ for $\alpha_2,6$-sialic acid-positive cysts: Fig. 4). Smoking increases the incidence of epithelial sialic acid containing ciliated cysts in the Fallopian tube.
Discussion

This study revealed that the expression of \textit{ST6GAL1}, which catalyzes \(\alpha\)2,6-sialic acid to block galectin-1 binding, in the Fallopian tube normally increases during the mid-secretory phase of the menstrual cycle. We also demonstrated that the expression of \textit{ST6GAL1} as well as \textit{ST3GAL3}, \textit{ST3GAL5}, and \textit{ST3GAL6} was significantly decreased in the Fallopian tube from women with ectopic pregnancy. Women who smoked demonstrated an increased \textit{ST3GAL5} expression as well as decreased expression of mid-secretory phase \textit{ST6GAL1} in the Fallopian tube. Both \(\alpha\)2,3- and \(\alpha\)2,6-sialic acids were localized to the apical surface of epithelium and the stroma in human Fallopian tubes. Although there was no obvious change in the localization of sialic acids during menstrual phase and with or without ectopic pregnancy, the number of sialic acid-containing ciliated cysts, which is known to be increased during abnormal ciliogenesis, significantly increased in women with smoking history. This suggests that sialic acids and the enzymes responsible for their insertion into glycoconjugates might have a role in the Fallopian tube function.

We identified that the six members of the ST3GAL family and the two members of the ST6GAL family were expressed in the Fallopian tube. Although some sialyltransferases are expressed in the female reproductive tissues including the placenta
and ovary, there was no information about their expression in human Fallopian tubes. While there are substrate specificities for each enzyme they collectively target glycoproteins, oligosaccharides, and glycolipids as acceptor substrates (Ishii et al, 1998; Kitagawa et al, 1994). ST6GAL1 utilizes the Gal\(\beta_1,3/4\)GlcNAc structure on glycoproteins and oligosaccharides as acceptor substrates (Harduin-Lepers et al, 2001) and it is ubiquitously expressed (Krzewinski-Recchi et al, 2003). We here demonstrated that \textit{ST6GAL1} transcripts were increased in the Fallopian tube in the secretory phase of the menstrual cycle. This suggests that the glycan structure may change during menstrual cycle. What happens in the endometrium during the implantation window has not been assessed; however there seems to be the potential for more \(\alpha_2,6\)-sialylation in the Fallopian tube epithelium during the secretory phase.

It is not certain whether this functions to inhibit ectopic implantation of the blastocyst. Certainly an increase in the addition of sialic acid will augment the epithelial negative charge. This might be important as it has been shown that the blastocyst also has predominately negatively charged molecules on its surface (Nilsson et al, 1975). What is clear is that there is less sialylation in the decidua in early pregnancy (Jones et al, 2010) and there are less sialylation enzymes expressed in the Fallopian tube in ectopic gestation. Reduced expression of \textit{ST6GAL1}, \textit{ST3GAL3}, \textit{ST3GAL4}, and
*ST3GAL5*, within the Fallopian tube samples from women with an ectopic pregnancy suggests that the reduced sialylation may be associated with implantation in the Fallopian tube.

Galectin-1 and galectin-3 are β-galactoside-binding animal lectins with high affinity for lactosamine residues (Galβ1,3/4GlcNAc). In ectopic pregnancy we saw increased galaectin-1 and reduced galectin-3 that is consistent increased galectin-1 activity. Galectins are expressed in implantation sites suggesting their involvement in the establishment of pregnancy (Nio-Kobayashi et al, 2015). Galectin-1 expression rather increased in cases of ectopic implantation and trophoblast cell-derived molecules enhanced the epithelial galectin-1 expression (Nio-Kobayashi et al, 2015). The sugar binding affinity for galectin-1 is blocked by α2,6-sialylation on terminal galactose and ST6GAL1 catalyzes this reaction. This suggests that the increased *ST6GAL1* in the secretory phase may block galectin-1 binding to inhibit the implantation process in the Fallopian tube. Although we cannot determine if the decrease in *ST6GAL1* in the Fallopian tube from women with ectopic implantation was a cause or effect of ectopic implantation, we found the reduction in *ST6GAL1* in the Fallopian tube of women with smoking history, a risk factor for tubal ectopic pregnancy. It is possible that loss of α2,6-sialylation and enhanced galectin-1-binding is involved in the ectopic implantation
of blastocysts in the Fallopian tube.

Although the expression of some $\beta$-galactoside sialyltransferases, especially the expression of $ST6GAL1$, altered during the menstrual phase and ectopic pregnancy, the localization of both $\alpha2,3$ and $\alpha2,6$-sialic acids revealed by lectin histochemistry did not change. The histochemical assay has not been validated as a tool where staining intensity reflects abundance in the Fallopian tube so we cannot comment on sialylation levels using this technique. However, there may be other sialyltransferases expressed that use non-galactose acceptors, as we cannot distinguish the acceptor sugar structure by the present lectin histochemistry. Further experiments, for example by use of mass spectrograph, are required to reveal whether whole glycan structures changes during menstrual phase and with or without ectopic implantation.

A key to reveal the function of galectins and glycoconjugates in the blastocyst implantation and pathogenesis of ectopic pregnancy is to clarify the ligand glycoconjugates, especially carrying $\alpha2,6$-sialylated glycoproteins. Mucin 1 (MUC1) is a possible candidate for the ligand glycoprotein of galectins in the Fallopian tube and uterus, since MUC1 is known to be recognized by galectins (Jeschke et al. 2009; Mori et al. 2015). MUC1 is a highly glycosylated protein and a decreased expression of surface MUC1 allows blastocyst to implant on the endometrium in normal pregnancy as
well as on the Fallopian tube epithelium in tubal ectopic pregnancy (Aplin et al, 2001; Savaris et al, 2008; Al-Azemi et al, 2009). It is reported that cigarette smoking alters the glycosylation of MUC1 being involved in epithelial-mesenchymal transition in airway (Zhang et al. 2014). Investigation of the change in glycan structure on MUC1 in the endometrium of non-fertile women, and in the Fallopian tube epithelium from women with ectopic implantation would be a great interest.

We also reported the formation of sialic acid-containing ciliated cysts in the epithelium of the Fallopian tubes. These ciliated cysts were observed in all sections examined but their number was significantly increased in the Fallopian tube from women who were confirmed smokers when compared to confirmed non-smokers. Cigarette smoking is one of the major risk factors for tubal ectopic implantation (Shaw et al, 2010). The intracellular ciliated cysts have been observed in ciliated epithelium of the oviducts from various animals including humans (Hagiwara, 2000), and are considered as markers of abnormal ciliogenesis: an inhibition of migration of duplicated centrioles by cytochalasin D induces the intracellular vacuoles containing cilia in the cultured oviducts of quails (Boisvieux-Ulrich et al, 1990). These data suggests that the increased number of ciliated cysts reflects impaired ciliogenesis in the Fallopian tube epithelium of women who smoked. We have previously shown that women who smoke
have alterations in cell turnover and potential loss of cilia in the luminal epithelial cells of the Fallopian tube (Horne et al. 2014). The results from this study add weight to the evidence that smoking results in structural changes of the Fallopian tube epithelial cells. While such cilia dysfunction might slow transit of a blastocyst we also suggest that this is linked to functional changes that may promote ectopic implantation.

The transcript abundance of two sialyltransferases significantly altered in the Fallopian tube from women who smoked. As well as a reduction in ST6GAL1 as discussed above, ST3GAL5 (ganglioside GM3 synthase) was increased. There is evidence that ganglioside GM3 synthesis is involved in ciliary development. St3gal5 knockout mice have impaired hearing because of a deformity in hair cells in the organ of Corti (Yoshikawa et al, 2009), implying that ganglioside GM3 is essential for the normal ciliary structure in this organ. It is not clear whether increased ST3GAL5 is linked to the abnormal ciliary structures in smokers, perhaps as a consequence of abnormal ciliogenesis or potentially a cause of inclusion cysts. Further experiments are required to reveal precise function of sialylation and its role in ciliogenesis, ciliary function, and ectopic implantation.

In conclusion, the present study demonstrates that decreased sialylation resulting in loss of surface negativity is involved in the pathogenesis of tubal ectopic pregnancy.
Decreased $\alpha_{2,6}$-sialylation may enhance the binding of galectin-1 to increase receptivity for the blastocyst in the Fallopian tube epithelium. Smoking is associated with less $\alpha_{2,6}$-sialylation-involved gene expression as well as being associated with increased ciliated cysts representing abnormal ciliary function. Although the precise roles of sialic acids are still unclear, sialylation may play an important role in pathogenesis of tubal ectopic pregnancy. We suggest that smoking alters both tubal transport (ciliary action) and enhances the potential for implantation with sialic acids being markers of both processes.
**Materials & Methods**

*Human Fallopian tube collection*

Ethical approval for this study was obtained from the Lothian Research Ethics Committee (LREC 10/S1102/40) with informed written consent from all of the women participating in this study. Serum samples and the Fallopian tube biopsies from the ampulla region of the Fallopian tube were collected at the time of hysterectomy for benign gynecological conditions or during surgical management of tubal ectopic pregnancy.

Women were 18–45 years of age. The menstrual phase of each patient at the time of hysterectomy was determined by histologic examination and staging of an endometrial biopsy taken with the Fallopian tube (non-pregnant samples only) and by measurement of serum estradiol and progesterone levels as described previously (Duncan et al, 2011). Fallopian tube samples from ectopic pregnancies were confirmed free of trophoblast contamination as described previously (Nio-Kobayashi et al, 2015). Patients were identified as smokers or non-smokers based on history from the patients combined with serum cotinine assessment as previously described (Horne et al, 2014). The serum cotinine levels were more than 100 ng/mL in the women with smoking history but negligible in non-smoking women.
Biopsies of the human Fallopian tubes were divided into equivalent portions and either immersed in RNAlater (Ambion, TX, USA) at 4°C overnight and then flash frozen at −80°C for RNA extraction, or fixed in 10% neutral-buffered formalin overnight at 4°C followed by storage in 70% ethanol and subsequent embedding in paraffin for histological staining.

**Quantitative RT-PCR (qRT-PCR)**

The Fallopian tube tissues used for a quantitative gene expression analysis were examined from confirmed non-smoking women (total 14; proliferative phase: n=5; mid-secretory phase: n=9), from women with a confirmed history of smoking (total 6; proliferative phase: n=3; mid-secretory phase: n=3), or from non-smoking women with ectopic pregnancy (n=13). Total RNA was extracted from the frozen human Fallopian tube using RNeasy Mini Kit (Qiagen Ltd., Crawley, UK) according to the manufacturer’s protocol. RNA (200 ng) was used to prepare cDNA using the TaqMan Reverse Transcription regents (Applied Biosystems, Foster City, CA, USA).

The sequences of the primer sets used for this study are listed in Table 1. Primers were pre-validated by standard PCR and by generating standard curves using qRT-PCR. Each reaction buffer contained 5.0 µL 2×PowerSYBR® Green PCR Master Mix
(Applied Biosystems), 0.5 µL primer pair (5 µM), 3.5 µL of nuclease free H₂O, and 1.0 µL cDNA, and each reaction was conducted in duplicate. The qRT-PCR cycling program consisted of a denaturing step (95°C for 10 min), annealing and extension step (95°C for 15 sec and 60°C for 1 min repeated for 40 cycles), and a dissociation step (95°C, 60°C, and 95°C for 15 sec each) using a 7900 Sequence Detection System (Applied Biosystems). The relative expression levels of each target to the housekeeping gene (G6PDH), previously validated using geNorm analysis (Primerdesign Ltd, Southampton, UK), were quantified using the ΔCt methods. After testing for normality, all statistical analyses were performed by unpaired t-tests using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA), and P<0.05 was regarded as significant. Values in the tables represent the mean ± SEM of relative expression of the target genes to G6PDH.

Lectin histochemistry

Fixed human Fallopian tube tissues from confirmed non-smokers (total 7; proliferative phase: n=2; mid-secretory phase: n=5), from confirmed smokers (total 7; proliferative phase: n=2; mid-secretory phase: n=5), and with ectopic pregnancy (n=8) were used for histological analysis. The sections, at 5 µm thickness, were de-waxed and washed in
phosphate-buffered saline (PBS). Sections were blocked using Carbo-free blocking solution for 60 min at room temperature following an incubation with Avidin/Biotin blocking solution (Vector Laboratories Inc., Burlingame, CA). Then the sections were incubated with the biotinylated *Maackia amurensis* (MAM) or *Sambucus sieboldiana* (SNA) (Seikagaku corporation, Tokyo, Japan) that specifically recognizes α2,3- or α2,6-sialic acids catalysed of glycoconjugates, which are catalysed by sialyltransferases including ST3GAL and ST6GAL families, diluted in 1:250 in PBS at 4°C overnight. The reaction sites were visualized using Vectastain ABC Elite kit for 60 min followed by ImmPACT™ DAB Peroxidase Substrate Kit (Vector Laboratories Inc.) for 5 min. The sections were counterstained with haemotoxylin and observed under a light microscope (BX51; Olympus corporation, Tokyo, Japan).

Photos of whole regions of all tissue sections stained for MAM or SNA were taken and the number of MAM- or SNA-positive ciliated cysts was counted throughout the section. The epithelium area was measured using Image J (http://imagej.nih.gov/ij/) and the number of cysts per 100,000 µm² epithelium was calculated. The difference in the number of ciliated cysts during the mid-secretory phases (n=5 from non-smokers and smokers) between the samples was analysed by unpaired t-tests using GraphPad Prism 6
software, and $P<0.05$ was regarded as significant. Values in the graphs represent the mean ± SEM.
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Conflict of Interest

There is nothing to be declared.
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Figure legends

Figure 1. Changes in mRNA expression of sialyltransferases in human Fallopian tube from women with or without smoking history.

The expression of $ST6GAL1$ is significantly low (A), while that of $ST3GAL5$ is significantly high (B) in the mid-secretory phase Fallopian tube from confirmed smokers than confirmed non-smokers.

Figure 2. Localization of $\alpha_2,3$- and $\alpha_2,6$-sialic acids in human Fallopian tube.

Lectin histochemistry using plant lectins which specifically recognize $\alpha_2,3$-sialic acids ($Maackia amurensis$: MAM) or $\alpha_2,6$-sialic acids ($Sambucus sieboldiana$: SNA), which are catalyzed by sialyltransferases including ST3GAL and ST6GAL families, shows abundant localization of both $\alpha_2,3$- and $\alpha_2,6$-sialic acids in the stroma (A, B). Apical surface of the epithelium is selectively labeled with both MAM (C) and SNA (D). Cilia of ciliated cells contain abundant $\alpha_2,3$- and $\alpha_2,6$-sialic acids (E, F) and apical surface of non-ciliated secretory cells is also labeled with both MAM and SNA (arrows in E, F). Another SNA reaction is seen in small vesicles in the cytoplasm of both ciliated cells and secretory cells (F).
Figure 3. Formation of sialic acid-containing ciliated cysts in the Fallopian tube epithelium.

MAM- and SNA-positive round structures are found in various depths of the epithelium (arrows in A, B). They are identified as ciliated cysts due to abnormal ciliogenesis. Condensed MAM reaction is found at the concaving bottom of the ciliary tuft (arrows in C), and then displays jag-like shape with contact to the luminal surface of the epithelium (arrow in D). In other cells, MAM-positive cysts are detached from the luminal surface (arrow in E), or embedded in the basal region of the epithelium (arrow in F). Asterisks show the nuclei of the ciliated cells with cysts.

Figure 4. The effect of smoking on the number of ciliated cysts in the Fallopian tube epithelium.

The number of both MAM- and SNA-positive ciliated cysts is increased in the Fallopian tube from smoking women when compared to non-smoking women during the mid-secretory phase (n=5).
A: ST6GAL1

B: ST3GAL5

Figure 1 Nio-Kobayashi et al.
Figure 2 Nio-Kobayashi et al.
Figure 3 Nio-Kobayashi et al.
Figure 4 Nio-Kobayashi et al.