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Cervical mucins carry α(1,2)fucosylated glycans that partly protect from experimental vaginal candidiasis

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Abstract Cervical mucins are glycosylated proteins that form a protective cervical mucus. To understand the role of mucin glycans in *Candida albicans* infection, oligosaccharides from mouse cervical mucins were analyzed by liquid chromatography-mass spectrometry. Cervical mucins carry multiple α(1-2)fucosylated glycans, but α(1,2)fucosyltransferase Fut2-null mice are devoid of these epitopes. Epithelial cells in vaginal lavages from Fut2-null mice lacked *Ulex europaeus* agglutinin-1 (UEA-I) staining for α(1-2)fucosylated glycans. Hysterectomy to remove cervical mucus eliminated UEA-I and acid mucin staining in vaginal epithelial cells from wild type mice indicating the cervix as the source of UEA-I positive epithelial cells. To assess binding of α(1-2) fucosylated glycans on *C. albicans* infection, an *in vitro* adhesion assay was performed with vaginal epithelial cells from wild type and Fut2-null mice. Vaginal epithelial cells from Fut2-null mice were found to bind increased numbers of *C. albicans* compared to vaginal epithelial cells obtained from wild type mice. Hysterectomy lessened the difference between Fut2-null and wild type mice in binding of *C. ablicans in vitro* and susceptibility to experimental *C. albicans* vaginitis *in vivo*. We generated a recombinant fucosylated MUC1 glycanpolymer to test whether the relative protection of wild type mice compared to Fut2-null mice could be mimicked with exogenous mucin. While a small portion of the recombinant MUC1 epitopes displayed α(1-2)fucosylated glycans, the predominant epitopes were sialylated due to endogenous sialyltransferases in the cultured cells. Intravaginal instillation of recombinant MUC1 glycanpolymer partially reduced experimental yeast vaginitis suggesting that a large glycanpolymer, with different glycan epitopes, may affect fungal burden.

Keywords Fucosyltransferase · *Candida albicans* · *Secretor* gene · Cervical mucins · Hysterectomy · ABO/Lewis blood group

Abbreviations

CFU	Colony forming unit
Fut2	*a*(1,2)fucosyltransferase "Secretor" gene
UEA-I	Ulex europaeus agglutinin I

Introduction

Vulvovaginal candidiasis is a mucosal infection caused by opportunistic *Candida* species, typically *Candida albicans*. A polymorphism in approximately 20% of humans at the *FUT2*, or *Secretor* gene (OMIM 182100) abolishes α(1,2) fucosyltransferase activity (EC 2.4.1.69) in the Golgi apparatus of mucosal glandular cells [1]. Loss of α(1-2) fucosylated glycans in the mucosal secretions of 'non-secretors' is associated with a 2.4–4.4 fold increased relative risk for recurrent vaginitis by *C. albicans* accounting for an

Domino and Hurd contributed equally to this study.

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estimated 67% of the attributable risk in women with the nonsecretor phenotype [2, 3]. While current anti-fungal medications effectively target yeast metabolism for acute treatment, recurrent infections occur in 5–10% of reproductive age women with a mean time to recurrence of 4–10 months even under optimal management [4].

The immune response elicited during an episode of recurrent vulvovaginal candidiasis appears different from classical host-defense mechanism, as infection occurs despite normal *Candida*-specific Th1-type cell-mediated immunity [5, 6] and T-cell immunodeficient knockout mice show no difference in susceptibility to vaginal candidiasis [7]. These and other studies have led to a hypothesized major role for *Candida*-epithelial cell interactions in the mechanism of vaginal susceptibility vs. resistance [8]. Due to the relative lack of cell-mediated immunity during recurrent vulvovaginal candidiasis, protection is believed to be acquired locally, possibly involving incompletely defined carbohydrate adhesion molecules [9, 10] or epithelial cell mediated growth inhibition of Candida [11, 12].

Several different fucosylated glycans have been implicated in mediating interactions between pathogens and host epithelial cells. Microbe adhesin molecules are lectin-like proteins that facilitate these interactions. Fucose-specific adhesins have been identified on germ tubes of *C. albicans* [13]. *In vitro* experiments using exogenous carbohydrates isolated from human breast milk [14] and antibodies against the H blood group antigen [15] demonstrate that *C. albicans* specifically bind α(1-2)fucosylated glycans. Many more adhesin molecules exist in the cell wall of *Candida* and the binding specificities of most have not been determined.

In our animal model of nonsecretors, Fut2-null mice, we reported an increased susceptibility to experimental vaginitis [16]. *Fut2* is expressed in secretory cells of uterine and endocervical glandular epithelium, but expression was not detected at the major site of Candidal adhesion and invasion, i.e. vaginal squamous epithelium [17]. Despite this discrepancy, *Ulex europaeus agglutinin I* (UEA-I) lectin staining demonstrated the presence of α(1-2) fucosylated glycans at the apical surface and lumen of the vagina of wild type mice [16]. In this study, we tested the hypothesis that secreted α(1,2) fucosylated mucins descend from the endocervix into the vagina, coat exposed epithelial cells with α(1-2) fucosylated glycans, and potentially alter microbe adhesion *in vitro* and fungal burden *in vivo*.

Materials and methods

Animals

Female C57BL/6 J wild type (Jackson Laboratory, Bar Harbor, ME; stock no. 000664) and Fut2-null mutant mice [18] backcrossed for 12 generations to C57BL/6 J (Jackson Laboratory stock no. 006262, designated B6.129P2-Fut2^{tm1Sdo}), 8–10 weeks of age were maintained under Specific Pathogen Free conditions and handled according to institutionally approved guidelines. Mice were maintained in pseudoestrus using a 5 mg, 21 day controlled release 17β-estradiol pellet (Innovative Research of America, Sarasota, FL) 72 h prior to subsequent experiments unless stated otherwise.

Histological staining

Vaginal washings were collected from female wild type and Fut2-null mice and processed for immunohistochemistry using either the α(1-2) fucose-specific lectin UEA-I conjugated to biotin (EY laboratories, Inc., San Mateo, CA), as previously described [18], or Alcian Blue, pH 2.5.

Preparation of cervical mucin oligosaccharides

Wild type and Fut2-null mice were euthanized 4 days after induction of pseudoestrus with 0.2 mg/week estrogen injections. Cervical mucus samples were collected and stored at -80°C. Mucus from approximately six mice was pooled and dissolved in 150 µl 'extrGuHCL' (guanidinium chloride 6 M/EDTA 5 mM/NaH_2PO_4 0.01 M/ pH 6.7 adjusted with TRIS base). Following the addition of protease inhibitor (4 µl PMSF,100 mM in 2-propanol), the samples were homogenized with a mini mortar and stirred gently over night at 4°C, followed by reduction with dithiothreitol (25 mM final concentration) in 330 µl 'red-GuHCL' (guanidinium chloride 6 M/EDTA 5 mM/TRIS 0.1 M/ pH 8.0). Alkylation with iodacetamide (63 mM final concentration) of the proteins was performed in dark conditions at 20°C over night. Samples were dialyzed against water over night (10000 MWCO, Slide-A-Lyzer, Pierce, Rockford, IL), followed by addition of 300 µl sample buffer (0.75 M TRIS-HCl pH 8.1, 2 % SDS, 0.01% bromophenol blue, 60% glycerol) before being concentrated down to 30–70 µl on 100 kD cut off membranes (Millipore, Billerica, MA). The samples were analyzed on SDS/PAGE composite gels [19] and wet blotted to PVDF Immobilon PSQ membrane (Millipore) in transfer buffer (25 mM TRIS, 192 mM glycine, 0.04% SDS, 20% MeOH) for 4 h at 400 mA. The blots were stained with Alcian Blue, visualized mucin bands excised, and the oligosaccharides released with reductive β-elimination [19].

LC-MS of oligosaccharides

Sample injection and LC was performed by using a PAL CTC autosampler (CTC Analytics AG, Switzerland) and a Agilent 1100 Series degasser and binary pump (Agilent

Technologies, CA). The samples were resuspended in 15 µl of water and 2 µl were injected onto graphitized carbon columns (20 cm × 0.18 mm id) packed in-house with 5 µm Hypercarb particles (Thermo-Hypersil, Runcorn, UK). Oligosaccharides were eluted with a H_2O/acetonitrile (ACN) gradient containing 8 mM NH_4HCO_3 (0–36% ACN 3–46 min, followed by a 10-min wash step in 80% ACN). The flow rate was 2 µl/minute and maintained by splitting the liquid flow from the pump with a fused silica restrictor. MS was performed by using a Thermo LTQ linear ion trap mass spectrometer equipped with an Ion Max ion source (Thermo, San Jose, CA) with a 50 µm fused emitter operating in negative ion mode. The capillary temperature was 250°C, the capillary voltage was 26 V, and the electrospray voltage was 3.5 kV. For MS/MS experiments, the normalized collision energy 35%, with an activation time of 30 ms. MS was performed with four scan events: full scan with mass range *m/z* 380–2000, followed by successive MS/MS scans after collision induced fragmentation for the three most intense ions in each full scan.

In vitro epithelial cell adhesion assay

Wild type and Fut2-null female mice received either an abdominal ovariectomy (control surgery) or ovario-hysterectomy (including removal of the cervix) following a Jackson Laboratories protocol for these surgeries. Following a 1–2 week recovery, pseudoestrus was induced and maintained with 0.2 mg/week intraperitoneal estrogen injections. Vaginal epithelial cells were freshly collected from control surgery and hysterectomized wild type and Fut2-null mice (n=5 per group) by lavage using approximately 100 µl PBS per mouse. Cells were pooled, washed in PBS, counted and resuspended at a concentration of 8×10^4 cells ml^{-1}. An *in vitro* filter adhesion assay described by Zhao and colleagues was used [20]. *C. albicans* (3153A) grown to stationary phase in liquid salts-proline-biotin (SPB) medium supplemented with 12.5 g/l glucose and 1 g/l*N*-acetyl-D-glucosamine (GlcNAc) [21] for 2 days at 30°C in an orbital shaking incubator were quantified using a hemocytometer. For this assay, blastoconidia (2×10^6) were grown in 25 mL sterile Erlenmeyer flasks containing 4 mL SPB medium supplemented with 1 g/L GlcNAc and incubated at 37°C with 200 rpm shaking for 3 h to promote germ tube formation. Vaginal epithelial cells (2×10^4, 250 µl) were added to each flask, incubated at 37°C with 200 rpm shaking for 30 min to allow adhesion to occur. Cells were vacuum filtered across 12 mm pore size Nucleopore Track-Etch membrane filters (Whatman, NJ) and washed with 25 mL PBS. Filters were inverted onto glass microscope slides, dried and heat fixed. Slides were stained with crystal violet, washed with tap water and dried and the number of germ tubes adhering to 100 epithelial cells were counted. The experiment was performed in triplicate with similar results.

Inoculation of mice with experimental vaginal candidiasis

Female wild type and Fut2-null mice received either an abdominal ovariectomy or ovario-hysterectomy. Following a 2 week recovery, pseudoestrus was induced using a 5 mg, 21 day controlled release 17β-estradiol pellet (Innovative Research of America) and mice underwent experimental vaginal candidiasis as described previously [16]. *C. albicans* (3153A), originally a clinical isolate now propagated in the laboratory, was grown to stationary phase in 1% phytone peptone (Becton Dickinson, Cockeysville, MD) supplemented with 0.1% glucose for 16–18 h at 30°C in an orbital shaking incubator, washed in PBS and quantified using a hemocytometer. 4 days after inducing pseudoestrus, mice were intravaginally inoculated with 5×10^5 stationary-phase *C. albicans* (3153A) in 10 µl PBS. At 2 days post-inoculation mice were sacrificed, their lower reproductive tracts (vagina and cervix of ovariectomized mice, and vagina of hysterectomized mice) were removed en-bloc, weighed, homogenized in PBS, serially diluted and plated on Sabourand dextrose agar plates and incubated at 35°C for 48 h, after which colony forming units (CFU) were determined.

Treatment of experimental vaginal candidiasis with recombinant MUC1

The human FUT2 cDNA [1] was transferred to the pcDNA6 plasmid with a Blasticidin resistance using EcoRI/XbaI. The plasmid was transfected into CHO-K1 cells permanently expressing MUC1-IgG_{2a} with 32 tandem repeats [22] using Lipofectamine 2000 (Invitrogen, Baltimore, MD). Stable clones were generated using 10 µg/mL Blasticidin and 250 µg/mL G418 (Invitrogen, Baltimore, MD). Clones (FUT2/MUC1-Ig(32TR)) were grown in T flasks in Iscove's Modified Dulbecco's Medium with 10% fetal bovine serum and single high-expressing clones were selected and adapted to grow in suspension in the protein-free medium ProCHO4-CDM (Lonza Biologicals) supplemented with ProHT supplement (Lonza Biologicals) in spinner flasks. A perfusion bioreactor culture of FUT2/MUC1-Ig(32TR) was performed in a 1 l culture volume in ProCHO4-CDM with ProHT supplement, using the following set-points: pH=6.9, pO_2=40% and T=37ºC and a perfusion rate of 0.5–0.8 ml/min.

Wild type mice underwent abdominal ovariectomy and induced into pseudoestrus as described above after a 2 week recovery. After 4 days of pseudoestrus, mice were intravaginally inoculated with 5×10^5 stationary-phase *C. albicans* (3153A) which had been pre-incubated for 15 min with either PBS or 0.02 mg of fucosylated Muc1 (FUT2/MUC1-Ig

(32TR)) mucin (eight mice per group). At 3 days post-infection mice were sacrificed, the vagina and cervix removed en-bloc, weighed, homogenized in PBS, serially diluted and plated on Sabourand dextrose agar plates and incubated at 35°C for 48 h, after which CFU were determined.

Statistics

Comparisons between groups in each experiment were made using two-way analysis of variance (ANOVA) using the program SPSS 13 (Chicago, Illinois). Statistical significance was defined as a P value of <0.05.

Results

Cervical mucins carry multiple α(1,2)fucosylated glycans, but Fut2-null mice are devoid of these epitopes

To understand the role of mucin glycans in relation to *C. albicans* infections, cervical mucins were isolated from wild type and Fut2-null mice and the *O*-linked oligosaccharides released and analyzed by mass spectrometry (liquid chromatography electrospray ionization mass spectrometry [LC-ESI-MS] and tandem mass spectrometry) [19, 23, 24]. The sequences and/or monosaccharide composition of neutral and sialylated oligosaccharides from wild type and Fut2-null mice were identified. The core structures were mainly core 1 (Galβ1-3GalNAc) and core 2 (Galβ1-3 (**GlcNAcβ1-6**)GalNAc). No sulfated oligosaccharides were detected. Deduced sequences and/or monosaccharide compositions corresponding to the fucose-containing oligosaccharides together with their relative abundance are listed in Table 1.

In wild type mice, the most abundant fucose-containing epitope among the deduced sequences was the Fuc-Gal- epitope (blood group H-type epitope), an epitope suggested to be made by the Fut2 transferase. These were absent in the oligosaccharides obtained from Fut2-null mice. Oligosaccharides with the Fuc-GlcNAc- epitope, made by Lewis-type transferases, were found on both wild type and Fut2-null mice mucins. Sequences, which are likely precursor oligosaccharides for the fucose-containing oligosaccharides were also detected. In particular, the oligosaccharide Galβ1-3GalNAcol, which is a precursor substrate for the Fut2 transferase to generate one of the most common fucosylated glycans, increased in relative abundance in the Fut2-null mouse. Several doubly charged ions corresponding to larger neutral and sialylated oligosaccharides (10–12 monosaccharide residues) were identified on mucins from both wild type and Fut2-null mice. These oligosaccharides separated into several peaks on the column, suggesting numerous glycoforms. For example octasaccharides with the molecular mass of 1553 Da were composed of at least ten different glycoforms (Table 1). Due to their large size, and the relatively low abundance of each individual glycoform, complete sequences could not be interpreted. From these data, we conclude that the mucin molecules in Fut2-null mice lack α(1-2)fucosylated glycans resulting in a very different glycan profile compared to cervical mucins from wild type animals.

Epithelial cells in vaginal washings from hysterectomized mice lack acid mucins and α(1-2)fucosylated glycans

Using lectin histochemistry on tissue sections, we previously observed UEA-I staining associated with endocervical glands and ectocervical squamous cells [16]. In the present study, we postulated that removal of the cervix by hysterectomy would allow us to separate the effects of the cervix from vaginal effects on fungal burden. In these experiments, groups of wild type and Fut2-null mice underwent either control surgery (ovariectomy alone) or ovario-hysterectomy (ovariectomy and total hysterectomy) to remove the entire cervix along with uterus. To determine the length of time necessary for cervical cells and secretions to clear from the lower reproductive tract, vaginal lavage was performed every other day post-surgery and the cells obtained processed for cytologic analysis. By 9 days post-surgery, Alcian blue pH 2.5 staining for acid mucins was absent in vaginal lavages from both surgical groups. Beginning on day 10, a strong pseudoestrus was then induced with estradiol subcutaneous pellets. The cytological appearance of epithelial cells obtaining by vaginal lavage showed a range of sizes from round, relatively small squamous cells to large, flat squamous cells (not shown). Very few leukocytes were seen in vaginal lavages indicating a satisfactory pseudo-estrus effect. On day 14, Alcian blue staining of epithelial cells from vaginal lavages was observed only in wild type mice with the cervix/uterus still present (control surgery) (Fig. 1a). Alcian blue staining in wild type mice was most intense on round, relatively small squamous cells, compared to large squamous cells. Alcian blue-positive cells were absent from the washings of hysterectomized mice (Fig. 1a). Washings from hysterectomized mice were found to contain only large, flat squamous cells with no Alcian blue staining consistent with the interpretation that epithelial cells associated with the cervix or near the cervix are the source of the Alcian blue-positive squamous cells. Similar results on Alcian blue staining were obtained by hysterectomy in Fut2-null mice (supplemental Fig. 1).

In parallel cytology slides, vaginal lavages from control surgery and hysterectomy surgery mice were also stained with UEA-I for α(1-2)fucosylated glycans. After control surgery, wild type mice expressed UEA-I positive glycans associated with squamous epithelial cells, while Fut2-null mice did not (Fig. 1b). UEA-I staining was heterogeneous

Table 1 Fucosylated oligosaccharides and nonfucosylated precursor ions detected in wild type and Fut2-null mice

Molecular mass	Ret.time (min)	Sequence/composition[a]	No. of isoforms	Fuc epitope	Relative abundance[b]	
					Wild type	Fut2-null
385	14.8	Gal-3GalNAcol	1		+	++++
531	30.8	Fuc-Gal-3GalNAcol	1	Fuc(α1-2)Gal-	+	–
676	19.4	Gal-3**(NeuAc-6)**GalNAcol	1		+	++
734	31.3	Fuc-Gal-3**(GlcNAc-6)**GalNAcol	1	Fuc(α1–2)Gal-	+	–
750	21.1	Gal-3**(Gal-GlcNAc-6)**GalNAcol	1		+	++
750	22.4	Gal-GlcNAc-Gal-3GalNAcol	1		+	+++
822	36.6	Fuc-Gal-3**(NeuAc-6)**GalNAcol	1	Fuc(α1–2)Gal-	++	–
896	18.8	Gal-3**(Gal-(Fuc-)GlcNAc-6)**GalNAcol	1	Fuc(α1–3/4)GlcNAc-	+	++
896	22.9	Gal-(Fuc-)GlcNAc-Gal-3GalNAcol	1	Fuc(α1–3/4)GlcNAc-	+	–
896	26.0	Gal-3**(Fuc-Gal-4GlcNAc-6)**GalNAcol	1	Fuc(α1–2)Gal-	+	–
896	31.5	Fuc-Gal-3(**Gal-GlcNAc-6**)GalNAcol	1	Fuc(α1–2)Gal-	+	–
1042	32.7	Fuc-Gal-3(**Fuc-Gal-4GlcNAc-6**)GalNAcol	1	Fuc(α1–2)Gal-	+	–
1187	25.7	Fuc-Gal-GlcNAc-Gal-3**(NeuAc-6)**GalNAcol	1	Fuc(α1–2)Gal-	+	–
1187	30.4	NeuAc-Gal-3**(Fuc-Gal-4GlcNAc-6)**GalNAcol	1	Fuc(α1–2)Gal-	+	–
1187	36.4	Fuc-Gal-3(**NeuAc-Gal-GlcNAc-6**)GalNAcol	1	Fuc(α1–2)Gal-	++	–
1333	30.2	NeuAc-Gal-3**(Fuc-Gal-(Fuc-)GlcNAc-6)**GalNAcol	1	Fuc(α1–3/4)GlcNAc-+Fuc(α1–2)Gal-	+	–
749	21.9	2 Gal, GlcNAc, GalNAcol	1		+	–
1042	31.5	2 Fuc, 2 Gal, GlcNAc, GalNAcol	1		+	–
1187	21.9–26.3	NeuAc, Fuc, 2 Gal, GlcNAc, GalNAcol	3		+	+
1478	27.9, 28.9	2 NeuAc, Fuc, 2 Gal, GlcNAc, GalNAcol	2		+	–
1553	27.5–33.2	NeuAc, Fuc, 3 Gal, 2 GlcNAc, GalNAcol	10		++	–
1627	24.1–24.8	2 Fuc, 4 Gal, 3 GlcNAc, GalNAcol	2		–	++
1715	33.4–35.0	NeuAc, Fuc, 4 Gal, 2 GlcNAc, GalNAcol	3		+	–
1773	23.2–23.9	2 Fuc, 4 Gal, 3 GlcNAc, GalNAcol	2		–	+++
1844	30.7–36.1	2 NeuAc, Fuc, 3 Gal, 2 GlcNAc, GalNAcol	5		+	–
2209	33.4–38.0	2 NeuAc, Fuc, 4 Gal, 3 GlcNAc, GalNAcol	6		++	–
2355	35.3–42.1	2 NeuAc, 2 Fuc, 4 Gal, 3 GlcNAc, GalNAcol	5		++++	–

Compositions and sequences were elucidated by LC-ESI-MS and LC-ESI-MS/MS

[a] The saccharides marked in bold are located on C-6 of GalNAcol. The following assumptions have been made based on previously published data confirming typical mucin monosaccharide components in mouse:[39] all deoxyhexoses are fucose (Fuc); hexoses are galactose (Gal); *N*-acetylhexosamines are *N*-acetylglucosamines (GlcNAc), *N*-acetylhexosaminitols are *N*-acetylgalactosaminitols (GalNAcol); NeuAc are *N*-acetylneuraminic acid.

[b] Measured as LC-MS chromatogram peak areas compared to the largest peak area of the oligosaccharides listed in the table. Oligosaccharides marked (−) were not detected in the sample.

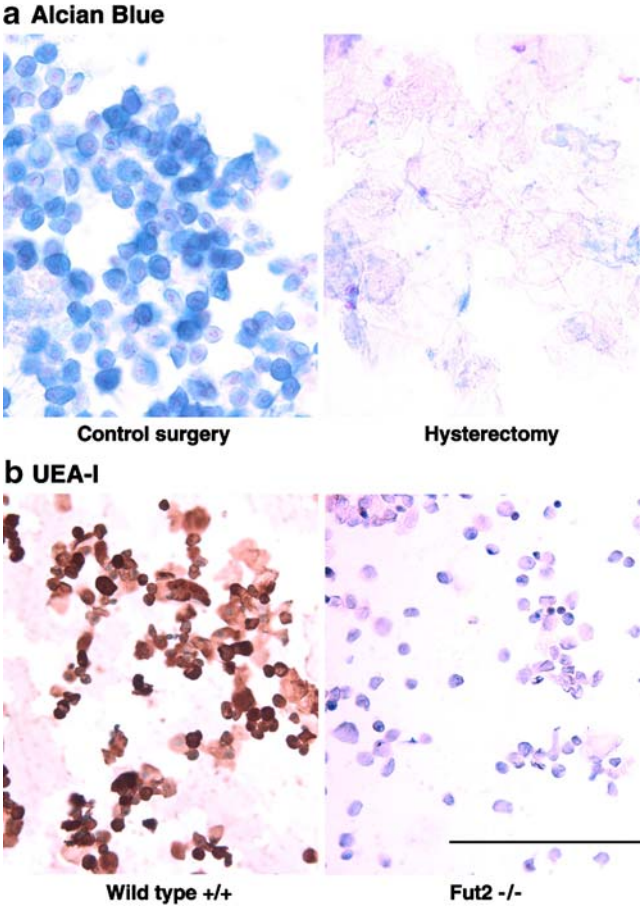

Fig. 1 Vaginal epithelial cells obtained by vaginal lavage from wild type and Fut2-null mice stained with UEA-I and Alcian blue pH 2.5. Wild type and Fut2-null mice underwent either control surgery (ovariectomy alone) or hysterectomy (ovariectomy and total hysterectomy) to remove the entire cervix along with uterus. (**a**) Intense Alcian blue pH 2.5 mucin staining was present on epithelial cells after control surgery, but absent in vaginal epithelial cells from hysterectomized mice. (**b**) Specific UEA-I lectin staining was associated with vaginal epithelial cells from wild type mice, but absent in washings Fut2-null mice. Bar=100 μm. (see Supplemental Fig. 1 for positive and negative controls in all groups)

with the most intensely stained cells being round and relatively small, whereas larger epithelial cells showed almost no UEA-I staining. In hysterectomized mice, neither wild type nor Fut2-null mice showed UEA-I positive staining (supplemental Fig. 1). Taken together, these data show that hysterectomy removes Alcian blue-positive and UEA-I positive cells from the vagina, and the most likely source of Alcian blue-positive and UEA-I positive epithelial cells is the cervix.

Increased binding of C. albicans to epithelial cells from vaginal lavages of Fut2-null mice

Groups of wild type and Fut2-null mice underwent either control surgery (ovariectomy alone) or ovario-hysterectomy (ovariectomy and total hysterectomy) as described above. After a post-operative recovery period of two weeks, pseudoestrus was induced with estrogen pellet. Using a previously published *in vitro* adhesion assay [20], vaginal squamous cells were collected by lavage and incubated with germinating *C. albicans*. The *in vitro* adhesion assay chosen utilizes germinating *C. albicans* since this stage of growth is the most clinically relevant. Epithelial cells were incubated with germinating *C. albicans* for 30 min, vacuum filtered across 12 micrometer pore size filters, and extrensively washed. Following staining with crystal violet, the number of yeast organisms adherent to 100 squamous cells were counted by light microscopy. Germinating *C. albicans* showed greatest adherence to squamous cells isolated from Fut2-null mice with an intact cervix. These had a mean of nine yeast bound per epithelial cell, compared with means around four yeast bound per epithelial cell from the other groups (P=<0.01, Fig. 2). Removal of the cervix by hysterectomy from wild type mice did not alter the total number of *C. albicans* binding to vaginal squamous cells.

Hysterectomy reduces susceptibility of Fut2-null mice to experimental vaginal candidiasis

In comparison to the *in vitro* adhesion data in the previous experiment, we wanted to determine the effect of removal of the cervix on fungal burden *in vivo*. Groups of wild type and Fut2-null mice underwent either control surgery (removal of ovaries) or ovario-hysterectomy surgery (ovariectomy and total hysterectomy) as described above. After a

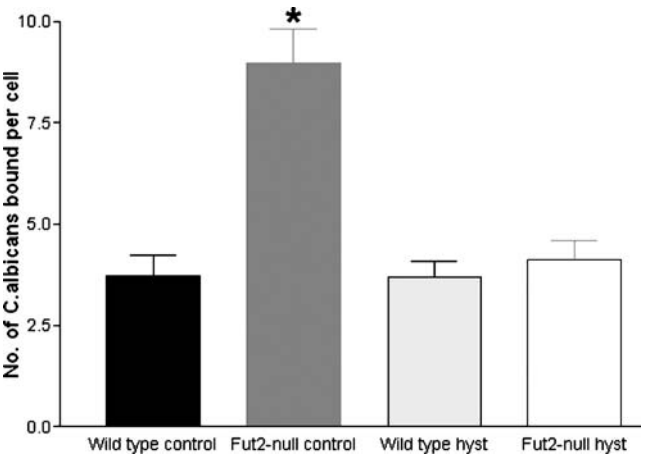

Fig. 2 Analysis of adherence of vaginal epithelial cells to *C. albicans* in an *in vitro* adhesion assay. Following estrogen treatment, vaginal epithelial cells from wild type and Fut2-null mice after control surgery or hysterectomy surgery (n=5) were incubated with *C. albicans* in an *in vitro* adhesion assay. Error bars show S.E.M. The experiment was performed three times with similar results (a representative fig. is presented) and analyzed using two-way ANOVA. Statistically significant difference (p=<0.01) indicated by *

post-operative recovery period of two weeks, pseudoestrus was induced with estrogen and *C. albicans* inoculated vaginally. In our initial experiments, the time course for fungal burden was monitored on a daily basis using vaginal swabs. We observed peak fungal burden at days 2–3 post-inoculation (data not shown). In subsequent experiments, mice were euthanized 2 days post-inoculation and fungal burden quantified by removing the entire vagina, homogenizing, and measuring CFU of *C. albicans* per g of vaginal tissue. Consistent with our previously reported increased susceptibility of Fut2-null mice to experimental vaginal candidiasis [16], Fut2-null mice showed a 3-fold increase in susceptibility to *C. albicans* compared to wild type mice after control surgery ($p=< 0.05$) (Fig. 3). Unexpectedly, the increased susceptibility of Fut2-null mice compared to wild type mice was eliminated by hysterectomy (Fig. 3). While the mechanism of the increased susceptibility of Fut2-null mice is not known, we hypothesis that *C. albicans* binds more strongly to non-α(1-2)fucosylated glycans on epithelial cells associated with the cervix, consistent with the interpretation of the *in vitro* cell binding experiments. Hysterectomy with removal of the cervix was observed to reduce this susceptibility normalizing the difference between wild type and Fut2-null mice, but the precise mechanisms are not know, nor the glycan structures that specifically binds *C. albicans* known.

Generation and analysis of recombinant MUC1 glycanpolymer

The relative protection of wild type mice compared to Fut2-null mice to experimental yeast vaginitis may be due to the

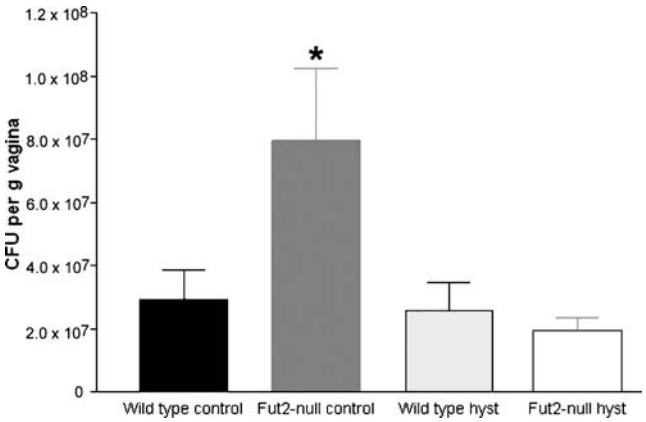

Fig. 3 Quantification of colonization of *C. albicans* in wild type and Fut2-null mice after control surgery or hysterectomy surgery. Following surgery and estrogen treatment, wild type and Fut2-null mice (n=11–13 per group) were inoculated vaginally with *C. albicans* to induced experimental vaginal candidiasis. After 2 days, mice were euthanized and the vagina was removed, homogenized, and fungal burden determined. Error bars show S.E.M. The experiment was performed in triplicate with similar results. For statistical analysis, the three trials were combined and subjected to two-way ANOVA. Statistically significant differences (p=<0.05) are indicated by *

secretion of cervical mucins. To test this hypothesis, we administered exogenous mucins displaying α(1-2)fucosylated glycans. We generated a recombinant MUC1 glycanpolymer by transfecting human FUT2 cDNA into CHO-K1 cells (which lack a functional Fut2 gene) permanently expressing the extracellular MUC1 with 32 tandem repeats fused to mouse-IgG2a [22]. A perfusion bioreactor culture of FUT2/MUC1-Ig(32TR) cells were performed and the resulting recombinant MUC1 glycanpolymer purified and analyzed for carbohydrate composition (Table 2). Just as for the cervical mucins (Table 1), the NeuAca2-6GalNAc substructure was relatively abundant. However, in contrast to the cervical mucins, where the core 1 Galb1-3GalNAc was largely fucosylated, the recombinant mucin was largely sialylated. The recombinant MUC1 mucin was determined to contain 4% Fuc-2Gal-3GalNAc oligosaccharides (Table 2). The activity of the introduced FUT2 transferase was thus not sufficient to override the endogenous sialyltransferases in CHO cells to make a mucin more similar to the mouse cervical mucins [25]. The recombinant MUC1 mucin thus displayed a smaller proportion of α(1-2)fucosylated glycans relative the cervical mucins.

To test the recombinant MUC1 glycanpolymer as a potential therapeutic treatment for yeast vaginitis, we "treated" mice in the experimental yeast vaginitis model with intravaginal instillation of recombinant MUC1 glycanpolymer. Ovariectomized wild type mice underwent pseudoestrus and intravaginal inoculation with *C. albicans* (3153A) which had been pre-incubated for 15 min with either PBS or 0.02 mg of recombinant MUC1. For 2 days mice underwent daily intravaginal instillation with PBS or recombinant MUC1. Mice were euthanized at 3 days post-inoculation and fungal burden determined by excising the entire vagina and quantifying CFU per g of vaginal weight (Fig. 4). A trend to reduced fungal burden in the recombinant MUC1 group was observed although this failed to reach statistical significance. This suggests that a large glycanpolymer may affect fungal burden but the possibility remains that mucins could affect positively or negatively multiple steps in adhesion and/or proliferation of *C. albicans*.

Discussion

Small numbers of *C. albicans* are normally found as part of the human vaginal flora and is tolerated by the vaginal mucosal immune system [26]. Perturbations in the vaginal flora due to pH and other factors allow this opportunistic organism to multiply leading to symptomatic infection [8]. A woman's hormonal changes during the ovulatory cycle have profound effects on the potential for infection. At a molecular level, estrogen is required for candidal proliferation and

Table 2 Relative amounts of O-glycans on a recombinant Fut2 MUC1 tandem repeat (32 repeats) fused with mouse IgG Fc

Structures Cho/MUC1/Fut2	m/z	Corrected Area[a]	Rel. abundance %
Gal-3GalNAcol	385	2678	0.3
Fuc-Gal-3GalNAcol	531	45669	4.3
Gal-3**(NeuAc-6)**GalNAcol	676a	1975	0.2
NeuAc-3Gal-3GalNAcol	676b	737228	69
NeuAc-3Gal-3**(NeuAc-6)** GalNAcol	967	284894	27

[a] Correction factors from [40].

adhesion *in vivo*, reduces epithelial-cell-mediated anti-candidal activity, and causes a decrease in immunoglobulins in vaginal secretions [27–29]. We postulate that estrogen-regulated Fut2 is involved in host-microbe interaction with *C. albicans* since: 1) Epidemiological evidence indicates that ABO/Lewis histo-blood group non-secretors are more susceptible to recurrent vulvovaginal candidiaiss [2, 3]; 2) Fut2-null mice display an increased susceptibility to experimental vaginal candidiasis [16]; and 3) Fucose has been implicated in *C. albicans* adhesion from various *in vitro* studies [13–15].

Previously, we have shown cell-specific localization of Fut2 within secretory glands of the endocervix. This finding is consistent with its predicted role in fucosylating glycans, principally mucins, within mucosal secretions. Since Fut2 expression was not detected in vaginal squamous epithelial cells, while UEA-I lectin staining was found within the vaginal lumen and on exfoliated epithelial cells of wild type mice, we proposed that UEA-I reactivity was due to the presence of α(1-2)fucosylated glycans secreted from the endocervix [16]. In this study, staining of vaginal washings from estrogen-treated hysterectomized wild type and Fut2-null mice with Alcian blue pH 2.5 and UEA-I supports this hypothesis. We showed that cervical mucins carry a number of glycans with the Fucα1-2Gal epitope as made by the Fut2 enzyme. This epitope is attached to GalNAc-Ser/Thr, is the most abundant sub-structure, and as expected is absent in Fut2-null mice. In humans, fucosylated blood group antigen [30] and mucin expression [31] within cervical mucus varies during the ovulatory cycle.

Removal of the cervix and uterus from Fut2-null mice was found to decrease the adhesion of germinating *C. albicans* to isolated vaginal epithelial cells *in vitro*. In testing the overall effect of removal of cervical secretions on fungal burden *in vivo* by surgically removing the cervix and uterus, we found that hysterectomy lessened the difference between wild type and Fut2-null mice. Removal of the cervix and uterus likely did more than remove mucins, it also removed other cervical mucus components that may promote yeast growth making the vaginal environment less conducive to experimental vaginitis. Since all mice underwent oophorectomy followed by pseudoestrus with exogenous estrogen, we attempted to minimize differences due to hormone levels.

The relationship of cervical mucus changes during the ovulatory cycle to infection by micro-organisms is complex since cervical mucus provides a chemical, as well as physical, barrier to infection and absence of these factors may promote the opportunity for microbes to proliferate [32, 33]. Under estrogen control, cervical mucins act as a hydrated scaffold for spermatozoa only around the time of ovulation while at other times preventing passage of spermatozoa or ascending infection. For *Neisseria gonorrhoeae*, ascending infection is highest during the early proliferative phase of the menstrual cycle and is less prevalent among women who use oral contraceptives [34]. In contrast, *C. albicans* infection is more common in the luteal phase of the menstrual cycle, with use of oral contraceptive and hormone therapy, and under non-hormonal factors such as antibiotic use, uncontrolled diabetes mellitus, and HIV infection [35]. Hence, it is possible that one set of cervical secretions allow for growth/proliferation of fungus, while another differentially affects adhesion.

Results from this study suggest that it is the epithelial cells from cervix that carries fucosylated glycans, but it is not known if this is due to their endogenous expression or the binding of fucosylated glycoconjugates. In vaginal cells collected by lavage, the binding of germinating *C. albicans*

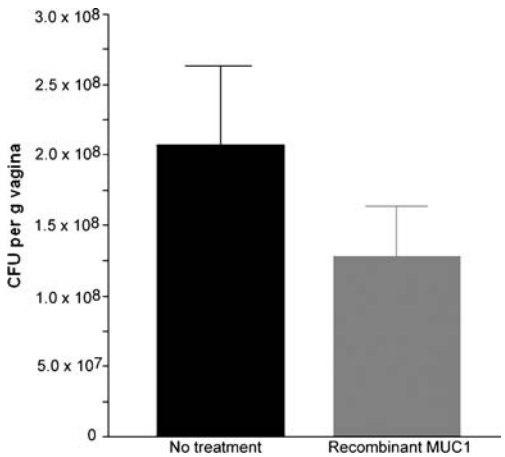

Fig. 4 Treatment of experimental vaginal candidiasis with fucosylated MUC1. Control surgery wild type mice underwent experimental vaginal candidiasis. *C. albicans* aliquots were pre-incubated with either PBS or fucosylated extracellular MUC1 (FUT2/MUC1-Ig (32TR)) prior to inoculation (eight mice per group). Once a day for the next two days, an additional intravaginal treatment was performed with PBS or fucosylated MUC1. Mice were euthanized at 3 days post-inoculation and fungal burden determined. Error bars show S.E.M. (no statistically significant difference)

in vitro differs between wild type and Fut2-null mice. Future experiments may further examine the mechanism by exposing vaginal cells from wild type mice to cervical secretions from Fut2-null mice and measuring *C. albicans* binding *in vitro* and testing the effects of recombinant mucins on binding and proliferation of *C. albicans*. It is possible that soluble mucins and other components from cervix secretions bind *C. albicans* separate from the binding to the epithelial cells. For instance, the major mucin secreted from cervix, fucosylated Muc5b [23], may bind *C. albicans* and compete with the binding to the vaginal cells. Furthermore, vaginal cells collected by lavage may not represent the binding properties of epithelial cells of the vaginal epithelium. Instead, exfoliated epithelial cells may act as a sink that are trapping *C. albicans* and slowly removing them from the vagina.

There is limited clinical information on whether removal of the cervix affects susceptibility to yeast vaginitis in humans. One epidemiological study suggests hysterectomized patients harbor more aggressive strains of Candida because they are less likely to respond to one course of therapy compared to non-hysterectomized patients [36]. However, this is a limited study that did measure additional factors, e.g., use of estrogen, type of hysterectomy, or vaginal pH, which is more acidic in hysterectomized women, because of absence of endocervical secretions [37, 38]. Further research is required to assess whether leaving the cervix in place with a supracervical hysterectomy compared to a total hysterectomy would affect the risk of recurrent yeast vaginitis.

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