

Expression of the transmembrane mucins, MUC1, MUC4 and MUC16, in normal endometrium and in endometriosis

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STUDY QUESTION: Are the transmembrane mucins, MUC1, MUC4 and MUC16, differentially expressed in endometriosis compared with normal endometrium?

SUMMARY ANSWER: This study revealed that transmembrane mucin expression does not vary significantly in normal endometrium during the menstrual cycle and is not altered in endometriosis relative to the epithelial marker, cytokeratin-18 (KRT18).

WHAT IS KNOWN ALREADY: Increased serum levels of the transmembrane mucin fragments MUC1, MUC4 and MUC16 that normally dominate the apical surface of simple epithelia are found in several pathological conditions, including endometriosis. Altered mucin expression in gynecologic diseases may promote infertility or endometrial pathologies.

STUDY DESIGN, SIZE, DURATION: This was a laboratory-based study of samples from 12 endometriosis patients as well as non-endometriosis control samples obtained from 31 patients.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Total RNA was isolated from endometrial biopsies of ectopic and eutopic endometrium from women with endometriosis and control patients from different stages of the menstrual cycle. Quantitative (q)-RT-PCR analyses were performed for the mucins, *MUC1*, *MUC4* and *MUC16*, relative to the epithelial marker, *cytokeratin-18 (KRT18)*, or β -actin (*ACTB*). Frozen sections from endometrial biopsies of proliferative and mid-secretory stage women with endometriosis were immunostained for MUC1, MUC4 and MUC16.

MAIN RESULTS AND THE ROLE OF CHANCE: qRT-PCR analyses of *MUC1* and *MUC16* mRNA revealed that these mucins do not vary significantly during the menstrual cycle nor are they altered in women with endometriosis relative to the epithelial marker, *KRT18*. *MUC4* mRNA is expressed at very low levels relative to *MUC1* and *MUC16* under all conditions. There was little difference in *MUC1* and *MUC16* expression between eutopic endometrial and ectopic endometriotic tissues. *MUC4* expression also was not significantly higher in the ectopic endometriotic tissues. Immunostaining for all three mucins reveals robust expression of MUC1 and MUC16 at the apical surfaces of endometrial epithelia, but little to no staining for MUC4.

LIMITATIONS, REASONS FOR CAUTION: qRT-PCR analysis was the main method used for mucin detection. Additional studies with stage III–IV endometriotic tissue would be useful to determine if changes in MUC1 and MUC16 expression occur, or if MUC4 expression increases, at later stages of endometriosis.

WIDER IMPLICATIONS OF THE FINDINGS: We report a comprehensive comparative profile of the major transmembrane mucins, MUC1, MUC4 and MUC16, relative to the epithelial marker, KRT18, in normal cycling endometrium and in endometriosis, and indicate constitutive expression. Previous studies have profiled the expression of individual mucins relative to β -actin and indicate accumulation in the luteal phase. Thus, these differences in interpretation appear to reflect the increased epithelial content of endometrium during the luteal phase.

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Key words: mucins / endometrium / endometriosis / cytokeratin-18

Introduction

The transmembrane mucins, MUC1, MUC4 and MUC16, belong to a family of very large, heavily glycosylated proteins that are characterized by a variable number of tandem repeat (VNTR) motifs. These mucins are expressed on the apical surface of nearly all simple epithelial tissues as well as various tumor cells and serve to keep epithelial surfaces lubricated and hydrated, protecting them from pathogens and environmental challenges (Hatrup and Gendler, 2008). Uterine membrane-tethered mucins play a key role in embryo implantation, a process facilitated by the loss or reduction of the mucin barrier (Braga and Gendler, 1993; Brayman *et al.*, 2004; Dharmaraj *et al.*, 2009). Dysregulated mucin expression is associated with various disease states (Hollingsworth and Swanson, 2004; Rose and Voynow, 2006; Vlad *et al.*, 2006; Margarit *et al.*, 2010; Chang *et al.*, 2011; He *et al.*, 2011) and high levels of transmembrane mucin expression reduce drug efficacy and protect cells from apoptosis (Hollingsworth and Swanson, 2004; Ren *et al.*, 2004). Transmembrane mucin expression is hormonally regulated during the estrous/menstrual cycle in many species (Hey *et al.*, 1994; Surveyor *et al.*, 1995; Hild-Petito *et al.*, 1996; Hoffman *et al.*, 1998; Julian *et al.*, 2005; Gipson *et al.*, 2008) and altered expression of membrane-tethered mucins is associated with infertility (Koscinski *et al.*, 2006; Margarit *et al.*, 2010). In addition, increased serum levels of transmembrane mucin fragments are found in gynecological pathologies such as endometrial cancer and endometriosis (He *et al.*, 2011; Szubert *et al.*, 2012). Endometriosis is a benign chronic inflammatory disorder associated with an increased risk of progression to ovarian cancer and impaired fecundity (Lessey *et al.*, 2013). The Center for Disease Control reports that 11.8% of all women (~17 million in the USA alone) between the ages of 15 and 44 have endometriosis. Identifying approaches to control transmembrane mucin expression provides novel opportunities to enhance embryo implantation success, improve protective functions of the female reproductive tract and potentially reduce endometriotic spread and/or enhance the efficacy of existing therapies.

MUC1 expression has been extensively studied in human endometrium (Aplin *et al.*, 1994, 1998; DeLoia *et al.*, 1998; Horne *et al.*, 2005; Wang *et al.*, 2008); however, there is limited information available on MUC4 and MUC16 expression in human uterine tissues. Studies from Gipson and colleagues demonstrate MUC16 (CA-125) expression at the apical surface of luminal epithelia with little change during the menstrual cycle (Gipson *et al.*, 2008), in contrast to changing patterns observed for MUC1 (Hey *et al.*, 1994). Recently, serological studies indicated that CA 125 levels are useful markers of endometriosis (Patrelli *et al.*, 2011; Socolov *et al.*, 2011; Szubert *et al.*, 2012). Genetic polymorphisms in MUC4 are associated with endometriosis in certain populations (Chang *et al.*, 2011), but little information on protein and mRNA expression is available. An isolated report has shown MUC4 expression by immunostaining of secretory stage samples from women in the secretory stage of the cycle and undergoing *in vitro* fertilization (IVF) (Koscinski *et al.*, 2006). However, careful comparative studies of the expression of

these three major membrane-tethered mucins have not been performed in the cycling endometrium and endometriosis.

In the current study, we report a comprehensive expression profile of MUC1, MUC4 and MUC16 in normal endometrium during the menstrual cycle and in eutopic and ectopic endometrium of women with and without endometriosis. Mucin expression profile studies so far have been performed relative to the housekeeping gene, β -actin (ACTB). Here, we use the epithelial-specific markers, cytokeratin-18 mRNA (*KRT18*) or pan-cytokeratin antibodies, as controls for changes in the content of the uterine epithelial population that occur during the menstrual cycle (Moll, 1983).

Materials and Methods

Sample collection

All patient samples were collected according to the Institutional Review Board for Baylor College of Medicine and affiliated hospitals, Greenville Hospital System and University of North Carolina School of Medicine and processed at Rice University with approval from the Institutional Review Board at Rice University. Sample collection was performed as described previously (Hawkins *et al.*, 2011; Plante *et al.*, 2012). All tissue samples were collected with patient consent and approvals from the Institutional Review Board for Baylor College of Medicine and affiliated hospitals, Greenville Hospital System and University of North Carolina School of Medicine and processed at Rice University with approval from the Institutional Review Board at Rice University. Tissue collection was performed as described previously (Hawkins *et al.*, 2011; Plante *et al.*, 2012). Normal endometrium tissue samples from S.L.Y. (Plante *et al.*, 2012) were obtained from a tissue library, with participants' cycles ranging from 25 to 35 days. Samples were randomized to a specific proliferative cycle day or a specific day post-luteinizing hormone (LH) surge. Endometrial histology and dating was performed according to Noyes criteria (Noyes *et al.*, 1950). Matched samples of endometrium and endometriosis obtained were not LH timed but confirmed histologically according to Noyes criteria (Noyes *et al.*, 1950). Samples were categorized as proliferative ($n = 6$), early secretory ($n = 4$), mid-secretory ($n = 6$) and late secretory ($n = 4$), and stage-matched eutopic and ectopic endometrial samples (total $n = 6$; early secretory, stages I-II, $n = 1$; secretory, stage II, $n = 3$; proliferative, stage II, $n = 1$; mid-secretory, stage III, $n = 1$).

For the purpose of immunohistochemistry, samples from normal secretory endometrium, and proliferative and mid-secretory endometriosis grade stage II were used. These samples obtained from S.M.H. (Hawkins *et al.*, 2011) were from women with regular menstrual cycles, who were free from hormones for at least 30 days before surgery. Menstrual cycle phase was determined by patient-provided information and confirmed by pathology according to standard criteria (Noyes *et al.*, 1950). Samples were categorized as non-endometriosis proliferative ($n = 5$), non-endometriosis secretory ($n = 6$) and endometriotic samples (total $n = 6$; proliferative, $n = 2$; secretory, $n = 2$; interval phase, $n = 1$; inactive phase, $n = 1$).

RNA isolation, DNase treatment and qRT-PCR

Total RNA was isolated as described (Hawkins *et al.*, 2011; Plante *et al.*, 2012). RNA was diluted with nuclease free water to a volume of 40 μ l and

DNase treated (Ambion, Austin, TX, USA; AM1906) according to the manufacturer's instructions. Reverse transcriptase reactions were performed using 1 µg of total RNA in a 20 µl reaction using qScript cDNA Super mix (Quanta; 95048) incubated for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. Real-time PCR was performed using the primer sequences shown in Table 1 and SYBR Green Super mix (Quanta Bioscience). Samples were cycled for 30 s at 95°C, 30 s at 59°C and 30 s for 72°C for 40 cycles for *MUC1*, *MUC16*, *KRT18* and *ACTB*. For *MUC4*, and for accompanying *KRT18*, samples were cycled for 30 s at 95°C, 15 s at 58°C and 15 s for 72°C for 40 cycles. The relative amounts of *mucin* mRNA were identified using the comparative threshold cycle method and normalized to that of either *KRT18* or *ACTB*. Primer sets not previously described were designed using Primer-BLAST (NCBI) and were validated according to protocols in the BioRad CFX96 manual.

Immunofluorescence analysis

Frozen sections from mid-secretory stage of cycling endometrium were thawed at room temperature for 20 min, rehydrated with 1 × PBS for 10 min and fixed with 4% (wt/vol) paraformaldehyde for 10 min. Slides were washed three times for 10 min each with 1 × PBS, blocked for 1 h with [1% (wt/vol) BSA in PBS] and washed three times with 1 × PBS. Primary antibody was added and incubated overnight at the indicated dilutions: rabbit polyclonal antibody to wide spectrum cytokeratin, 1:50 (Abcam; ab9377); mouse monoclonal anti-MUC4, 1:250 (kindly provided by Dr S.K. Batra, University of Nebraska); rabbit anti-MUC1, 1:50 (CT-1, ref.); mouse monoclonal anti-MUC16 (OC125), 1:200 (kindly provided by Dr Robert Bast, MD Anderson Cancer Center). Mouse and rabbit IgG controls (Jackson ImmunoResearch Laboratories) at 250 ng each as well as a secondary antibody only were used as controls. Slides were washed three times for 10 min each with 1 × PBS, then incubated with 1:400 dilution of secondary antibody, Alexa-fluor 647 goat anti-mouse (Invitrogen; A21235) or Alexa-fluor 488 goat anti-rabbit (Invitrogen; A11034) in 1% (wt/vol) BSA in PBS for 1 h in the dark, and washed three times for 10 min with 1 × PBS. Samples were mounted with Prolong-Antifade with DAPI as per manufacturer's instructions (Invitrogen) and subjected to analyses by confocal microscopy (LSM710).

Tissue samples from proliferative stage endometriosis were embedded in O.C.T. and stored at -80°C. Tissue blocks were incubated at -20°C overnight prior to sectioning in a Leica cryotome. Ten micron tissue sections were then processed as described above. Tissue sections were imaged using a Nikon AI-Rsi confocal microscope.

The pancreatic adenocarcinoma cell line, HPAF was plated in an 8-well chamber slide in RPMI + 10% (vol/vol) FBS until confluent. Cells were fixed in 4% (vol/vol) paraformaldehyde for 10 min, then in 0.02% (vol/vol) Triton X-100 for 2 min. Cells were blocked in filtered 3% (wt/vol) BSA for 1 h, followed by overnight incubation at 4°C with

MUC4 antibody (8G7 from Dr Batra) at a 1:100 dilution in filtered 3% (wt/vol) BSA. Cells were washed three times for 5 min each in PBS, and then incubated with goat anti-mouse AlexaFluor 647 at a dilution of 1:400 in filtered 3% (wt/vol) BSA for 1 h at room temperature, followed by two 5 min washes in PBS. Cells were then washed in PBS + DAPI for 5 min. Coverslips were mounted using ProLong Gold anti-fade agent and cells were imaged on a Zeiss LSM 710 confocal microscope (Supplementary data, Fig. S1).

Statistical analysis

Statistical analyses were performed using a two-tailed student's *t*-test (GraphPad InStat program; GraphPad Software, Inc., San Diego, CA, USA).

Results

Mucin mRNA profiles in normal endometrium across the menstrual cycle

Levels of *MUC1* and *MUC16* mRNAs have been previously profiled in human endometrial tissues (Hey et al., 1994; Gipson et al., 1997, 2008; Hebbar et al., 2005). These studies did not account for variations in uterine epithelial cell populations during the menstrual cycle. We therefore examined mucin mRNA expression levels by qRT-PCR in the different stages of the cycling endometrium relative to the epithelial marker, cytokeratin-18, *KRT18* (Fig. 1). The mucins, *MUC1*, *MUC4* and *MUC16*, were expressed in all stages of the cycle with very low expression of *MUC4* detected in all cases. There was no significant change in *MUC1* and *MUC16* mRNA expression across the cycle when compared with *KRT18*. Since this was in apparent conflict with previous reports, we also compared *MUC1* and *MUC16* expression relative to *ACTB* used by others as a load control in similar studies. In agreement with previous reports (Hey et al., 1994; Gipson et al., 2008), we observed a rise in *MUC1* and *MUC16* mRNA expression relative to *ACTB* during the secretory phase; however, no significant changes were observed in the expression of these mucin mRNAs relative to *KRT18* (Fig. 2A and B, respectively). Thus, the apparent rise in *MUC1* and *MUC16* reported previously parallels the increased epithelial content of the endometrium during the secretory phase.

Mucin mRNA profiles in normal endometrium and endometrium of women with endometriosis

Mucin glycoproteins have been assessed as potential biomarkers or diagnostic tools in endometriosis because of their utility as markers of

Table 1 Oligonucleotide primer sequences used in this study.

Gene	Forward	Reverse	Amplicon	Reference
<i>MUC1</i>	5' AGAGAAGTTCAGTGCCAGC	5' TGACATCCTGTCCCTGAGTG	114 bp	Dharmaraj et al. (2010)
<i>MUC4</i>	5' GCCCAAGCTACAGTGTGACTC	5' ATGGTGCCGTTGTAATTTGTT	102 bp	Argueso et al. (2002)
<i>MUC4-CT</i>	5' CCATGGAGGCCAGTGCCAGC	5' GTGCTCACAGTGCTCGCCCC	94 bp	^a This Paper
<i>MUC16</i>	5' GCCTCTACCTTAACGGTTACAATGAA	5' GGTACCCCATGGCTGTTGTG	114 bp	Argueso et al. (2003)
<i>ACTB</i>	5' GATGAGATTGGCATGGCTTT	5' CACCTTACCCTGGTCCAGTTT	100 bp	Dharmaraj et al. (2010)
<i>KRT18</i>	5' GACACCAATATCACACGACTG	5' GGCTTGTAGGCCTTTACTTC	108 bp	^a This Paper

^aRefer to materials and methods for primer validation.

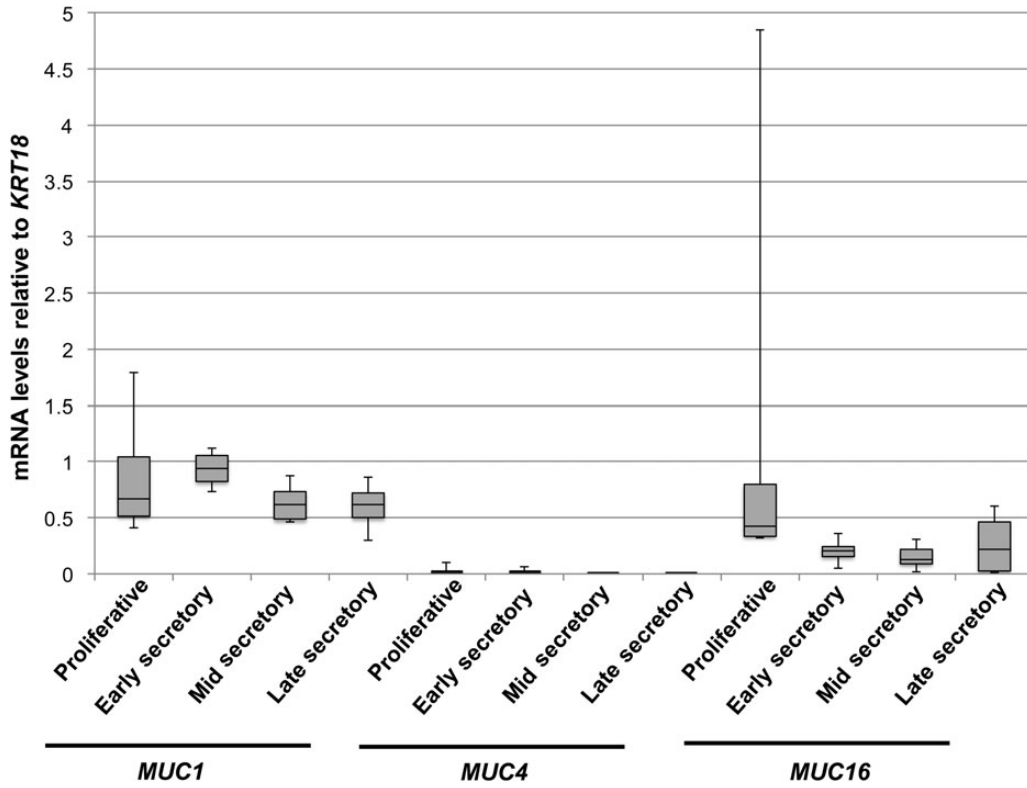


Figure 1 Mucin mRNA profile in normal endometrium. Box plots were used to express levels of transmembrane mucin mRNA levels in the cycling human endometrium. Mucin mRNA expression was determined relative to that of epithelial marker, cytokeratin-18 (*KRT18*) by qRT-PCR as described in Materials and Methods. Mucins were expressed at similar levels at all stages of the cycle with very low expression of *MUC4*. The box plot shades the second and third quartiles of the data. The black line in the box is drawn at the median value. The whiskers above and below show the maximum and minimum values, respectively.

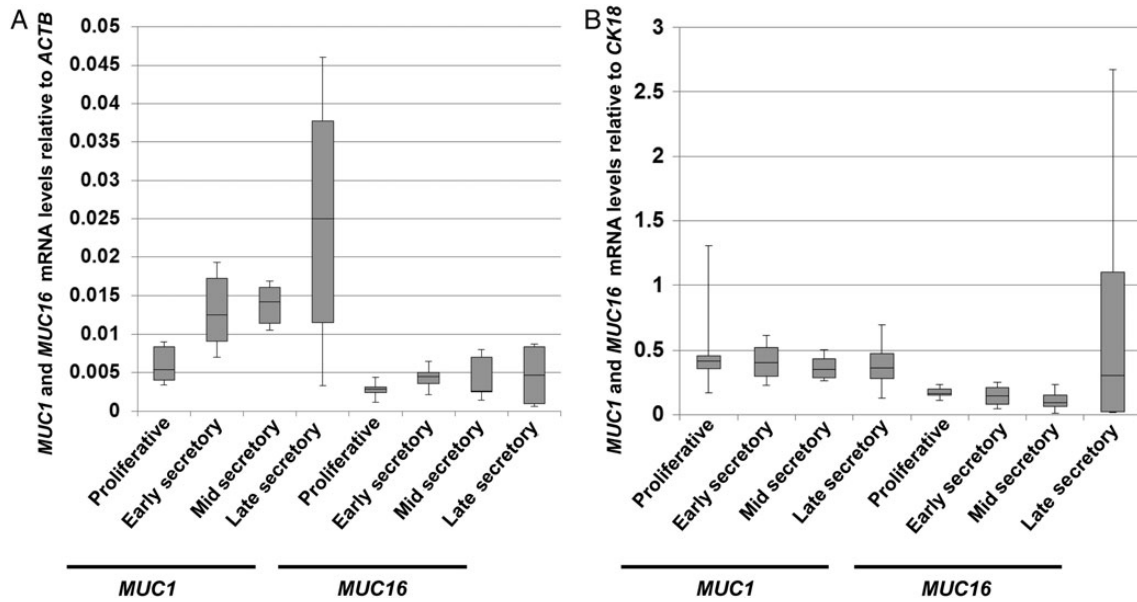


Figure 2 Mucin mRNA levels relative to those of *KRT18* (A) and *ACTB* (B). *MUC1*, *MUC16*, *KRT18* and *ACTB* mRNA levels were determined by qRT-PCR as described in Materials and Methods. Box plots were used to express these data as described in the legend to Fig. 1. No significant differences in mucin expression were observed across the cycle when compared with *KRT18* mRNA levels.

malignant diseases (May et al., 2010). We sought to profile *MUC1*, *MUC4* and *MUC16* mRNA expression in normal endometrium and in endometrium of women with endometriosis again relative to the epithelial marker, *KRT18* mRNA. *MUC1* and *MUC16* mRNA expression did not vary significantly between these conditions (Fig. 3). *MUC4* mRNA was much more difficult to detect than *MUC1* and *MUC16* in normal endometrium and in endometrium from endometriosis patients (Fig. 3). Primers to the cytoplasmic tail (CT) region of the *MUC4* gene were therefore designed, in addition to the primers to the tandem repeat (TR) region, to detect *MUC4* mRNA expression to account for any variability in *MUC4* mRNA expression (Table I). We confirmed minimal levels of *MUC4* mRNA expression in both normal endometrium and that of women with endometriosis (Fig. 4). Collectively, these data indicated little variation in *MUC1*, *MUC4* and *MUC16* mRNA expression in the eutopic endometrium of women with endometriosis when compared with normal endometrium.

Mucin mRNA profiles in stage-matched endometriotic tissue

We then sought to compare mucin expression in stage-matched eutopic and ectopic samples from endometriosis patients relative to *KRT18* mRNA (Fig. 5). qRT-PCR analyses revealed that *MUC1* (Fig. 5A), *MUC4* (Fig. 5B) and *MUC16* (Fig. 5C) mRNA expression did not vary between eutopic and ectopic stage-matched endometriotic tissues. *MUC4* mRNA expression was not significantly higher in ectopic endometriotic tissue (Fig. 5B) and was still very low relative to that of *MUC1* and *MUC16*. Collectively, these results were in accordance with the above

experiment and showed little variation in mucin expression in stage-matched eutopic and ectopic endometriotic tissue.

Transmembrane mucin protein detection by immunohistochemistry

To determine the localization of each mucin, immunohistochemistry was performed on frozen sections from normal and mid-secretory endometriosis grade Stage II endometrium (Fig. 6A–L). Robust *MUC1* and *MUC16* localization was confirmed on the apical surface of epithelia (Fig. 6A–D). Localization of *MUC16* protein expression to epithelia was confirmed using the pan-cytokeratin antibody (Fig. 6I–L). We performed immunohistochemistry for *MUC4* only in endometrium from endometriosis patients since *MUC4* mRNA expression was barely detectable in normal cycling endometrium. Confirming the results from *MUC4* qRT-PCR analyses, a lack of reactivity with the *MUC4*-specific antibody was observed in sections of mid-secretory endometriotic tissue (Fig. 6E–H). Pan-cytokeratin staining was performed to identify epithelial cells in each case (Fig. 6F and J). *MUC4* immunohistochemistry on frozen sections from proliferative stage endometrium from endometriosis patients was also performed (Supplementary data, Fig. S1). Similar to the results obtained in mid-secretory endometriosis grade tissues, *MUC4* protein expression was undetectable (Supplementary data, Fig. S1B) while *MUC1* and *MUC16* were expressed (Supplementary data, Fig. S1A). Recognition of *MUC4* by the 8G7 antibody was confirmed by positive immunostaining for *MUC4* in the pancreatic adenocarcinoma cell line, HPAF (Supplementary data, Fig. S1C and D). Collectively, these data confirm that *MUC1* and *MUC16* expression are restricted to endometrial epithelial cells and that *MUC4* protein is not present at appreciable levels in endometriosis.

Discussion

Mucin expression and distribution changes in several gynecological pathologies, including endometrial cancer and endometriosis (He et al.,

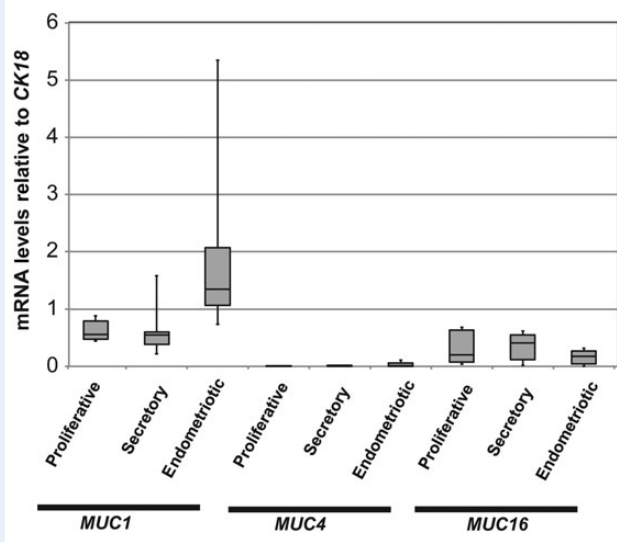


Figure 3 Relative abundance of mucin mRNAs in normal endometrium and in endometrium of women with endometriosis. *MUC1*, *MUC4* and *MUC16* mRNA in normal endometria at different stages of the menstrual cycle and in endometria of women with endometriosis relative to *KRT18* mRNA were determined as described in Materials and Methods. Box plots were used to express the data as described in the legend to Fig. 1. No significant differences were observed between these conditions.

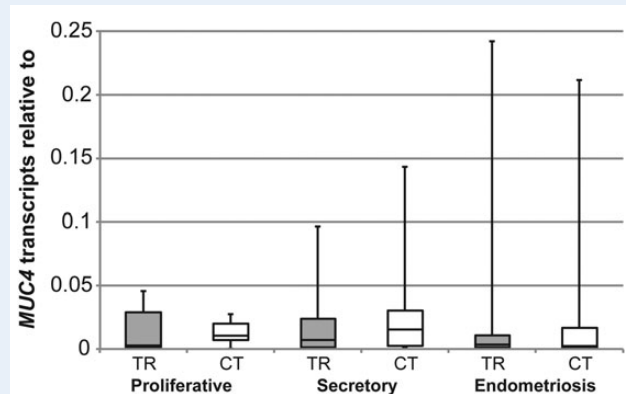


Figure 4 A comparison of *MUC4* mRNA detection by primers against tandem repeat (TR) and cytoplasmic tail (CT) regions of the *MUC4* gene. *MUC4* mRNA in normal endometria at different stages of the menstrual cycle and in endometria of women with endometriosis relative to *KRT18* mRNA using primers to the TR and CT regions. Box plots were used to express these data as described in the legend to Fig. 1. No significant differences were observed using either set of primers.

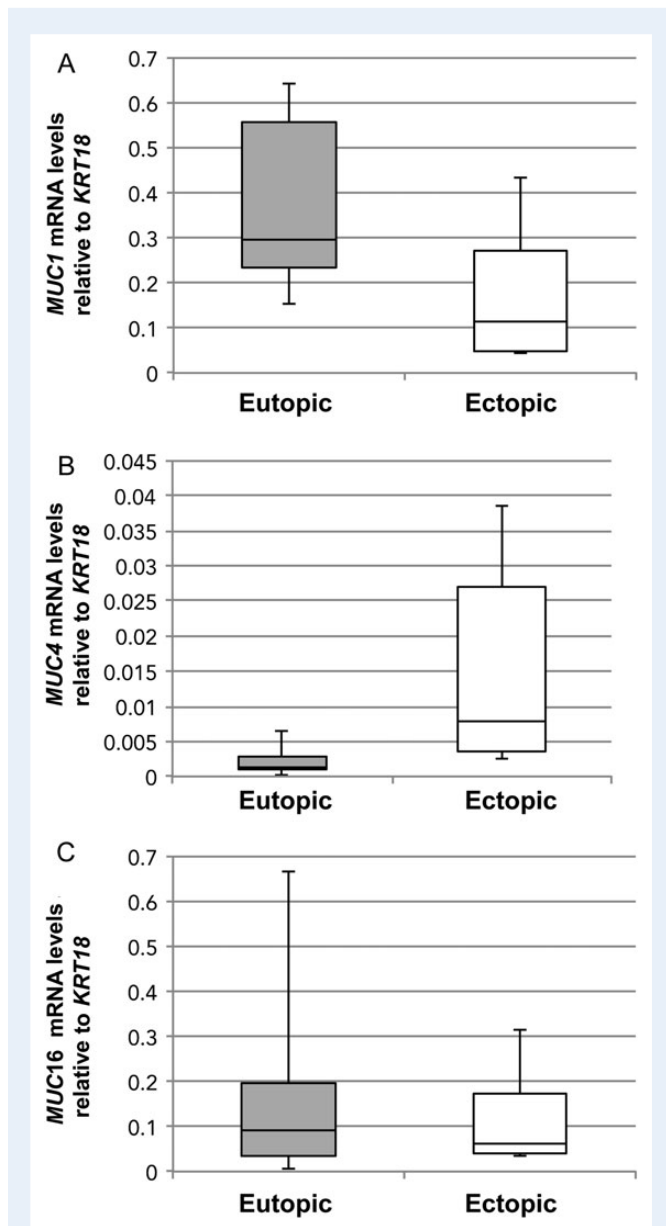


Figure 5 (A) MUC1, (B) MUC4 and (C) MUC16 mRNA expression in stage-matched eutopic and ectopic endometria of endometriosis patients. Six menstrual stage-matched samples, either from secretory or proliferative stage of the menstrual cycle and stage II or III (one sample) endometriosis were analyzed by qRT-PCR for the different mucins as described in Materials and Methods. Box plots were used to express these data as described in the legend to Fig. 1. Changes in levels of MUC4 expression was observed in endometriotic tissue when compared with eutopic endometriotic tissue.

2011). Endometriosis is a prevalent endocrine-driven disease occurring in almost 45% of all women receiving laparoscopic examination and in ~10% of all women presenting with clinical symptoms (Lessey, 2000; Winkel, 2003; de Ziegler *et al.*, 2010). Symptoms range from mild to severe pelvic pain and infertility (Winkel 2003; de Ziegler *et al.*, 2010) and are cyclical following the rise and fall of ovarian steroid hormones through the menstrual cycle that act on endometriotic tissue as well as the endometrium (Winkel, 2003). The etiology of endometriosis

remains unclear, but a common theory is that retrograde menstruation leads to the deposition of endometrial tissue on the ovaries and within the peritoneal cavity. Retrograde menstruation is common in menstruating women, but the reasons that some women get endometriosis and others do not remain an area of active investigation.

The current study focused on quantitative analyses of membrane-tethered mucin (MUC1, MUC4 and MUC16) expression in normal endometrium and eutopic and ectopic endometrium of endometriosis patients. This is the first comprehensive study of these three key membrane-associated mucins in these contexts. Only three reports have examined MUC4 expression by immunohistochemistry in human endometrium (Gipson *et al.*, 1997; Koscinski *et al.*, 2006; Alameda *et al.*, 2007) with no agreement on the expression pattern of MUC4. Gipson *et al.* (1997) reported a lack of MUC4 expression in normal endometrium while Koscinski *et al.* (2006) report isolated patches of MUC4 expressed in luminal and glandular epithelium using the same 8G7 antibody utilized in the present studies in infertile and women undergoing IVF. A lack of information on MUC4-specific antibody used by Alameda *et al.* (2007) hinders interpretation of their results which indicate endometrial MUC4 protein expression in a subset of women. There also is limited information on MUC16 expression in the cycling endometrium, although qRT-PCR has been used to assess levels of MUC16 mRNA levels during the cycle (Gipson *et al.*, 2008). Previous reports have indicated that MUC1 and MUC16 vary in the cycling endometrium with maximal expression in the secretory phase (Hey *et al.*, 1994; Gipson *et al.*, 2008). These studies relied on the use of *ACTB* or 28S RNA as RNA load controls, i.e. an RNA species found in all endometrial cells. When MUC1 and MUC16 mRNA levels were compared relative to *ACTB* mRNA, our results were in agreement with previous reports (Hey *et al.*, 1994; Gipson *et al.*, 2008).

We used *KRT18* mRNA as a control for potential changes in the epithelial populations in endometrial samples (Moll, 1983). Our data indicate that endometrial MUC1 and MUC16 mRNA expression does not change significantly relative to *KRT18* and rather seems to vary in parallel with the changes in the epithelial populations in the endometrium. While it is possible that *KRT18* is hormonally regulated, this has not been described. A number of agents including progesterone and proinflammatory cytokines modulate transmembrane mucin expression in uterine cell types and endometrium of various animal models (Hoffman *et al.*, 1998; Brayman *et al.*, 2006, 2007; Dharmaraj *et al.*, 2010). Nonetheless, the current studies indicate that changes in mucin expression are not observed during the normal menstrual cycle nor in endometriotic tissues. Several reports also indicate that serum MUC1 levels are not changed in endometriosis (reviewed in May *et al.* 2010). One report describes a reduction of MUC1 in endometriosis (Margarit *et al.* 2010). Endometriosis has been suggested as a precursor for ovarian cancer (Worley *et al.*, 2013) and the serum MUC16 levels test (detected as CA-125) is a widely used test for ovarian cancer. Elevated serum MUC16/CA-125 has also been used as a marker for endometriosis, particularly for Stage III–IV disease (Mol *et al.*, 1998; Vodolazkaia *et al.*, 2012). Our data indicate that MUC16 mRNA expression does not vary in endometriotic tissue through the cycle relative to the levels observed in normal endometrium. Nonetheless, our samples were primarily obtained from patients presenting with stage I–II endometriosis and thus we can only support the notion that MUC16/CA 125 is not a useful marker for early-stage disease.

Previous reports have examined MUC4 expression in the human endometrium (Gipson *et al.*, 1997; Koscinski *et al.*, 2006; Alameda

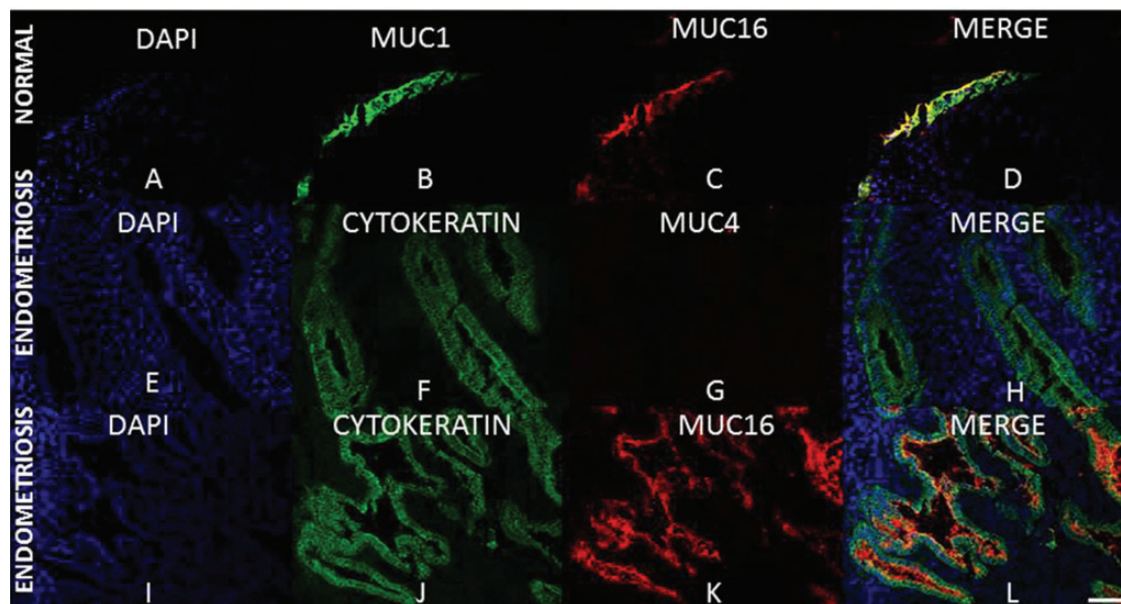


Figure 6 MUC1, MUC4 and MUC16 expression in normal endometrium and in endometriosis. Frozen sections were stained with antibodies to the indicated mucins as described in Materials and Methods. Pan-cytokeratin staining was used to detect epithelia in each case (**F, H, J** and **L**). DAPI staining was used to detect nuclei as described in Materials and Methods. Robust staining for both MUC1 (**B** and **D**) and MUC16 (**C, D, K** and **L**) were observed in all cytokeratin positive (epithelial) cells. No MUC4 staining was detected (**G** and **H**). DAPI: blue; pan-cytokeratin or MUC1: green; MUC4 or MUC16: red. Magnification for all fields is as indicated in (**L**), 50 μm .

et al., 2007). Using an established qRT-PCR protocol that detects a region of *MUC4* mRNA encoding a portion of the ectodomain, we found that *MUC4* mRNA expression is at very low levels under all conditions examined. Consistent with this, *MUC4* protein was undetectable by immunohistochemistry in proliferative and mid-secretory stage endometriosis. Thus, we conclude that full-length *MUC4* is not expressed at appreciable levels in normal endometrium and endometriosis. Although we found a trend toward higher *MUC4* mRNA levels in ectopic versus eutopic endometrium of stage-matched endometriosis patients, this change was not significant. It would be interesting to determine whether a significant accumulation of *MUC4* expression occurs in advanced stages of endometriosis.

Progesterone resistance is now considered a key player in the etiology of endometriosis (Brosens *et al.*, 2012). Nuclear receptors and nuclear receptor coregulator dysregulation are associated with the progression of endometriosis either due to an increase in estrogen or due to signaling by inflammatory molecules (Han and O'Malley, 2014). Several of these factors, such as PR-A and PR-B, PPAR γ and TNF α regulate the expression of transmembrane mucins in various systems (Brayman *et al.*, 2006; Dharmaraj *et al.*, 2010; Wang *et al.*, 2010; Morgado and Carson, unpublished observations). Thus, an understanding of mucins in endometriosis is important. There is great need for endometriosis biomarkers to aid clinical diagnosis and also to monitor the disease. Changes in mucin glycoforms have been suggested to characterize endometrial disease (Sivridis *et al.*, 2002; Seeber *et al.*, 2008). Although we have found no alterations in mucin expression in endometriosis, a comprehensive profile of transmembrane mucin expression, including changes in the oligosaccharide moieties carried by the mucin core proteins, in normal and endometriotic tissues may yet allow for development of meaningful

biomarkers. In addition, changes in the oligosaccharide structures carried by mucins may have important biological consequences such as binding of selectins (Carson *et al.*, 2006) or suppression of the immune system (Daniels *et al.*, 2002; Hauselmann and Borsig, 2014). Future studies should focus on how glycoforms of MUC1 and MUC16 change in endometrial disease states in ways that might promote disease progression.

Supplementary data

Supplementary data are available at <http://humrep.oxfordjournals.org/>.

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Authors' roles

N.D., P.J.C. and M.M. performed all the qRT-PCR and immunostaining experiments, participated in the experimental design and co-wrote the manuscript. S.M.H., S.L.Y. and B.A.L. secured the patient samples and participated in the experimental design, critical discussions and manuscript editing. D.D.C. participated in the experimental design and manuscript preparation.

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Conflict of interest

None declared.

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