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Cytokine Stimulation of MUC4 Expression in Human Female Reproductive Tissue Carcinoma Cell Lines and Endometrial Cancer

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ABSTRACT

MUC4, a transmembrane glycoprotein, interferes with cell adhesion, and promotes EGFR signaling in cancer. Studies in rat models have demonstrated steroid hormonal regulation of endometrial MUC4 expression. In this study, qRT-PCR screening of mouse tissues determined that *Muc4* mRNA also was robustly expressed in mouse uteri. Previous studies from our labs have demonstrated *MUC4* mRNA was expressed at levels <1% of *MUC1* mRNA in human endometrium and endometriotic tissue. Multiple human endometrial adenocarcinoma cell lines were assayed for *MUC4* mRNA expression revealing extremely low basal expression in the Ishikawa, RL-95-2, AN3CA, and KLE lines. Moderate to high expression was observed in HEC50 and HEC-1A cells. *MUC4* mRNA expression was not affected by progesterone and/or estrogen treatment, but was greatly stimulated at both mRNA and protein levels by proinflammatory cytokines (IFN-γ and TNF-α), particularly when used in combination. In endometrial tissue, *MUC4* mRNA levels did not change significantly between normal or cancerous samples; although, a subset of patients with grade 1 and 2 tumors displayed substantially higher expression. Likewise, immunostaining of human endometrial adenocarcinoma tissues revealed little to no staining in many patients (low MUC4), but strong staining in some patients (high MUC4) independent of cancer grade. In cases where staining was observed, it was heterogeneous with some cells displaying robust MUC4 expression and others displaying little or no staining. Collectively, these observations demonstrate that while MUC4 is highly expressed in the mouse uterus, it is not a major mucin in normal human endometrium. Rather, MUC4 is a potential marker of endometrial adenocarcinoma in a subset of patients. J. Cell. Biochem. 116: 2649–2657, 2015.

KEY WORDS: MUC4; INTERFERON-γ; TUMOR NECROSIS FACTOR-α; ENDOMETRIUM

Transmembrane mucin proteins are large, heavily glycosylated proteins that dominate the apical cell surfaces of polarized epithelial cells. These complex proteins protect and hydrate mucosal surfaces largely due to the great abundance of oligosaccharides characteristic of the ectodomain (reviewed in [Kufe, 2009]). Under normal physiological conditions, mucins are expressed solely on the apical surface where they limit access of foreign material to the cell membrane due to their large, hydrophilic nature. Transmembrane mucins generally are anti-adhesive and inhibit cell-cell and cell-extracellular matrix interactions [Hattrup and Gendler, 2008]. As a result of these anti-adhesive properties, reduction of mucin expression in uterine epithelia is critical to permit embryo implantation (reviewed in [DeSouza et al., 1999]). In many species, ovarian steroid hormones that synchronize the development of the receptive phase also regulate uterine transmembrane mucin expression (reviewed in [Meseguer et al., 1998]). Uterine Muc4 expression has been examined in multiple animal models [Idris and Carraway, 1999; Ferrell et al., 2003; Jasper et al., 2011]; however, only limited information on MUC4 expression in human endometrium has been reported [Gipson et al., 1997; Alameda et al., 2007].

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Previous work in our lab has demonstrated very low basal levels of *MUC4* mRNA in human endometrium independent of menstrual cycle stage [Dharmaraj et al., 2014].

Dynamic changes in the expression of proinflammatory cytokines and growth factors also occur during the menstrual cycle (reviewed in [Kelly et al., 2001]). These cytokines, particularly interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα), dramatically stimulate MUC1 expression in multiple contexts including cancer [Lagow and Carson, 2002; O′Connor et al., 2005; Albertsmeyer et al., 2010]. These same cytokines are widely expressed in the tumor microenvironment (reviewed in [Hanahan and Weinberg, 2011]). Inflammation is considered to be an enabling characteristic due to its contributions to the progression of tumor development and heightened malignancy. The current view holds that the suppression mechanisms of inflammatory cytokines are disrupted in tumor cells allowing the tumors to survive and grow [Mantovani et al., 2008] For example, MUC4 is not normally expressed in the pancreas under normal or inflammatory states, but becomes detectable in cancers where it can serve as a tumor biomarker [Andrianifahanana et al., 2001].

In the present study, we examined MUC4 expression in human endometrium and human endometrial, breast, and cervical cancer cell lines under basal growth conditions and in response to ovarian steroid hormones or pro-inflammatory cytokines. Our results indicate that MUC4 expression is stimulated by proinflammatory cytokines in a variety of cellular contexts. In contrast to mice, MUC4 protein is not present at appreciable levels in the healthy uterine environment in humans, but accumulates in subsets of cells in more advanced stages of endometrial cancer. Thus, MUC4 is not likely to play major roles in normal human uterine physiology, but rather serves as a marker of endometrial carcinoma in a subset of patients.

MATERIALS AND METHODS

MOUSE TISSUE COLLECTION

Multiple organs from male and female FvB/N mice were collected, frozen, and stored at −80° C prior to RNA extraction. The stage of estrous cycle of female mice was determined by the vaginal smear method [McLean et al., 2012]. Animal protocols were in accordance with the guidelines for humane treatment of laboratory animals by the National Institutes of Health and the Institutional Animal Care and Use Committee at Rice University.

HUMAN TISSUE SAMPLES

All patient samples were collected according to the Institutional Review Boards for Baylor College of Medicine and affiliated hospitals, Greenville Hospital System, and the University of Texas, M.D Anderson Cancer Center 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; Okoye et al., 2015].

CELL CULTURE AND TREATMENTS

The MCF7 cell line [Soule et al., 1973] was provided by Dr. Sang Jun Han (Baylor College of Medicine). HEC50 [Kuramoto et al., 1991], KLE [Richardson et al., 1984], and AN3CA [Dawe et al., 1964] cell lines were provided by Dr. Russell Broaddus (University of Texas M.D. Anderson Cancer Center). The HeLa [Scherer et al., 1953] cell line was provided by Dr. Laura Segatori (Rice University). Ishikawa cell line [Nishida et al., 1985] was provided by Bruce Lessey (Greenville Health System). HEC-1A [Kuramoto, 1972] and T47D [Keydar et al., 1979] cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). HEC-1A/PRB cells were made previously by stably transfecting HEC-1A cells with hPRB and PUR plasmids [Brayman et al., 2006]. MCF7 cells were maintained in phenol red-free Dulbecco′s modified Eagle′s medium/Ham′s F12 medium (DMEM)/F12 (Life Technologies) supplemented with 5% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals) and 400 μg/ml G418 (Geneticin) (Life Technologies). Ishikawa, HEC50, and KLE cells were maintained in phenol red-free DMEM/F12 supplemented with 10% (v/v) FBS. HEC-1A and HEC-1A/PRB cells were maintained in DMEM/F12 supplemented with 10% (v/v) charcoal-stripped FBS (Hy-Clone). HEC-1A/PRB cells were maintained in DMEM/F12 supplemented with 10% (v/v) charcoal-stripped FBS and 175 ng/ml puromycin (Life Technologies). RL-95-2 cells were maintained in DMEM/F12 supplemented with 5% (v/v) FBS. AN3CA cells were maintained with DMEM (Life Technologies) supplemented with 5% (v/v) FBS. HeLa cells were maintained in DMEM supplemented with 10% (v/v) FBS. T47D cells were cultured in RPMI (Life Technologies) supplemented with 10% (v/v) FBS. All cells were maintained at 37° in a humidified atmosphere of air:CO_2 (95:5, v/v).

While HEC-1A cells produce the most *MUC4* mRNA, they do not express functional IFNγ receptors (P. Chapela and D.D. Carson, unpublished observations) and, therefore, were not useful for many of the studies of cytokines described below. Therefore, the HEC50 cell line, which expresses functional cytokine receptors, was used as the representative endometrial cell line in subsequent experiments.

For treatments, cells were seeded in 6-well tissue culture plates and maintained until cells reached 80% confluence. Cells then were incubated in serum-free media for 24 hrs prior to treatment. Three wells were used for each treatment as biological replicates. For pro-inflammatory cytokine treatments of HEC50, MCF7, and HeLa cell lines, cells were treated with 200 IU IFNγ (Roche) and 25 ng/ml TNFα (Roche), either alone or in combination. Ten μg/ml bovine serum albumin (Sigma) was used as a vehicle control for protein treatments. For steroid hormone treatments of MCF7 cells, 10 nM 17-β-estradiol (Sigma) or 400 nM progesterone (Sigma) was used. The vehicle control for the hormone treatments was 0.1% (v/v) ethanol. RNA was extracted after 24 hrs for qRT-PCR analyses, and cell lysates were collected 48 hrs after treatment for western blot analyses (see below).

RNA ISOLATION AND QRT-PCR

Tissues collected from mice were homogenized by mortar and pestle. RNA was extracted using 1 ml TRIzol reagent (Life Technologies) and resuspended in 40 μl nuclease-free water. DNA-free kit (Ambion) was used to degrade remaining genomic DNA in accordance with manufacturers′ directions. One microgram of RNA was reverse-transcribed using cDNA Supermix (Quantas) as per the manufacturer′s protocol and amplified using a CFX96 Real Time System (BioRad). For qRT-PCR, iQ SYBR Green Supermix (BioRad) was used for the reactions. All samples were run as

technical triplicates. For mouse tissues, *Muc4* mRNA was detected using the primer pair 5′TGGCTGTGTCTGAGCTGCCTG and 5′TTGGGATGTTCTGGTGCTGCT designed at NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). Reactions were standardized to *Krt18* mRNA to compare Muc4 to the epithelial content of the samples using primers previously published [Brayman et al., 2006]. Transcript levels were calculated by ΔCt method [Livak and Schmittgen, 2001]. For human cell lines, the RNA was isolated after 24-hr treatment as described above. Previously published primers for *MUC4* were used in qRT-PCR reactions [Argueso et al., 2002]. Reactions from cell lines were standardized to *ACTB* mRNA. *ACTB* transcripts appeared unaffected by treatments because threshold cycles did not vary more than ½ cycle between treatments (data not shown). RNA from healthy human endometrium was isolated from 50–100 mg of frozen tissue using mirVana kit (Applied Biosystems). RNA was extracted from endometrial tumor sections homogenized in TriReagent (Molecular Research Center), precipitated with isopropanol, and isolated using RNeasy spin columns (Qiagen). All samples were treated with DNA-free kit (Ambion) as described above. Reactions from human endometrium were standardized to *KRT18* mRNA to control for the epithelial content of the tissue [Dharmaraj et al., 2014].

WESTERN BLOTTING

Cells were lysed using sample extraction buffer [Thathiah et al., 2003]. Lysates were stored at −20°. For detection of progesterone receptor, cell lysates were boiled in Laemmli sample buffer [Laemmli, 1970] then separated by SDS-PAGE by a 4% (w/v) acrylamide stacking gel and a 10% (w/v) acrylamide separating gel and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore). Membranes were blocked in 3% (w/v) BSA (Sigma) in PBS. Progesterone receptor antibody, Ab-8 (Thermo Scientific), was used at a concentration of 200 ng/ml to detect progesterone receptor isoforms. Two cell lines were used as controls to determine the progesterone receptor isoform present. T47D cells produce both isoforms (PRA and PRB), while HEC1A-PRB cells are stably transfected with PRB only.

To detect MUC4, cell lysates were boiled in reducing sample buffer (Pierce) and separated on a 2% (w/v) agarose—10% (w/v) urea gel layered on a preformed 10% (w/v) polyacrylamide gel. After transfer to PVDF, the membrane was cut along the agarose/polyacrylamide interface. The agarose portion of the membrane containing MUC4 then was blocked in 5% (w/v) milk and incubated with a MUC4 antibody (8G7), kindly provided by Dr. S. Batra (University of Nebraska Medical Center), used at a dilution of 1 μg/ml as described [Moniaux et al., 2004]. The lower portion of the membrane corresponding to the polyacrylamide separating gel was blocked in 3% (w/v) BSA in PBS and probed for β-actin, serving as the load control (Abcam 8226), used at a concentration of 100 ng/ml. Densitometry was performed using Image J software [Schneider et al., 2012].

LUCIFERASE ASSAY

The method and constructs used for luciferase assay were described previously [Brayman et al., 2006]. Briefly, MCF7 cells were seeded in serum-free media in 6-well tissue culture plates. Once 80% confluence was reached, cells were transfected using Lipofectamine 2000, and Opti-MEM (Life Technologies) according to the manufacturer's instructions, with two plasmids: 1 μg of the firefly luciferase plasmid containing a consensus response element for either progesterone receptor or estrogen receptor, and 10 ng of a *Renilla* luciferase plasmid driven by a cytomegalovirus (CMV) promoter [Zhou et al., 1998; Wang et al., 2010]. After 24 hrs, the cells were treated with either 10 nM estrogen, or 400 nM progesterone dissolved in ethanol. Ethanol was used at a final concentration of 0.1% (v/v) as a vehicle control. Luciferase activity was evaluated 24 h after treatment using the Dual Luciferase Reporter Assay System (Promega). Luminescence was measured in 96-well plates using a Tecan Infinite M1000 PRO microplate reader with icontrol software v.1.10.4.0 (Tecan). Firefly luciferase signal was normalized to *Renilla* luciferase internal control. Then data were standardized to the vehicle control for each treatment.

IMMUNOFLUORESCENCE

Frozen human endometrial tissues were embedded in Optimal Cutting Temperature (O.C.T.) medium and sectioned on a cryostat. Samples were fixed in 4% (v/v) paraformaldehyde (PFA), permeabilized in 0.02% (v/v) Triton X-100, and blocked in filtered 3% (w/v) BSA in PBS. Then samples were incubated with MUC4 antibody (8G7) at a concentration of 10 μg/ml and wide-spectrum anti-cytokeratin antibody (Abcam ab9377) at a concentration of 12 μg/ml at room temperature for 90 min. Samples were rinsed in PBS 3 times for 5 min each at room temperature, followed by incubation with Alexa Fluor 488 goat anti-rabbit IgG, and Alexa Fluor 647 goat anti-mouse at a 1:400 dilution (Life Technologies) for 1 h at room temperature. Samples were imaged using a LSM Zeiss 710 confocal microscope.

STATISTICAL ANALYSIS

InStat/Prism was the statistical software package used for all calculations. For tissue culture experiments, each tissue culture well constituted a single biological replicate. For tissue samples, each patient was a single biological replicate. Samples were not pooled. For qRT-PCR, three technical replicates were used for each biological sample. Mean value and standard deviation were calculated using the observed value for each biological replicate within a treatment group. All statistics were determined by a two-tailed Students *t*-test comparing control to treatment response.

RESULTS

Muc4 mRNA EXPRESSION IN MOUSE TISSUES

Initially, we sought to determine the endogenous expression of *Muc4* in different tissues in mice. No anti-mouse Muc4 antibodies are available. Therefore, we used *Muc4* mRNA expression for this purpose. *Muc4* was normalized to *Krt18* mRNA as an index of the epithelial content of the tissues. As shown in Table I, *Muc4* was detected in lung and small intestine at levels similar to *Krt18* mRNA. *Muc4* levels were much lower, but detectable, in testis, and kidney, but not detectable in heart, liver, pancreas, or spleen. The highest

TABLE I. Relative *Muc4* mRNA Levels in Mouse Tissues

Tissue	Relative Muc4 mRNA levels*
Heart	ND
Kidney	0.4% ± 0.02%
Liver	ND
Lungs	111% ± 20%
Pancreas	ND
Small Intestine	93% ± 26%
Spleen	ND
Testis	0.4%
Uterus	3190% ± 1571%

*Values represent *Muc4* mRNA as a percent of cytokeratin 18 (*Krt18*) mRNA values determined by qRT-PCR as described in Materials and Methods. Uteri were taken from 2 proestrous stage females. For testes, 1 male was used. All other samples are the mean values ± SEM for separate determinations from 4 different animals (2 male, 2 female). ND, not detectable.

levels of *Muc4* mRNA in tissue were detected in uterine tissue. Vaginal smears were used to confirm that mice in proestrus when tissue was harvested. We made no attempt to compare *Muc4* expression in uterine tissue at other stages of the cycle. We next sought to determine MUC4 expression in human endometrial cell lines and tissues.

MUC4 mRNA EXPRESSION IN HUMAN ENDOMETRIAL CELL LINES

Similar to the results obtained with normal human endometrium [Dharmaraj et al., 2014], basal levels of *MUC4* transcripts in most human endometrial cancer cell lines were very low (Table II). HEC-1A cells displayed the highest levels of MUC4 expression, at 0.03% the level of β-actin (*ACTB*) transcripts. The cell line that contained the second highest level was HEC50, expressing *MUC4* mRNA at 10% of the levels found in HEC-1A. KLE, Ishikawa, and RL95-2 cells produce *MUC4* transcripts at much lower levels (1–3%) relative to HEC-1A. *MUC4* transcripts were near background levels in AN3CA cells.

OVARIAN STEROID HORMONES DO NOT ALTER STEADY-STATE LEVELS OF MUC4 mRNA

In order to evaluate a possible response to ovarian steroid hormones, the presence, and functionality of the steroid hormone receptors were determined in the MCF7 cell line. MCF7 cells expressed primarily the A isoform of progesterone receptor (Fig. 1A). The functionality of both progesterone and estrogen receptors was verified by steroid hormone response reporter assays (Fig. 1B and D). Once we confirmed the steroid hormone receptors were present and functional, we could evaluate the effect female steroid hormones may have on steady-state transcript levels of *MUC4*. *MUC4* mRNA levels remained unchanged after treatment with progesterone or estradiol alone and in combination (Fig. 1C). Therefore, it was concluded that the human *MUC4* gene was not responsive to ovarian steroids in MCF7 cells. This is consistent with our previous study showing no change in MUC4 in endometrium during the normal menstrual cycle [Dharmaraj et al., 2014].

TABLE II. Relative MUC4 mRNA Expression in Human Uterine Epithelial Cell Lines.

Cell Line	Normalized to HEC1A*
Increasing differentiation	
Ishikawa	0.015
HEC-1A	1.000
RL-95	0.010
AN3CA	0.001
HEC50	0.109
KLE	0.033

**MUC4* mRNA levels were determined by ΔC_t method. Transcripts were normalized to *ACTB* mRNA by qRT-PCR as described in Materials and Methods. The value obtained for HEC-1A cells was the highest in the group and was set to 1.0 for comparison. The values shown are calculated from average values of triplicate independent determinations in each case where the standard deviations were 5% or less. The arrow to the left of the table indicates the relative levels of differentiation of each cell line [Dawe et al., 1964; Hannan et al., 2010; Richardson et al., 1984; Kuramoto et al., 1991].

PRO-INFLAMMATORY CYTOKINES INCREASE STEADY-STATE LEVELS OF MUC4 mRNA

A series of studies indicate that MUC1 gene expression is robustly stimulated by proinflammatory cytokines [Lagow and Carson, 2002; O′Connor et al., 2005; Albertsmeyer et al., 2010; Dharmaraj et al., 2010]. Less has been done on regulation of MUC4 expression in this regard. Nonetheless, it appears that proinflammatory cytokines stimulate MUC4 expression in certain cellular contexts [Perrais et al., 2001; Wasserberg et al., 2003]. We examined the effects of interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα), which are also present in the endometrium as well as other female reproductive tract tissues under both normal and pathological states [Hunt et al., 1992; Tabibzadeh, 1994]. Although some cell lines displayed significant increases in *MUC4* mRNA in response to individual cytokines, the most consistent, and robust response was observed in HEC50, MCF7, and HeLa cell lines when receiving combined treatment with IFNγ and TNFα. The degree of response in these cell lines varied with MCF7 cells displaying approximately a 150-fold increase (Fig. 2A) and HEC50 cells a ten-fold increase to the combined cytokines (Fig. 2A and B). HeLa cells demonstrated almost a 1000-fold increase (Fig. 2C). While the basal levels of MUC4 varied greatly among these cell lines, cytokine stimulation elevated the absolute values for *MUC4* mRNA to similar levels in all cell lines. Thus, the large differences in degree of cytokine stimulation largely reflect the differences in the basal levels of *MUC4* mRNA among the different cell lines tested.

PRO-INFLAMMATORY CYTOKINES INCREASE MUC4 PROTEIN LEVELS

Due to the increased *MUC4* mRNA expression in response to treatment with pro-inflammatory cytokines, we determined if there was a corresponding effect on MUC4 protein expression in a representative uterine cell line. After HEC50 cells were treated with IFNγ and TNFα for 48 hrs, cellular lysates were analyzed for MUC4 expression by western blotting. In good agreement with the mRNA response, we observed about a 10-fold increase in MUC4 protein upon cytokine treatment (Fig. 3). Thus, the changes in mRNA were reflected by a substantial elevation in the cellular MUC4 content. Western blots for MCF7 and HeLa cell lines were unable to detect any MUC4 protein, at basal levels or after cytokine treatment (data not shown).

MUC4 EXPRESSION IN ENDOMETRIAL CANCERS

MUC4 mRNA expression showed no correlation to cancer grade (data not shown); however, there were patients with endometrial

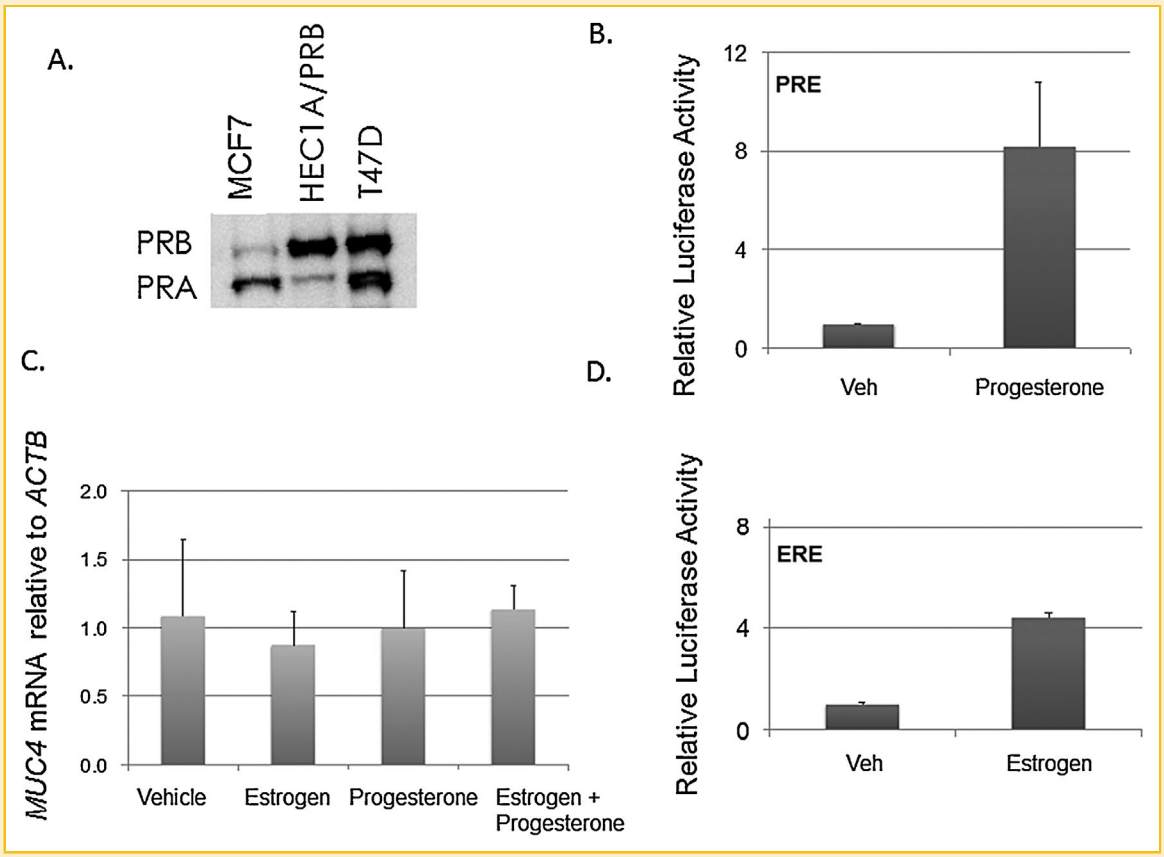

Fig. 1. MUC4 mRNA levels do not change in response to ovarian steroid hormone treatments. A. Progesterone receptor (PR) expression in different cell lines. Western blotting shows the presence of both PR isoforms in the three indicated cell lines. B. MCF7 cells express functional progesterone receptor (PR). Luciferase assays with a consensus response element for PR (PRE) showed increased activity after 24 h of treatment with 400 nM progesterone versus vehicle (Veh). C. *MUC4* mRNA levels in steroid hormone-treated cells. MCF7 cells were incubated with vehicle control, 10 nM 17-β-estradiol (Estrogen), 400 nM progesterone or a combination of both steroids for 48 hr and *MUC4* mRNA levels determined relative to that of *ACTB* by qRT-PCR as described in Materials and Methods. D. MCF7 cells express functional estrogen receptor (ER). Luciferase assays with a consensus response element for ER (ERE) showed increased activity after 24 hr of treatment with 10 nM 17-β-estradiol (estrogen) versus vehicle (Veh). Values shown are the means ± SD of triplicate determinations for three independent samples in all cases.

carcinoma who displayed substantially (>10 fold) higher MUC4 mRNA levels than normal endometrium. Therefore, we separated cancer patients as low and high expressors (Fig. 4). Low expressors were defined as tumors having MUC4 mRNA levels ≤2 SD higher than the mean value obtained for normal endometrium. High expressors were defined as tumors having MUC4 mRNA levels >2 SD higher than the mean value obtained for normal endometrium.

We also stained frozen sections with both a MUC4 antibody as well as a pan-cytokeratin antibody to reveal all sites containing epithelial cells (Fig. 5). As shown previously, MUC4 protein was not detected in normal tissue (panel A). MUC4 expression in MUC4 low expressors ranged from undetectable by immunostaining, to intermittent recognition of a subset of cytokeratin positive cells (panel B, arrows). Much stronger staining was observed in MUC4 high expressors (panel C, arrows). Nonetheless, even in these patients the pan-cytokeratin staining revealed that while many tumor cells were MUC4 positive, many were not. Thus, there is heterogeneity within even high MUC4 -expressing tumors.

DISCUSSION

A simple epithelial lining covers the endometrium with a prominent glycocalyx that dominates the apical cell surface [Schlafke and Enders, 1975]. This surface provides a hydrated, non-adhesive interface with the uterine lumen into which a variety of proteins and small molecules are secreted that can provide nourishment and support for the developing embryo [Bazer and Johnson, 2014]. This surface also must provide a protective function against enzymatic and microbial attack, a particularly important function following *coitus* when a significant microbial load is introduced interrupting the environment created by normal flora [Hickey et al., 2012]. Understanding the nature of the molecules that exist at this surface, their precise functions, and how their expression is controlled is important for developing strategies to control endometrial functions.

Previous studies in rats and pigs detected Muc4 protein in the uterus and observed fluctuations throughout the estrous cycle

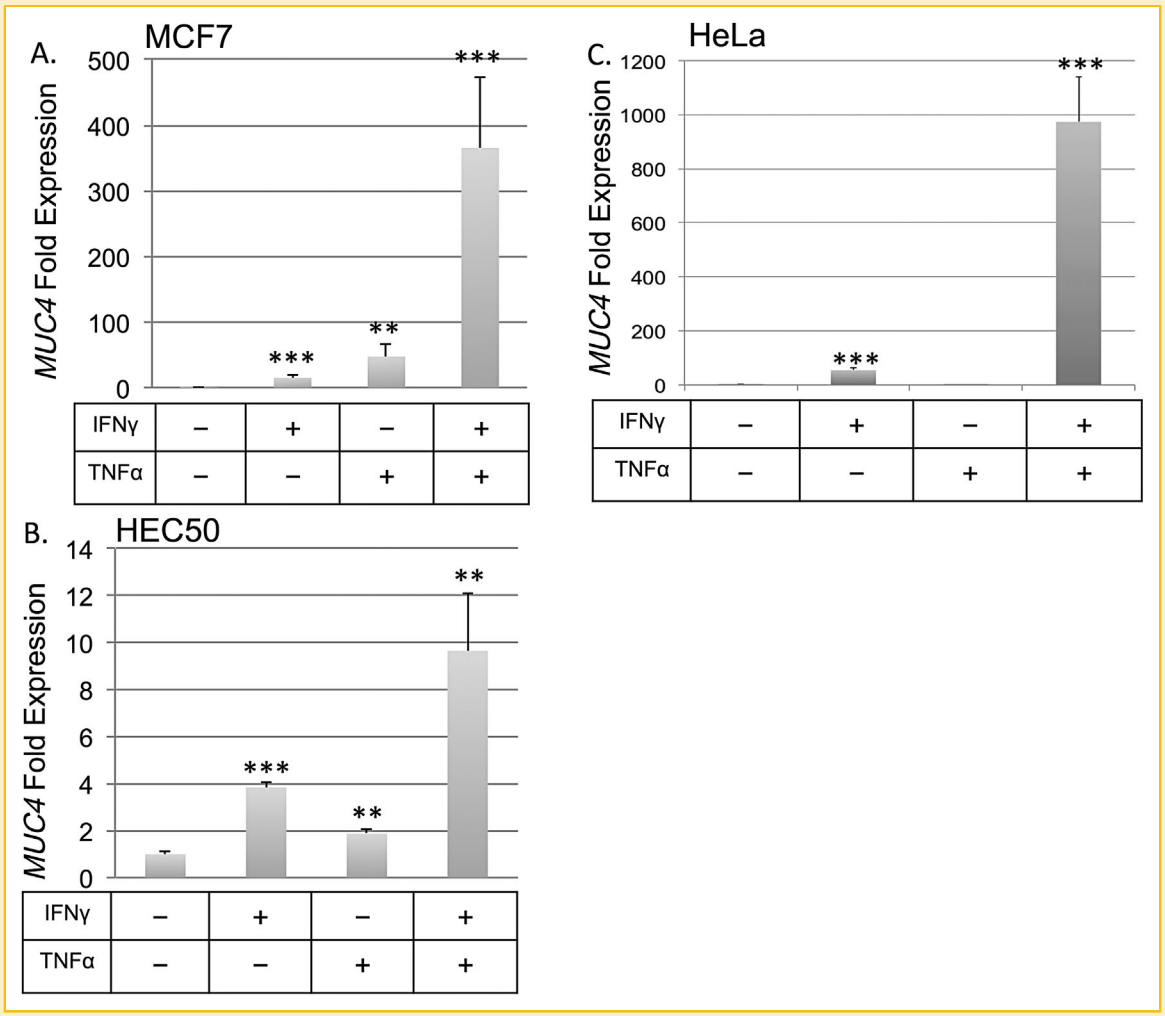

Fig. 2. MUC4 mRNA increases in response to IFNγ and TNFα. A. *MUC4* mRNA relative to *ACTB* was determined in MCF7 cells after 24 hr treatment with either IFNγ (200 IU/ml), or TNFα (25 ng/ml) alone or in combination as indicated. B. *MUC4* transcripts relative to *ACTB* in HEC50 cells after 24 hr treatment with cytokines as indicated. C. *MUC4* transcripts relative to *ACTB* in HeLa cells after 24 hr treatment with cytokines as indicated. The bars represent the means ± SD of triplicate determinations of independent samples in each case. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

[McNeer et al., 1998; Ferrell et al., 2003]. Through qRT-PCR, we determined that *Muc4* transcripts are highly expressed in the proestrus mouse uterus. In the rat, Muc4 (also called ASGP-1 and ASGP-2) expression is elevated by estrogen, a response antagonized by progesterone [McNeer et al., 1998]. However, previous studies conflict on whether or not human endometrial epithelia express MUC4 [Gipson et al., 1997; Alameda et al., 2007]. Previous studies in our lab found no evidence of changes in MUC4 expression in cycling human endometrium [Dharmaraj et al., 2014]. In the current studies, we found low basal level of *MUC4* transcripts in most uterine epithelial adenocarcinoma cell lines. This suggests that the normal function that Muc4 serves in the rodent uterus is not preserved in human endometrium and may have been supplanted by other transmembrane mucins. MUC1, MUC8, and MUC16 are all readily detected in human endometrial epithelia [Kabawat et al., 1983; Hey et al., 1994; D′Cruz et al., 1996; Gipson et al., 1997; Dharmaraj et al., 2014] and may provide the normal protective, hydration, and barrier functions needed by this tissue. In addition, rodents, unlike humans, do not menstruate, and therefore do not have the extreme need to replenish the endometrial lining cyclically. Loss of MUC4 expression in human endometrium may reflect the loss of the need to have this mucin perform normal homeostatic functions in this tissue.

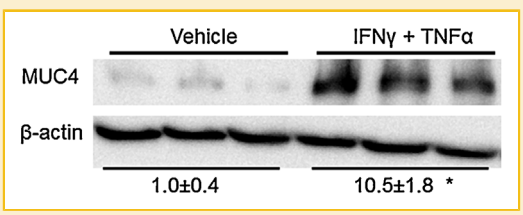

Fig. 3. MUC4 protein increases in response to cytokines. Treatment of HEC50 cells with pro-inflammatory cytokines (200 IU IFNγ, 25 ng/ml TNFα) for 48 hr increased the quantity of cell-associated MUC4. MUC4 protein was compared to that of the β-actin load control. Values shown below the figure are the average of the three samples ± SD. $^{*}P < 0.001$.

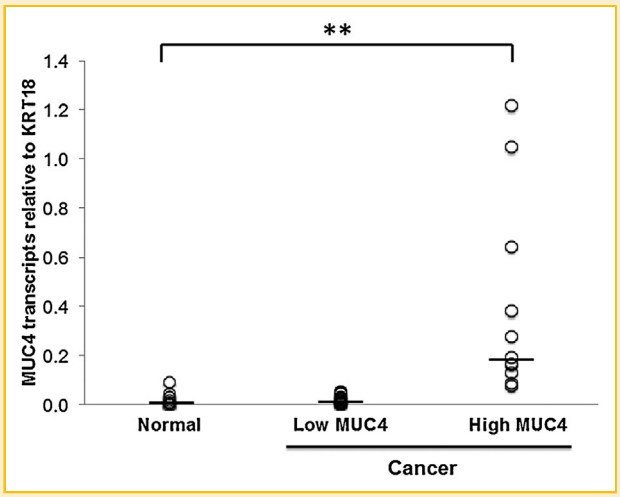

Fig. 4. MUC4 mRNA levels in endometrial cancer tissues. *MUC4* mRNA relative to *KRT18* was determined in RNA samples isolated from healthy human endometrium (Normal; n = 11) and endometrial adenocarcinoma tissues (grades 1–3). Each circle represents the value obtained for an individual patient sample. The median values are marked with a horizontal line. Although individual patients with endometrial cancers displayed highly elevated *MUC4* levels, there was no difference in *MUC4* levels between grades. Therefore, cancer tissues were grouped as follows, independent of grade: Low MUC4, cancer samples with *MUC4* mRNA levels <2 SD greater than the median of normal (n = 21); High MUC4, cancer samples with MUC4 mRNA values >2 SD greater than the median of normal (n = 10).

Pro-inflammatory cytokines strongly elevate the steady-state levels of *MUC4* mRNA in multiple female reproductive tissue cell lines. *MUC4* expression was stimulated to similar absolute levels in multiple cell lines even though the basal level of expression varied widely. The response to pro-inflammatory cytokines, similar to that seen for other membrane-bound mucins, suggests a common response to inflammation for membrane bound mucin genes [Lagow and Carson, 2002; O'Connor et al., 2005; Albertsmeyer et al., 2010]. Proinflammatory cytokines produced at sites of tissue damage or infection would rapidly promote expression of transmembrane mucins providing a physical barrier against further damage or tissue penetration thereby providing a normal protective function. Previous studies have demonstrated strong cooperative responses to proinflammatory cytokines [Lagow and Carson, 2002; Dharmaraj et al., 2010]. These observations demonstrate that combinations of cytokines at low concentrations can profoundly elevate mucin expression. Thus, these responses are likely to continue to occur at sites distal to the site of cytokine production. This would provide regional protection around sites of trauma or infection.

Abnormal elevation of cytokines in pathologic states also would be expected to elevate mucin expression. The appearance of MUC4 in endometrial cancer indicates that normally this gene is efficiently silenced in the uterus and only activated in advanced pathological states. This may be due to a loss of polarity in the epithelial cells. MUC4 translation is suppressed by *MIR200c*, one of the microRNAs involved in maintaining polarity in epithelial cells [Radhakrishnan et al., 2013]. Reduced expression of *MIR200c* accompanies epithelial-mesenchymal transition in endometrial cancer [Castilla et al., 2011]. This may contribute to elevated MUC4 expression in a subset of endometrial cancers. While MUC4 is not expressed by normal pancreas or in pancreatitis, it is elevated in pancreatic cancer [Chakraborty et al., 2008]. Tumor heterogeneity also may account for the incongruity between the qRT-PCR data and the immunofluorescence staining (reviewed in [Le Gallo and Bell, 2014]). We found that subsets of cells (pan-cytokeratin-positive) within these tumors displayed strong MUC4 staining while other tumor cells were negative. However, *MUC4* mRNA expression in the tumor as a whole would be diluted by the non-expressing cells in the population. Thus, in addition to pancreas, human endometrium is another example in which ectopic MUC4 expression can occur in disease. Collectively, these studies demonstrate that while rodent uteri robustly express Muc4, MUC4 is not expressed by human endometrium under most conditions. Nonetheless, *MUC4* gene expression is activated in advanced endometrial cancer in some patients and is strongly driven by cytokines in multiple contexts of female reproductive tissue epithelia.

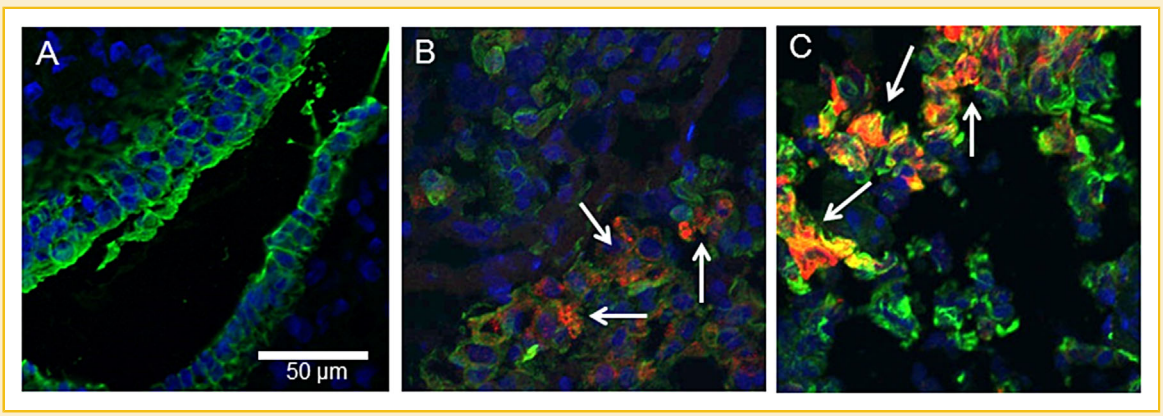

Fig. 5. MUC4 accumulates in subsets of cells in advanced stage endometrial cancer. Frozen sections of human endometrial tissues were stained with MUC4 antibody (red), pan-cytokeratin antibody (green) to identify all epithelial cells within the section and DAPI (blue) as described in Materials and Methods. A) Normal endometrium during secretory phase; B) Low MUC4 expressing tumor, Grade 3, Stage 3A; C) High MUC4 expressing tumor, Grade 3, Stage 3A. Scale bar indicates 50 μm. Arrows in panels B and C indicate cells that are positive for both cytokeratin and MUC4. Note that in cases where MUC4 is detected, there are many cytokeratin-positive cells that remain MUC4-negative.

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