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Mucin gene expression in human male urogenital tract epithelia

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Abstract

BACKGROUND—Mucins are large, hydrophilic glycoproteins that protect wet-surfaced epithelia from pathogen invasion as well as provide lubrication. At least 17 mucin genes have been cloned to date. This study sought to determine the mucin gene expression profile of the human male urogenital tract epithelia, to determine if mucins are present in seminal fluid, and to assess the effect of androgens on mucin expression.

METHODS AND RESULTS—Testis, epididymis, vas deferens, seminal vesicle, prostate, bladder, urethra and foreskin were assessed for mucin expression by RT-PCR and immunohistochemistry. Epithelia of the vas deferens, prostate and urethra expressed the greatest number of mucins, each expressing 5–8 mucins. Messenger RNA of MUC1 and MUC20, both membrane-associated mucins, were detected in most tissues analyzed. Conversely, MUC6 was predominantly detected in seminal vesicle. MUC1, MUC5B and MUC6 were detected in seminal fluid samples by immunoblot analysis. Androgens had no effect on mucin expression by cultured human prostatic epithelial cells.

CONCLUSIONS—Each region of urogenital tract epithelium expressed a unique mucin gene repertoire. Secretory mucins are present in seminal fluid, and androgens do not appear to regulate mucin gene expression.

Keywords

Mucin; MUC; Male urogenital tract; male reproductive tract epithelia

Introduction

The wet-surfaced epithelia of the body, including those of the urogenital, gastrointestinal, and respiratory tracts, and the ocular surface, are the primary sites of pathogenic infection. Wet-surfaced epithelia of the body, in addition to providing defense mechanisms against infection, must be lubricated to prevent luminal surfaces from adhering to one another. A class of molecules that provides both pathogen barrier and lubricating, disadhesive functions to the wet-surfaced epithelia of the body are large, high molecular weight, hydrophilic glycoproteins known as mucins (for review, see (Jeffery and Li, 1997; DeSouza *et al.*, 1998; Lagow *et al.*, 1999; Corfield *et al.*, 2001; Gipson and Argueso, 2003; Gipson *et al.*, 2004)). Mucins by definition have tandem repeats of amino acids in their protein backbone rich in serine and threonine that provide sites for O-linked glycosylation. Carbohydrates usually make up a majority of the mass of the mucin molecule (Gendler and Spicer, 1995; Moniaux *et al.*, 2001). To date, at least 17 mucin genes have been cloned (Gendler *et al.*, 1987; Gum *et al.*, 1989; Gum *et al.*, 1990; Lan *et al.*, 1990; Porchet *et al.*, 1991; Bobek *et al.*, 1993; Dufosse *et*

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al., 1993; Toribara *et al.*, 1993; Arias *et al.*, 1994; Meezaman *et al.*, 1994; Shankar *et al.*, 1994; Guyonnet Duperat *et al.*, 1995; Williams *et al.*, 1999; Pratt *et al.*, 2000; Williams *et al.*, 2001; Yin and Lloyd, 2001; Gum *et al.*, 2002; Pallesen *et al.*, 2002; Chen *et al.*, 2004; Higuchi *et al.*, 2004): MUC1–4, 5AC, 5B, 6–13, 15–17, 19, and 20. Based on sequencing data, two classes of mucins have been identified. These include the membrane-associated mucins (MUC1, 3, 4, 11/12, 13, 15, 16, 17, and 20) and the secreted mucins. The latter category includes both the large gel-forming (MUC2, 5AC, 5B, 6, and 19) and small soluble mucins (MUC7 and 9).

Membrane-associated mucins have hydrophobic domains towards the carboxy terminus of the protein that allow them to span the cell membrane. The extracellular domain of membrane-associated mucin molecules may extend up to 500 nm from the epithelial cell surface (Bramwell *et al.*, 1986), forming a dense glycocalyx along the apical surface of the epithelia that is the cells' protective barrier. Experimental data indicate that the extracellular domain of membrane-associated mucins also provides a disadhesive character to the apical, luminal surfaces of epithelia (Hilkens *et al.*, 1992; Ligtenberg *et al.*, 1992; Komatsu *et al.*, 1997). The studies further indicate that the negatively charged carbohydrate residues on the mucin protein backbone provide the disadhesive character (Hilkens *et al.*, 1992; Ligtenberg *et al.*, 1992). The extracellular domain of membrane-associated mucins can be detected in mucosal secretions (Ellingham *et al.*, 1997; Jumblatt *et al.*, 1999; Pflugfelder *et al.*, 2000; Zhao *et al.*, 2001; Argueso *et al.*, 2002; Spurr-Michaud *et al.*, 2004) as well as in sera of breast tumor (Burchell *et al.*, 1984; Linsley *et al.*, 1986) and ovarian tumor (Yin *et al.*, 2002) patients, indicating that the extracellular domain is shed from apical surfaces (Rossi *et al.*, 1996) or that splice variants are secreted in which the membrane-spanning and cytoplasmic domain are not translated (Gendler, 2001; Moniaux *et al.*, 2001). The shed membrane-associated mucins may contribute to protection/lubrication of the epithelia in both soluble and membrane-tethered forms. Recent data indicate that membrane-associated mucins are multifunctional molecules, providing not only barrier and lubrication functions but also signal transduction through their juxtamembrane regions (Pandey *et al.*, 1995; Yamamoto *et al.*, 1997; Carraway *et al.*, 2002; Ren *et al.*, 2002). Several membrane-associated mucins have EGF-like domains near their membrane-spanning domain, and phosphorylation sites have been detected in the MUC1 cytoplasmic tail (Zrihan-Licht *et al.*, 1994; Pandey *et al.*, 1995; Quin and McGuckin, 2000).

The secreted mucins, gel-forming and small soluble, are produced by epithelial goblet cells and associated submucosal glands. The common features of gel-forming mucins are cysteine-rich D domains on either side of large long domains of tandem repeats. Three D domains are located in the amino terminal region of the protein and one in the carboxy region (Perez-Vilar and Hill, 1999). MUC6, however, lacks the carboxy D domain. The D domains provide sites for homo-multimerization of gel-forming mucin molecules; the large homo-multimers provide the viscosity of the mucosal secretions. The small soluble mucins lack D domains and subsequently remain as monomers (Bobek *et al.*, 1993).

Although mucins of the female reproductive tract have been studied (for review, see (Carson *et al.*, 1998; Gipson, 2001)), little is known about the mucin gene repertoire of the male urogenital tract. As assayed by in situ hybridization and immunohistochemistry, ejaculatory epithelial ducts and seminal vesicles expressed MUC6, while the prostate and bladder samples tested did not (Bartman *et al.*, 1998; Leroy *et al.*, 2003). In other male urogenital tissues, MUC1 message and protein have been detected in human testis and epididymis (Franke *et al.*, 2001), and MUC1, 3, and 4 have been reported in the normal urothelium (N'Dow *et al.*, 2000). Mucin gene expression in the human prostate, however, is controversial. In contrast to the MUC6 studies cited above (Bartman *et al.*, 1998; Leroy *et al.*, 2003), Northern blot and immunostaining assays detected MUC1 and MUC6 in the human prostate (Ho *et al.*, 1993; Gold *et al.*, 1994). Furthermore, Ho et al. (Ho *et al.*, 1993) did not detect MUC2 or 3 in the

prostate, while Durrant and colleagues (Durrant *et al.*, 1994) reported MUC2 expression using an antibody to the tandem-repeat of the mucin. A comprehensive analysis of mucin gene expression in the human testis, epididymis, vas deferens, seminal vesicle, prostate, bladder, urethra, and foreskin has not been reported. Inconsistencies such as those reported for MUC2 and MUC6 expression in the prostate (Ho *et al.*, 1993; Durrant *et al.*, 1994) validate assaying human specimens for both mucin mRNA and protein. In addition, there have been no reports on specific mucin components of seminal plasma. Similarly, androgen regulation of mucin gene expression in male urogenital tract epithelia has not been studied. A comprehensive analysis of mucins in the male urogenital tract will provide a baseline for future studies that aim to clarify which mucins may be involved in the various activities of the different regions of the male urogenital tract, where they may contribute to protection against infections from sexually transmitted pathogens, provide disadhesive functions to facilitate sperm passage and provide lubrication to maintain luminal patency.

Materials and methods

Tissue and seminal plasma acquisition and preparation

All discarded human tissue specimens of surgical and post-mortem origin collected from the human male urogenital tract were obtained in accordance with good clinical practice, Institutional Review Board and informed consent regulations of the Schepens Eye Research Institute and the Brigham and Women's Hospital, and the doctrines of the Declaration of Helsinki. Normal discarded tissues were collected from the urogenital tract during autopsy of male patients or as surgical specimens from prostatectomy, orchiectomy, malignancy of the bladder, vasectomy, and circumcision. Tissues collected included testis, epididymis, vas deferens, seminal vesicle, prostate, bladder, urethra, and foreskin. The tissues were snap frozen in liquid nitrogen for RNA isolation, or frozen in optimum cutting temperature [OCT] compound (IMEB, Inc.; San Marcos, CA) or fixed in 4% paraformaldehyde for immunofluorescence microscopy within 45 min of surgical removal or 38 h post-mortem.

Semen samples were collected from five male partners of women who had been pregnant within the previous two years. After 48 h of sexual abstinence, semen samples were collected by masturbation and submitted for semen analysis. Samples were diluted 1:1 with sterile PBS, mixed thoroughly, and centrifuged for 10 min at 400 × g. Seminal plasma was removed, aliquoted, and stored at −70°C.

Cell culture

We sought to characterize non-cancerous prostatic epithelial cells for mucin gene expression in order to identify potential transcriptional regulators of mucin expression. The primary human, adult prostatic epithelial cells used were PrEC (Cambrex Bio Science Walkersville, Inc.; Walkersville, MD). Cells were grown in Clonetics™ Prostate Epithelial Growth Media [PrEGM] (Cambrex Bio Science Walkersville, Inc.) containing bovine pituitary extract, insulin, hydrocortisone, gentamicin sulfate amphotericin-B, retinoic acid, transferrin, triiodothyronine, epinephrine, and human recombinant epidermal growth factor, per the manufacturer's instructions. Cells were cultured in six-well plates at 2.5×10^3 cells/cm^2 at 37° C in a 5% carbon dioxide atmosphere to confluence. To assess the potential regulatory effects of androgens and serum on mucin gene expression, media was supplemented with one of the following: 10% (v/v) calf serum (Invitrogen; Rockville, MD), 10^{-6} M dexamethasone, 10^{-8} M dihydrotestosterone [DHT] (Steraloids, Inc.; Newport, RI), or 1 nM or 5 nM mibolerone, a non-metabolizable androgen (BioMol Research Laboratories, Inc.; Plymouth Meeting, PA). Dexamethasone, DHT, and mibolerone were dissolved in ethanol (less than or equal to 0.01% v/v final concentration). Duplicate wells for each treatment condition, including an untreated control group, were harvested at 1, 3, and 6 days. The media was changed every 48 h.

RNA isolation and reverse transcription

Total RNA was extracted from pulverized, frozen tissues using TRIzol reagent (Invitrogen) in accordance with the manufacturer's recommended protocol. Cell culture lysates were homogenized in 1× Lysis Buffer (PE-Applied Biosystems), and total RNA was isolated using the ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems User Guide, 2001). As previously described (Argueso *et al.*, 2002), 2 μg aliquots of total RNA were treated with DNase I (Amplification Grade; Invitrogen) and reverse transcribed using random hexamer primers and Superscript II reverse transcriptase (Invitrogen), per the manufacturer's instructions.

Polymerase chain reaction

RNA isolated from tissue specimens was assayed by conventional RT-PCR at 35 cycles, to look for expression of MUC1, 2, 3, 4, 5AC, 5B, 6, 7, 13, 16, 17, 19, 20, with glyceraldehyde-3-phosphate dehydrogenase [GAPDH] as a quality control. PCR primers and amplification parameters for the mucin genes are indicated in Table I (Finkbeiner *et al.*, 1993;Bernacki *et al.*, 1999;Argueso *et al.*, 2003). MUC3 primers used in this screening recognized a homologous region in both MUC3A and 3B genes. Studies of the incompletely characterized mucins, MUC8, 9, and 11, were not conducted. Real-time PCR compatible primers (*Taq*Man chemistry, Applied Biosystems) for membrane-associated mucin MUC15 (Pallesen *et al.*, 2002) were designed using Primer Express software (Applied Biosystems) and are as follows: MUC15 Forward: TACAGCCAGCCCCACCTAATT; MUC15 Reverse: GAAAACAGATGGGTTAAGTGTGACAA. Nucleotide database searches using BLASTN (http://www.ncbi.nlm.nih.gov/blast/, provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) verified the specificity of the MUC15 primer set. The identity of the purified 81-bp MUC15 PCR product was confirmed by the DNA Sequencing Center for Vision Research, the Ocular Molecular Genetics Institute of the Massachusetts Eye and Ear Infirmary (Boston, MA).

Androgen receptor expression in RNA isolated from PrEC cells was determined by RT-PCR, using primers and amplification parameters as previously described (Table I) (Lau *et al.*, 2000). Samples lacking cDNA were run in each assay as negative controls, and cDNA from different tissues known to express one of the mucin genes or from human prostatic tissue, known to express the androgen receptor, were run as positive controls. Amplified products were run on a 2% agarose gel and visualized with ethidium bromide.

Real-time PCR

Relative mucin gene expression in the PrEC cell line was assayed with double-labeled fluorogenic primers and probes (TaqMan; Applied Biosystems), as previously described (Argueso *et al.*, 2002), using a sequence detection system (ABI Prism 7900HT; Applied Biosystems). MUC1, 4, 5AC, (Table I) and GAPDH PCR primers and probes used in this study have been reported (Argueso *et al.*, 2002). Equivalent PCR amplification efficiencies for GAPDH, the endogenous control, and the target mucin genes were confirmed.

The ΔC_T method (Applied Biosystems) was employed for the relative quantitation of mucin gene expression (Argueso *et al.*, 2002; Gipson *et al.*, 2003). Quantitative mRNA expression of each mucin gene amplified in the cell line was expressed relative to the amount of MUC1 mRNA present in a calibrator sample ($\Delta\Delta C_T$ method), namely the untreated control culture group of each time point assayed. Samples were amplified in triplicate in a 50 μl total volume reaction for 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60° C. Controls (no template) included in each real-time PCR assay confirmed that the amplification reagents were not contaminated with DNA. Real-time PCR results were

statistically compared using the Fisher Protected Least-Significant Difference (Fisher's PLSD) test (StatView, Version 5.0, SAS Institute; Cary, NC). $P < 0.05$ was considered significant.

Immunofluorescence microscopy

Morphological characteristics of each of the male urogenital tract tissues were analyzed on hematoxylin- and eosin-stained sections. Mucins were immunolocalized in the testis, epididymis, seminal vesicle, prostate, bladder, urethra, and foreskin by immunofluorescence [IF] microscopy as previously described (Gipson *et al.*, 1992; Inatomi *et al.*, 1995). Vas deferens tissue was not available for immunofluorescence analysis. Tissues were incubated for 1 h at room temperature in primary antibody. Antibodies and their dilutions used are indicated in Table II. All antibodies, with the exception of the MUC4 antibody designated 528, have been previously described. The antibody was made to a synthetic peptide (SSIVPGTFHPTLSEAC) from the deduced amino acid sequence of a MUC4 genomic clone (Gipson *et al.*, 1999). Peptides were synthesized by solid-phase procedure (F moc chemistry) and then glutaraldehyde-conjugated to keyhole limpet hemocyanin in the Peptide Synthesis Core of the Reproductive Endocrine Sciences Center, Massachusetts General Hospital. Antibodies against the conjugated 528 peptide were produced in chickens by Avian Antibodies, Inc. (Carlisle, MA) and affinity purified prior to use.

Samples lacking primary antibody were run as negative controls. Sections were incubated in secondary antibody in the fluorescein-conjugated donkey-anti mouse IgG or donkey-anti chicken IgY (both at 1:50 dilution from Jackson Immuno Research; West Grove, PA) and were coverslipped with Vectashield, a propidium iodide-containing antifade mounting medium that also acts as a nuclear counterstain (Vector Laboratories; Burlingame, CA) (Argueso *et al.*, 2002).

Sections incubated with the MUC6 primary antibody, CLH5, were pretreated with neuraminidase as previously reported (Gipson *et al.*, 1992). Antigen retrieval (15 min at 97°C in 10 mM citrate buffer, pH 6.0) was necessary prior to incubation with the commercially available MUC4 antibody, 1G8.

Agarose gel electrophoresis and immunoblot analysis

Total protein (125 μg) from seminal plasma was denatured and separated under reducing conditions in a 1% (w/v) agarose gel in electrophoresis buffer (Tris/Glycine/SDS), a modification of Thornton et al. (Thornton *et al.*, 2000). Appropriate controls were also included in the assays to ensure the specificity of the protein detected. The proteins were then vacuum blot transferred to a nitrocellulose membrane, blocked with 5% nonfat dry milk (w/v) in Tris-buffered saline with 0.1% Tween 20 [TTBS] or PBS, and probed with the following antibodies: 214D4 (MUC1), 8G7 (MUC4), CLH2 (MUC5AC), 799W (MUC5AC), and CLH5 (MUC6) (Table II). Following incubation with peroxidase-conjugated-goat-anti-mouse IgG1 (MUC1, 4, 5AC, 6) or anti-chicken IgY (MUC5B) secondary antibody, binding was detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce; Rockford, IL).

Results

In order to determine the mucin profile for native human male urogenital tract epithelia, human tissue from multiple individuals, as available, were analyzed for message (see Table III) and protein. On average, 3 to 7 specimens were assayed for mucin message and 3 to 9 specimens for mucin protein, whereas only 1 to 2 specimens of vas deferens and epididymis were available (discarded epithelial specimens from these tissues were difficult to collect). Five seminal plasma samples were also collected for immunoblot analysis.

Mucin gene and protein expression profile

Epithelia from all major regions within the male urogenital tract, from testis to foreskin, were analyzed for mucin message and protein. Gene transcripts for MUC1, 3, 4, 5AC, 6, 13, 15, 17, and 20 were detected by RT-PCR (Table III, Figure 1). Human epithelia known to express each mucin gene, and their corresponding negative controls, validated all RT-PCR assays – human trachea (MUC1, 4, 5B, 7, 13, 16, 19, 20), small intestine (MUC2, 3, 17), stomach (MUC5AC, 6) and salivary gland (MUC15) (data not shown). The housekeeping gene, GAPDH, was amplified in all of the samples, and only those samples with quality starting mRNA were assayed for mucin gene expression. MUC2, 5B, 7, 16, and 19 message were not detected in male urogenital tract epithelia. Of the nine (9) mucin gene transcripts detected, well-characterized antibodies were only available for localization of MUC1, 4, 5AC, 5B, and 6 proteins. The results of immunolocalization of these mucin proteins in the male urogenital tract generally support the RT-PCR data, with exceptions noted below.

Testis, epididymis, and vas deferens—Testicular tissue expressed mRNA for MUC1 and 20 in 2 out of 3 samples and for MUC4, 5AC, and 6 in 1 out of 3 samples amplified. The mucin antibodies used in this study did not bind to the testicular sections assayed, indicating that, despite the presence of MUC1 message in the testis, the corresponding protein was not detected. By contrast, MUC1 mRNA (2 out of 2 samples) and protein (1 out of 1 sample) were detected in the caudal epididymis. In fact, antibodies to MUC1 bound relatively uniformly and intensely to the stereocilia on the apical membrane of the ductal epithelium (Figure 2B, C). Of all the male urogenital tract epithelia analyzed, the binding intensity of MUC1 protein was the greatest in the pseudostratified epithelium of the caudal epididymis. MUC6 and 15 gene transcripts were also detected in 1 of 2 epididymis samples, making this the urogenital tract epithelium that expressed the fewest mucins (Table III). Only one sample of vas deferens was available for assay, and it was found to express MUC1, 3, 4, 5AC, 15, 17, and 20. Well-preserved epithelium from the vas deferens was not available for immunofluorescence analysis.

Seminal vesicle and prostate—MUC1, 6, and 20 mRNA was detected in 5 out of 5 seminal vesicle samples, and MUC4, 15, and 17 in 1 to 2 out of 5 samples. Three seminal vesicle sections displayed positive apical membrane binding for MUC1. Unlike the epididymis, where there was uniform binding of the MUC1 antibody, the binding pattern for MUC1 in the luminal epithelium of the seminal vesicle was patchy (Figure 3B). MUC6 protein was detected throughout the cytoplasm of some seminal vesicle epithelial cells, and other cells had little if any antibody binding to MUC6 (Figure 3C).

Mucin gene transcripts for MUC1, 3, 4, and 20 were amplified in 6 out of 6 prostate samples, and MUC5AC expression was detected in 4 of 6 prostate specimens. Regional variations in mucin protein localization, as with seminal vesicle, were noted in the prostate. Antibodies to both MUC1 and 4 bound to the apices of the epithelial cell membranes lining the lumen of the prostate gland (Figure 4B, C). MUC5AC protein was also detected in the cytoplasm towards the apical surface of the glandular epithelium (Figure 4D).

Bladder, urethra, and foreskin—As determined by RT-PCR analysis, the human bladder expressed MUC1, 3, 4, 5AC, 13, 15, 17, and 20. The immunohistochemical data on MUC1, 4, and 5AC supported the RT-PCR findings. The bladder samples assayed were contracted after fixation, as evidenced by the rounded appearance of the apical epithelial cells, the overall thickness of the transitional epithelium and the appearance of crypt-like foldings. MUC1 localized to the apical cytoplasm of the transitional epithelium at the tops of the artificial folds lining the lumen of the bladder (Figure 5B). Curiously, MUC5AC antibody bound to clusters of apical cells of the epithelium and these surface cells were filled with the mucin (Figure 5C).

Urethral epithelium expressed MUC1, 3, 4, 5AC, 13, 15, 17 and 20 (Table III). MUC1 protein distribution was prominent along the apical layer of the stratified epithelium of the urethra and, to a lesser extent, in the subapical cells of the epithelium (Figure 6B). On the other hand, MUC4 binding was more evenly distributed throughout the stratified epithelium (Figure 6C). The only secretory mucin detected in the urethra was MUC5AC, where it was localized to the glands of Littré present in the lamina propria of the urethra (Figure 6D). The foreskin, discarded following circumcision, was the most external human male urogenital tissue assayed. MUC1, 3, 4, 5AC, 13, 15, 17, and 20 gene transcripts were amplified, and MUC1 and 4 protein (1 out of 2 samples) were detected in the foreskin, confirming the transcriptional data.

MUC1 and 20 were detected in most of the male urogenital tract epithelia, with the exception of epididymis (MUC1 only). Representative agarose gels of PCR products amplified from vas deferens, prostate, and urethra are presented in Figure 1. A summary of RT-PCR data for all of the tissue types analyzed can be found in Table III.

Mucins present in seminal plasma

Immunoblot analysis for presence of MUC1, 4, 5AC, 5B, and 6 was performed on five samples of seminal plasma from fertile men. MUC1, 5B, and 6 proteins were detected, but differences in binding intensities were noted between individuals in equally loaded gels (Figure 7). The MUC1 antibody (214D4) bound to all five seminal plasma samples assayed, with two samples showing greater intensity. Two bands were observed in one sample indicating polymorphism of the gene (Gendler and Spicer, 1995). MUC6 binding (CLH5 antibody) was the most variable, with MUC6 protein strongly detected in only two out of five samples, with one more intense than the other. Compared to MUC1 and 6, MUC5B binding (799W antibody) was relatively uniform amongst the samples and displayed the characteristic smear observed in immunoblot detection of highly glycosylated gel-forming mucins such as MUC5B (Gipson *et al.*, 2001). Despite detection of MUC4 and 5AC message by RT-PCR in urethral tissues, protein was not found in the seminal plasma samples assayed (data not shown). Positive and negative controls included in the immunoblot analysis confirmed the specificity of mucin proteins detected in seminal plasma (Figure 7).

Mucin mRNA expression in primary prostatic epithelial cells [PrEC]

Primary prostatic epithelial cells, PrEC, were used for assessing androgen regulation of mucin expression in the prostatic epithelium. Dihydrotestosterone [DHT] and mibolerone, a non-metabolizable androgen, were tested. The expression of androgen receptor and GAPDH, an internal mRNA quality control, in PrEC cells was confirmed by RT-PCR (Figure 8) prior to relative quantitation by real-time PCR of mucin transcripts in confluent cultures.

Androgen regulation of three of the five mucins detected in the native prostatic epithelium (MUC1, 4, and 5AC), were analyzed. The mRNA values of MUC1 mucin in the untreated control group served as the calibrator for the corresponding time point's treatment groups (baseline relative expression = 1). The untreated control group expressed only MUC1 at 1, 3 and 6 days of culture (Figure 9A). MUC5AC remained undetectable throughout the time course of the experiment (data not shown). Neither DHT nor mibolerone, at any concentration, effected mucin expression. To test the PrEC cells response to dexamethasone and serum, potent regulators of mucins in ocular surface epithelia (Gipson *et al.*, 2003; Hori *et al.*, 2004), PrEC cells were treated with 10^{-6} M dexamethasone or switched to serum-containing media (10% (v/v)) for the duration of the experiment. Unlike ocular surface epithelia, dexamethasone had no effect on mucin gene expression, but as in ocular surface epithelia, both MUC1 and MUC4 transcripts were significantly upregulated in the serum treatment groups. MUC1 expression peaked at 1 day post-treatment, with an 18-fold increase (Figure 9A), whereas MUC4 expression peaked with a 3.3-fold increase at 6 days post-treatment (Figure 9B).

Discussion

The data obtained in this study provide baseline information on mucin gene expression profiles of the male urogenital tract epithelia. The data also indicate that each region of the male urogenital tract epithelium analyzed has a unique mucin gene repertoire. These variations in mucin expression may reflect specific functions of each region. The fact that testis and epididymis epithelia are most distal from the external environment (and thus the pathogen source) and have spermatozoa associated with their epithelial surfaces may be reflected in their mucin expression patterns. For example, spermatozoa are moved gently through the lumen of the epididymis epithelium by surface cilia or stereocilia that are richly invested with MUC1, which may prevent their adherence. The occlusion of the epididymis that occurs in gonorrheal and chlamydial infection (Ness *et al*., 1997) may be due in part to the presence of fewer mucins in this region. Similar occluding and cicatrizing pathological processes occur at the ocular surface and in these cases mucin-secreting cells are lost (Nelson *et al.*, 1983; Argueso *et al.*, 2002).

Several more membrane-associated mucins are expressed in the vas (ductus) deferens and urethra, regions in which sperm transport is rapid and driven by muscular contraction. Forces on the surface of these epithelia may be harsher, requiring better lubrication and disadhesive properties. In addition, these tubular organs are closer to the external environment than the epididymis and thus more subject to infection.

The glands emptying into the vas deferens and urethra have unique secretory products that may have caustic effects on these epithelia, thus requiring mucin protection. For example, seminal vesicle epithelium produces MUC6 that may protect its surface from its alkaline secretions. MUC6 expression is limited to the stomach and seminal vesicles, which has led Toribara et al. to hypothesize that MUC6 shields against harsh acid or alkaline environments better than other gel-forming mucins because this mucin has fewer exposed unglycosylated regions (specifically an absence of the unglycosylated carboxy-terminal D domain) that would be subject to acid/alkaline degradation (Toribara *et al.*, 1997). Similarly, prostatic epithelium has a large mucin gene repertoire, including several membrane-associated mucins and the gel-former MUC5AC. A large repertoire of mucins may protect this epithelium from secreted lytic enzymes. Bladder epithelium also expresses several membrane-associated mucins as well as MUC5AC, a gel-forming mucin. It too exists in a caustic, urea-rich environment, and the redundancy of expression of membrane-associated mucins, including MUC1, 3, and 4, confirming the previous findings by N'Dow and colleagues (N'Dow *et al.*, 2000) may indicate that a mucin-rich protective surface barrier is required by bladder epithelium.

It is not surprising that gel-forming mucins MUC5B and MUC6 are constituents of seminal plasma, since sperm motility studies indicate that cervical mucins protect sperm from the vaginal environment and assists their transport through the female reproductive tract (Eriksen *et al*., 1998). Mucins in the male urogenital tract may serve similar functions, especially along the urethra where both urine and sperm are conducted out of the body. Our immunoblot data detecting MUC5B protein in seminal plasma is of particular interest, since MUC5B was not detected by RT-PCR or immunohistochemistry in any of the urogenital tract epithelia analyzed. Perhaps the Cowper's Glands, which were not available for study, could account for this discrepancy. However, only 5% of seminal secretions originate from these secondary glands; prostate and seminal vesicle secretions account for the majority of the seminal volume (reviewed in (Owen and Katz, 2005)). Unlike MUC5B, MUC4 and MUC5AC proteins were not detected in the seminal plasma samples despite finding their transcripts in the male urogenital epithelia, especially in the prostate and urethra. Although it is unclear why these mucin proteins were not found, proteases, such as trypsin and prostate specific antigen [PSA],

have been found in seminal fluid (Paju *et al.*, 2000) and may destroy the antigen binding sites on these mucins.

By comparison to the endocervical epithelium of the female reproductive tract, where three of the large gel-forming mucins, MUC5AC, 5B, and 6, were detected in some epithelial cells (Gipson *et al.*, 1997), no such protective redundancy was identified in any cells of male urogenital tract epithelia examined. With the exception of seminal vesicle and epididymis epithelia, MUC5AC was detected in all epithelia assayed, whereas seminal vesicle expressed only MUC6 (mRNA and protein). Our data corroborate previous findings, regarding seminal vesicle epithelial expression of MUC6 (Ho *et al.*, 1995; Bartman *et al.*, 1998; Leroy *et al.*, 2003).

MUC2 and 6 expression in the prostate is controversial. Durrant et al. detected MUC2 by immunohistochemistry using the monoclonal antibody 996/1 (Durrant *et al.*, 1994), while Ho et al. and Gold et al. failed to identify MUC2 expression in the prostate by Northern blot and immunostaining techniques (Ho *et al.*, 1993; Gold *et al.*, 1994). A similar dichotomy exists for MUC6 reports – detected by Northern blot and immunostaining (Ho *et al.*, 1993; Gold *et al.*, 1994) but not by in situ hybridization or immunohistochemistry (Bartman *et al.*, 1998; Leroy *et al.*, 2003). Our data indicate that neither MUC2 nor MUC6 is present in prostatic epithelium.

The mucins in the human female reproductive tract are hormonally regulated. Cervical MUC4 and 5B expression peaks when progesterone levels are low (prior to midcycle) in normally cycling women (Gipson *et al.*, 1999), and the highest levels of MUC5B protein are detected in cervical mucus at midcycle (Gipson *et al.*, 2001). Unlike female reproductive tract mucins, prostatic epithelial mucins MUC1 and 4 do not appear to be under hormonal control. O'Connor et al. reported similar findings for MUC1 when PrEC cells were cultured with DHT (O'Connor *et al.*, 2005).

To our knowledge, the data reported in this manuscript provide the most inclusive mucin profile of the human male urogenital tract epithelia to date. Even so, inherent limitations of the study exist. Since the samples consisted of discarded surgical and post-mortem epithelia, the quantity of usable specimens collected varied from 1 to 9 individuals per epithelia. Moreover, epithelia from all areas of interest within the male urogenital tract were not available for collection. Efferent ducts, ejaculatory ducts, and Cowper's gland, a potential major mucin source, were not available for study.

Further study of mucins in the male urogenital tract may yield information regarding the functional importance of specific mucins in the maintenance of normal reproductive health, including defense against pathogens. The mucin profile and expression level in male urogenital tract epithelia in diseased states, such as infections with sexually transmitted pathogens, will thus be of interest, particularly if downregulation occurs. Effects of inflammation resulting from infections on mucin expression have not been investigated in these epithelia, and, since information on regulators of mucin expression is beginning to be known, therapeutic agents targeted at regulation may be feasible.

Summary Sentence

Tissue-specific mucin repertoires were observed in the human male urogenital tract epithelia; MUC1 and MUC4 expression in prostatic epithelium is not under direct androgenic control.

Acknowledgments

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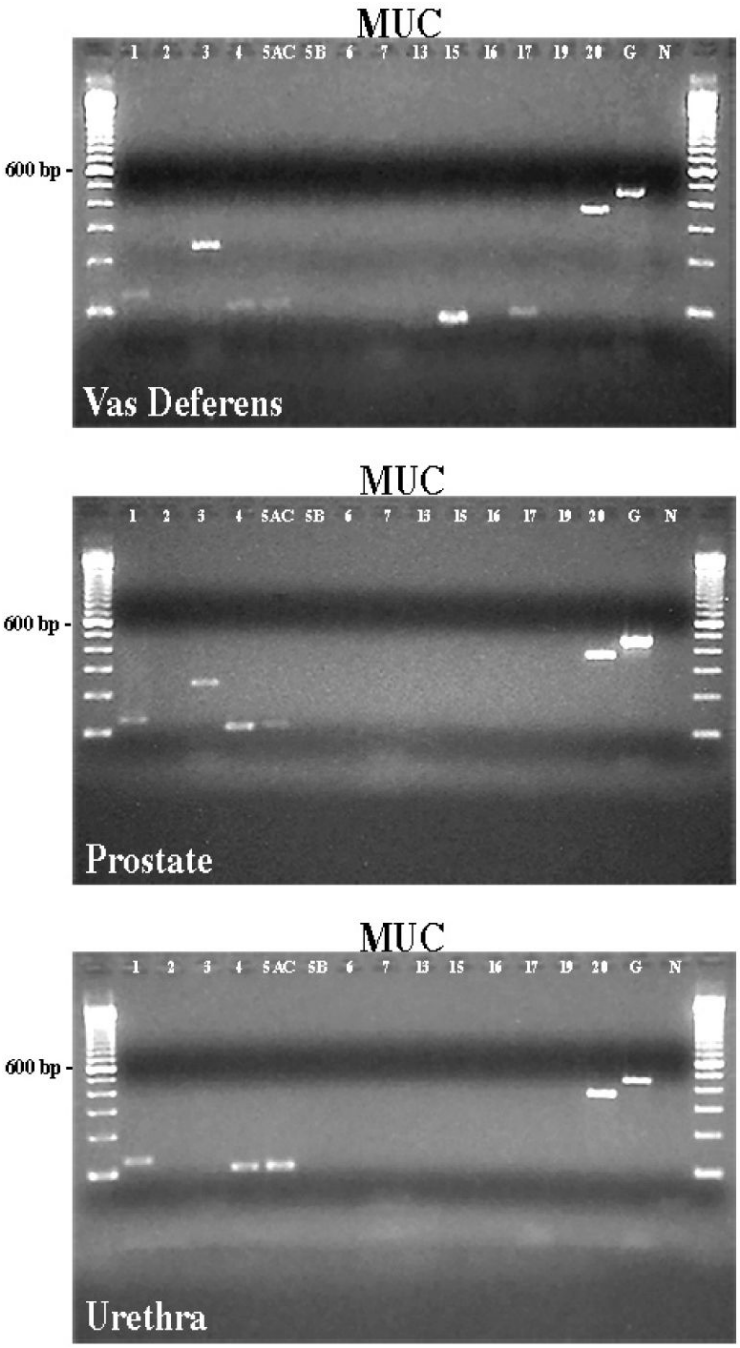

Figure 1.

Representative RT-PCR analyses of mucin mRNA from human vas deferens, prostate, and urethra. **1**, MUC1 (104bp); **2**, MUC2 (441bp); **3**, MUC3 (234bp); **4**, MUC4 (101bp); **5AC**, MUC5AC (103bp); **5B**, MUC5B (405bp); **6**, MUC6 (303bp); **7**, MUC7 (901bp); **13**, MUC13 (73bp); **15**, MUC15 (81bp); **16**, MUC16 (114bp); **17**, MUC17 (91bp); **19**, MUC19 (94bp); **20**, MUC20 (360bp); **G**, GAPDH (452bp); **N**, no RT control.

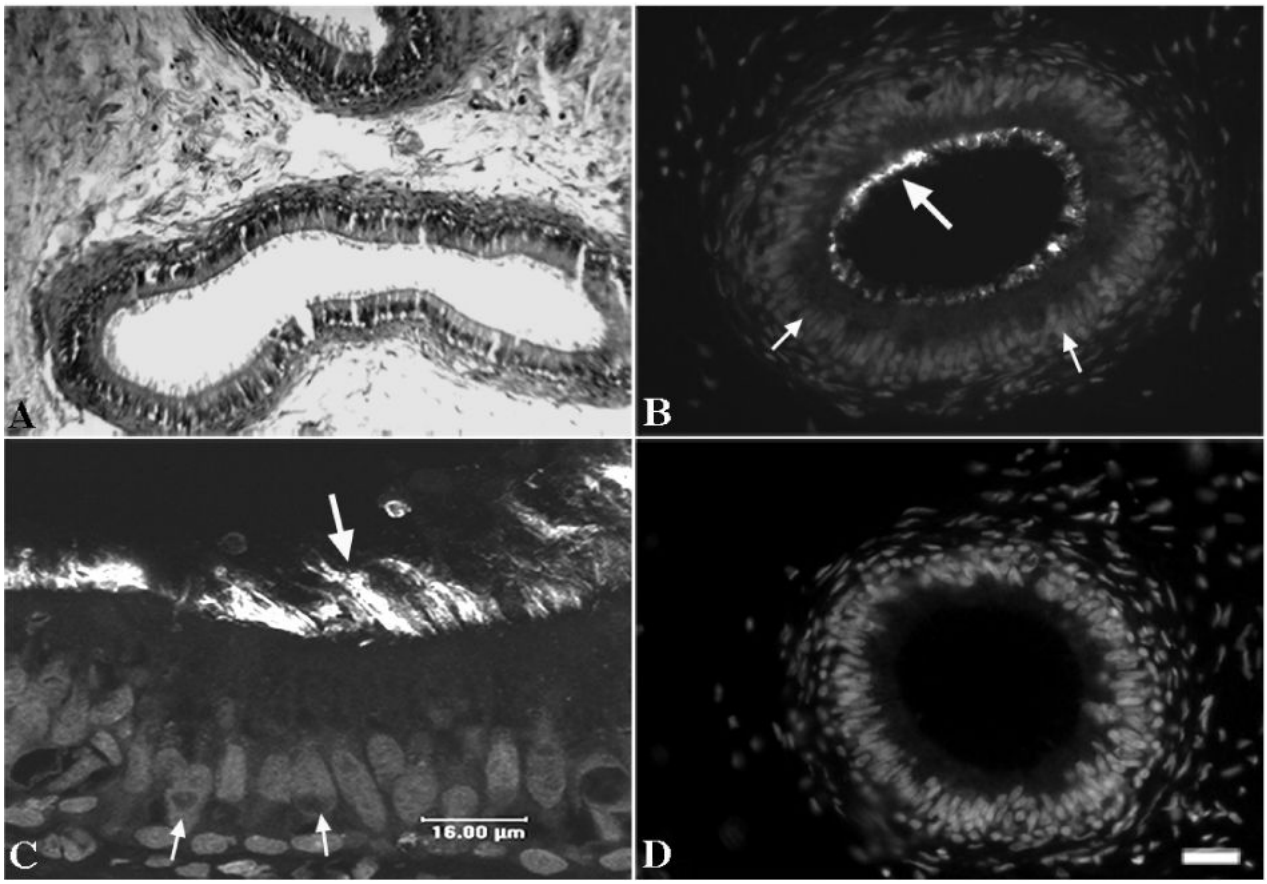

Figure 2.

Immunofluorescence localization of mucin protein in epithelial cells of the epididymis. (**A**) Light micrograph showing histology of epididymis tissue. (**B**) MUC1 protein (HMFG-2 antibody) is localized to the apical luminal surface (large arrow) of the epithelia lining the lumen of the epididymis. Nuclei (small arrows) were stained with propidium iodide to demonstrate epithelial borders. (**C**) Confocal microscopy image of MUC1 (HMFG-2 antibody) on the epididymis epithelium. MUC1 is specifically located on the stereocilia (large arrow) of the pseudostratified epithelium. Small arrows indicate cell nuclei. (**D**) Section of epididymis illustrating lack of binding of secondary antibody alone (negative control). Scale bars: A, B, D = 10 µm; C = 16 µm.

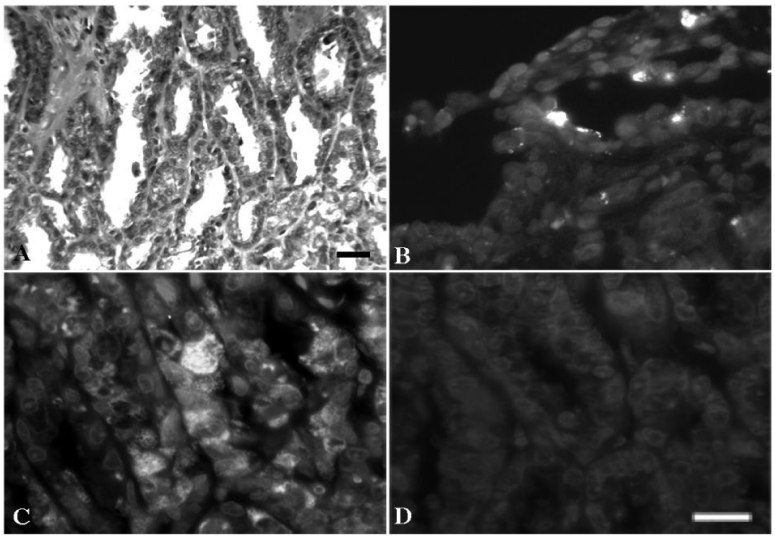

Figure 3.

Localization of mucin protein in seminal vesicle epithelia. (**A**) Histology of seminal vesicle epithelium as determined by hematoxylin and eosin staining. (**B**) Patchy MUC1 (HMFG-2 antibody) immunolocalization to the luminal epithelium of the seminal vesicle. (**C**) MUC6 protein (CLH5 antibody) detected in the cytoplasm of a majority of the seminal vesicle epithelial cells. (**D**) Control section showing lack of nonspecific binding of secondary antibody to a section of seminal vesicle. Scale bars: A; B − D = 10 μm. Intense binding illustrates positive binding for mucin protein. Nuclei of epithelia were counterstained with propidium iodide to demonstrate epithelial cells.

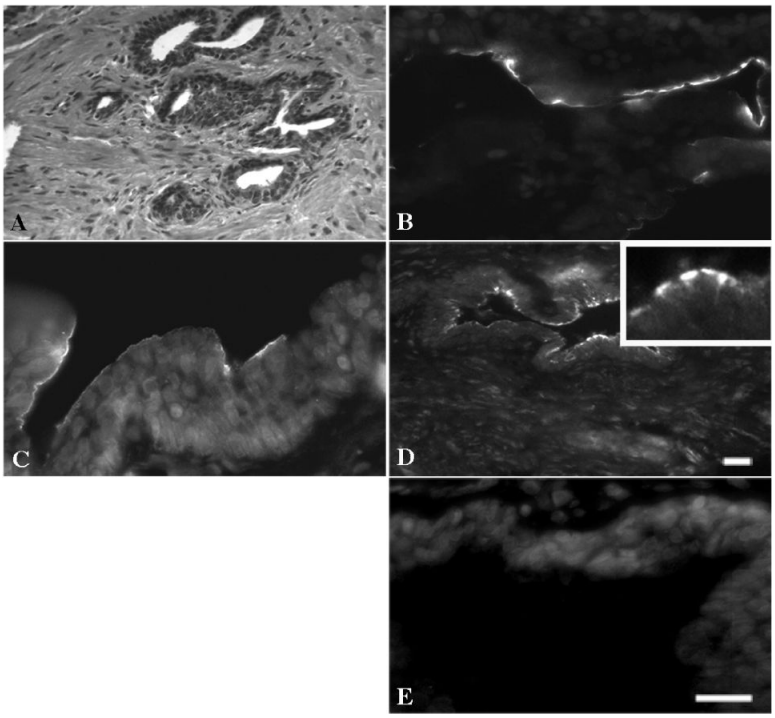

Figure 4.

Immunolocalization of mucin protein in prostate epithelium. (**A**) Histology of prostate depicting the integrity of the specimen's epithelium. MUC1 antibody (HMFG-2) (**B**) and MUC4 antibody (1G8) (**C**) bound to the apical portions of the prostate epithelium. MUC4 appears to localize to both apical and subapical cells, probably due to a plane of section. (**D**) MUC5AC protein (791 antibody) in apical cytoplasm of luminal epithelium. Inset is a higher magnification of MUC5AC positive cells showing cytoplasmic localization (**E**) Negative control showing nonspecific binding of secondary antibody to prostate epithelium. Epithelial nuclei were stained with propidium iodide to demonstrate epithelial borders. Scale bars: A, D; B, C, E = 10 μm.

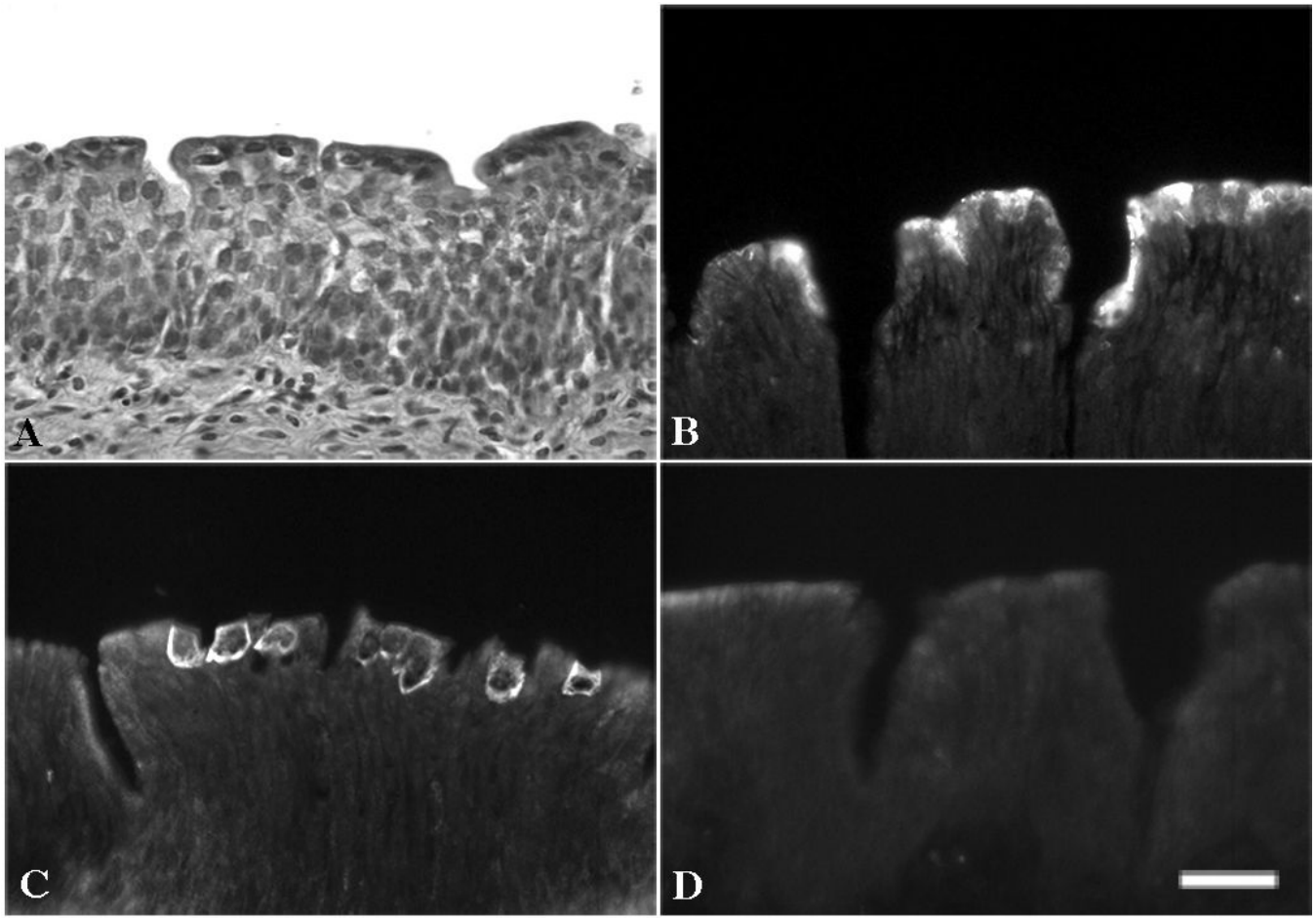

Figure 5.
Immunolocalization of mucin protein in bladder epithelium. (**A**) Light micrograph showing histology from a representative section. (**B**) MUC1 protein (HMFG-2 antibody) localized to the apices of the crypts of the transitional epithelium. (**C**) MUC5AC (791 antibody) was localized in the cytoplasm of apical cells in the epithelium. (**D**) Control showing slight nonspecific binding to bladder epithelium (no primary antibody). Nuclei were stained with propidium iodide. Scale bar = 10 μm.

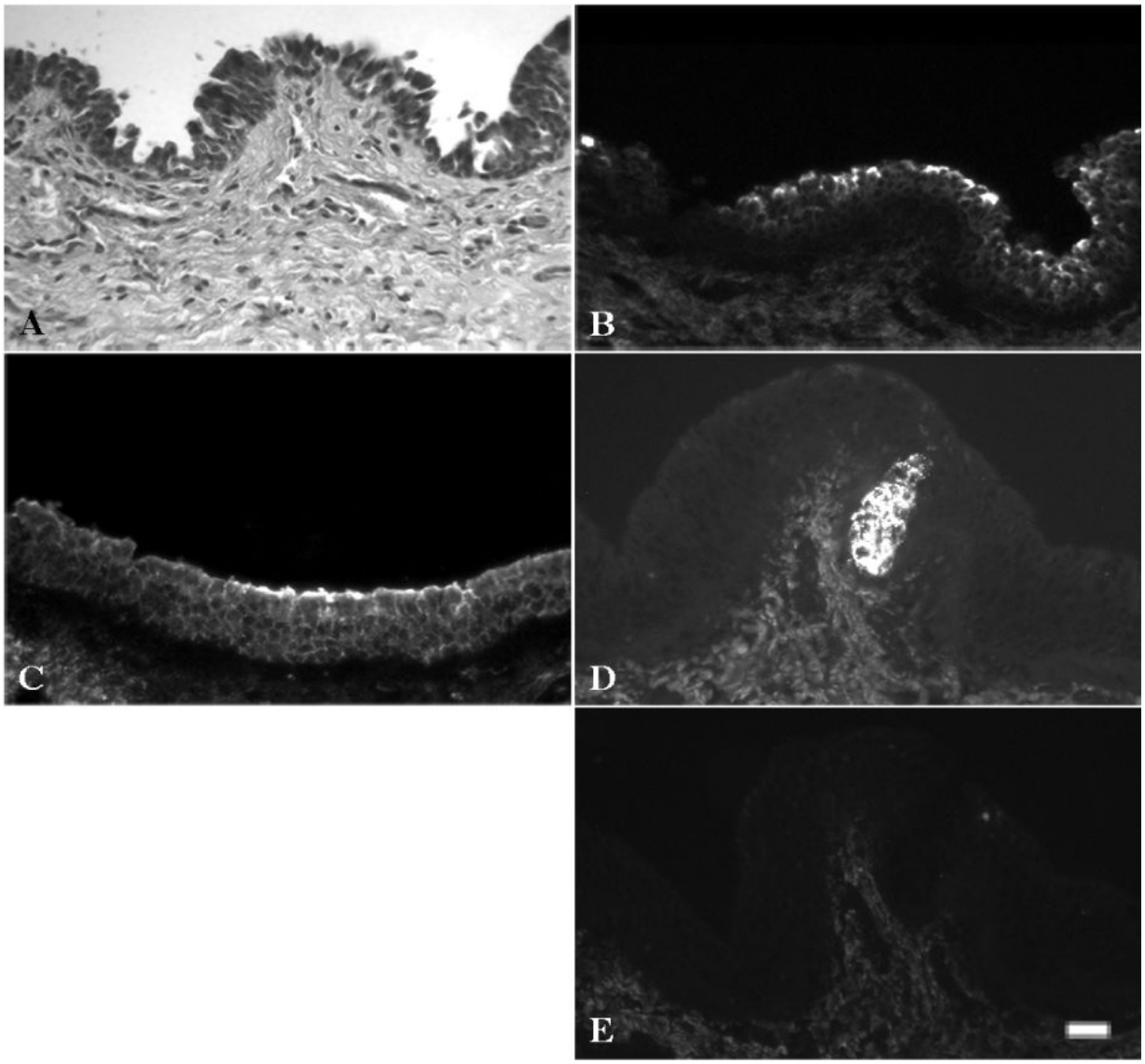

Figure 6.

Distribution of mucin protein in urethral epithelium. (**A**) Histological characterization of urethra sample. Prominent apical binding of MUC1 (HMFG-2 antibody) (**B**) and MUC4 (528 antibody) (**C**), with some localization of both mucin proteins to the subapical portions of the stratified epithelium. (**D**) MUC5AC (CLH2 antibody) strongly bound to the glands of Littré, with little to no binding detected on the apical surfaces of the urethral epithelium. (**E**) Negative control depicting nonspecific binding of antibody to IgG-containing cells in a section of urethral epithelium. Nuclei were stained with propidium iodide. Scale bar = 10 μm.

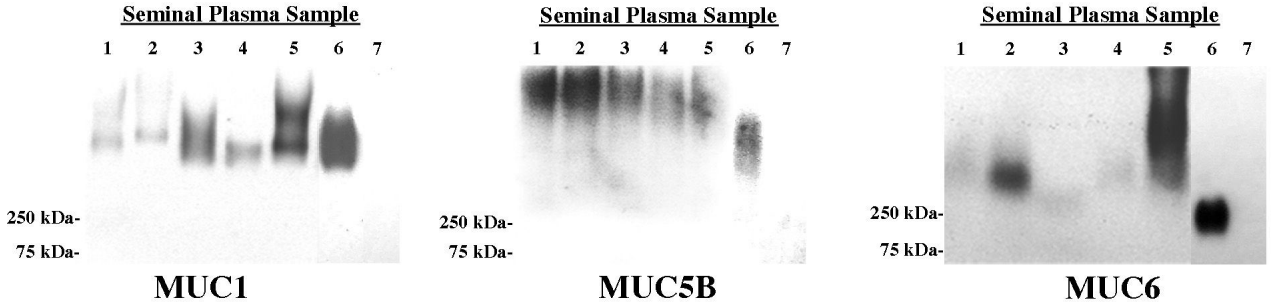

Figure 7.

Representative immunoblots demonstrating the presence of MUC1, 5B, and 6 in five samples of seminal plasma. Varying levels of mucin protein were observed in the samples. 125 μg of total protein was loaded in each lane and run on a 1% (w/v) agarose gel under reducing conditions. Lanes 6 and 7 indicate positive and negative controls, respectively. For MUC1, 25 μg of total protein from an immortalized human corneal cell line was run as a positive control and 25 μg of CHO protein was included as a negative control. For MUC5B, 1 μg of purified human cervical mucus (positive control) and 125 μg of urine (negative control) were assayed. For MUC6, fundus (1 μg) served as the positive control, while urine (125 μg) was run as a negative control. MUC4 and 5AC were not detected in the samples (data not shown).

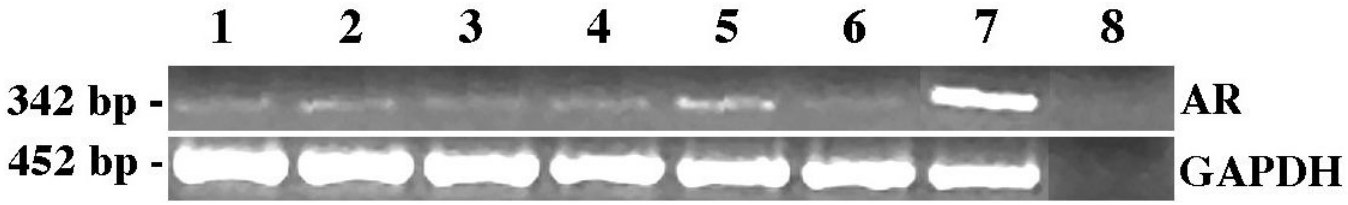

Figure 8.

Androgen receptor [AR] and GAPDH mRNA expression (RT-PCR) in confluent PrEC cells. cDNA were electrophoresed on 2% agarose gels and visualized with ethidium bromide. PCR products of expected amplicon size were amplified from RNA from PrEC cells from all conditions tested. **1**, untreated; **2**, serum; **3**, dexamethasone; **4**, DHT; **5**, 1 nM mibolerone; **6**, 5 nM mibolerone; **7**, prostatic tissue (positive control); **8**, no RT control. Note that the experimental levels of the receptor in the untreated and treated PrEC cells are less than that of the human prostatic tissue.

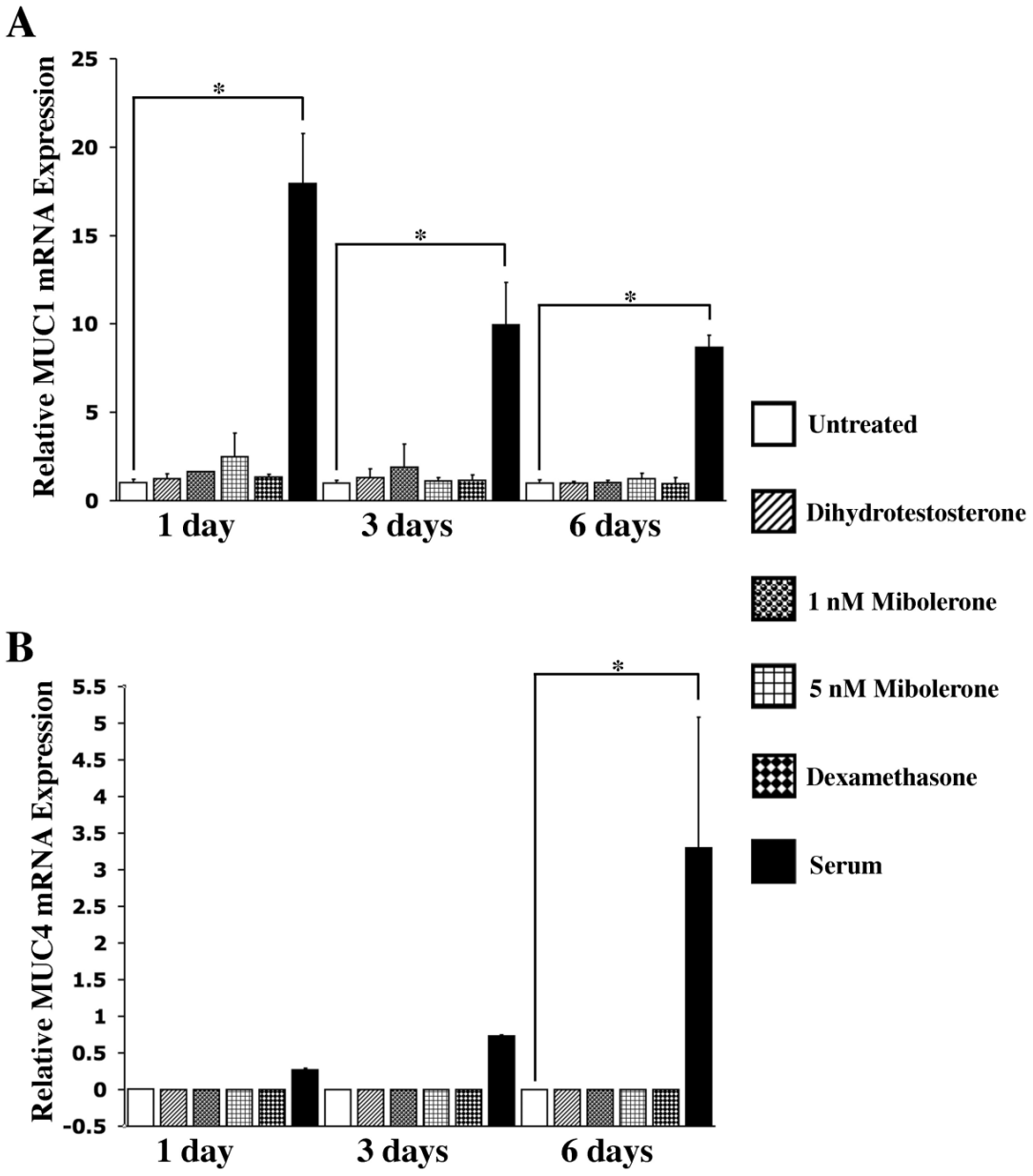

Figure 9.

Real-time PCR assay of relative expression of membrane-associated mucins MUC1 (**A**) and MUC4 (**B**) in primary prostatic epithelial cells, PrEC, treated with the androgens DHT and mibolerone, dexamethasone and serum. The level of MUC1 in the untreated control for each time point was used as the calibrator (expression set at 1). Duplicates were harvested for all experiments conducted. Serum significantly upregulated MUC1 at days 1, 3, and 6, and MUC4 at day 6 ($P < 0.05$). Data are mean ± SEM (error bars).

TABLE I

Primers used for PCR analysis.

Gene	Primer Sets	Product Size	Traditional Amplification	PCR Parameters	Reference	GenBank Accession No.
MUCI	F: 5'-GTG CCC CCT AGC AGT ACC G-3' R: 5'-GAC GTG CCC CTA CAA GTT GG-3'	104 bp	1 cycle:	95°C for 10 min;	Argueso et al., IOVS 2002; 44:1004–1011	NM_002456
	* TaqMan Probe for Real-lime PCR: AGC CCC TAT GAG AAG GTT TCT GCA GGT AAT G		35 cycles:	95°C for 15 sec; 60°C for 1 min		
MUC2	F: 5'-CAA GCA CAG CAC CGA TTG CTG AGT-3' R: 5'-CAC CTG GTG CGT AGT ACG TGT CGT T-3'	441 bp	1 cycle:	94° C for 5 min	Finkbeiner et al., Am J Respir Cell Mol Biol 1993; 9:547–556	NM_002457
			35 cycles:	94° C for 1 min; 60° C for 1 min; 72° C for 1 min		
			1 cycle:	72° C for 7 min		
MUC3	F: 5'-CCT CAT TGC AAA CTT CAC TC 3' R: 5'-AGC CCA CAT TTT CTG TAC TG-3'	234 bp	1 cycle:	94° C for 2 min	Bernacki et al., Am J Respir Cell Mol Biol 1999; 20:595–604	AF007194
			35 cycles:	94° C for 1 min; 55° C for 2 min; 72° C for 3 min.		
			1 cycle:	72° C for 7 min		
MUC4	F: 5'-GCC CAA GCT ACA GTG TGA ACT CA-3' R: 5'-ATG GTG CCG TTG TAA TTT GTT GT-3'	101 bp	1 cycle:	95°C for 10 min;	Argueso et al., IOVS 2002; 44:1004–1011	AF058803
	* TaqMan Probe for Real-time PCR: CGG CCA CAT CCC CAT CTT CTT CAC		35 cycles:	95°C for 15 sec; 60°C for 1 min		
MUC5AC	F: 5'-TCC ACC ATA TAC CGC CAC AGA-3' R: 5'-TGG ACC GAC AGT CAC TGT CAA C-3'	103 bp	1 cycle:	95°C for 10 min;	Argueso et al., IOVS 2002; 44:1004–1011	Z48314
	* TaqMan Probe for Real-time PCR: CTC GCT GGC CAT TGC TAT TAT GCC C		35 cycles:	95°C for 15 sec; 60°C for 1 min		
MUC5B	F: 5'-GAC ATT GAC CGC TTC CAG G-3' R: 5'-GAG ATT CCC AAA GCG TGC ATG-3'	405 bp	1 cycle:	94° C for 5 min	Gipson et al., Biol Reprod 1999; 60:58–64	U78553
			35 cycles:	94°C for 40 sec; 58°C for 1 min; 72°C for 1 min		
			1 cycle:	72°C for 7 min		

Gene	Primer Sets	Product Size	Traditional Amplification	PCR Parameters	Reference	GenBank Accession No.
MUC6	F: 5'-AGG AGG AGA TCA CGT TCA AG-3" R: 5'-TGT CAT CTG CAG TCC TTC AG-3'	303 bp	1 cycle:	94°C for 5 min	Gipson et al., Biol Reprod 1999; 60:58–64	U97698
			35 cycles:	94°C for 40 sec; 64°C for 1 min; 72°C for 2 min		
			1 cycle:	72°C for 7 min		
MUC7	F: 5'-AAA TAG CAG TGT GGT CAA CC-3' R: 5'-GCA CTC ATG AAT CAC ATC T-3'	901 bp	1 cycle:	94°C for 2 min	Bernacki et al., Am J Respir Cell Mol Biol 1999; 20:595–604	LI3283
			35 cycles:	94°C for 1 min; 55°C for 2 min; 72°C for 3 min		
			1 cycle:	72°C for 7 min		
MUC13	F: 5'-TGC TTC TAT CCC TCC AAT GGA-3' R: 5'-TGG GTG AGG CTA GGT TGC A-3'	73 bp	1 cycle:	95°C for 10 min;	Gipson et al., IOVS 2003; 44:2496–2506	AF286113
			35 cycles:	95°C for 15 sec; 60°C for 1 min		
MUC15	F: 5'-TAC AGC CAG CCC CAC CTA ATT-3' R: 5'-GAA AAC AGA TGG GTT AAG TGT GAC AA-3'	81 bp	1 cycle:	94°C for 2 min	designed with Primer Express (Gipson) and confirmed by BLASTN and sequencing at DSCVR	BC020912
			35 cycles:	94°C for 1 min; 55°C for 2 min; 72°C for 3 min		
			1 cycle:	72°C for 7 min		
MUC16	F: 5'-GCC TCT ACC TTA ACG GTT ACA ATG AA-3' R: 5'-GGT ACC CCA TGG CTG TTG TG-3'	114bp	1 cycle:	95°C for 10 min;	Argueso et al., IOVS 2003; 44:2487–2495	AF361486
			35 cycles:	95°C for 15 sec; 60°C for 1 min	Gipson et al., IOVS 2003; 44:2496–2506	
MUC17	F: 5'-GGG CCA GCA TAG CTT CGA-3' R: 5'-GCT ACA GGA ATT GTG GGA GTT GA-3'	91 bp	1 cycle:	94°C for 2 min	Gipson et al., IOVS 2003; 44:2496–2506	AF430017
			35 cycles:	94°C for 1 min; 55°C for 2 min; 72°C for 3 min		
			1 cycle:	72°C for 7 min		

Gene	Primer Sets	Product Size	Traditional Amplification	PCR Parameters	Reference	GenBank Accession No.
MUC19	F: 5'-ACC ACA AGT ATC CCA GCC AG-3' R: 5'-AGC TGG TGG AAG TGA GGC TA-3'	94 bp	1 cycle: 35 cycles:	95°C for 10 min; 95°C for 15 sec; 60°C for 1 min	Chen et al., Am. J Respir. Cell Mol. Eiol. 30:155–165, 2004.	AY236870
MUC20	F: 5'-AAC TCC ACG CCC ACG CGC CT-3' R: 5'-GGA AGC ACA CAG ATG GGT GA-3'	360 bp	35 cycles:	94°C for 30 sec; 55°C for 30 sec; 72°C for 1 min	Higuchi el al., J. Biol. Chem. 279: 1968–1979, 2004.	AB098731
Androgen Receptor	F: 5'-CTC TCT CAA GAG TTT GGA TGG CT-3' R: 5'-CAC TTG CAC AGA GAT GAT CTC TGC 3'	342 bp	1 cycle: 35 cycles:	95°C for 6 min; 94°C for 1 min 55°C for 1 min 72°C for 1 min	Lau et al., Cancer Res. 60:3175–3182, 2000.	M23263

*Note: Real-time PCR amplifications were performed over 40 cycles.

TABLE II

Primary antibodies used for immunohistochemistry and immunoblot analysis.

Antigen	Antibody	Working Dilution	Epitope	Source / Reference
MUC1	HMFG-2	1:100	Tandem Repeat	Biodesign International, Saco, ME
MUC1	214D4	1:100	Tandem Repeat	J. Hilkens (Wesseling *et al.*, 1995)
MUC4	528	Undiluted	Peptide	I. K. Gipson
MUC4[d]	1G8	1:100	ASPG-2	Zymed Corp., South San Francisco, CA
MUC4	8G7	1:1000	Tandem Repeat[e]	S.K. Batra (Moniaux *et al.*, 2004)
MUC5AC[b]	791	1:3000	D3 Domain	Argüeso et al. (Argueso *et al.*, 2002)
MUC5AC	CLH2	Undiluted	Tandem Repeat	U. Mandel (Reis *et al.*, 1997)
MUC5B[a]	799W	1:7500	D4 Domain	Gipson et al. (Gipson *et al.*, 2001)
MUC6[c]	CLH5	Undiluted	Tandem Repeat	U. Mandel (Reis *et al.*, 2000)

[a] Cryostat sections.

[b] Paraffin sections.

[c] Neuraminidase treated paraffin sections.

[d] Antigen retrieval on paraffin sections.

[e] Independent of glycosylation / native MUC4.

TABLE III

Results of RT-PCR for mucins on male reproductive tissue.

	Testis	Epididymis	Vas Deferens	Seminal Vesicle	Prostate	Bladder	Urethra	Foreskin
MUC	PCR	PCR	PCR	PCR	PCR	PCR	PCR	PCR
1	+ (2/3)	+ (2/2)	± (1/1)	+ (5/5)	+ (6/6)	+ (2/3)	+ (5/7)	+ (1/4)
3	− (3/3)	−(2/2)	+ (1/1)	− (5/5)	+ (6/6)	+ (2/3)	+ (2/7)	+ (2/4)
4	+ (1/3)	− (2/2)	± (1/1)	+ (2/5)	+ (6/6)	+ (1/3)	+ (7/7)	+ (1/4)
5AC	+ (1/3)	− (2/2)	± (1/1)	− (5/5)	± (4/6)	± (1/3)	+ (3/7)	+ (2/4)
6	+ (1/3)	+ (1/2)	− (1/1)	+ (5/5)	− (6/6)	− (3/3)	− (7/7)	− (4/4)
13	− (3/3)	− (2/2)	− (1/1)	− (5/5)	+ (1/6)	± (1/3)	+ (2/7)	+ (1/4)
15	− (3/3)	+ (1/2)	+ (1/1)	+ (1/5)	− (6/6)	+ (1/3)	+ (2/7)	+ (1/4)
17	− (3/3)	− (2/2)	+ (1/1)	+ (2/5)	+ (2/6)	+ (1/3)	+ (3/7)	+ (3/4)
20	+ (2/3)	− (2/2)	+ (1/1)	+ (5/5)	+ (6/6)	+ (2/3)	+ (5/7)	+ (1/4)

Note: MUC2, 5B, 7, 16, and 19 were not detected in any epithelium assayed. A mucin gene was considered as expressed by an epithelium if any of the samples obtained showed a RT-PCR product for that gene. Samples scored as ± required long (4 sec) exposures.