Title

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Journal

REPRODUCTIVE SCIENCES, 21(3)

ISSN

1933-7191

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Publication Date

2014

Peer reviewed

Human Reproduction, Vol.29, No.6 pp. 1255–1270, 2014
Advanced Access publication on March 13, 2014 doi:10.1093/humrep/deu047

human reproduction

ORIGINAL ARTICLE ***Reproductive biology***

Seminal plasma induces global transcriptomic changes associated with cell migration, proliferation and viability in endometrial epithelial cells and stromal fibroblasts

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Submitted on November 12, 2013; resubmitted on January 30, 2014; accepted on February 12, 2014

STUDY QUESTION: How does seminal plasma (SP) affect the transcriptome of human primary endometrial epithelial cells (eEC) and stromal fibroblasts (eSF)?

SUMMARY ANSWER: Exposure of eEC and eSF to SP *in vitro* increases expression of genes and secreted proteins associated with cellular migration, proliferation, viability and inhibition of cell death.

WHAT IS KNOWN ALREADY: Studies in both humans and animals suggest that SP can access and induce physiological changes in the upper female reproductive tract (FRT), which may participate in promoting reproductive success.

STUDY DESIGN, SIZE, DURATION: This is a cross sectional study involving control samples versus treatment. SP (pooled from twenty donors) was first tested for dose- and time-dependent cytotoxic effects on eEC and eSF ($n = 4$). As exposure of eEC or eSF to 1% SP for 6 h proved to be non-toxic, a second set of eEC/eSF samples ($n = 4$) was treated under these conditions for transcriptome, protein and functional analysis. With a third set of samples ($n = 3$), we further compared the transcriptional response of the cells to SP versus fresh semen.

PARTICIPANTS/MATERIALS, SETTING, METHODS: eEC and eSF were isolated from endometrial biopsies from women of reproductive age undergoing benign gynecologic procedures and maintained *in vitro*. RNA was isolated and processed for microarray studies to analyze global transcriptomic changes. Secreted factors in conditioned media from SP-treated cells were analyzed by Luminex and for the ability to stimulate migration of CD14+ monocytes and CD4+ T cells.

MAIN RESULTS AND THE ROLE OF CHANCE: Pathway identifications were determined using the *Z*-scoring system in Ingenuity Pathways Analysis (*Z* scores $\geq |1.5|$). SP induced transcriptomic changes ($P < 0.05$) associated with promoting leukocyte and endothelial cell recruitment, and proliferation of eEC and eSF. Cell viability pathways were induced, while those associated with cell death were suppressed ($P < 0.05$). SP and fresh semen induced similar sets of pathways, suggesting that SP can model the signaling effects of semen in the endometrium. SP also induced secretion of pro-inflammatory and pro-chemotactic cytokines, as well as pro-angiogenic and proliferative growth factors ($P < 0.05$) in both eEC and eSF. Finally, functional assays revealed that conditioned media from SP-treated eEC and eSF significantly increased ($P < 0.05$) chemotaxis of CD14+ monocytes and CD4+ T cells.

LIMITATIONS, REASONS FOR CAUTION: This study is limited to *in vitro* analyses of the effects of SP on endometrial cells. In addition, the measured response to SP was conducted in the absence of the ovarian hormones estradiol and progesterone, as well as epithelial-stromal paracrine signaling. While this study focused on establishing the baseline cellular response of endometrial cells to SP, future work should assess how hormone signaling in the presence of appropriate paracrine interactions affects SP-induced genes in these cells.

WIDER IMPLICATIONS OF THE FINDINGS: The results of this study support previous findings that SP and semen contain bioactive factors capable of eliciting chemotactic responses in the uterus, which can lead to recruitment of leukocytes to the endometrium. Future directions will explore if similar changes in gene expression do indeed occur after coitus *in vivo*, and how the signaling cascades initiated by SP in the endometrium can affect reproductive success, female reproductive health and susceptibility to sexually transmitted diseases. The gene list provided by the transcriptome analysis reported here should prove a valuable resource for understanding the response of the upper FRT to SP exposure.

STUDY FUNDING/COMPETING INTEREST(S): This project was supported by NIH AI083050-04 (W.C.G./L.C.G.); NIH U54HD 055764 (L.C.G.); NIH 1F32HD074423-02 (J.C.C.); DOD W81XWH-11-1-0562 (W.C.G.); NIH 5K12-DK083021-04, NIH 1K99AI104262-01A1, The UCSF Hellman Award (N.R.R.). The authors have nothing to disclose.

Key words: semen / endometrium / reproduction / microarray / chemotaxis

Introduction

Human semen, comprising spermatozoa and seminal plasma (SP), is a composite fluid consisting of secretions from the seminal vesicles, prostate and bulbourethral glands (Mann, 1954; Guyton, 1991). Historically, the role of SP was thought to have been limited to providing a protective and nourishing medium for transporting sperm within the female reproductive tract (FRT) (Harvey, 1948; Owen *et al.*, 2005). However, emerging data from studies involving rodents, domestic species, primates and humans suggest a broader role for SP in participating in male-to-female signaling cascades in the FRT before, during and after fertilization (Robertson, 2005, 2007).

Studies in rodents (Robertson *et al.*, 1992; Sanford *et al.*, 1992), pigs (Bischof *et al.*, 1994) and humans (Sharkey *et al.*, 2012a,b) indicate that SP induces an inflammatory response in the lower FRT. For example, pro-inflammatory genes including interleukin (IL)8, IL6, colony stimulating factor (CSF)2, monocyte chemoattractant protein 1 (MCP1, or chemokine ligand [CCL]2) are induced in ectocervical cells exposed to SP (Sharkey *et al.*, 2007). Because factors deposited into the lower FRT can rapidly access the upper FRT (Cicinelli *et al.*, 2000; Barnhart *et al.*, 2001), it is likely that SP also exerts biologically relevant effects on uterine endometrium. In line with this hypothesis, pro-inflammatory cytokines including IL1β, IL6 and leukemia inhibitory factor (LIF) were induced in endometrial epithelial cells (eEC) and stromal fibroblasts (eSF) following exposure to SP (Gutsche *et al.*, 2003). SP can additionally stimulate potent angiogenic and proliferative effects in both lower and upper FRT tissues (Sales *et al.*, 2012; Sutherland *et al.*, 2012; Kaczmarek *et al.*, 2013). Together, these studies suggest that SP-derived bioactive factors can signal a variety of responses in cells of both the lower and upper FRT.

While the successful use of artificial insemination and IVF in assisted reproduction technology indicate that the presence of SP is not a necessary component for pregnancy, SP can significantly enhance reproductive success. A randomized placebo-controlled clinical trial demonstrated that vaginal capsules containing SP significantly enhanced implantation rates (Coulam *et al.*, 1995). In a separate study, deposition of semen into the upper vaginal tract of women with tubal occlusion or with no Fallopian tubes during IVF treatment resulted in increased implantation rates (Bellinge *et al.*, 1986). Furthermore, women exposed to semen through intercourse before and after frozen embryo transfer exhibited improved implantation success (Tremellen *et al.*, 2000). Thus, although SP is not necessary for implantation, clinical evidence supports that exposure of the FRT to SP promotes implantation success. These studies are further supported by numerous *in vivo* animal studies demonstrating the ability of SP to increase pregnancy rates (reviewed in (Robertson, 2007)).

In vivo data suggest that semen components can access the upper FRT through peristalsis (Kunz *et al.*, 1997) but the molecular effects of SP on cells from this tissue are poorly understood. Because the uterine endometrium of the upper FRT is critical for gamete and embryo transport, implantation, and pregnancy (Aghajanova *et al.*, 2008), and can also serve as a portal of entry for a variety of microbial pathogens (McGowan, 2006), understanding the effects of SP on the endometrium may provide new insights into optimizing reproductive success and lead to a better understanding of how sexually transmitted diseases establish infection through the FRT. The endometrium is composed of two predominant cell types: the eEC and the eSF. The eEC line the uterine lumen and comprise the first endometrial cell type to interact with SP, while the eSF reside in the underlying stroma, a compartment which SP may access during phases of the menstrual cycle when luminal components traverse the epithelial basement membrane (Someya *et al.*, 2013). Although eEC are the first cells to encounter luminal contents of the endometrium, eSF make up the majority of the cellular volume that comprises the endometrium (Giudice, 2003; Aplin *et al.*, 2008). Herein we report the effect of SP on the global transcriptome of human eEC and eSF using an *in vitro* model. The results suggest that SP is a potent inducer of genes involved in inflammation, cell proliferation and cell viability.

Materials and Methods

Tissue specimens and processing

Human endometrial tissue samples were obtained in accordance with the guidelines of the Declaration of Helsinki. Written, informed consent was obtained from all subjects. The study was approved by the Committee on Human Research of the University of California, San Francisco (UCSF) CHR# 10-02786. Endometrial tissue samples were processed on the day of collection and primary cell cultures were initiated immediately after tissue processing. Subjects were premenopausal women (ages 23–42 years) and confirmed not to be pregnant. Details of their clinical history

Table I Individual subject characteristics for endometrial biopsies obtained for this study.

Sample ID	Cells used	Diagnosis	Phase	Age (years)
2074	Epithelial	Oocyte donor	ESE	27
2172	Epithelial	Oocyte donor	ESE	24
2069	Epithelial	Dysmenorrhea, AUB, fibroids	PE	42
2079	Epithelial	Dysmenorrhea, AUB, fibroids	LSE	34
2018	Stromal	Oocyte donor	ESE	26
2056	Stromal	Oocyte donor	ESE	23
2059	Stromal	Oocyte donor	ESE	28
2043	Stromal	Ovarian cyst	PE	27

Diagnosis abbreviations: AUB, abnormal uterine bleeding; Phase abbreviations: PE, proliferative endometrium; ESE, early secretory endometrium; LSE, late secretory endometrium.

and cycle phase at the time of tissue sampling are described in Table I. Tissue samples were obtained through the National Institutes of Health Specialized Cooperative Centers Program in Reproduction and Infertility Research (SCCPIR) Human Endometrial Tissue and DNA Bank at UCSF under established standard operating procedures (Sheldon *et al.*, 2011). Endometrial tissue samples included biopsies (obtained using the Pipelle® Endometrial Suction Curette, Cooper Surgical, Trumbull, CT, USA) from subjects undergoing oocyte retrieval or examination for benign gynecological conditions. Endometrial tissue was digested with 6.4 mg/ml collagenase type I and 100 U/ml hyaluronidase in Hanks Buffered Salt Solution with Ca^{++} and Mg^{++}. Red blood cells were lysed in a solution containing 0.155M NH_4Cl, 0.01M $KHCO_3$ and 0.1 mM EDTA, adjusted to pH 7.3. The dissociated cellular elements were then treated with DNase (4 mg/ml), and filtered through a 40 μm cell strainer (BD Biosciences, San Jose, CA, USA) to separate single cells from fragments of endometrial epithelial sheets and glands (Chen *et al.*, 2013). Selective attachment to plastic dishes was used as the final step to separate endometrial epithelial and stromal cells (Kirk *et al.*, 1978).

Seminal plasma and semen processing

Protocols for use of human semen were approved by the Committees on Human Research at UCSF under CHR # 11-06115. Semen samples obtained from 20 de-identified donors from the UCSF Center for Reproductive Health were allowed to liquefy for 2 h, frozen at −20°C, and then simultaneously thawed and combined. The fertility status of these donors was unknown as required by the confidentiality rules established in the approved IRB. The pooled samples were then centrifuged at 176*g* for 30 min at 4°C to remove spermatozoa. The supernatant was aliquoted, frozen and served as the SP stock. For studies involving the use of fresh whole semen (inclusive of sperm), a sample was obtained from one donor and liquefied for 2 h prior to use.

Endometrial epithelial and stromal cell culture

The eSF fraction was established in primary culture and serially passaged as previously described (Irwin *et al.*, 1989) in serum-containing fibroblast growth medium (SCM: 75% phenol red-free Dulbecco's Modified Eagle's Medium (DMEM)/25% MCDB-105 supplemented with 10% charcoal-striped fetal bovine serum (FBS) and 5 μg/ml insulin). After achieving confluency in passage P1 to establish purity, 10^5 eSF were plated in uncoated 24-well plastic dishes. Confluency was achieved after 2–4 days. The eEC were plated on Matrigel™ coated dishes (BD Biosciences) with defined keratinocyte serum-free medium (KSFM) (Gibco, Grand Island, NY, USA), and achieved 50–75% confluence within 10–14 days. At 50–75% confluency, eEC were detached using Accutase (EMD Millipore, Billerica, MA, USA), pelleted, and resuspended in KSFM +1% FBS as previously reported (Chen *et al.*, 2013). After cell counting, 10^5 eEC were plated (passage = P1) into Matrigel-coated dishes (24-well size, BD Biosciences). eEC achieved confluency in 2–4 weeks. To establish eEC and eSF purity, immunofluorescence staining of cell-specific markers (E cadherin and keratin 18 for eEC; platelet derived growth factor B (PDGFRB) and vimentin for eSF) was performed. Details of these experiments are shown in the Supplementary data and Supplementary data, Fig. S1.

SP toxicity evaluation

To first establish the non-toxic doses of SP on these cells, given that *in vitro* exposure of tissue culture cells to SP can result in marked cytotoxicity (Allen *et al.*, 1986, 1987; Fiore *et al.*, 1997), we treated the cells with multiple concentrations of SP (1, 10, 20 or 50%) for various lengths of time (0.5, 3 or 6 h) and then assessed viability. To this end, a water-soluble tetrazolium salt (WST)-based assay (Clonetech, Mountainview, CA, USA) was utilized to assess metabolic activity for each cell type at each treatment condition. At confluency, eEC and eSF were exposed to either vehicle (veh) (KSFM for eEC; SCM with 2% serum for eSF), or to 1, 10, 20 or 50% SP for 0.5, 3 or 6 h. After exposure to SP for the appropriate period of time, the premixed WST reagent was added to each well such that the final WST concentration was 10%. After 2 h, the optical density of the WST solutions was measured at 450 nm. Toxic concentrations of Nonoxynol-9 (20, 50 and 100 μg/ml) were used as positive controls for reducing cell viability.

RNA isolation

Total RNA was isolated from cultured eEC and eSF using the Nucleospin RNA purification kit (Machery Nagel, Bethlehem, PA, USA) following the manufacturer's protocol including DNase treatment (Qiagen, Valencia, CA, USA). The purity and integrity of all RNA samples were confirmed through Nanodrop (Nanodrop, Wilmington, DE, USA) and Bioanalyzer (Agilent, Santa Clara, CA, USA), respectively. All RNA samples were free of DNA, protein and buffer contamination, and met the level of qualification necessary for downstream microarray analysis.

Microarray analysis

eEC and eSF were exposed to either veh, 1% SP, or 1% fresh semen for 6 h. Cells were then collected for RNA and microarray analysis, while conditioned medium was collected for secreted factor and functional analyses. RNA from cultured eEC and eSF ($n = 4$; different patients for each cell type) was processed for analysis on the Affymetrix Human Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA), with updated annotations, probing 36 079 transcripts and 21 014 genes, as previously reported (Spitzer *et al.*, 2012). Briefly, RNA was reverse transcribed and amplified into cDNA and sense-strand cDNA targets, and then fragmented, labeled, and hybridized to Affymetrix Human Gene 1.0 ST arrays. The quality of the amplified cDNA and fragmented cDNA was assessed using the Bioanalyzer, and only samples meeting sufficient yields and quality standards were used for hybridization. Intensity values of different probe sets (genes) were imported into GeneSpring GX 11.02 software (Agilent) and processed using the robust multiarray analysis algorithm for background adjustment, normalization and log2 transformation of perfect match values. RMA16 was utilized as the background correction algorithm for ST array technology. Differential expression analysis was performed for the following comparisons between eEC or eSF treated with veh or 1% SP: (i) eEC_{veh} versus eEC_{SP}; (ii) eSF_{veh} versus eSF_{SP}; and (iii) eEC_{veh} versus eSF_{veh}. Analysis output included only genes with ≥1.5-fold change and $P < 0.05$ by two-way analysis of

variance (ANOVA) with Benjamini–Hochberg multiple-testing correction for false discovery rate. The use of a 1.5-fold cutoff for biologically relevant analysis is in line with previous reports (Yuan *et al.*, 2005; Dalman *et al.*, 2012).

Principal component analysis (PCA) and hierarchical clustering were performed as previously described (Spitzer *et al.*, 2012). Briefly, the unbiased PCA algorithm in GeneSpring was applied to all samples, using all 21 014 genes on the Affymetrix Human Gene 1.0 ST array chip to identify similar expression patterns and underlying cluster structures. Hierarchical cluster analysis was conducted using only differentially expressed genes from all samples and among all experimental conditions. The smooth correlation distance measure algorithm (GeneSpring) was then used to identify samples with similar patterns of gene expression.

Biological functions and canonical pathway analyses

Utilizing the transcript cluster ID and fold changes of up- and down-regulated genes, each pairwise comparison was imported into Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA). Detailed pathway analysis was performed using the Core Analysis function on IPA to interpret data in the context of biological functions, pathways and networks. Biological functions are composed of molecular and cellular functions, and canonical pathways include both signaling and metabolic pathways. Significance of the biological functions and the canonical pathways were tested by the Fisher exact test *P*-value, and the relevant *Z*-scores, which predict activation or suppression of a given pathway given the differential expression of genes in the comparison, were calculated (St-Pierre *et al.*, 2013).

Quantitative real-time PCR validation of microarray data

In order to validate patterns of gene expression revealed by the microarray data, a selected set of differentially expressed genes were chosen for quantitative real-time PCR (qRT–PCR). cDNA used for the array analysis was diluted to 10 ng/μl in nuclease-free water, and confirmed for the absence of RNA/protein contaminants by Nanodrop. Total cDNA (20 ng) was combined with SYBR green and 1 μM custom-made primers (Fluidigm, South San Francisco, CA, USA) directed toward human *AREG*, *BCL6*, *CSF3*, *FGF2*, *IL8*, *IL11* and *VEGFA*. Amplification was performed using the Stratagene MX3005P (Agilent) Thermocycler. Dissociation curves for both target and housekeeping genes were utilized to ensure the absence of primer dimers and other non-specific amplification. Primers were designed by Fluidigm and optimized for qRT–PCR following the Fluidigm Biomark guidelines on mRNA amplification, including primer amplification efficiency, amplicon size and appropriate dissociation temperatures governing mRNA amplification. Our amplification conditions are compliant with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) (Bustin *et al.*, 2009). The comparative (delta-delta) Ct method was used to measure relative gene expression for each cell type following veh versus SP treatment (ABI User bulletin 2).

Luminex multiplex cytokine assays

Conditioned media from veh- and 1% SP-treated eEC and eSF were centrifuged at 13 000*g* for 5 min to remove cellular debris. Supernatants were analyzed for secreted cytokines using a custom multiplex Luminex kit (EMD Millipore) which included IL1A, ILB, IL2, IL4, IL5, IL6, IL8 and IL10; tumor necrosis factor alpha (TNFA); interferon gamma (IFNG), granulocyte macrophage colony stimulating factor (CSF2); macrophage inflammatory protein 1α (CCL3) and β (CCL4); monocyte chemoattractant protein 1 (CCL2) and 3 (CCL7); fractalkine (CX3CL1); Regulated Upon Activation, Normal T-cell Expressed, and Secreted Chemokine (c-c motif) ligand 5 (RANTES; CCL5); fibroblast growth factor (FGF); granulocyte colony stimulating factor (CSF3); vascular endothelial growth factor (VEGF[A]); and chemokine (c-x-c) ligand 1 (GRO; CXCL1). All protocols were based on manufacturer's specifications. Briefly, conditioned media were incubated overnight in

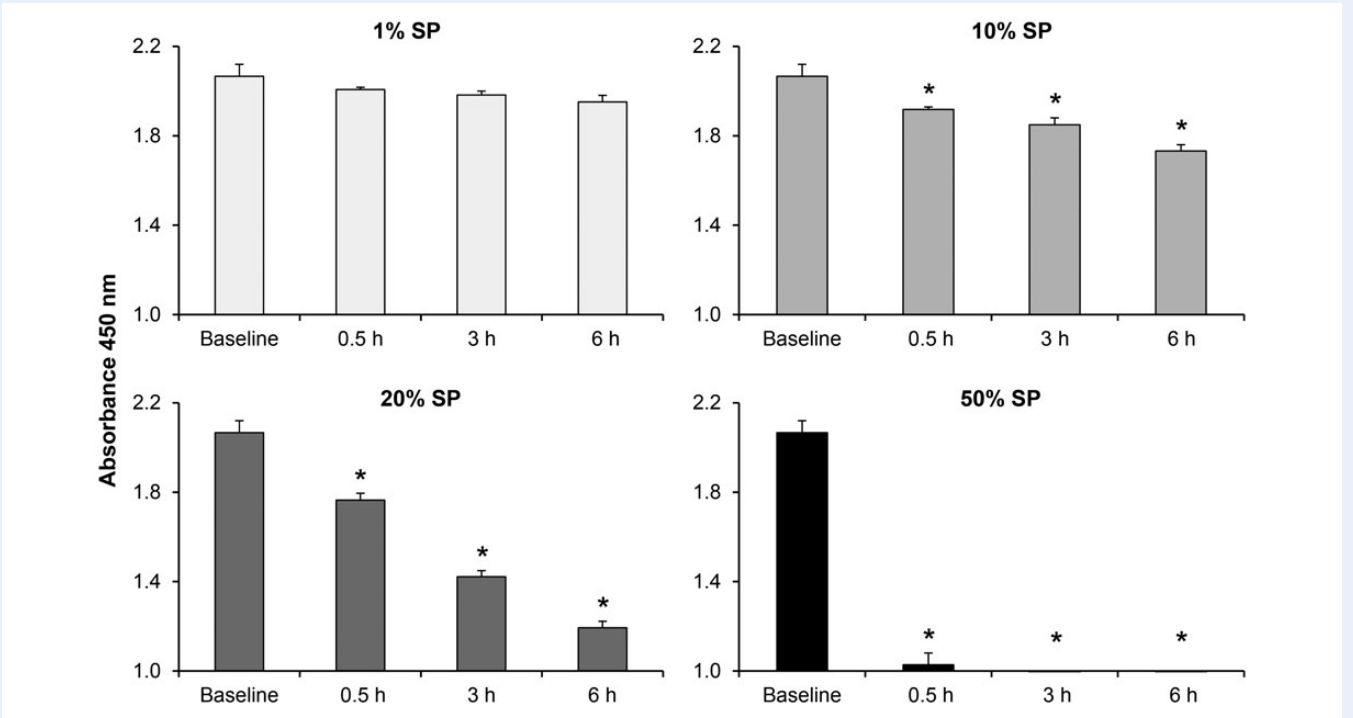

Figure 1 Time and concentration-dependent toxicity analysis on the effect of seminal plasma (SP) on viability of endometrial epithelial cells (eEC). Viability of eEC was measured by absorbance following treatment with water-soluble tetrazolium salt (WST). $n = 4$. Asterisk indicates $P < 0.05$ compared with baseline.

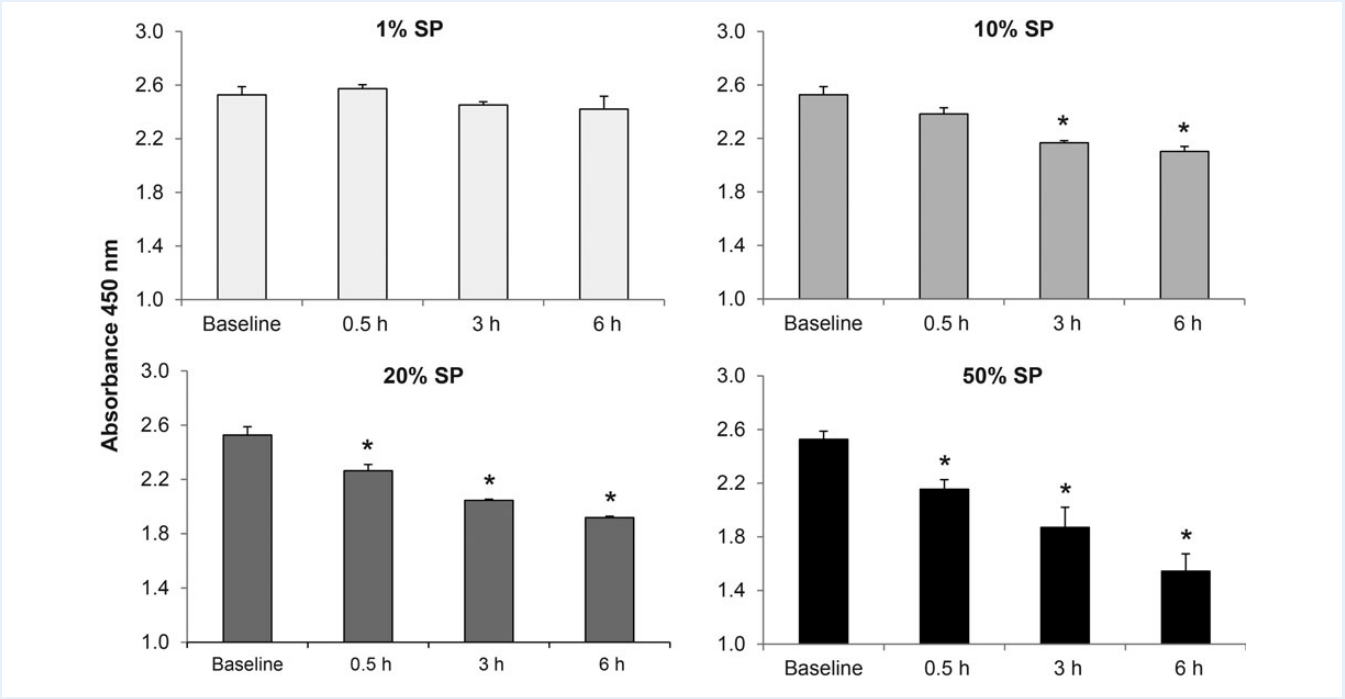

Figure 2 Time and concentration-dependent toxicity analysis on the effect of SP on viability of endometrial stromal fibroblasts (eSF). Viability of eSF was measured by absorbance following treatment with WST. $n = 4$. Asterisk indicates $P < 0.05$ compared with baseline.

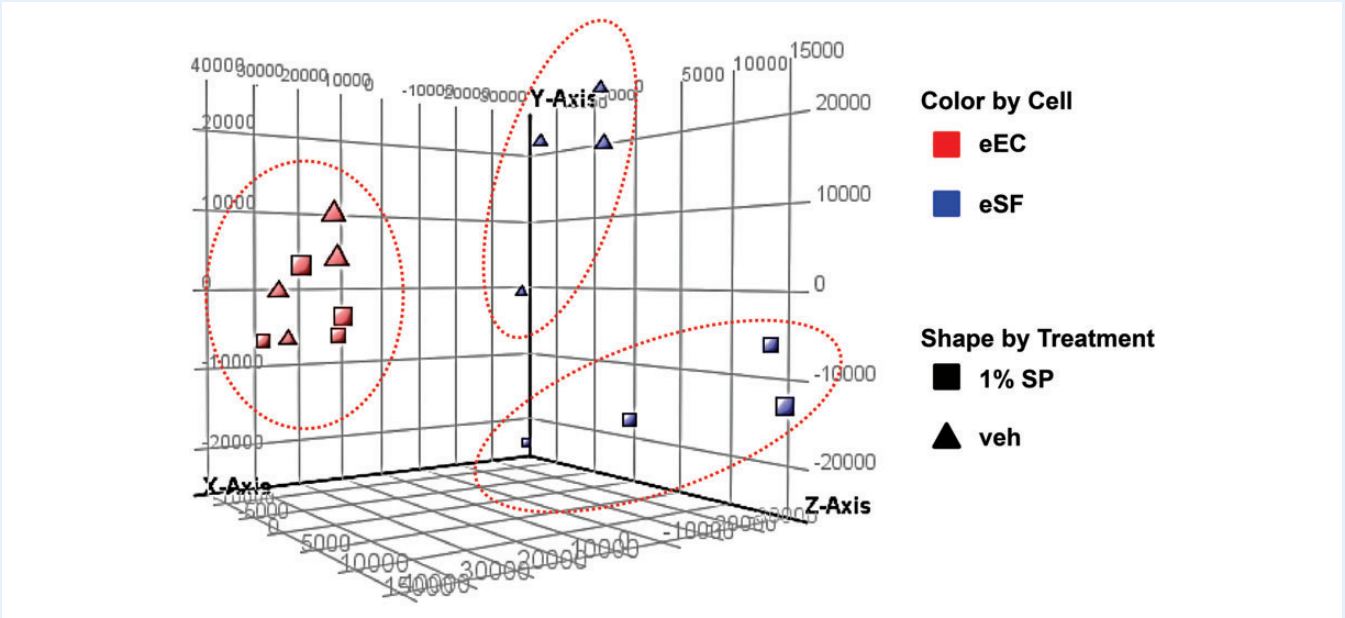

Figure 3 Clustering analysis of eEC and eSF in response to 1% SP or veh. Principal component analysis clustering: The first delineation is by color (eEC represented by red and eSF represented by blue), and the second delineation is by treatment (vehicle (veh) represented by triangles and SP represented by squares).

pre-wet Luminex plates with antibody-coated, fluorescent-dyed capture microspheres specific for each analyte. Assay wells were then washed and incubated with bead-panel detection antibodies, followed by the addition of streptavidin-phycoerythrin. The washed microspheres with bound analytes were resuspended in sheath fluid and analyzed on a Bioplex (Biorad, Hercules, CA, USA) bead sorter. Standard curves and high/low range positive controls were used to determine the concentrations of each cytokine. Additional controls for background noise and interference included unconditioned media with and without phenol red. To ensure the appropriate level of sensitivity, samples with <50 beads for each cytokine target were excluded from the analysis. Each sample was run in duplicate, and results for each sample were repeated independently on at least two different plates. Data were adjusted for media volume and normalized to cell number.

Migration assays

Buffy coats from human blood were purchased from the Stanford Blood Bank (Palo Alto, CA, USA) and processed by Ficoll-density gradient centrifugation to remove dead cells. Monocytes were positively selected with CD14 microbeads (Miltenyi Biotech, Auburn, CA, USA), and CD4+ T cells were isolated by positively selecting the CD14− population with CD4 microbeads (Miltenyi Biotech). Both CD14+ monocytes and CD14−CD4+ T cells were allowed to equilibrate in Roswell Park Memorial Institute medium (RPMI) 1640 with 10% FBS overnight. Cells were then cultured in serum-free (0% FBS) RPMI for an additional 12 h before initiation of migration experiments as per manufacturer recommendations.

CD14+ monocyte migration was conducted using the Millipore QCM 5 μm migration assay (EMD Millipore) following the manufacturer's recommendations. Briefly, cells were pelleted and resuspended in phosphate-buffered saline with 1% bovine serum albumin, and 10^5 cells were added to the upper migration chamber. Serum-free KSFM or 2% SFM (500 μl) were added to the bottom chamber as negative controls. Standard curves were established with FBS, a known chemoattractant for monocytes and T cells (Muinonen-Martin *et al.*, 2010; Maffei *et al.*, 2013). IL8 and CCL2 were utilized as additional chemoattractant positive controls for monocytes and T cells (Uguccioni *et al.*, 1995; Kunstfeld *et al.*, 1998; Jinquan, 2003; Smythies *et al.*, 2006) and established 6 h as the equilibrium period during which maximal migratory activity occurs. Sample wells contained conditioned media pooled from either eEC or eSF cultures ($n = 4$ each). Migrated cells that crossed the membrane and adhered to the opposite side were stained with the provided staining solution and counted. To ensure consistency, four random non-overlapping fields at $\times 200$ magnification were counted and averaged. Experiments were conducted in triplicate, and repeated twice to ensure reproducibility of results.

CD4+ T cell migration was conducted using the QCM 3 μm non-adherent migration assay (EMD Millipore), designed to measure cell migration of non-adherent cells, including T cells. As with monocyte migration experiments, cells were incubated in serum-free (0% FBS) RPMI and then added (10^5/well) to the upper migration chambers while conditioned media from veh- or SP-treated eEC/eSF were added to the lower chamber. After 6 h, migrated cells were collected in the bottom chamber. T cell migration was assessed by quantifying the metabolic activity of migrated T cells using WST reagent.

Statistics

Differential expression analysis of microarray data was conducted using Genespring 12.1. Secreted cytokine data were analyzed using *t*-tests for equal variance utilizing Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA). For statistical analysis of the cellular toxicity and

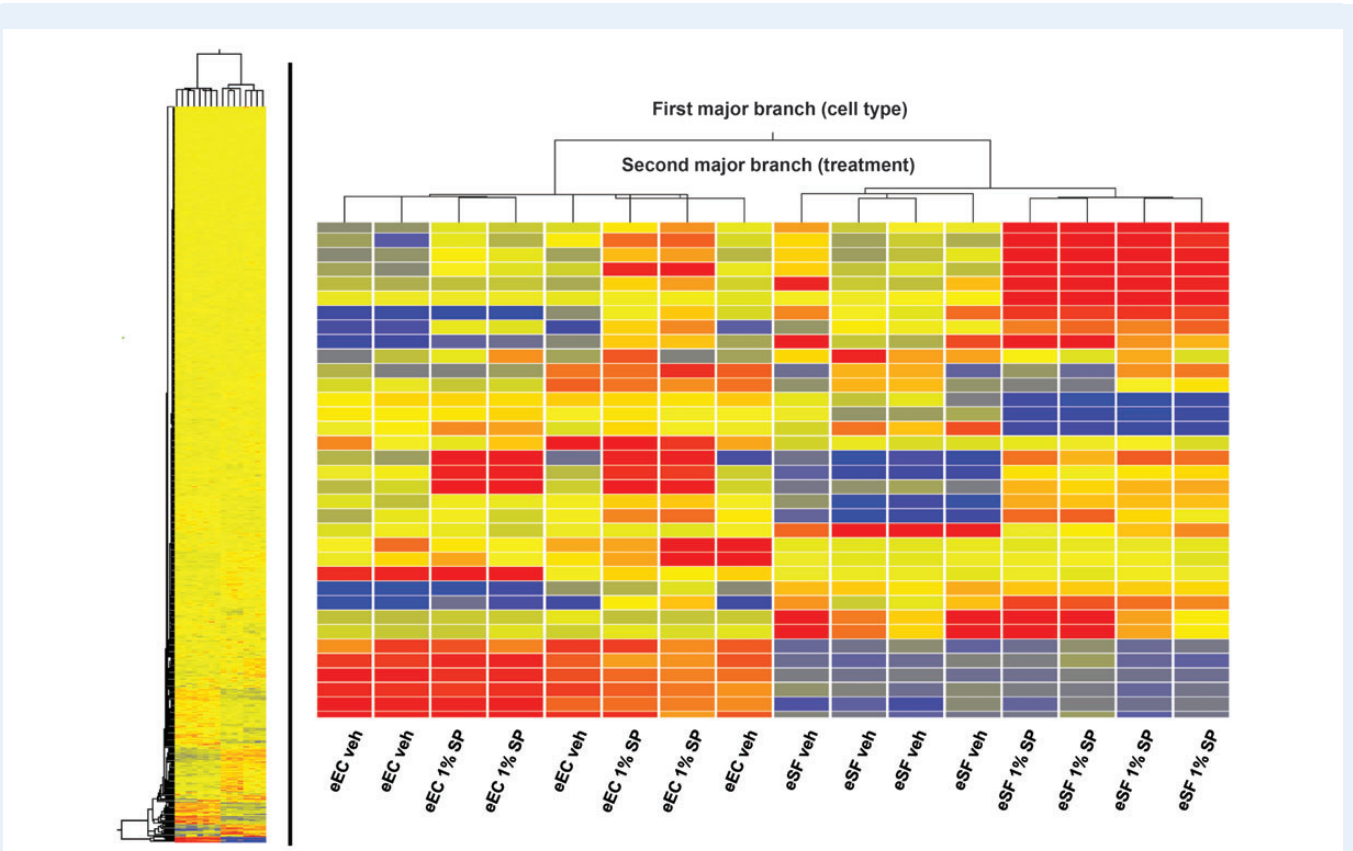

Figure 4 Hierarchical clustering. The left panel shows complete hierarchical clustering analysis between eEC and eSF in response to 1% SP or veh. The right panel shows a magnified inset of the most differentially expressed genes from the complete hierarchical cluster analysis.

migration data, ANOVA was conducted with R Commander (2011) with Tukey's *post hoc* analysis (http://www.r-project.org/). For pathway analysis of biological functions, IPA was used to identify pathways that were affected by differentially expressed genes in each treatment group with a >1.5-fold change. Identified pathways were analyzed for *Z*-scores to assess the activation (>1.5) or inhibition (<-1.5) of respective pathways (Mazan-Mamczarz *et al.*, 2008).

Results

SP toxicity evaluation

Before conducting a detailed analysis on the effect of SP on endometrial cells, we identified a treatment regime that did not result in cellular toxicity for both eEC and eSF. In eEC, the baseline level of viability was not significantly different after 0.5, 3 or 6 h of treatment with veh (Fig. 1). While 1% SP did not decrease the viability of eEC at all time points tested, higher concentrations of SP led to reduction in viability ($P < 0.05$), particularly after 6 h of treatment (Fig. 1). The same pattern of SP-induced toxicity was observed for eSF, where 1% SP was not toxic while 10, 20, and 50% SP caused significant reductions ($P < 0.05$) in viability following 6 h of treatment (Fig. 2). Morphological assessment of eEC and eSF cells revealed that concentrations of SP causing decreased viability corresponded with cell detachment (data not shown). Mean ($\pm$ SEM) pH values of the conditioned media from all tested SP treatment conditions did not deviate significantly from baseline (pH 7.435 ± 0.02 for eEC and 7.45 ± 0.04 for eSF), demonstrating that the observed toxicity was not due to pH changes (data not shown). Based on these data, we chose for subsequent microarray analyses 1% SP treatment for 6 h as the non-toxic treatment condition for both eEC and eSF.

Transcriptome analysis of cultured eEC and eSF

The CEL files obtained from the complete array studies showing differential gene expression assessed by cell type (eEC versus eSF) and treatment condition (veh versus 1% SP or fresh semen) have been uploaded to the Gene Expression Omnibus (GEO) at the National Center for Bioinformatic Information (NCBI), http://www.ncbi.nlm.nih.gov/geo/.

PCA and hierarchical clustering analysis of SP-treated eEC and eSF

In microarray analysis, a three-dimensional distribution profile known as the PCA can be constructed from the variance for each gene. Genes with similar expression profiles cluster together in this space. Using every gene on the Affymetrix array and an unbiased approach, the complete set of arrayed samples clustered into two major subdivisions, eEC and eSF. Within each subdivision, the eSF clustered further into eSF_{SP} and eSF_{veh}, while the eEC_{SP} and eEC_{veh} showed less heterogeneity, suggesting that SP-induced differences were more pronounced in eSF (Fig. 3). Unsupervised hierarchical clustering analyses based on the combined

Table II Selected pathways significantly affected by seminal plasma (SP) in endometrial epithelial cells (eEC).

Category	Functions annotation	Activation state	*Z*-score
Cellular movement	Leukocyte migration	Increased	2.319
Cellular movement	Chemotaxis of myeloid cells	Increased	2.169
Cellular movement	Chemotaxis of phagocytes	Increased	2.387
Cellular movement	Cell movement of myeloid cells	Increased	2.431
Cellular movement	Cell movement of phagocytes	Increased	2.597
Cellular movement	Chemotaxis of granulocytes	Increased	1.934
Cellular movement	Chemotaxis of neutrophils	Increased	1.934
Growth and proliferation	Proliferation of cells	Increased	3.501
Growth and proliferation	Proliferation of tumor cells	Increased	2.685
Growth and proliferation	Colony formation of cells	Increased	2.396
Growth and proliferation	Proliferation of breast cell lines	Increased	2.218
Growth and proliferation	Proliferation of epithelial cell lines	Increased	1.965
Growth and proliferation	Proliferation of connective tissue cells	Increased	1.958
Growth and proliferation	Proliferation of lymphatic system cells	Increased	1.954
Growth and proliferation	Proliferation of endothelial cells	Increased	1.562
Cell death and survival	Cell viability	Increased	3.101
Cell death and survival	Cell viability of tumor cell lines	Increased	2.177
Cell death and survival	Cell viability of neurons	Increased	2.000
Cell death and survival	Cell viability of endothelial cells	Increased	1.936
Cell death and survival	Cell viability of leukocytes	Increased	1.961

$P < 0.05$; *Z*-score >|1.5|.

gene list ($P < 0.05$, >1.5-fold change) derived from pairwise comparisons yielded a dendrogram of sample clustering and a heatmap of gene expression. The clustergram (Fig. 4), similar to the PCA, revealed a main branching between cell types (eEC and eSF), and from each cell type to another branch based on treatment (SP versus veh).

IPA of differentially expressed pathways following SP exposure

In eEC, SP induced significant ($P < 0.05$, Z-score > 1.5) activation of pathways that positively regulate immune cell trafficking, cell viability/survival, and cellular growth and proliferation (Table II). In addition, pathways that promote cell death were inhibited ($P < 0.05$). In eSF, SP activated pathways that positively regulate cellular movement, growth and proliferation ($P < 0.05$, Z-score > 1.5), and inhibited pathways involving cellular necrosis, apoptosis and cell death ($P < 0.05$, Z-scores > 1.5) (Table III). Examples of differentially expressed genes and their associated pathways identified by IPA are shown in Table IV.

Because the SP used in these studies had undergone a series of processing steps including freeze/thaw cycles, we next confirmed that the pathways induced by SP were also induced by fresh semen. Treatment of eEC and eSF ($n = 3$ for each) with 1% fresh semen from one donor stimulated the same major pathways as those stimulated by 1% SP, demonstrating that the SP-induced pathways were not an artifact of the freeze/thaw process and did not require the presence of spermatozoa (Supplementary data, Figs 2 and 3). These results validated the use of SP for studies of the effect of semen on endometrial cells.

Table III Selected pathways significantly affected by SP in endometrial stromal fibroblasts (eSF).

Category	Functions annotation	Activation state	Z-score
Cellular movement	Invasion of cells	Increased	3.16
Cellular movement	Chemotaxis of endothelial cells	Increased	2.39
Cellular movement	Chemotaxis of cells	Increased	2.36
Cellular movement	Cell movement	Increased	2.20
Cellular movement	Cell movement of neutrophils	Increased	2.20
Cellular movement	Chemotaxis of tumor cell lines	Increased	2.18
Cellular movement	Movement of vascular endothelial cells	Increased	2.18
Cellular movement	Migration of cells	Increased	2.00
Cellular movement	Infiltration of cells	Increased	1.98
Cellular movement	Chemotaxis vascular endothelial cells	Increased	1.95
Cellular movement	Cell movement of granulocytes	Increased	1.95
Cellular movement	Invasion of endothelial cells	Increased	1.94
Cellular movement	Cell movement of myeloid cells	Increased	1.93
Cellular movement	Cell movement of phagocytes	Increased	1.87
Cellular movement	Chemotaxis of leukocytes	Increased	1.50
Growth and proliferation	Proliferation of cells	Increased	3.92
Growth and proliferation	Proliferation of tumor cell lines	Increased	2.99
Growth and proliferation	Proliferation of muscle cells	Increased	2.68
Growth and proliferation	Proliferation of breast cell lines	Increased	2.59
Growth and proliferation	Proliferation of tumor cells	Increased	2.33
Growth and proliferation	Proliferation of neuroblasts	Increased	2.00
Growth and proliferation	Colony formation	Increased	1.70
Growth and proliferation	Proliferation of fibroblasts	Increased	1.67
Growth and proliferation	Proliferation of connective tissue cells	Increased	1.58
Cell death and survival	Cell survival	Increased	1.75
Cell death and survival	Cell viability of neurons	Increased	1.63
Cell death and survival	Cell viability	Increased	1.63
Cell death and survival	Apoptosis of endothelial cells	Decreased	−1.65
Cell death and survival	Necrosis	Decreased	−2.56
Cell death and survival	Apoptosis	Decreased	−2.89
Cell death and survival	Cell death	Decreased	−2.97

$P < 0.05$; Z-score >|1.5|.

Table IV Selected genes up-regulated in eEC and eSF by SP.

eEC SP versus eEC vehicle	FC	eSF SP versus eSF vehicle	FC
Immune cell trafficking		Cellular movement	
CSF3	2.95	*IL8*	14.47
FGF2	2.57	*IL1B*	6.88
EDNRB	2.41	*CXCL1*	6.55
CXCL2	1.86	*IL1A*	5.48
CXCL3	1.84	*NRG1*	4.65
IL33	1.78	*ANGPTL4*	4.55
CXCL5	1.77	*FGF2*	4.22
VEGFA	1.61	*VEGFA*	3.63
Growth and proliferation		Growth and proliferation	
IL11	4.36	*AREG*	8.29
FGF2	2.57	*HGF*	4.82
EREG	1.78	*FGF2*	4.23
		ANPEP	3.42
Cell death and survival		*LIF*	2.67
IL11	4.36	*BCL6*	−2.72
FGF2	2.57		
ANGPTL4	1.56	Cell death and survival	
IL33	1.78	*AREG*	8.29
NRG1	1.64	*IL1B*	6.88
HGF	1.61	*CXCL1*	6.55
VEGFA	1.61	*IL1A*	5.48
		VEGFA	3.64
		ANXA1	−1.54
		BCL2L1	−1.65
		BCL6	−2.72

$P < 0.05$, Fold Change (FC) >1.5.
Gene Definitions: CSF3, granulocyte colony stimulating factor; FGF2, fibroblast growth factor 2; EDNRB, endothelin receptor type B; CXCL2, chemokine (C-X-C motif) ligand 2; CXCL3, chemokine (C-X-C motif) ligand 3; IL33, interleukin 33; CXCL5, chemokine (C-X-C motif) ligand 5; VEGFA, vascular endothelial growth factor; IL11, interleukin 11; EREG, epiregulin; ANGPTL4, angiopoietin-like 4; NRG1, neuregulin 1; HGF, hepatocyte growth factor; IL8, interleukin 8; IL1B, interleukin 1B; CXCL1, chemokine (C-X-C motif) ligand 1; IL1A, interleukin 1A; AREG, amphiregulin; ANPEP, alanyl (membrane) aminopeptidase; LIF, leukemia inhibitory factor; BCL6, B-cell lymphoma 6 protein; ANXA1, annexina1; BCL2L1, BCL2-like 1.

For logistical reasons, and also because use of SP allowed for the combination of semen from 20 donors thereby decreasing issues related to donor variability, for subsequent assays we used SP instead of fresh semen.

qRT–PCR analysis of selected differentially expressed genes from microarray

To validate data obtained from microarray analysis, a selected set of differentially expressed genes representative of the SP-induced pathways (Table IV) were also analyzed by qRT–PCR. SP-mediated induction of CSF3, FGF2, IL11, VEGFA in eEC, and SP-mediated induction of AREG, BCL6, IL8, VEGFA in eSF, were confirmed by qRT–PCR (Fig. 5).

Cytokine and chemokine secretion in eEC and eSF in response to SP treatment

To determine whether selected genes that were identified as being up-regulated by SP in eEC and eSF by microarray analysis were also induced at the protein level, we examined a set of secreted factors by multiplex assays. These secreted factors were chosen based on their presence in the endometrium and the roles they play in uterine immunity and endometrial function (Kelly *et al.*, 2001; Jones *et al.*, 2004; Salamonsen *et al.*, 2007). We found that treatment of eEC with 1% SP significantly increased ($P < 0.05$) the production of CCL2, CSF2, CSF3, CXCL1, CX3CL1, IL1A, IL6, IL8, TNFA and VEGFA, while production of CCL4, CCL7 and FGF2 was not affected by SP (Table V). In eSF, SP significantly increased ($P < 0.05$) the production of CCL2, CCL7, CXCL1, CX3CL1, FGF2, IL6, IL8 and VEGFA. Of note, the levels of secreted proteins were normalized by subtracting out endogenous levels of the corresponding factor in 1% SP, which contained detectable levels of CCL2, CCL3, CCL4, CCL5, CSF3, CXCL1, CX3CL1, FGF2, IL8 and VEGFA. This normalization allowed us to distinguish the factors secreted by eEC and eSF in response to SP from the chemotactic and inflammatory factors endogenously present in SP itself.

SP upregulates secretion of chemotactic factors by eEC and eSF that recruit monocytes and T cells

Because several of the SP-induced secreted proteins are chemokines, we next tested whether conditioned media from SP-treated eEC and eSF induce chemotaxis of specific leukocyte populations. When exposed to veh alone for either cell type, CD14+ monocytes did not significantly migrate, whereas exposure to 1% SP induced a low level of migration ($P < 0.05$, Fig. 6A). Remarkably, conditioned media from SP-exposed eSF induced CD14+ monocyte migration more than 4-fold the level observed with 1% SP alone, while the conditioned media of eEC treated with 1% SP led to even higher levels of CD14+ monocyte migration ($P < 0.05$, Fig. 6). Similar migratory responses were observed for non-adherent CD4+ T cells, with the highest level of migration observed after treatment with conditioned medium from SP-treated eEC ($P < 0.05$, Fig. 6B). Collectively, these data suggest that SP induces both eEC and eSF to secrete chemotactic factors sufficient to recruit CD14+ monocytes and CD4+ T cells.

Discussion

The study presented herein provides a genome-wide gene expression analysis of specific endometrial cell types in response to SP. Although effects of semen and SP on human and animal FRT cells have been previously characterized (Robertson *et al.*, 2002, 2009; Robertson, 2005, 2007; Sharkey *et al.*, 2007, 2012a,b), we provide, for the first time to our knowledge, a set of primary array data for the response of both human eEC and eSF to SP. We ensured that the response to SP was not a response to SP-induced toxicity, a well-described *in vitro* phenomenon (Allen *et al.*, 1986, 1987; Fiore *et al.*, 1997), by conducting a careful dose- and time-dependent cytotoxicity analysis. We demonstrate that SP and fresh semen induced similar transcriptional responses in the cells, providing evidence that SP can be utilized to model the signaling effects of semen on endometrial cells. We

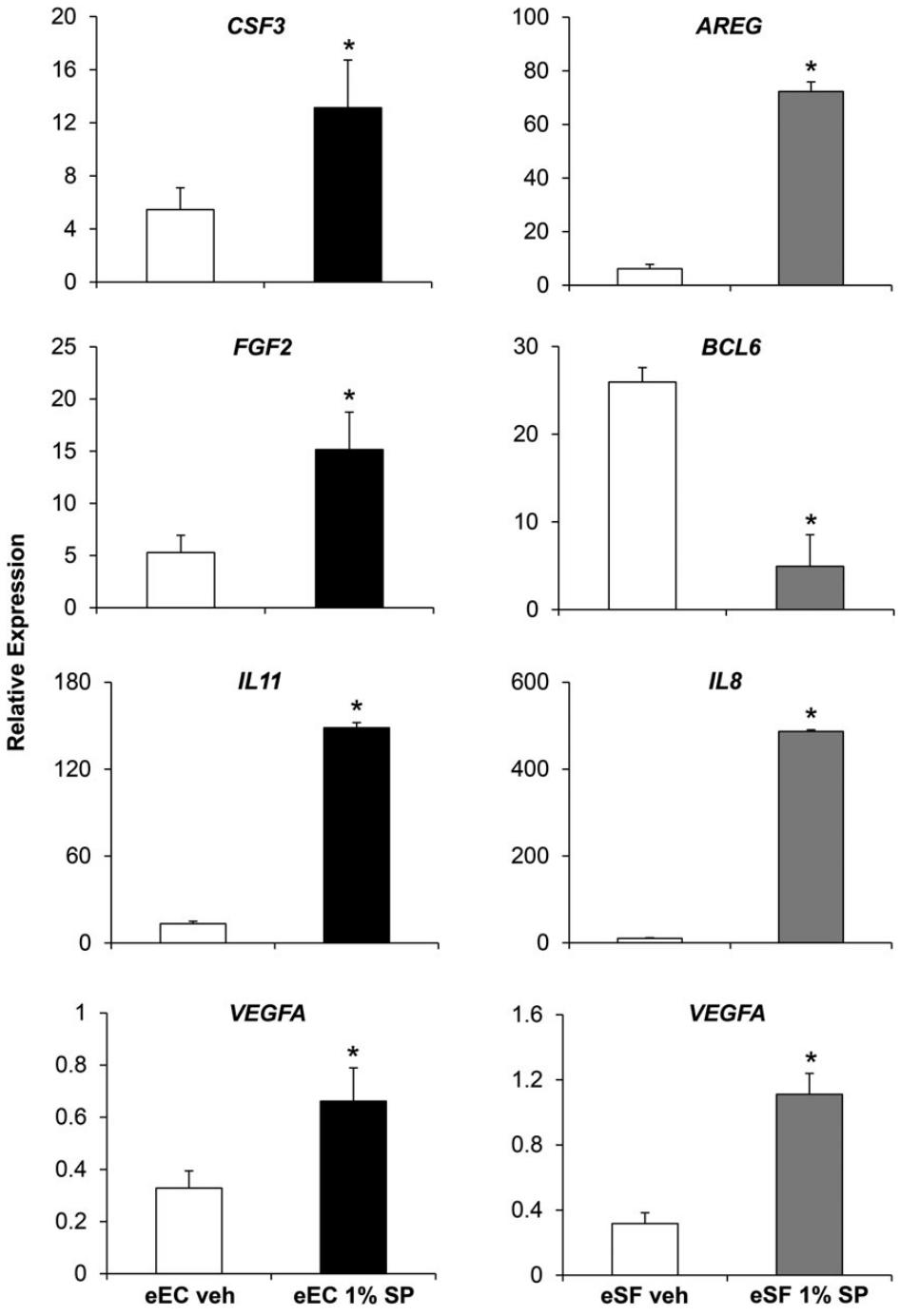

Figure 5 Quantitative real-time PCR analysis of select differentially expressed genes following SP treatment. Expression data are plotted as relative expression per cell type and treatment. $n = 4$. Asterisk indicates $P < 0.05$ compared with veh.

further provide complementary protein data for both eEC and eSF, and demonstrate that chemotactic factors are induced by SP at levels sufficient to recruit leukocytes, including monocytes and T cells. The pathways stimulated by SP, including cellular trafficking, proliferation and viability, are different than the reported effects of endometrial responses to foreign antigens (Giudice, 2003), progestin-based contraceptives (Guttinger *et al.*, 2007) or topical agents that may enter the FRT (Fichorova *et al.*, 2001). The pathways induced by SP can conceivably affect the endometrium in ways that could support reproductive success.

Access of SP to endometrial cells and the upper FRT

The notion that SP can affect the endometrium in a biologically relevant manner is based on the assumption that SP components can reach the

Table V The effect of SP on the production of selected cytokines in eEC and eSF.

Analytes	Treatment				
	eEC (pg/10^5 cells)		eSF (pg/10^5 cells)		(pg/ml)
	Veh	1% SP	Veh	1% SP	1% SP
CCL2	1347.6 ± 451.3	3303 ± 311.0*	154.3 ± 82.9	574.4 ± 173.6*	58.0 ± 13.6
CCL3	ND	ND	ND	ND	1 ± 0.03
CCL4	0.96 ± 0.12	0.98 ± 0.21	ND	ND	1 ± 0.3
CCL5	ND	ND	ND	ND	1.7 ± 0.4
CCL7	15.84 ± 15.5	33.24 ± 20.23	3.6 ± 1.7	21.0 ± 5.0*	ND
CSF2	3.3 ± 1.5	34.5 ± 14.5*	1.04 ± .5	1.48 ± 0.64	ND
CSF3	51.9 ± 15.44	2714.0 ± 1166.56*	ND	ND	30.2 ± 10.3
CXCL1	583.0 ± 95.37	6158.7 ± 1269.3*	1.7 ± 0.7	39.74 ± 11.68*	91.1 ± 12.9
CX3CL1	40.9 ± 11.3	183.2 ± 62.4*	15.1 ± 1.9	85.0 ± 29.7*	10.1 ± 1.1
FGF2	9.5 ± 2.4	13.34 ± 2.11	7.6 ± 1.5	15.52 ± 5.1*	14.4 ± 4.1
IL1A	3.66 ± 0.9	39.58 ± 13.8*	ND	ND	ND
IL4	ND	ND	ND	ND	ND
IL6	92.9 ± 41.8	741.2 ± 226.7*	3.8 ± 1.6	110 ± 14.6*	ND
IL8	1343.5 ± 218.3	5845.0 ± 1155.3*	56.7 ± 32.7	1303 ± 414.6*	13.265 ± 4.8
TNFA	20.6 ± 2.8	74.6 ± 23.0*	ND	ND	ND
VEGFA	96.4 ± 23.7	324.5 ± 72.7*	4.9 ± 3.69	291.6 ± 74.99*	830.0 ± 20.2

Common synonyms are as follows: CCL2 – MCP1; CCL3 – MIP1A; CCL4 – MIP1B; CCL5 – RANTES; CCL7 – MCP3; CSF2 – GM-CSF; CSF3 – GCSF; CXCL1 – GROA; CX3CL1 – Fractalkine; VEGFA – VEGF.
*$P < 0.05$.

upper FRT. Prior studies in humans have demonstrated that bioactive factors deposited into the vaginal canal of the lower FRT can induce effects in the upper FRT (Hartman, 1957; Egli *et al.*, 1961; Barnhart *et al.*, 2001). The rapid ascension of SP components into the upper FRT is thought to be due to the activity of sub-endometrial and myometrial peristaltic waves (Leyendecker *et al.*, 1996; Kunz *et al.*, 1997). Although these data suggest that SP can access the endometrial lumen and the epithelial lining (eEC), whether these factors can access the underlying stroma is more ambiguous, given that the polarized epithelium of eEC is connected by tight junctions and generally does not allow for passive diffusion of factors (Fish *et al.*, 1994). However, recent studies showed that progesterone can promote permeability in primary polarized eEC, which could potentially allow for passage of luminal factors (such as SP) through the polarized eEC to the underlying stroma during the progesterone-dominated secretory phase (Someya *et al.*, 2013). In addition, SP itself may increase access of eSF to SP components by virtue of its ability to induce TNFA, which has been shown to increase the permeability of eEC to luminal factors including human immunodeficiency virus (HIV)-1 (Nazli *et al.*, 2010). Thus, it is possible that SP can gain access to both eEC and eSF following coitus to participate in signaling mechanisms in the FRT.

SP is a potent inducer of chemotaxis and cell migration

SP stimulated the expression of multiple genes associated with chemotaxis, including CSF3, VEGFA, and FGF2 (in eEC), and IL8, CXCL1, FGF2, and VEGFA (in eSF). Pro-inflammatory mediators, including CCL2, CSF2, IL6, and TNFA (from eEC); and CCL2 and IL6 (from eSF), were also increased by SP. A prior microarray analysis on the effect of SP on Ect1 immortalized ectocervical epithelial cells revealed induction of pro-inflammatory genes including IL8, IL6, CSF2 and CCL2 (Sharkey *et al.*, 2012a,b), all of which were induced in both eEC and eSF following SP exposure in our study. In cervical cells, the induction of IL6 and CSF2 was largely attributed to the activity of seminal tumor growth factor-beta (Sharkey *et al.*, 2012a,b). All together, these data suggest that expression of inflammatory mediators is increased by both ectocervical and endometrial cells following SP exposure. The induction of genes associated with inflammation and chemotaxis in the FRT is consistent with reports in multiple animal models, particularly in rodents (Robertson *et al.*, 1992; Sanford *et al.*, 1992), pigs (Bischof *et al.*, 1994) and humans (Sharkey *et al.*, 2012a,b), showing that SP induces an inflammatory state that ultimately recruits immune cells to the uterine microenvironment.

Several of the secreted factors induced by SP exposure, including CCL2, IL8 and VEGFA, are chemotactic for systemic monocytes (Gerszten *et al.*, 1999; Waltenberger *et al.*, 2000; Daly *et al.*, 2003), which, when recruited to tissues, can further differentiate in response to cues from the tissue microenvironment. For example, monocytes are capable of differentiating into phagocytic cells such as tissue dendritic cells (DC) (Chapuis *et al.*, 1997; Randolph *et al.*, 1998). A main function of DC is to act as antigen-presenting cells during infection. In the endometrium, DC have also been shown to participate in endometrial angiogenesis (Pollard, 2008; Lee *et al.*, 2011). Moreover, endometrial DC also play a role in converting T cells into T-regulatory cells (Pollard, 2008), which are vital for promoting an immune-tolerant environment for the embryo (Aluvihare *et al.*, 2004). T cells can also be directly recruited by SP-induced factors, since naïve T cells

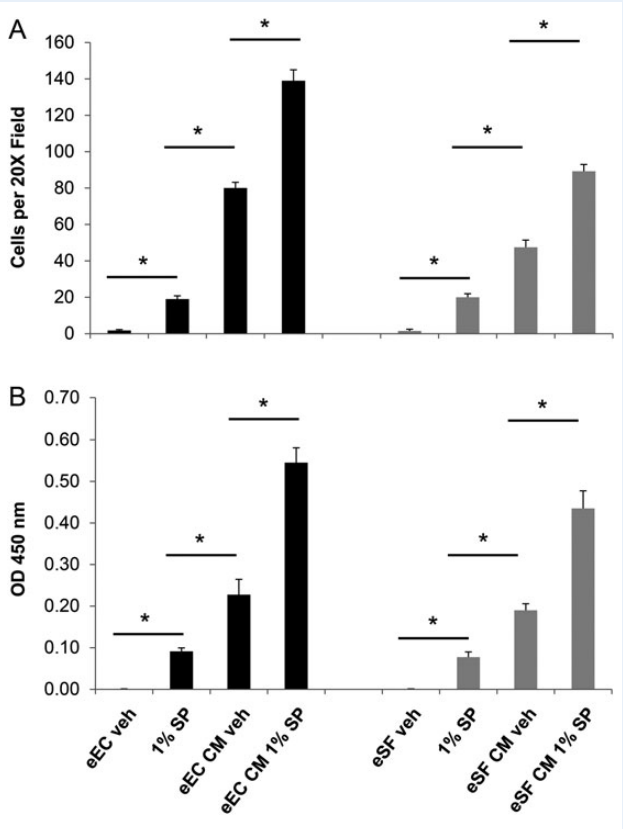

Figure 6 Migration of CD14+ monocytes and CD4+ T cells in response to 1% SP or conditioned media (CM) from eEC and eSF cultures. (**A**) Black bar indicates migrated cells per ×200 magnification viewing in response to eEC-conditioned media. Gray bar indicates migration in response to eSF-conditioned media. (**B**) Values correspond to optical density (450 nm), which reflects cellular migration in response to eEC-conditioned media (black) or eSF-conditioned media (gray). Asterisk and horizontal line indicate $P < 0.05$ within relevant *post hoc* comparisons.

migrate in response to IL8 (Taub *et al.*, 1996), a cytokine that was secreted by both eEC and eSF in response to SP. In addition, IL8 can recruit other immune cells, such as neutrophils and macrophages, which participate in the post-coitus clearance of micro-organisms and superfluous or morphologically abnormal sperm (Tomlinson *et al.*, 1992; Robertson, 2005). These studies, together with our observation that conditioned media from SP-treated eEC and eSF is highly chemotactic, suggest that SP-stimulated release of cytokines by the endometrium may recruit immune cells that then participate in modulating the immune milieu of the upper FRT.

In addition to immune cell recruitment, another major pathway that was induced by SP in both eEC and eSF was endothelial cell migration and invasion, in particular through pathways associated with VEGFA signaling. VEGFA is a secreted factor that promotes vascular permeability, stimulates angiogenesis (proliferation and vascular sprouting from existing vessels), and *de novo* synthesis of new blood vessels (vasculogenesis) (Ferrara *et al.*, 1997a,b; Neufeld *et al.*, 1999). VEGFA exerts its effects by recruiting mature and progenitor endothelial cells, which form the inner lining of blood vessels and together with pericytes and smooth muscle cells form the systemic vascular network. Expression of VEGFA is required for normal tissue growth, and proper vascular development is crucial for normal endometrial function. Endometrial VEGFA can be produced by both endometrial macrophages and eSF (Popovici *et al.*, 1999), and VEGFA production is increased in these cells during localized hypoxia which occurs during menses to stimulate the regeneration and repair of new tissue (Sharkey *et al.*, 2000). Our results are consistent with those findings and suggest that both eEC and eSF are further stimulated to increase expression and secretion of VEGFA upon exposure to SP. This effect may be due to SP-derived or SP-induced IL8, which has been shown *in vitro* to stimulate VEGFA production (Martin *et al.*, 2009). Secreted VEGFA can in turn play a role in recruiting endothelial cells for the purpose of angiogenesis and vasculogenesis in the endometrium.

The effect of SP on cellular proliferation and viability

Pathways associated with cellular growth and viability were up-regulated by both eEC and eSF in response to SP exposure. The ability of SP to up-regulate genes associated with eEC proliferation and survival could promote endometrial function, as the luminal epithelium serves as the site of initial attachment for the fertilized egg (Giudice, 2003; Aplin *et al.*, 2008). In eSF, SP induced pathways associated with fibroblast proliferation while SP inhibited pathways associated with cellular death. Prior studies detailing the phase-dependent viability of eSF during the menstrual cycle indicate that eSF viability is lowest during the late secretory phase and early proliferative phase, and highest during the proliferative and early/mid secretory phases. Stromal viability is likely crucial for the cyclic development of the endometrial lining as the uterus prepares for the receptive window of implantation. Thus, SP may contribute to eSF survival during a period where stromal viability is important for embryo attachment (Dmowski *et al.*, 2001).

SP also induced pathways associated with proliferation and viability of other cell types found in the endometrium. For example, pathways associated with endothelial cell proliferation and viability were induced by SP in both eEC and eSF, suggesting that SP may have a role in angiogenesis or vasculogenesis in the endometrium. Surprisingly, pathways associated with neuronal cell proliferation and survival were also stimulated by SP in both cell types. Neuronal cells exist in the endometrium, and aberrant innervations by neuronal complexes have been implicated in endometrial pathologies associated with pain (Newman *et al.*, 2013). However, it is poorly understood how patterns of normal innervations in the endometrium contribute to implantation success and if those patterns are affected by the menstrual cycle or by exogenous factors (e.g. SP) that access the uterine lumen. Because we did not examine induction of neurotrophic factors at the protein level or carry out functional studies on innervation, it is difficult to assess the significance of these changes in the transcriptome. Future studies should determine if SP and SP-conditioned eEC/eSF media can propagate neuronal growth.

Implications for HIV infection

Although our discussion focuses on how SP-induced alterations in gene expression can facilitate pregnancy, the data described herein also have implications for sexually transmitted pathogens, which are typically transported via semen. *In vitro* studies have shown that semen can directly enhance HIV-1 infection (Kim *et al.*, 2010) and that this effect may be mediated through semen-derived peptides that form amyloid fibrils that facilitate viral entry (Munch *et al.*, 2007; Hauber *et al.*, 2009; Kim *et al.*, 2010; Roan *et al.*, 2011). These amyloids are derived from the

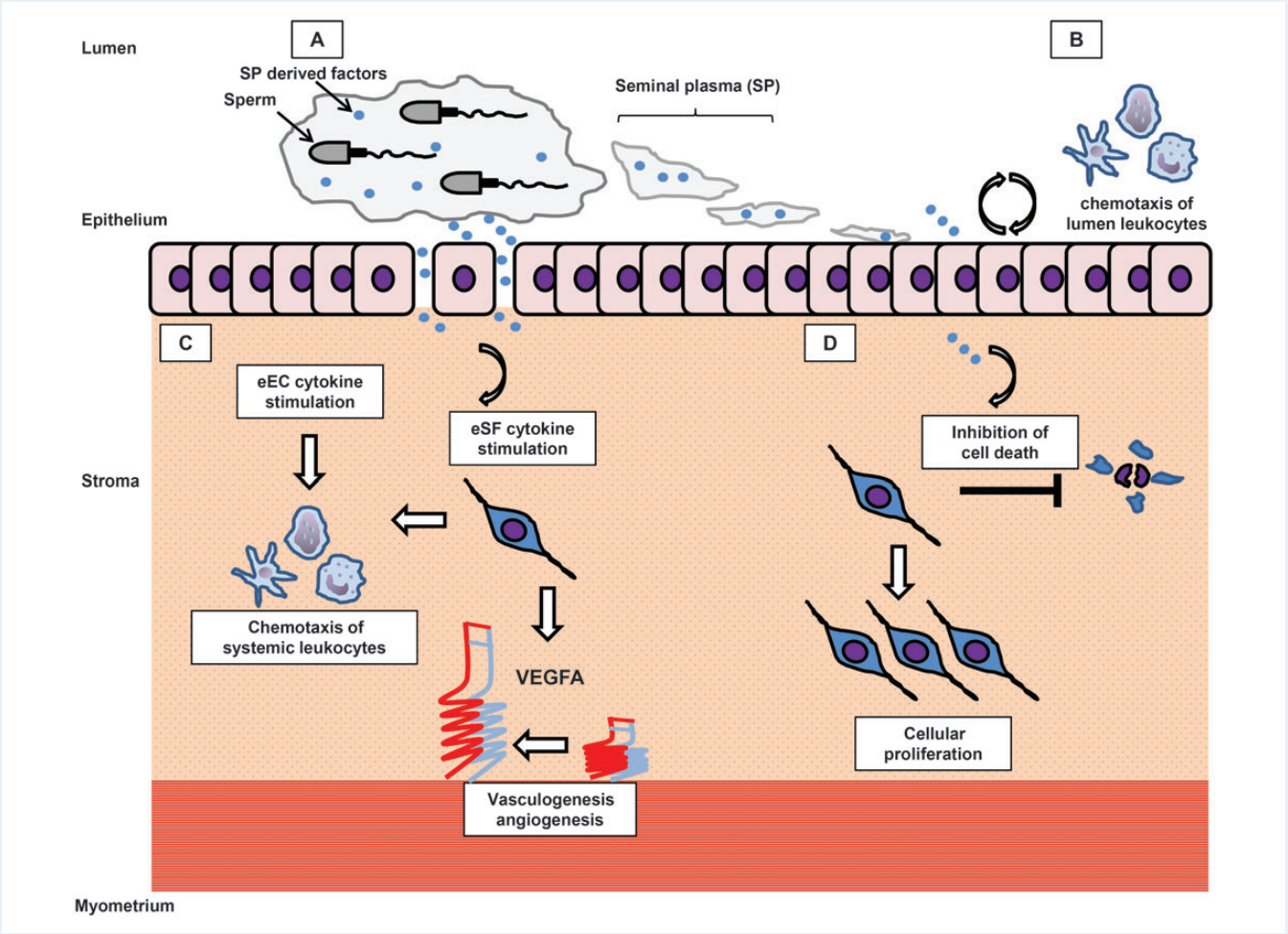

Figure 7 Schematic working model, built upon prior publications and data from this study, of how SP affects target cells in the endometrium. (**A**) SP-derived factors present in semen access the upper female reproductive tract (FRT) of the uterine lumen after being deposited into the lower FRT. (**B**) SP factors induce pro-inflammatory and pro-chemotactic responses in the luminal epithelium, resulting in recruitment of resident lumen leukocytes to the exposure site. (**C**) During the secretory phase or in the presence of luminal epithelial perturbations, SP factors act on both eEC and eSF to promote cytokine and vascular endothelial growth factor (VEGF)A production, which can potentiate recruitment of systemic leukocytes and vasculogenesis/angiogenesis in the underlying stroma. (**D**) SP can also promote eSF viability and proliferation.

abundant proteins prostatic acid phosphatase (Munch *et al.*, 2007; Arnold *et al.*, 2012) and the semenogelins (Roan *et al.*, 2011), the major components of the semen coagulum. Semen has also been shown to harbor high levels of IL7, which can enhance HIV infection by promoting proliferation of permissive CD4+ T cells (Introini *et al.*, 2013). Studies investigating the safety of vaginal microbicides have shown that pro-inflammatory pathways in the FRT can enhance HIV infectivity by recruiting HIV target cells to the site of exposure (Fichorova *et al.*, 2001). The current study suggests that SP induces potent pro-inflammatory, pro-chemotactic effects in the endometrium of the FRT. In contrast to the multi-layered stratified squamous epithelium of the lower FRT, the epithelium of the upper FRT contains only a single layer of columnar epithelium and therefore may provide a less formidable barrier for HIV entry. Whether SP-induced inflammation in the endometrium facilitates HIV transmission and, if so, how this compares to other reported mechanisms of semen-mediated enhancement of HIV infection will be important questions to answer in future investigations.

Limitations of interpretations

A limiting factor of this study is the absence of ovarian hormones in the cell culture model. Both eEC and eSF are exposed to dynamic levels of estrogen and progesterone *in vivo* throughout the menstrual cycle, which considerably affect the biological activity of these cells including immune environment modulation (Talbi *et al.*, 2006). Additionally, it is well reported that eEC require paracrine support from eSF to properly respond to ovarian hormone-driven differentiation and proliferative cues. Studies exploring the effects of SP and ovarian hormones on eEC in the presence of eSF paracrine support, for example through the use of eEC/eSF co-culture models (Chen *et al.*, 2013), should be considered in future endeavors.

Another factor to consider is that SP is not absolutely required for successful fertilization or implantation, as evidenced by the success of artificial insemination. However, a limited set of studies in humans show that implantation or implanted embryo quality is increased by exposure to SP

(Bellinge *et al.*, 1986; Coulam *et al.*, 1995; Tremellen *et al.*, 2000). In addition, SP components, such as VEGFA and glutathione peroxidase, are associated with sperm quality (Crisol *et al.*, 2012). Thus, while SP may not be unconditionally necessary for fertilization or implantation success, studies in both humans and animal models (Carp *et al.*, 1984; Mah *et al.*, 1985; Johansson *et al.*, 2004) suggest that there is a positive correlation between SP exposure and reproductive success.

Summary

In summary, we have demonstrated that eEC and eSF respond to SP at both the transcriptional and protein secretion levels. Our data support and build upon existing knowledge about the physiological role of SP signaling in the FRT. A model consistent with our findings and those previously published is illustrated in Fig. 7. SP, upon access to the uterine lumen (Fig. 7A), stimulates a pro-chemotactic response in eEC that can recruit leukocytes to the site of exposure (Fig. 7B). These leukocytes, including monocytes and CD4+ T cells, can differentiate into cell populations that may play roles in endometrial function and immunity (Hunt *et al.*, 2000). Although most studies on SP exposure to FRT cells have focused on epithelial cells, it is conceivable that SP-derived factors can access the underlying stroma during specific phases of the menstrual cycle, in the presence of physical perturbations, or as a result of endometrial exposure to SP. Stimulation of cytokine and VEGFA production in both eSF and eEC can recruit systemic leukocytes to the endometrium, a process necessary for cyclical endometrial growth and repair, vasculogenesis, and angiogenesis (Fig. 7C). In addition, exposure of eSF to SP can up-regulate expression of factors that support cellular proliferation and viability, as suggested in this study (Fig. 7D). Of note, most of the experimental data supporting this model (including that reported in this study) were based on *in vitro* experiments, although *in vivo* studies in both animal models (Robertson, 2007) and in humans (Sharkey *et al.*, 2012a,b) generally support the notion that SP-induced cytokines and chemokines recruit and regulate leukocytes in the FRT. Key follow-up studies that build on existing *in vivo* animal models or clinical human studies would support our current *in vitro* observations. In any case, the data herein describing the response of the two dominant endometrial cell types to SP should provide a valuable resource and reference for future research on the effects of semen on the human FRT.

Supplementary data

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

Acknowledgements

The authors thank Drs Barbara Shacklett, Ruth Greenblatt, Karen Smith-McCune and Shomyseh Sanjabi for helpful discussions. The authors also appreciate the advice of Thad Baker (EMD Millipore) for technical guidance regarding Luminex multiplex assays. Finally, the authors would like to acknowledge Dr James Smith, Liza Jalalian and the UCSF Fertility Clinic; Kim Chi Vo for her assistance regarding human endometrial tissue use; Linda Ta, Yanxia Hao and the University of California, San Francisco Genomics Core at the J. David Gladstone Institutes; the NIH Specialized Cooperative Centers Program in Reproduction and Infertility Research Human Endometrial Tissue and DNA Bank; and the University of Oulu, Oulu University Hospital, Oulu, Finland.

Authors' roles

J.C.C., lead author of study, participated in experimental design and data acquisition for all facets of the paper, drafting and revising the manuscript, and final manuscript approval. B.A.J. participated in experimental design of toxicity experiments, clinical support and tissue acquisition, drafting and revision of the manuscript, and final manuscript approval. D.W.E. participated in experimental design, optimization of leukocyte migration assays, drafting and revision of the manuscript, and final manuscript approval. T.T.P. participated in clinical tissue acquisition, experimental design, clinical interpretations, drafting and revision of the manuscript, and final manuscript approval. F.B. participated in experimental design and toxicity experiments, drafting of the manuscript, final manuscript approval. S.C. participated in experimental design, semen processing, leukocyte isolation, drafting of the manuscript, final manuscript approval. N.K. participated in experimental design, global microarray analysis of the fresh semen study, drafting of the manuscript, final manuscript approval. J.C.I. participated in experimental design, technical troubleshooting, data analysis, drafting of the manuscript, final manuscript approval. W.C.G. participated in experimental design, senior-level mentorship, technical troubleshooting, drafting of the manuscript, final manuscript approval. L.C.G. participated in experimental design, senior-level mentorship, technical troubleshooting, clinical interpretations, drafting of the manuscript, final manuscript approval. N.R.R., corresponding author of the study, participated in experimental design, troubleshooting and data analysis for all facets of the paper, drafting and revising the manuscript, and final manuscript approval.

Funding

This project was supported by NIH AI083050-04 (to W.C.G. and L.C.G.), the Eunice Kennedy Shriver NICHD Specialized Cooperative Centers Program in Reproduction and Infertility Research U54HD 055764 (to L.C.G.), The Ruth L. Kirschstein National Research Service Awards NICHD 1F32HD074423-02 (to J.C.C.), the 1PO1 AI083050-01 PPG and Department of Defense grant W81XWH-11-1-0562 (to W.C.G.), and the 5K12 DK083021-04 KURe, 1K99AI104262 – 01A1, and the Hellman Award at UCSF (to N.R.R.).

Conflict of interest

None declared.

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