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Title Page

Title: Idiopathic infertility in women is associated with distinct changes in proliferative phase uterine fluid proteins

Running title: The proteome of proliferative phase uterine fluid

Summary sentence: Proteomic analysis of proliferative phase uterine fluid in both fertile and infertile women, showed significant differences in the secreted proteins including ECM1 which was further studied for its cellular location and hormonal regulation.

Key words: endometrium; proteome; proliferative phase; uterine fluid; infertility; ECM1

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Grant support: Supported by NHMRC Fellowship to L.A.S. (#1002028), the Victorian Government's Operational Infrastructure Support Program, and the L.E.W. Carty Charitable Fund. H.C.F. supported by Monash University Medicine, Nursing and Health Sciences Faculty Postgraduate Research Scholarship.

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Abstract

The regenerative, proliferative phase of a woman's menstrual cycle is a critical period which lays the foundation for the subsequent, receptive secretory phase. Although endometrial glands and their secretions are essential for embryo implantation and survival, the proliferative phase, when these glands form, has been rarely examined. We hypothesized that alterations in the secreted proteome of the endometrium of idiopathic infertile women would reflect a disturbance in proliferative phase endometrial regeneration. Our aim was to compare the proteomic profile of proliferative phase uterine fluid from fertile (n=9) and idiopathic infertile (n=10) women. Proteins with ≥2-fold change ($P<0.05$) were considered significantly altered between fertile and infertile groups. Immunohistochemistry examined the endometrial localization of identified proteins. Western immunoblotting defined the forms of extracellular matrix protein 1 (ECM1) in uterine lavage fluid. Proteomic analysis identified

four proteins significantly downregulated in infertile women compared to fertile women, including secreted frizzled related protein 4 (SFRP4), CD44, and ECM1: two proteins were upregulated. Seven proteins were unique to the fertile group and six (including isoaspartyl peptidase/L-asparaginase (ASRGL1)) were unique to the infertile group. Identified proteins were classified into biological processes of tissue regeneration and regulatory processes. ASRGL1, SFRP4 and ECM1 localized to glandular epithelium and stroma, CD44 to stroma and immune cells. ECM1 was present in two main molecular weight forms in uterine fluid. Our results indicate a disturbance in endometrial development during the proliferative phase among infertile women, providing insights into human endometrial development and potential therapeutic targets for infertility.

Introduction

Following menses, the human endometrium undergoes rapid repair to re-epithelialize the surface and then proliferates and regenerates during the proliferative phase of a woman's menstrual cycle [1]. The importance of adequate endometrial development is highlighted by uterine gland knockout (UGKO) of mice and sheep in which the absence of endometrial glands and their secretions results in infertility due to failure of embryo implantation [2-4]. Pregnancy will only occur when there is a synchronized development between the embryo and the maternal uterine environment. Indeed, the endometrium must be receptive for embryo implantation to occur [5]. As such, it can be inferred that a disturbance in endometrial regeneration during the proliferative phase may prevent the endometrium from fully differentiating or becoming receptive to embryo implantation during the secretory phase. Furthermore, changes in the secretions from the endometrium found in uterine fluid in infertile women may highlight possible causes of a disturbance in endometrial regeneration.

Importantly, the endometrium is receptive for only approximately four days during the mid-secretory phase of a woman's menstrual cycle. Until now, most studies have focused on changes in protein expression between the proliferative and secretory phases of the menstrual cycle, or between fertile and infertile women during the window of receptivity. Additionally, many of these studies have been biased towards certain proteins and molecules of interest [6, 7]. A number of cytokines, chemokines and growth factors have been identified in the endometrium and uterine lavage fluid [6-9]. For example, VEGFA is downregulated in the uterine lavage fluid of infertile women compared to fertile women during the mid-secretory phase [8].

Examining the full proteome of the uterine microenvironment presents an unbiased approach in identifying essential factors regulating endometrial and uterine remodeling and fertility [10]. Changes in the proteomic profile of uterine lavage fluid are associated with changes in the phase of menstrual cycle and fertility status [11-13]. Parmar et al. examined changes in the proteomic profile of uterine fluid between the proliferative and mid-secretory phases of the menstrual cycle using 2D gel methodology [12]. Similarly, Hannan et al. [11], investigated the proteomic profiles of proliferative and secretory phase uterine fluid as well as uterine fluid collected from fertile and infertile women during the mid-secretory phase of the menstrual cycle identifying several proteins including forms of alpha-2-macroglobulin decreased in the mid-proliferative phase compared to the mid-secretory phase of fertile women, and of apolipoprotein-A4 increased in infertile women compared to fertile women during the mid-secretory phase. While these studies targeting mid-secretory changes with fertility status and cycle phase have highlighted the dynamic remodeling of the uterine environment, the full proteome of proliferative phase uterine fluid and how this may impact female fertility has not yet been defined.

We previously demonstrated, for the first time, that the cytokine profile of proliferative phase uterine lavage fluid is significantly altered in infertile women compared to fertile women of <35 years [9].

This indicated that in this group of women, the endometrium was developing in an environment of increased inflammation, potentially influencing the subsequent secretory potential of the endometrial glands and inadequately priming the endometrium for embryo implantation. Such findings are particularly important as it is now acknowledged that abnormal inflammation can have negative effects on endometrial receptivity and the establishment of pregnancy [14-16]. Observation of these changes in the cytokine profile of fertile and infertile proliferative phase uterine fluid, highlighted the need to fully investigate the proteome of proliferative phase uterine fluid and how this may reflect on endometrial regeneration and fertility competence.

In this study, we compare the proteome of proliferative phase uterine fluid collected from fertile and infertile women using state-of-the-art technology and validate a number of proteins as being of endometrial origin. In particular extracellular matrix protein 1 (ECM1) identified in the proteome analysis, was examined further in terms of its secretion from endometrial cells.

Materials and Methods

Ethical approval

All human endometrial tissue and uterine fluid samples were collected under ethics approval (approval #03066B) from Monash Health Human Ethics Committee and written informed consent was obtained from each patient.

Patient details and sample collection

Human endometrial biopsies and uterine lavage were obtained during the proliferative phase of the menstrual cycle (unless otherwise stated) from fertile women and women with unexplained

(idiopathic) primary infertility who were undergoing hysteroscopy or dilatation and curettage. Fertile women all had proven parity and had no uterine abnormalities. Primary infertile women were screened for non-endometrial causes of their infertility, for example, ovarian dysfunction, tubal patency, endometriosis and male factor infertility which serve as exclusion factors. Patients with uterine abnormalities such as endometriosis and endometritis or who had received steroid hormone therapy in the last 6 months were excluded. The menstrual cycle stage was categorized by experienced pathologists according to established criteria [17].

Endometrial biopsies obtained by curettage from fertile (n=19) and primary infertile women (n=19) were either formalin-fixed overnight (16 ± 1hr) at 4°C and processed to wax, or placed in 1:1 Dulbecco's modified Eagle's tissue medium and Ham's F12 medium containing 1% L-glutamine culture medium (DMEM/F12 GIBCO Invitrogen, Mt Waverley, VIC, Australia) until epithelial and stromal cell isolation.

Uterine lavage was collected from fertile (n=23) and primary infertile women (n=24) by gently infusing 3ml of sterile saline trans-cervically into the uterine cavity using an infant feeding tube. The uterine lavage was withdrawn, centrifuged at 1000 revolutions per minute (rpm) to remove cellular debris, aliquoted and immediately stored at -80°C.

Proteomic analysis

To analyze the proliferative phase proteome of uterine fluid using mass spectrometry, uterine lavage fluid samples, collected during the proliferative phase (fertile n=9 and infertile women n=10) were prepared by filter aided sample preparation (FASP) (Protein Discovery) as described by Wisniewski et al. [18]. A urea sample solution was prepared using Tris hydrochloride (Tris HCl); 0.75g urea in 1ml Tris HCl solution per sample. One hundred µl of the urea sample solution was added to a spin

column with a relative molecular mass cut-off of 30,000 (30k), and centrifuged at 14,000 x g for 4 minutes to wet the column filter. A 10 mmol solution of tris(2-carboxyethyl) phosphine (TCEP) in urea sample solution was prepared, added to the spin column with 200µg protein to reduce the disulfide bonds and incubated with agitation for 20 minutes. The spin column was centrifuged at 14,000 x g for 15 minutes and the flow-through discarded. Two hundred µl of urea sample solution was added to the spin filter and centrifuged at 14, 000 x g for 15 minutes and the flow-through discarded. A 50mM iodoacetamide (IAA) solution was prepared by adding 100µl urea sample solution to one tube of pre-weighed IAA IAA. IAA alkylates the reduced disulfide bonds and prevents them from reforming. 10µl of the 10X IAA solution and 90ul of urea sample solution were added to the spin column and vortexed for 1 minute, then incubated without mixing for 20 minutes in the dark. The spin column was centrifuged at 14, 000 x g for 10 minutes. 100µl of urea sample solution was added to the spin column and centrifuged at 14, 000 x g for 15 minutes. This was repeated twice and the flow-through discarded. 100µl of 50mM ammonium bicarbonate solution was added to the spin column and centrifuged at 14,000 x g for 10 minutes, repeated twice. A digestion solution was prepared using trypsin diluted in ammonium bicarbonate solution to a concentration of 0.027µg/ml. 75µl of the digestion solution (enzyme-to-protein ratio 1:100) was added to the spin filter and vortexed for 1 minute. The tubes were wrapped in parafilm to minimize evaporation and incubated overnight with agitation at 37°C.

The spin filter was transferred to a new collection tube. 40µl of 50mM ammonium bicarbonate solution was added to the spin filter and centrifuged at 14,000 x g for 10 minutes, repeated once. The flow-through was transferred to a glass vial.

Mass Spectrometry and Bioinformatics

Peptides were acidified with formic acid (FA) to 1% final concentration. Solvent was removed in a CentriVap concentrator (Labconco) and peptides were resuspended in MilliQ water containing 3%

acetonitrile (ACN) and 1% FA. 5 µl of each sample were analyzed by nanoflow liquid chromatography tandem-mass spectrometry (LC-MS/MS) on a nanoAcquity system (Waters, Milford, MA, USA) coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) through a nanoelectrospray ion source (Thermo Fisher Scientific). Peptide mixtures were loaded on a 20mm trap column with 180mm inner diameter (nanoAcquity UPLC 2G-V/MTrap 5mm Symmetry C18) at 1% buffer B, and separated by reverse-phase chromatography using a 150 mm column with 75mm inner diameter (nanoAcquity UPLC 1.7mm BEH130 C18) on a 60 minutes linear gradient from 1% to 50% buffer B (A: Milli-Q water, 3% ACN, 0.1% FA; B: Milli-Q water, 80% ACN, 0.1% FA) at a 500nl/min constant flow rate. The Q-Exactive was operated in a data-dependent mode, switching automatically between one full-scan and subsequent MS/MS scans of the ten most abundant peaks. The instrument was controlled using Exactive series version 2.1 build 1502 and Xcalibur 3.0. Full-scans (m/z 350–1,850) were acquired with a resolution of 70,000 at 200 m/z. The 10 most intense ions were sequentially isolated with a target value of 10,000 ions and an isolation width of 3 m/z and fragmented using HCD with normalized collision energy of 27 and stepped collision energy of 15%. Maximum ion accumulation times were set to 50ms for full MS scan and 80ms for MS/MS. Underfill ratio was set to 2% and dynamic exclusion was enabled and set to 30 seconds. Raw files were processed and analyzed by Maxquant [19].

Statistical comparison of changes in the proteomic profile were made between proliferative phase fertile uterine fluid and infertile uterine fluid. Proteins were considered altered in uterine fluid from infertile women if there was a 2-fold difference between fertile and infertile groups and $P<0.05$ was considered statistically significant. To be considered for statistical analysis, peptides were present in at least half of the samples in any group. Unique proteins were those present in one group and absent in another.

Enrichment analysis

To identify functions and biological processes associated with the differentially expressed proteins, gene ontology (GO) was analyzed using the WEB-based Gene Set Analysis Toolkit (WebGestalt)

[20]. At least two genes needed to be present in a category and the top 10 enriched GO biological processes were reported. A hypergeometric test was used and Benjamin and Hochberg analysis applied to adjust for multiple tests.

Immunohistochemistry

Immunohistochemistry for CD44, isoaspartyl peptidase/L-asparaginase (ASRGL1), secreted frizzled related protein 4 (SFRP4) and extracellular matrix protein 1 (ECM1) was performed on 5μm sections of formalin-fixed paraffin-embedded endometrial tissue collected from fertile (n=8) and infertile (n=10) women (except for ECM1: fertile n=10; infertile n=9) during the proliferative phase. Fixed endometrial sections were dewaxed and rehydrated. Endogenous hydrogen peroxidase activity was quenched using 3% hydrogen peroxide (H_2O_2) for 10 minutes at room temperature (RT). Sections were rinsed in Tris-buffered saline (TBS) for five minutes at RT. Non-specific binding was blocked by pre-incubation of tissues with non-immune blocking solution; 10% normal horse serum, 2% normal human serum in TBS for CD44, ASRGL1 and SFRP4, and 6% goat serum in 1% TBS-Triton for ECM1 for 30 minutes at RT. Sections were incubated with an antibody against CD44 (Abcam, ab9524, final concentration 2.5ug/ml), ASRGL1 (Santa Cruz, sc-130472, final concentration 0.5ug/ml), SFRP4 (Abcam, ab122905, final concentration 0.625ug/ml), ECM1 (Abcam, ab126629, final concentration 0.091ug/ml) diluted in non-immune blocking solution (Supplemental Table S1). Negative isotype control of mouse IgG (Dako, X0931), goat IgG (R&D Systems, AB-108-C) or rabbit IgG (Dako, X0936) were used at equivalent concentrations. Primary antibodies and negative isotype controls were incubated overnight at 4°C. The sections were incubated for 30 minutes at RT with biotinylated horse antimouse secondary antibody (Vector Labs, BA-2000), biotinylated horse antigoat secondary antibody (Vector Labs, BA-9500) or biotinylated goat antirabbit secondary antibody (Vector Labs, BA-1000) at a dilution of 1:200v:v in non-immune blocking solution. Avidin-biotin conjugated with horseradish peroxidase (Vector Laboratories) at a dilution of 1:4000v:v in TBS was applied to each section and incubated for 30 minutes at RT. Positive immunostaining was visualized

using 3,3'-diaminobenzidine (Dako) and development was arrested using tap water. Sections were counterstained with hematoxylin (Sigma) at a 1:10v:v dilution in dH_2O for three minutes at RT. Sections were dehydrated and mounted. Localization of CD44, ASRGL1, SFRP4 and ECM1 was analyzed by a light microscope.

Assessment of immunostaining intensity

Immunostaining was examined using light microscopy. Positive staining for ASRGL1, SFRP4 and CD44 was assessed semi-quantitatively by two unbiased observers. Intensity of staining within the glandular epithelium (GE) and stroma was analyzed and allocated a score from 0 to 3, where 0 was no staining, 1 was minimal staining, 2 was strong staining and 3 was intense staining.

CD44/45 serial sections

To determine whether CD44 was expressed by leukocytes, serial 2μm sections of formalin fixed, paraffin embedded proliferative phase endometrial tissue collected from fertile (n=4) and infertile (n=4) women were immunostained with CD44 at 10μg/ml and CD45 (Dako, M0701) at 1μg/ml. The same procedure was followed as above. Non-specific binding was blocked by immune block, 2% human serum and 10% horse serum in TBS. The secondary antibody was biotinylated horse antimouse secondary antibody (Vector Labs, BA-2000) and negative isotype control was mouse IgG (Dako, X0931).

ECM1 ELISA with uterine fluid

The concentration of ECM1 in proliferative phase uterine lavage fluid from fertile (n=18) and infertile (n=18) women was measured by ELISA according to the manufacturer's instructions (Sigma, #RAB1038). Analysis of each sample and standard was performed in duplicate and on the same plate.

100µl of standards and samples, diluted 1:100, were added to a Human ECM1 antibody coated plate and incubated for 2.5 hours at RT with gentle shaking. The samples and standards were washed four times. 100µl of detection antibody was added to each well and incubated for one hour at RT with gentle shaking. Following washing, 100µl of HRP-streptavidin was added to each well and incubated for 45 minutes at RT with gentle shaking. Wells were washed four times. 100µl of Colorimetric TMB substrate reagent was added to each well and incubated at RT in the dark with gentle shaking for 30 minutes. 50µl of Stop Solution was added to each well and the plate was read immediately using the CLARIOstar plate reader at 450nm with a 5-parameter fit standard curve generated. A pilot study determined that sample ECM1 concentrations lay within the functional range of the standard curve with samples diluted 1:100. The minimum detectable dose of ECM1 for this assay is 24pg/ml and the intra-assay reproducibility is CV <10% according to the manufacturer.

Western blot analysis of ECM1 in uterine lavage fluid

Proliferative phase uterine lavage fluid samples (fertile, n=3; infertile, n=3) were assayed for total protein by Nanodrop. 10µg total protein was resolved on a 4-15% Mini Protean TGX Stain-free gel (Bio-rad, #456-8083) and then transferred to 0.2µm PVDF membrane (Bio-rad, #1704156) using the Biorad Transblot Turbo Transfer system. The membrane was blocked in 0.2% Tween-20, 5% skim milk in TBS solution and then incubated overnight at 4°C with the same ECM1 antibody used for immunohistochemistry, (1:1000 in blocking solution). After washing in TBS and 0.2% Tween-20 in TBS, HRP-conjugated secondary (goat antirabbit antibody, 1:2000 in blocking solution, Dako #P0448) was applied for 45 minutes at RT. Following this, the membrane was washed and ECL applied. Bands were visualized using the ChemiDoc imaging system (Bio-rad) and Image Lab software (Bio-rad).

Isolation of primary endometrial epithelial and stromal cells

Primary endometrial epithelial and stromal cells were isolated from tissue biopsies collected from fertile (n=7) and primary infertile (n=3) women. Endometrial tissue samples were washed with

phosphate buffered saline (PBS, Gibco, Life Technologies), finely minced with scissors and digested in PBS with 7.5 international units (IU)/ml collagenase III (Sigma) and 100 mg/ml DNase I (Worthington). Following incubation in a water bath at 37°C with shaking at 130rpm for 20 minutes, the extent of digestion was assessed and the tissue further chopped if necessary. Tissue was incubated for a further 20 minutes at 37°C with shaking. Digestion was stopped by the addition of excess DMEM/F12 (Gibco, Life Technologies). The digested tissue was filtered through 45μm nylon mesh to collect endometrial epithelial cell fragments, which were retained on the mesh, and then through an 11μm nylon mesh to collect stromal cells. Endometrial epithelial cell fragments were collected from the 45μm nylon mesh by gentle washing with PBS, pelleted by centrifugation, resuspended in DMEM/F12 supplemented with 10% fetal calf serum (FCS, Gibco, Life Technologies) and 1% penicillin/streptomycin (Sigma), and sequentially seeded in a 24-well plate. Endometrial stromal cells were pelleted by centrifugation, resuspended in DMEM/F12 supplemented with 10% charcoal-stripped FCS (cSFCS, Gibco, Life Technologies) and 1% penicillin/streptomycin and sequentially seeded in T25 cell culture flasks.

ECM1 ELISA with media from primary endometrial epithelial and stromal cells

Primary endometrial epithelial (fertile, n=3; infertile, n=2) and stromal cells (fertile, n=5; infertile, n=3) were isolated and cultured from endometrial tissue biopsies as described above. Once confluent, 500,000 stromal cells/well were seeded into a 6-well plate. At 80% confluency, endometrial epithelial and stromal cells were starved with 0.5% csFCS DMEM/F12 for eight hours and then maintained in 1% FCS DMEM/F12 for 72 hours. Following this, media were collected and centrifuged to remove cellular debris and frozen at -20°C. The concentration of ECM1 in media collected from primary endometrial epithelial and stromal cells was measured using the above ECM1 ELISA kit (Sigma). The same protocol was followed although media collected from epithelial and stromal cells were diluted 1:5 with sample diluent buffer. All samples were assayed on the same plate.

Estrogen dose response treatment of ECC1 cells

The endometrial epithelial carcinoma cell line, ECC1 (fully validated) [21], was cultured in 10% FCS DMEM/F12 in 6-well plates until 80% confluency. ECC1s were serum starved for eight hours with 0.5% csFCS DMEM/F12 and then primed for 24 hours with either 10^{-9}M, 10^{-8}M or 10^{-7}M estradiol 17β (henceforth referred to as estrogen) in 0.5% csFCS DMEM/F12, or 0.5% csFCS alone (control), n=5/treatment. The media were removed and epithelial cells were treated for a further 48 hours with these same treatment groups. The media were collected, centrifuged to remove cell debris and stored at -80°C.

ECM1 ELISA with media from estrogen treated ECC1s

The concentration of ECM1 in media collected from ECC1s treated with either control, 10^{-9}M, 10^{-8}M or 10^{-7}M estrogen (n=5/treatment) was measured using the above ECM1 ELISA kit (Sigma). The same protocol was followed, although the ECC1 conditioned media were diluted 1:2 with sample diluent buffer.

Western blot analysis of ECM1 in estrogen treated ECC1 media

ECM1 was analyzed in ECC1 media (n=5/treatment, treatments as above) by Western immunoblotting using the same method as that used for the uterine fluid. Media were concentrated 12-fold and equal volumes (20ul) of ECC1 media were resolved on a 4-20% Mini Protean TGX Stain-free gel (Biorad).

Statistics

Data were analyzed using GraphPad Prism. Mann-Whitney U test was used to identify differences between fertility status, comparing fertile and infertile women, and treatment groups. The Mann-

Whitney U test was used to compare ECM1 concentration between fertile and infertile groups, immunostaining scores between individual groups, and ECM1 concentrations in media collected from primary endometrial and stromal cells. The Kruskal-Wallis and Dunn's post hoc tests were used to analyze changes in ECM1 expression in the media of estrogen treated ECC1s compared to control. In all cases a P-value of <0.05 was considered statistically significant.

Results

Proteomics

Analysis of proliferative phase uterine lavage fluid by mass spectrometry identified approximately 1500 proteins present in uterine fluid collected from fertile and primary infertile women. Four proteins were consistently downregulated $\geq$2-fold in proliferative phase uterine fluid from infertile women compared to fertile women (Table 1), while two proteins were upregulated in infertile women compared to fertile women (Table 1). Seven proteins were unique to fertile women while six proteins were unique to infertile women and absent in fertile women (Table 2).

Enrichment analysis

The web-based integrated data mining system, WebGestalt, was used to identify biological processes that were enriched for the proteins identified in the mass spectrometry analysis (Table 3). Proteins downregulated in infertile women were involved in processes including the negative regulation of molecular function, cell communication, signaling and peptidase activity. Processes of development, including gland morphogenesis, were also enriched for proteins downregulated in infertile women. There were no processes enriched for the proteins upregulated in infertile women and hence are not included in Table 3. Biological processes of cilium morphogenesis, protein localization, reproduction

and catabolic processes were enriched for proteins unique to proliferative phase uterine fluid from fertile women. Proteins unique to infertile women are involved in metabolic and cellular processes.

Immunohistochemistry

The localization of four proteins identified in the proteomic analysis were examined in proliferative phase endometrial tissue collected from fertile and infertile women (Figure 1 and 2). CD44 was localized predominantly to the stroma and immune cells of tissue from fertile and infertile women (Figure 1A,B). There was no change in immunostaining score of CD44 in the stroma between fertile and infertile women (P=0.9841), and a range of staining scores was observed (Figure 1C). There was minimal CD44 staining observed in the GE of tissue from fertile and infertile women (Figure 1A, B), with no significance difference (P=0.6109) seen in staining intensity between fertile and infertile women (Figure 1C). Half of the samples from fertile women showed GE staining for CD44, while 3/10 samples from infertile women showed CD44 staining in the GE.

SFRP4 was localized to the stroma and GE of proliferative phase endometrial tissue from both fertile and infertile women (Figure 1D,E), but with a wide range of immunostaining scores (Figure 1F). The median SFRP4 immunostaining score was decreased in the GE of tissue from infertile women, although this was not statistically significant (P=0.3931). There was no significant difference in the staining intensity of SFRP4 in the stromal compartment between fertile and infertile women (P=0.9137).

ASRGL1 was localized to both the GE and stroma of proliferative phase uterine tissue from both fertile and infertile women (Figure 1G,H). Again, there were a range of difference ASRGL1 immunostaining scores (Figure 1I) but no significant difference between fertile and infertile women in the GE (P=0.7451) and stromal (P=0.3239) compartments.

To examine further the observed localization of CD44 in immune cells (Figure 1A,B), CD44 (Figure 1J) and the leukocyte marker CD45 (Figure 1K) were examined in serial sections of endometrial tissue. Co-localization of CD44 and CD45 is indicative of the presence of CD44 in some leukocytes (Figure 1J,K), in addition to stromal cells.

ECM1 showed a range of different staining patterns (Figure 2 and Supplemental Figure S1) and was mostly localized to the stroma and GE (Figure 2A) of proliferative phase tissue from both fertile and infertile women. ECM1 showed intense localization to the basal lateral membranes of GE cells (Figure 2B). Of the 19 tissues examined, two showed intensely localized large aggregations of ECM1 within the stroma as seen in Figure 2C and 2D. Most tissues, both fertile and infertile, had diffuse stromal ECM1 staining (Figure 2A) or small intense spots of ECM1 throughout the stroma (Figure 2E). ECM1 appeared to accumulate in a layer beneath the luminal epithelium (Figure 2F). These staining patterns did not distinguish infertile from fertile.

ECM1 validation in uterine fluid

To validate the presence of ECM1 in proliferative phase uterine fluid, an ELISA was performed. An independent sample set to that used in the mass spectrometry analysis, in addition to four randomly selected samples from fertile and infertile women used in the mass spectrometry were used in this validation. There was a trend showing a decrease in ECM1 concentration in proliferative phase uterine fluid from infertile women compared to fertile women ($P=0.0790$) (Figure 3A). Although not significant, this was the same pattern as observed in the mass spectrometry analysis.

ECM1 was also detected in proliferative phase uterine lavage fluid by Western immunoblotting (Figure 3B). Analysis of six proliferative phase lavage fluids from fertile (n=3) and infertile (n=3) women showed that there are two main forms of ECM1 in uterine lavage fluid with mass of approximately 75kDa and 60kDa.

Secretion of ECM1 by primary endometrial epithelial and stromal cells

To identify the cellular source of secreted ECM1 in uterine fluid, an ELISA was used to measure the concentration of ECM1 in primary endometrial epithelial (fertile, n=3; infertile, n=2) and stromal (fertile=5; infertile= 3) cell conditioned media (Figure 4). These samples were collected between days 3 to 16 of the menstrual cycle due to the limited availability of appropriate patient samples. As such, of the ten endometrial biopsies collected, five were of proliferative phase, three were of unknown phase and two did not fall within the proliferative phase. Both epithelial and stromal cell conditioned media contained ECM1 indicating secretion. There was no significant difference in ECM1 concentration in epithelial and stromal cell conditioned media between samples collected from fertile and infertile women: however, this could be due to the low sample size.

The effect of estrogen treatment on ECM1 secretion from ECC1s

ECC1s were treated with three doses of estrogen to determine any effect of estrogen on ECM1 secretion. The media from these cells were collected and ECM1 content measured by ELISA. There were no significant differences in ECM1 concentration in the media of control ECC1s and estrogen treated ECC1s (Figure 5A). This suggests that estrogen does not influence the secretion of ECM1 from ECC1s. Two main forms of ECM1 were detected in ECC1 media, measuring approximately 79kDa (Band 1) and 46kDa (Band 2) (Figure 5B). Minor differences in molecular weight from those in uterine fluid (Figure 3) are likely due to differences in posttranslational modifications.

Discussion

This study shows for the first time that the proteome of the proliferative phase uterine microenvironment is altered in idiopathic infertile women compared to fertile women. Analysis of the proteomic profile of proliferative phase uterine lavage revealed that a number of proteins were either up- or downregulated in infertile women compared to fertile women, or were unique to either fertile or infertile. Such dysregulation in proteins in uterine lavage of infertile women during the proliferative phase of the menstrual cycle, suggests a disturbance in the regeneration of the endometrium, reflected in its secretory products during this time.

The proliferative phase of a woman's menstrual cycle is vital in the cyclical development of the endometrium and lays the foundation for the endometrium to subsequently differentiate and become receptive to embryo implantation during the receptive, secretory phase. Endometrial glands develop during the proliferative phase, and along with their secretions, are essential for embryo implantation and development. Animal studies of uterine gland knockout mice and sheep have shown that the absence of uterine glands and their secretions in these animals leads to infertility and the inability to support a conceptus [2-4]. Endometrial glands develop during each proliferative phase in humans highlighting the importance of endometrial development and the uterine microenvironment in determining a woman's fertility. We have previously shown through histological analysis of the GE area of proliferative phase endometrial tissue that there is no change in GE area between fertile and infertile women [9]. There was, however, a significant alteration in the cytokine profile of proliferative phase uterine fluid in infertile women, whereby, C-C motif chemokine 11 (CCL11), transforming growth factor alpha (TGFα), interleukin-1 alpha (IL-1α) and interferon gamma (IFNγ) were increased in infertile women of <35 years compared to age-matched fertile women. There were no changes between fertile and infertile women in the older age group. This was the first comprehensive study of the proliferative phase uterine microenvironment indicating that these cytokines were contributing to a disturbance in endometrial regeneration in this younger cohort. Here

we show that in addition to cytokines, other totally unrelated proteins are also altered in infertile women.

A number of proteins, including CD44, SFRP4 and ECM1 were significantly downregulated in the proliferative phase uterine lavage of infertile women compared to fertile women. Gene ontology analysis highlighted their roles in development and gland morphogenesis, which is particularly relevant to their potential roles in the development of the endometrium and uterine environment. These three proteins, along with ASRGL1 were all localized within proliferative phase endometrial tissue from fertile and infertile women.

CD44 is a glycosylated cell-surface protein involved in processes such as cell migration, lymphocyte homing, T cell activation and cell-cell interactions. CD44 is also a receptor for hyaluronan. We showed CD44 as present within the stroma of the proliferative phase endometrium of fertile and infertile women with low expression also in the endometrial glands. Interestingly, CD44 positive leukocytes are also present as shown by its co-localization with the leukocyte marker, CD45. This suggests a role for CD44 in leukocyte recruitment in the proliferative phase endometrium. Previous conflicting studies have shown low or no expression of CD44 in the stroma of the proliferative phase endometrium [22, 23], while others support our results of stromal CD44 expression during the proliferative phase [24]. These conflicts could be due to antibodies used or fixation techniques. Similar to our study, strong staining for infiltrating lymphocytes during the proliferative phase has been shown, while there is increased staining in the glandular epithelium during the secretory phase compared to the proliferative phase [23]. The role of CD44 in endometriosis has also been investigated because of its role as an adhesion molecule and potential involvement in disease progression [25]. CD44 has a number of isoforms with CD44 standard, variant 5 and variant 6 measured in the supernatant of endometrial cultures from women with and without endometriosis.

Furthermore, soluble CD44 is present in follicular fluid, indicating its possible secretion into the cavities of the Fallopian tubes and thereby into the uterine cavity [26].

SFRP4 was significantly downregulated in infertile women compared to fertile women in proliferative phase uterine lavage, and was localized in proliferative phase endometrial tissue from both fertile and infertile women. SFRP4 is a secreted glycoprotein with roles in biological processes including angiogenesis, embryonic development and apoptosis [27-29]. The SFRP family are extracellular modulators of Wnt signaling that compete with frizzled (Fzd) for the binding of Wnts, thus preventing the Wnt-Fzd interaction and antagonizing Wnt action. Normally, the binding of the Fzd to the Wnt ligand activates different signaling pathways [30]. SFRP4 can inhibit Wnt7a induced pathways and also endometrial cancer growth [31]. Within a cohort of endometrial cancer specimens, decreased SFRP4 mRNA expression was associated with higher tumor grade [32]. Furthermore, Wnt7a and Wnt5a are particularly important for endometrial gland development [33-35]. A loss or downregulation of SFRP4, as reflected in proliferative phase uterine fluid of infertile women, could lead to excessive Wnt7a signaling, resulting in aberrant endometrial growth and development.

ASRGL1 was unique to the proliferative phase uterine fluid of infertile women but localized to the stroma and GE of proliferative phase endometrium of fertile and infertile women. ASRGL1 is an enzyme involved in the production of L-aspartate [36, 37]; however, little is known regarding its role in the endometrium or uterine microenvironment. Most studies have revolved around the use of ASRGL1 as a biomarker of endometrial cancer. In normal endometrium ASRGL1 immunolocalized to the glands but was not found in the stroma, while a loss of ASRGL1 in endometrial tumor cells was associated with increasing survival in endometrial cancer [38]. ASRGL1 mRNA is also higher in uterine, mammary and ovarian cancers compared to normal tissues [39]. In our study, secreted ASRGL1 was unique to the infertile group, suggesting that increased ASRGL1 expression may render the uterine environment hostile and disrupt endometrial development.

The significant downregulation of ECM1 in the proteomic evaluation of proliferative phase uterine fluid of infertile women compared to fertile women was validated by ELISA and Western immunoblotting. ECM1, originally identified in the conditioned medium of the murine osteogenic stromal cell line MN7 [40], is an 85kDa secreted glycoprotein. Most studies of ECM1 have focused on two skin conditions. Loss of function mutations in the ECM1 gene lead to the rare autosomal recessive disorder, lipoid proteinosis typified by alterations in the basement membrane and reduplication of the basement membrane of the skin [41, 42]. Circulating IgG autoantibodies to ECM1 result in the humoral autoimmune condition, lichen sclerosus. Interestingly, ECM1 has two central tandem repeats, thought to allow the ECM1 protein to act as a biotransporter and be involved in the binding and interaction of various regulatory factors [43]. Its binding partners include perlecan [44-46] and MMP9, for which binding to ECM1 negatively regulates enzymatic activity [47]. Other binding partners include fibulin 1C and fibulin 1D [48], fibulin 3 [49], fibronectin, type IV collagen and laminin 5 [49, 50] and cartilage oligomeric matrix protein [51]. In de-epithelialized human skin ECM1 colocalized with collagen IV, laminin 332 and perlecan, indicating a role in the suprastructural networks of the basement membrane and extracellular matrix [50]. In this study, some areas of colocalization were seen as small defined spots and in our examination of the human endometrium, similar spots were evident. As in the skin [50], ECM1 may act as a bridging molecule in the endometrium, maintaining the integrity of the basement membrane network. Any disruption in this anchoring or binding of ECM1 may influence the regeneration of the endometrium following menses. The binding of ECM1 to multiple proteins suggests that its role in endometrial development could be multifunctional.

ECM1 exists in two forms in proliferative phase uterine fluid (~75kDa and ~60kDa). The four known splice forms of ECM1 potentially give rise to secreted proteins with calculated masses of ~58kDa (isoform 1a), ~44kDa (isoform 2 or 1b), ~19kDa (isoform 3) and ~61kDa (isoform 4) together with a

number of known natural variants. Some mutations are associated with suppressed ECM1 secretion in conditions such as lipoid proteinosis [41, 42]. ECM1 proteins contain three potential N-glycosylation sites increasing the weight of the secreted protein. Unusually, N-glycosylation of ECM1 can negatively regulate its secretion [41]. ECM1 proteins of 61kDa, 68kDa and as high as 84kDa [41, 46, 52] are present in conditioned medium from 293-EBNA cells (human embryonic kidney cells expressing the Epstein- Barr virus nuclear antigen), normal breast tissue and breast cancer tissue lysate, and conditioned medium from human fibrosarcoma HT1080 cells, respectively. The >75kDa form in uterine fluid may represent altered glycosylation or complexing with another protein or binding partner, for example, MMP9 or perlecan [44-47].

ECM1 is secreted from both primary endometrial epithelial and stromal cells. Future investigations with a greater cohort of epithelial samples may establish the cellular origin of the disturbed secretion. ChIP-qPCR demonstrated ECM1 as a unique target for estrogen receptor alpha in the non-receptive HEC1A endometrial epithelial cell line following estrogen treatment [53] whereas it was a unique target for progesterone receptor A in the RL95-2 cells, a receptive endometrial cell line model following progesterone treatment. In the present study, estrogen, the dominant proliferative phase hormone, did not alter secretion of ECM1 from the endometrial epithelial cell line, ECC1. Further investigation into the relationship between ECM1, estrogen and progesterone stimulation is needed.

In our previous studies, CXCL1 increased trophoblast ECM1 mRNA expression by 6 fold and was immunolocalized to human first trimester implantation sites [54], suggesting a role for ECM1 in implantation and vascularization. Its expression in blood vessels during embryogenesis, in the heart and placenta of adults [55] and its overexpression in cancers and relationship to a metastatic phenotype and altered blood vessel formation [52], further supports a role for ECM1 in the remodeling and development of the endometrium. Therefore downregulated ECM1 in the proliferative phase uterine fluid of infertile women could lead to a disturbance in the proliferation and

migration of endometrial cells, development of blood vessels, and regulation of immune cells, all of which are essential for implantation of the embryo and a successful pregnancy.

In conclusion, this study identified a number of proteins, including CD44, SFRP4, ASRGL1 and ECM1 that are significantly altered in proliferative phase uterine lavage fluid from idiopathic infertile women compared to fertile women. Since these four proteins were localized to both the fertile and infertile proliferative phase endometrium, their dysregulation noted in uterine lavage fluid may indicate functional actions on the endometrium. Further validation of ECM1, confirmed its downregulation in proliferative phase uterine fluid of infertile women and for the first time, investigated the different forms of secreted ECM1 in uterine lavage fluid and media from endometrial epithelial cells. This study has emphasized the potential importance of the proliferative phase of the endometrium in determining its subsequent differentiation to acquire receptivity, and hence female fertility.

Authors' roles

HCF planned and performed experimental work and prepared manuscript. JE provided intellectual input. NJ performed ECC1 estrogen treatments. GI and AW performed mass spectrometry analysis. LJRR and BJV recruited patients and collected samples. LAS and TAE provided intellectual input throughout the experimentation and analyses, and editing of the manuscript. All authors edited and approved the manuscript.

Acknowledgements

We particularly thank the patients who provided consent for use of their endometrial tissue and uterine lavage fluid, and Sister Judi Hocking for its collection. The authors acknowledge the facilities,

and scientific and technical assistance of the Monash Histology Platform, Monash University and the Systems Biology and Personalised Medicine Division at the Walter and Eliza Hall Institute.

Conflict of Interest

The authors declare no conflict of interest.

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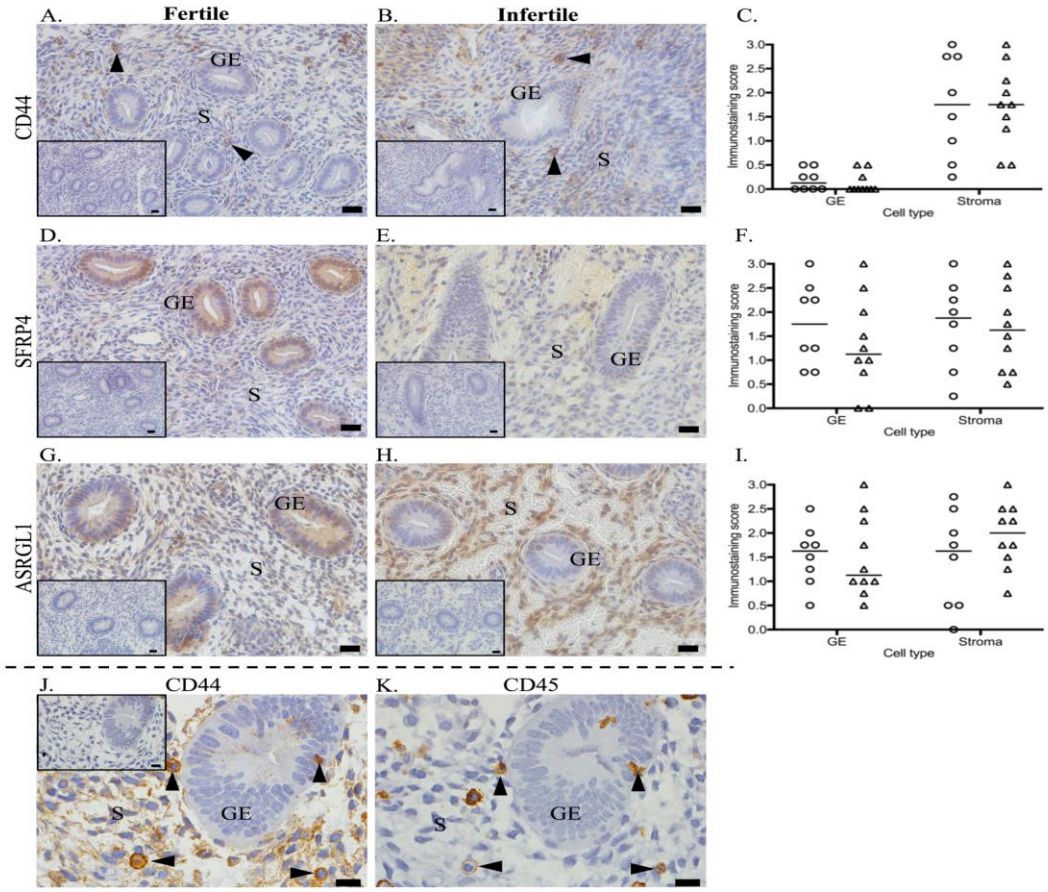

Figure 1. Immunolocalization and staining intensity scores of proteins identified in the proteomic analysis of proliferative phase uterine fluid in endometrial tissue. Immunolocalization of CD44 in proliferative phase endometrial tissue from fertile (A) and infertile (B) women, and associated immunostaining scores (C). Immunolocalization of SFRP4 in proliferative phase endometrial tissue from fertile (D) and infertile (E) women, and associated immunostaining scores (F). Immunolocalization of ASRGL1 in proliferative phase endometrial tissue from fertile (G) and

infertile (H) women, and associated immunostaining scores (I). CD44 (J) and CD45 (K) were immunolocalized on serial sections to examine the expression of CD44 by leukocytes (arrows). GE = Glandular epithelium; S= Stroma; arrows represent colocalization of CD44 and CD45. Scale bars (A,B,D,E,G.H): 20μm. Scale bars (J,K): 10μm. Negative isotype controls (inset, A,B,D,E,G,H,J). Line represents median (C,F,I). Open circles = fertile; open triangles = infertile.

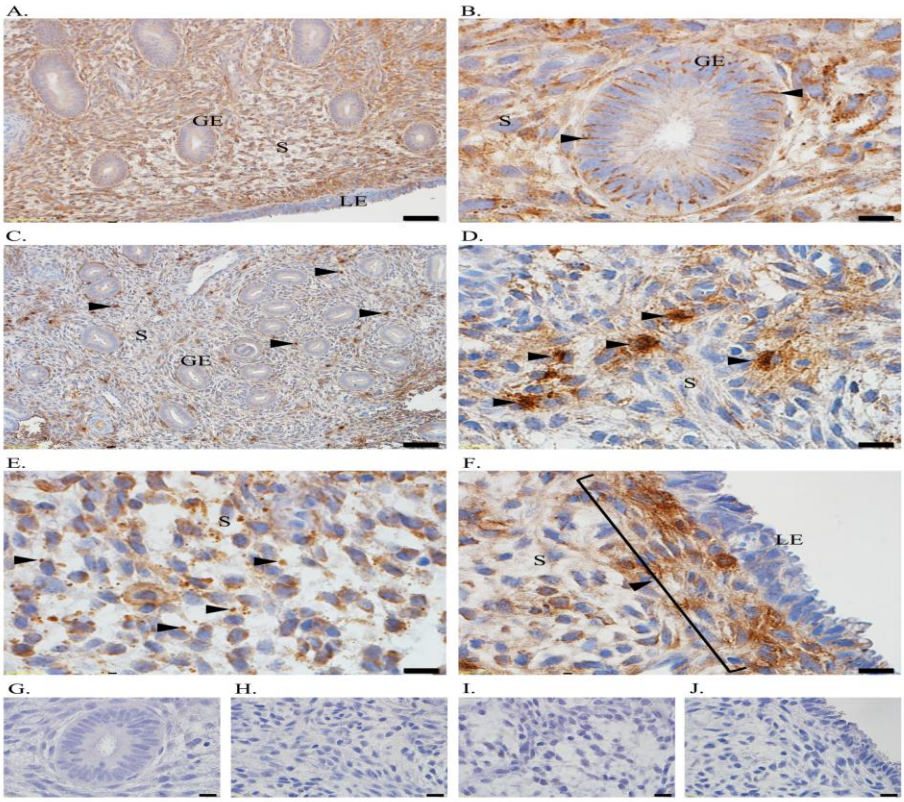

Figure 2. Images depicting range of ECM1 immunolocalization staining patterns in proliferative phase endometrial tissue. ECM1 localization in both the stroma and glands (A). ECM1 localization in lateral membranes of glandular epithelial cells shown by arrows (B). Aggregation of ECM1 protein shown by arrows (C,D). Intense small aggregations of ECM1 in stroma (E). Accumulation of ECM1 directly beneath luminal epithelium shown by arrow (F). Four tissues are shown A and B (negative isotype control is G); C and D (negative isotype control is H); E (negative isotype control is I); F

(negative isotype control is J). Scale bars (A, C): 50µm. Scale bars (B, D, E, F, G, H, I, J): 10µm. GE = Glandular epithelium; S= Stroma; LE=Luminal epithelium.

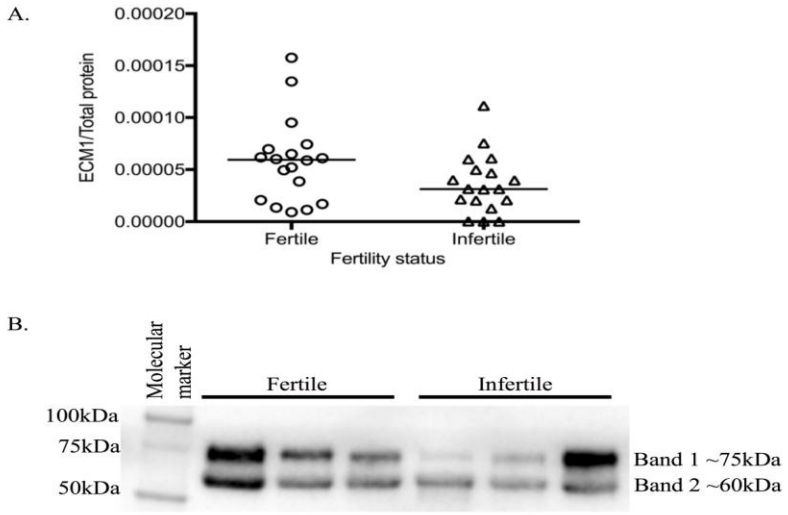

Figure 3. Validation of ECM1 in proliferative phase uterine fluid. The concentration of ECM1 in proliferative phase uterine fluid collected from fertile (n=18) and infertile (n=18) women. Line represents median (A). Western blot showing ECM1 in proliferative phase uterine lavage fluid collected from fertile (n=3) and infertile (n=3) women (B).

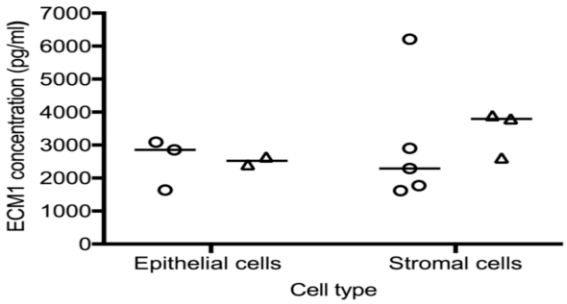

Figure 4. ECM1 concentration in media collected from primary endometrial epithelial cells isolated from fertile (open circles) and infertile (open triangles) women, and primary endometrial stromal cells isolated from fertile (open circles) and infertile (open triangles) women. Line represents median.

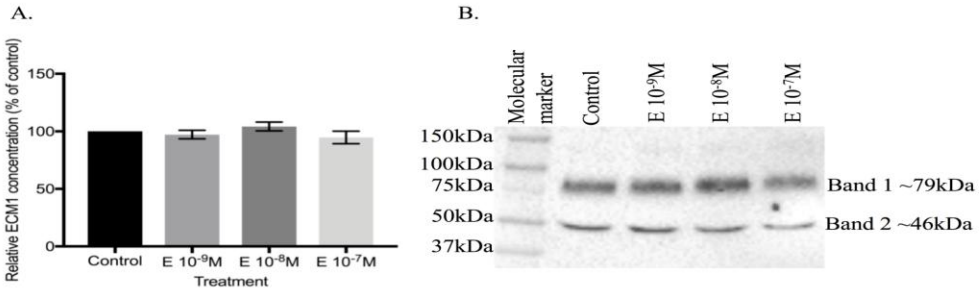

Figure 5. The effect of estrogen (E) dose response treatments on the secretion of ECM1 from endometrial epithelial cells. The concentration of ECM1 in media from estrogen treated ECC1s. Data are normalized to control and are mean ± SEM (A). Western immunoblot analysis of ECM1 in media from estrogen treated ECC1 cells (B).

Table 1. Proteins downregulated and upregulated ≥2-fold in proliferative phase uterine fluid collected from infertile women (n=10) compared to fertile women (n=9). P<0.05 was considered statistically significant.

Protein	**Gene name**	**Protein ratio infertile/fertile**	**Protein P value infertile/fertile**
Downregulated			
Extracellular matrix protein 1	ECM1	0.486	0.0000671
Transforming growth factor-beta-induced protein ig-h3	TGFBI	0.393	0.042
Secreted frizzled-related protein 4	SFRP4	0.492	0.0255
CD44 antigen	CD44	0.474	0.0293
Upregulated			
Protein-glutamine gamma – glutamyltransferase 2	TGM2	2.109	0.0085
Ig gamma-4 chain C region	IGHG4	2.548	0.0000599

Table 2. Proteins unique to proliferative phase uterine fluid collected from fertile (n=9) or infertile women (n=10).

Fertile		**Infertile**	
Protein	**Gene name**	**Protein**	**Gene name**
Filamin-A	FLNA	Neuroblast differentiation-associated protein	AHNAK
Pregnancy zone protein	PZP	Isoaspartyl peptidase/L-asparaginase	ASRGL1
Oviduct-specific glycoprotein	OVGP1	UMP-CMP kinase	CMPK1
Endoplasmin	HSP90B1	Phosphoglucomutase-1	PGM1
Annexin A6	ANXA6	Cornulin	CRNN
40S ribosomal protein S3	RPS3	Suprabasin	SBSN

Septin-2	SEPT2		

Table 3. Biological processes in which proteins found in proliferative phase uterine fluid were enriched.

Grouping	**Biological Process**	**Number of proteins**	**Enrichment (raw P value)**	**Enrichment (adjusted P value)**
Downregulated in infertile	Negative regulation of molecular function	3	0.0004	0.005
	Gland morphogenesis	2	0.0003	0.005
	Negative regulation of cell communication	3	0.0004	0.005
	Circulatory system development	3	0.0005	0.005
	Anatomical structure formation involved in morphogenesis	4	0.0001	0.005
	Cardiovascular system development	3	0.0005	0.005
	Negative regulation of peptidase activity	2	0.0005	0.005
	Negative regulation of signaling	3	0.0004	0.005
	Negative regulation of signal transduction	3	0.0003	0.005
	Tissue development	4	0.0001	0.005
Unique to fertile	Macromolecule catabolic process	4	0.0004	0.0483
	Cilium assembly	2	0.0006	0.0483
	Cilium morphogenesis	2	0.0009	0.0483
	Protein localization	4	0.0036	0.0495
	Negative regulation of molecular function	3	0.0029	0.0495
	Actin filament organization	2	0.0043	0.0495
	Multi-multicellular organism process	2	0.0029	0.0495

	Regulation of hydrolase activity	3	0.004	0.0495
	Maintenance of location	2	0.004	0.0495
	Multi-organism reproductive process	3	0.0037	0.0495
Unique to infertile	Carbohydrate derivative biosynthetic process	2	0.0136	0.2448
	Carbohydrate derivative metabolic process	2	0.0587	0.3522
	Small molecule metabolic process	3	0.0395	0.3522
	Catabolic process	2	0.1432	0.4296
	Organonitrogen compound metabolic process	2	0.1059	0.4296
	Organic substance catabolic process	2	0.1263	0.4296
	Cellular nitrogen compound metabolic process	2	0.6371	1
	Organic substance biosynthetic process	2	0.5808	1
	Cellular process	4	1	1
	Biosynthetic process	2	0.5902	1