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Modulating the endometrial epithelial proteome and secretome in preparation for pregnancy: the role of ovarian steroid and pregnancy hormones

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Abbreviations:

CI, cell index

CL, cell lysate

CM, culture media

DMEM, Dulbecco's Modified Eagle's Medium

E, estrogen

ECM, extracellular matrix

EP, estrogen plus progesterone

hCG, human chorionic gonadotropin

ITS, insulin-transferrin-seleniumMS/MS

NC, nitrocellulose

Nsc, significant normalized spectral count

pEEC, primary endometrial epithelial cells

Rsc, ratio (fold change) of normalized spectral counts

SS, soluble-secreted

SS/Ex, soluble secreted/extracellular vesicles

TF, transferrin

TTPBS, Tween-Tris Phosphate Buffered Saline

ABSTRACT

Dialogue between an appropriately developed embryo and hormonally-primed endometrium is essential to achieve implantation and establish pregnancy. Importantly, the point-of-first-contact between the embryo and the maternal-endometrium occurs at the endometrial luminal epithelium (LE). Implantation events occur within the uterine cavity microenvironment regulated by local factors. Defects in embryo-endometrial communication likely underlie unexplained infertility; enhanced knowledge of this communication, specifically at initial maternal-fetal contact may reveal targets to enhance fertility. Using a human endometrial luminal-epithelial (LE) cell line (ECC1), this targeted proteomic study reveals unique protein changes in both cellular (98% unique identifications) and secreted (96% unique identifications) proteins in the transition to the progesterone-dominated secretory (receptive) phase and subsequently to pregnancy, mediated by embryo-derived human chorionic gonadotropin (hCG). This analysis identified 157 progesterone-regulated cellular proteins, with further 193 significantly altered in response to hCG. Cellular changes were associated with metabolism, basement membrane and cell connectivity, proliferation and differentiation. Secretome analysis identified 1059 proteins; 123 significantly altered by progesterone, and 43 proteins altered by hCG, including proteins associated with cellular adhesion, extracellular-matrix organization, developmental growth, growth factor regulation, and cell signaling. Collectively, our findings reveal dynamic intracellular and secreted protein changes in the endometrium that may modulate successful establishment of pregnancy.

1 INTRODUCTION

The endometrium is a highly dynamic tissue that undergoes cyclical remodeling and differentiation each menstrual cycle throughout a women's reproductive life, under the influence of the ovarian steroid hormones. Estrogen dominates the proliferative phase, when the endometrium is regenerated after menses, while progesterone is essential for the cellular differentiation that defines the secretory phase. This differentiation promotes preparation of an endometrium receptive for embryo implantation (**Fig. 1**). Successful implantation and pregnancy are achieved only when the endometrium and the fertilized embryo develop synchronously [1, 2]. The endometrium is receptive to an embryo only during a brief 4-day period in the mid-secretory phase spanning day 20-24 of a normalized menstrual cycle (**Fig. 1**) when significant molecular and cellular remodeling of the endometrium, and embryo-endometrial interactions via secreted proteins within the uterine cavity, are optimal [1, 3, 4]. Understanding the changes associated with 'receptivity' and their regulation in the endometrial luminal cells, the first point of interaction between the mother and the embryo, is critical if we are to improve fertility in certain infertile women and/or develop new contraceptives that target the endometrium.

Numerous expression profiling studies of the uterine endometrium throughout the menstrual cycle, using discovery-driven methods, have revealed that proliferative and secretory phases can be distinguished at a global level by transcript, microRNA, and proteomic profiling [5-9]. However, the few proteomic studies have used now outdated technologies that detect predominantly high abundance proteins. Both cellular and secreted proteins are differentially regulated between the receptive and non-receptive phases of the menstrual cycle [9-19]. Previous studies identifying 'cellular' changes have analyzed tissue biopsy material which is

comprised of multiple cell types; teasing out specific alterations within the endometrial luminal epithelial cells (eLEs), that provide the first contact with the blastocyst, from these studies is therefore impossible. Secreted proteins have been identified in aspirate, lavage and primary endometrial epithelial cell cultures [13, 14]. Importantly, such secreted proteins contribute to the extracellular embryo-maternal interactions [20] that are important during the implantation process [1, 18, 21, 22]. Human chorionic gonadotrophin (hCG), one of the earliest proteins secreted by the pre-implantation embryo [23-26] (**Fig. 1**) is known as a critical signaling hormone for establishment and maintenance of pregnancy [24, 27, 28]. While effects of hCG on secretion of a specific subset of cytokine and growth factors from endometrial epithelial cells have been determined [29-32], the global eLE protein changes in response to hCG have not been examined.

In this study, using the human endometrial ECC1 cell line which is best representative of eLE, we have specifically analyzed both the cellular and secreted proteins in response to estrogen, (characteristic of the proliferative phase) combined estrogen and progesterone (representing the secretory phase) and these hormones together with hCG mimicking the presence of an embryo. This targeted approach has identified numerous proteins, many previously unknown, associated with endometrial remodeling and particularly those associated with the receptive state. This study provides new understanding of the protein changes induced by both maternal and embryonic hormones, essential for successful implantation and establishment of pregnancy.

2 MATERIALS AND METHODS

2.1 Endometrial epithelial cell line and primary cell isolation and culture

Primary endometrial epithelial cells are difficult to obtain in sufficient quantity for extensive study. Furthermore, most epithelial cells in primary tissues cultures are derived from endometrial glandular epithelium. Since the first point of contact of the embryo is with the endometrial luminal epithelium, the human ECC1 cell line was used as a model for this study. This is an endometrial adenocarcinoma epithelial cell line [33] that closely resembles the luminal epithelium. These ECC1 cells were validated by Karyotype analysis [33, 34] according to the ATCC guidelines [35], with allele match in STR profile of 100%. They were cultured and maintained in a 1:1 mix of DMEM and Hams F-12 medium (DMEM/F-12) (Invitrogen-GIBCO, Carlsbad, USA) supplemented with 10% FCS (Invitrogen-GIBCO), 1% (v/v) Penicillin Streptomycin (Pen/Strep) (Invitrogen-GIBCO), and incubated at 37°C with 5% CO_2 [36]. Validation was performed also with primary human endometrial epithelial cultures.

Ethical approval was obtained for all human sample collections from Human Ethics Committees at Southern Health (#03066B) and Monash Surgical Private Hospital (#04056) and written informed consent was obtained from all women. Endometrial tissue was obtained by dilatation and curettage from women with no known endometrial abnormalities, undergoing minor gynaecological surgical procedures, such as laparoscopic sterilization or investigation of tubal patency or first trimester termination of pregnancy [29]. All women had regular menstrual cycles and no contraceptive or steroid treatment for at least 3 months prior to surgery (mean age 34.6, mean BMI 28.5). Primary endometrial epithelial cells (pEECs)

were prepared from endometrial curettage as previously described [29]. Briefly, endometrial tissues were finely minced and digested in enzymatic solution containing DNAse (25 µg/mL, Roche, Basel, Switzerland) and collagenase type III (150 µg/mL, Sigma Aldrich, USA) and phosphate buffered saline (PBS, Invitrogen). Tissue dissociation was performed in a shaking water bath (130 rpm, 37 °C) for 40 min, stopped by additional DMEM/F12 and vacuum-filtered through 45 µm and 11 µm filters. pEECs were retrieved from the filters, centrifuged at 300×*g* for 5 min, resuspended in DMEM/F12 supplemented with 10% charcoal-stripped (cs) FCS and 1% Pen/Strep and plated into 24-well plates. Cells were grown to at least 80% confluence before hormonal treatments. Cultures in which endometrial stromal cells could be visually identified were discarded. Such epithelial cells are fully viable over the time frame of the experiment as previously described [29, 37] .

2.2 Hormonal treatment of endometrial epithelial cells (ECC1 and pEECs)

ECC1 cells and pEECs as required, were treated with estrogen in the absence/presence of progesterone to mimic the proliferative and secretory phases of the menstrual cycle respectively [22]. hCG was also added to 'secretory-phase' cultures to mimic the embryonic signal at the time of conception. ECC1 cells (1×10^6 per 150 mm^3 culture dish, total of 15 dishes/treatment, n = 3 separate experiments) were washed three times with PBS and cultured for 24 hrs in serum-free media (DMEM/F12 supplemented with 0.5% insulin-transferrin-selenium (ITS) solution (Invitrogen-GIBCO), and 1% (v/v) Pen/Strep). pEECs (n = 6 separate preparations from individual women, 80% confluence), were similarly washed and cultured for 24 hr in DMEM/F12 supplemented with 0.5% charcoal-stripped (cs) FCS. After 24 hr, media were replenished with the indicated media and all cells primed with estrogen (10^{-8} M, Sigma-Aldrich) for 24 hr. Cells were then divided into three groups and treated with

(i) E: estradiol-17-beta/estrogen (10^{-8} M), (ii) EP: E (10^{-8} M) and progesterone (P, medroxyprogesterone-17-acetate, 10^{-7} M, Sigma-Aldrich), or (iii) EP and hCG (10 IU/ml) [29, 38]. Conditioned media (CM) were harvested after 24 hr of hormonal treatment and treated as detailed below. Cells were also harvested at the 24 hr hormonal treatment time point.

2.3 ECC1 cell viability and adhesive capacity

To confirm ECC1 cell viability is not affected by hormonal treatments (E, EP or hCG) within the short time frame of this study, it was assessed and measured using the trypan blue assay and Cell Countess automated counter (Life Technologies) according to manufacturer's instructions. Viability (n = 3 biological replicates) was expressed as mean percentage ± SEM of viable cells. Cell adhesive capacity was determined using XCelligence™ real-time cell analysis [22] and expressed as relative cell index (mean ± SEM) over 24 hr (n = 3 biological replicates).

2.4 Cell lysate preparation

Following hormonal treatments (24 hr), ECC1 cells were washed with ice-cold PBS and lysed on ice (15 mins) with SDS sample buffer (4% (w/v) SDS, 20% (v/v) glycerol, 0.01% (v/v) bromophenol blue, 0.125 M Tris-HCl, pH 6.8), while pEEC cells were lysed on ice (15 mins) in RIPA lysis buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0), prior to the addition of equal volume of 2x SDS sample buffer. Cell lysates were then scraped off culture plates and transferred to sterile microfuge tubes. Both cell lysates were subjected to ultracentrifugation at 386,000*g* for 30

min at 4°C (TLA-100 rotor, Beckman Coulter), and soluble supernatants aspirated for downstream use, or frozen at -80°C.

2.5 Soluble-secretome isolation

Soluble secretome purification was performed as previously described with slight modification [36, 39, 40] (**Fig. 2**). ECC1 cell and/or pEEC CM were centrifuged at 500*g* for 5 min, 2000*g* for 10 min and 10,000*g* for 30 min to remove floating cells, cell debris and shed microvesicles (sMVs) respectively. Subsequently, CM was ultracentrifuged at 100,000*g* for 1 hr to remove exosomes [40, 41]. Soluble-secreted fractions (SS) were prepared by filtration of the supernatant through a 0.1 μm syringe filter membrane (Pall, Cornwall, UK) and concentrated to ~1 mL by centrifugal ultrafiltration (3K NMWL Ultra-15, Merck-Millipore) at 3000*g* [36, 42]. Seppro™ transferrin IgY microbeads (Genway Biotech, San Diego, CA) were used to deplete the abundant transferrin (TF) additive (from ITS) in SS fractions. SS was snap frozen and stored at -80°C.

2.6 Protein quantification and validation

All lysates and SS fractions were quantified using 1D-SDS-PAGE / SYPRO® Ruby protein staining densitometry, as previously described [36, 40]. For immunoblotting (15 μg of protein), membranes were probed with primary antibodies [rabbit anti-YBX3 (Origene; 1:1500), goat anti-SOD1 (C-17) (Santa Cruz Biotechnology; 1:1000), rabbit anti-LIMS1/Pinch-1/2 (H-300) (Santa Cruz Biotechnology; 1:1000), rabbit polyclonal anti-Met (Santa Cruz Biotechnology; 1:500), mouse anti-PTBP3/ROD1 (F-30) (Santa Cruz Biotechnology; 1:1000), rabbit polyclonal anti-LGALS1 (H-45) (Santa Cruz Biotechnology;

1:1000), rabbit polyclonal anti-MAT2B (Abcam; 1:1500)] for 1 hr at room temperature (RT) in 0.05% Tween-Tris PBS (TTBS: 50 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween 20, PBS) followed by incubation with either IRDye 800CW goat anti-mouse, IRDye 800CW donkey anti-goat, or IRDye 680RD goat anti-rabbit IgG (1:15000, LI-COR Biosciences) for 1 hr at RT in TTBS. Immunoblots were visualized using the Odyssey Infrared Imaging System (v3.0, LI-COR Biosciences, Nebraska USA).

2.7 Proteomic analyses

Proteomic experiments were performed on triplicate biological replicates, with duplicate technical replicates for each analysis.

Cell lysates: Lysate proteins (10 μg) were separated by short-range (8 min) SDS-PAGE (4-12% Bis-Tris SDS-PAGE) and visualized by Imperial Protein Stain (Thermo Fisher Scientific). A total of two individual gel bands (~5-6 mm each) were excised representing the entire gel, destained (50 mM ammonium bicarbonate/acetonitrile), reduced (10 mM DTT (Calbiochem) for 30 min), alkylated (50 mM iodoacetic acid (Fluka) for 30 min) and trypsinized (0.3 μg trypsin (Promega Sequencing Grade) for 16 h at 37°C), as described [43].

Secretome: SS samples (10 μg) were treated with ProteaseMAX™ Surfactant (0.15% w/v) (Promega) and 8 M urea. Proteins were reduced and alkylated as above, and in-solution digestion performed (0.3 μg trypsin), as described [44].

For all samples, peptides were desalted using reverse-phase C18 StageTips [45], and eluted in 85% (v/v) acetonitrile (ACN) in 0.5% (v/v) formic acid (FA). Peptides were lyophilized in a

SpeedVac and acidified with buffer containing 0.1% FA, 2% ACN. A nanoflow UPLC instrument (Ultimate 3000 RSLCnano, Thermo Fisher Scientific) was coupled on-line to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) with a nano-electrospray ion source (Thermo Fisher Scientific). Peptides (~2 μg) were loaded (Acclaim PepMap100 C18 5 μm 100Å, Thermo Fisher Scientific) and separated (Vydac MS C18-RP column, 25 cm, 75 μm inner diameter, 3 μm 300Å, Grace, Hesperia, CA) with a 120- min linear gradient from 0-100% (v/v) phase B (0.1% (v/v) FA in 80% (v/v) ACN) at a flow rate of 250 nL/min.

The mass spectrometer was operated in data-dependent mode where the top 20 most abundant precursor ions in the survey scan (300–2500 Th) were selected for MS/MS fragmentation. Survey scans were acquired at a resolution of 120,000 at m/z 400. Unassigned precursor ion charge states and singly charged species were rejected and peptide match disabled. The isolation window was set to 3 Th and fragmented by CID with normalized collision energies of 25. Maximum ion injection times for the survey scan and MS/MS scans were 20 ms and 60 ms, respectively, and ion target values were set to 3E6 and 1E6, respectively. Selected sequenced ions were dynamically excluded for 30 s. Raw mass spectrometry data is deposited in PeptideAtlas and can be accessed at http://www.peptideatlas.org/PASS/PASS00786.

2.8 Database searching and protein identification

Raw data was processed using Proteome Discoverer (v1.4.0.288, Thermo Fisher Scientific). MS2 spectra were searched with Mascot (Matrix Science, London, UK; v 1.4.0.288), Sequest (Thermo Fisher Scientific, San Jose, CA, v 1.4.0.288), and X!Tandem (v 2010.12.01.1) using a Human-Bovine sequence database (UniProt #178,618 entries) (July-2015). Data was

searched with a parent tolerance of 10 ppm, fragment tolerance of 0.6 Da, max delta CN 0.05 and minimum peptide length 6. Peptide spectral matches (PSM) were validated using Percolator based on q-values at a 1% false discovery rate (FDR) [46, 47]. With Proteome Discoverer, peptide identifications were grouped into proteins according to the law of parsimony and filtered to 1% FDR [48]. Scaffold (Proteome Software Inc., Portland, OR, v 4.3.4) was employed to validate MS/MS-based peptide and protein identifications from database searching. Initial peptide identifications were accepted if they could be established at greater than 95% probability (PEP 5%) as specified by the Peptide Prophet algorithm [49]. Protein probabilities were assigned by the Protein Prophet algorithm [48]. Protein identifications were accepted, if they reached greater than 99% probability and contained at least 2 identified unique peptides. These identification criteria typically established <1% false discovery rate based on a decoy database search strategy at the protein level. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone, were grouped to satisfy the principles of parsimony. Contaminants, bovine identifications, and reverse identification were excluded from further data analysis. UniProt was used for protein annotation (molecular function, enzyme category), and KEGG (http://www.genome.jp/kegg/pathway.html) and DAVID (http://david.abcc.ncifcrf.gov/) for pathway enrichment analyses.

2.9 Semi-quantitative label-free spectral counting

Significant spectral count normalized (Nsc) and fold change ratios (Rsc) were determined as previously described [36, 39-41]. The relative abundance of a protein within a sample was estimated using Nsc, where for each individual protein, significant peptide MS/MS spectra (i.e., ion score greater than identity score) were summated, and normalized by the total

number of significant MS/MS spectra identified in the sample. To compare relative protein abundance between samples the ratio of normalized spectral counts (Rsc, fold change) was estimated. Total number of spectra was only counted for significant peptides identified (Ion score ≥ Homology score). When Rsc is less than 1, the negative inverse value was used. The number of significant assigned spectra for each protein was used to determine protein expression differences. For each protein the Fisher's exact test was applied to significant assigned spectra. The resulting p-values (p<0.01 considered statistically significant) were corrected for multiple testing using the Benjamini-Hochberg procedure [50] and statistics performed as previously described [41].

2.10 Statistical analysis

All analyses were performed using GraphPad Prism (v6.05) software. Analysis of variance (ANOVA) and Tukey's post-hoc testing was applied to all experimental conditions between treatment groups and mock control. Student's t-test was used when two groups were compared. Unless otherwise stated, data are presented as mean ± SEM (n = 3 technical replicates), with statistics applied across the 3/6 biological replicates, with *p<0.05 and **p<0.01 considered statistically significant.

2.11 Immunohistochemistry

5 µm endometrial sections (5 proliferative, 5 secretory and 5 first trimester) were placed onto superfrost slides, dewaxed in Histosol (Sigma Chemical Co; St Louis, MO) and rehydrated through descending grades of alcohol (95–70%) to distilled water (dH_2O). Antigen retrieval was performed by microwave heating sections in 0.01M pH6 citrate buffer for 5 mins. Endogenous peroxidise activity was blocked by incubation in 3% hydrogen peroxide for 5

mins at room temperature. Non-specific binding was blocked by incubation of tissue sections in non-immune serum (10% horse serum, 2% human serum, Tris buffered saline [TBS]). Sections were subsequently incubated overnight at 4^{o}C with anti-PTBP3 antibody (0.5µg/ml, SC-100845, Santa Cruz Biotechnology) or mouse IgG (negative control, Dako). Sections were extensively washed in TBS-0.1% Tween-20 followed by incubation with biotinylated horse anti-mouse antibody (1:200, Dako) for 60 mins at room temperature. Sections were again washed with TBS-0.1% Tween-20 prior to application of avidin/biotin peroxidase detection system (ABC-HRP, Dako) for 30 mins at room temperature. Immunostaining was subsequently visualized by application of the peroxidase substrate 3, 3'-diaminobenzidine (DAB, Dako), which produces a brown precipitate. Tissue sections were counterstained with hematoxylin, dehydrated through ascending grades of ethanol (70-95%) and histosol and mounted on coverslips with DPX. Image were taken and processed using Olympus BX53 microscope and Cell Sens software (Olympus, Center Valley, Pennsylvania, USA).

3 RESULTS AND DISCUSSION

One in 8 couples are infertile, and success rates of artificial reproductive technologies remain low at <30%. Endometrial receptivity is now acknowledged as a key player in establishing pregnancy [1, 18, 21, 22, 51]. The data presented here defines the protein changes in the endometrial epithelial proteome and secretome, in response to both maternal and embryonic hormones, specifically estrogen, progesterone and hCG. Since these dynamic changes are essential for successful establishment of pregnancy, this new knowledge will inform new approaches to alleviating infertility in women and improving success rates of artificial reproductive technologies.

3.1 Proteomic analysis of the ECC1 lysate and secretome

Hormonal treatments mimicked physiological situations - proliferative phase; E alone, secretory phase receptive; EP, and conception cycle; hCG (in presence of EP), providing three groups for differential analysis. To define cellular and secreted protein changes between the three treatment groups we compared the proteome profiles of lysates and SS from ECC1 cells in response to these hormone treatments using GeLC-MS/MS [36, 39, 52] (**Fig. 3A**). For cell lysates, this resulted in 2169 (E), 2213 (EP) and 2092 (hCG) proteins, and 2485 proteins in total (identified in 2 biological samples with technical duplicates) (**Fig. 3B**) (**Supplementary Table S1**). Of the 157 proteins significantly differentially regulated in response to EP compared to E, 68 were enriched in expression (>2-fold, $p<0.05$) and 89 were decreased. These findings are overall in accord with gene array studies which show that progesterone modifies mRNA expression in both directions [5]. The addition of hCG to EP treated cells, resulted in 193 protein changes, with 67 being enriched in expression and 126 proteins with decreased levels (**Fig. 3B**).

When soluble secreted factors of ECC1 cells were examined, 928 (E), 787 (EP) and 816 (hCG) proteins respectively were identified consistently as differentially regulated (identified in 2 biological replicates with technical replicates) (**Fig. 3C**) (**Supplementary Table S2**). In the EP samples, 36 proteins were enriched in expression (>2-fold, $p<0.05$), while 87 were decreased, compared with the E treatment. In comparison between EP and hCG, 43 proteins were significantly differentially expressed, including 35 increased in expression in the presence of hCG, while 8 decreased in expression (**Fig. 3C**).

3.2 Receptivity and implantation potential are associated with regulation of cellular metabolism, RNA binding, and enzymatic activity in endometrial epithelial cells

Based on normalized spectral count ratios (Rsc), the dataset for proteins in cell lysates defined differentially regulated proteins that were significantly up- and down- regulated (>2-fold, $p<0.05$) in response to EP alone or in the presence of hCG (**Fig. 3B**). Bioinformatic interrogation of these proteins defined them as being involved in regulating cellular metabolism and RNA binding, and associated with basement membrane and cell connectivity, angiogenesis, proliferation, transport, and importantly embryo development (**Table 1**). Proteins associated with metabolism (MAT2B, NXN, NADH dehydrogenase 1 family), transport (SLC16A3), transcriptional regulation (NXN, MTA1), cell adhesion (SDCBP), and ligand-dependent activation of estrogen receptor target genes (CHTOP) were enriched in response to EP compared to E (**Table 1**). Interestingly, the NADH dehydrogenase 1 family (of which 3 are highly upregulated by progesterone in this study) are also selectively regulated during mating and sperm response [53]: since they are mitochondrial proteins involved in energy production, this could represent a response to the increased need for energy for the rapidly differentiating cells. Further, we noted the significant down-regulated expression of various integrins including ITGA3 and ITGB4-6 (Rsc -1.4 to -5.9), in response to EP. Members of the integrin family are essential for endometrium-embryo communication and implantation [54-56]. The presence and regulation of integrins in this study supports previous studies suggesting that integrin expression is hormone-dependent [55].

In the context of embryo-mediated signalling to enhance receptivity, proteins associated with metabolism (GNPDA2, MAT2B), basement membrane and cell connectivity (LGALS1, CLDN4), regulation of actin polymerization (ARPC3), implantation (RALA),

and proliferation and differentiation (PTBP3) were enriched in response to hCG compared to EP alone (**Table 1**). Furthermore, pathways significantly enriched in mRNA metabolic process, and RNA/nucleotide binding and regulation were identified in the hCG cellular dataset (GNA13, SEPT8, SF3B6). Interestingly, there were no further significant expression changes in cell adhesion (integrins), extracellular matrix, transport, ion channel, innate immunity, or basement membrane associated proteins (**Table 1**) beyond the changes induced by EP. Proteins down-regulated in expression in the hCG cellular dataset included metabolic enzymes (NDUF subunits) and two basement membrane proteins (HSPG2 and LAMB2) most likely reflecting the considerable cell and tissue remodelling during very early pregnancy.

To validate the changes in relative abundance of proteins using GeLC-MS/MS (relative spectral count ratios (Rsc)), Western blot analysis of cell lysates was performed for selected proteins; methionine adenosyltransferase 2 subunit beta (MAT2B), galectin-1 (LGALS1), and polypyrimidine tract-binding protein 3 (PTBP3) (**Fig. 4A**), revealing similar protein expression differences between ECC1 cell-derived lysates. These proteins were also validated using human primary endometrial epithelial cells (pEECs) treated with E, EP, and hCG (**Fig. 4B**). PTBP3 expression was highly elevated when the cells of origin were exposed to hCG (compared to EP), validating the findings from the ECC1 cell model used. Immunohistochemistry on endometrial tissue sections demonstrated that PTBP3 was predominant in vivo in epithelial cells, and that its staining intensity increased from proliferative to secretory phases and remained high in early pregnancy (**Fig 6**). This is consistent with proteomic profiling and Western Blot validation and indicates that PBTBP3 is being tightly regulated by steroid hormones (E, EP) and pregnancy hormone hCG. No significant change in expression for MAT2B or LGALS1 in response to hormonal

417 treatments for ECC1 cells and pEEC was observed. This difference in expression between
418 mass spectrometry and Western blotting may be due to epitope-specific differences in the
419 antibody, and therefore not able to distinguish differences in expression.
420
421 **3.3 Profiling secreted protein changes in response to progesterone and hCG**
422
423 Epithelial cell secreted proteins contribute substantially to the microenvironment of
424 implantation [4]. Indeed the pre-implantation embryo undergoes its final pre-implantation
425 development within the uterine cavity immersed in a complex milieu of factors contained
426 within uterine fluid. We therefore profiled the soluble-secretome of ECC1 cells treated with
427 EP and hCG. Based on normalized spectral count ratios (Rsc), this dataset showed a number
428 of differentially-regulated proteins that were significantly up- and down- regulated (>2-fold,
429 $p<0.05$) by these treatments (**Fig. 3C**). These proteins are known as involved in regulating
430 cellular adhesion, extracellular matrix (ECM) organization, regulation of developmental
431 growth, growth factor regulation, cell signaling, and immune response (**Table 2**) [13].
432 Proteins associated with cell adhesion and extracellular matrix (collagens, laminins, HSPG2,
433 DSC2, MUC5AC, BCAM), in addition to FBLN5 were identified. The matricellular protein
434 fibrilin-5 (FBLN5) is important in mediating cell-ECM interactions, associated with
435 endothelial cell adhesion, motility, and proliferation [57], and also promotes adhesion of
436 endothelial cells through interaction of integrin's and the RGD motif [58], all processes
437 associated with epithelial-embryo interactions at implantation. Further enrichment of proteins
438 associated with developmental regulation (RBP4, SEMA3F, SEMA3A), growth factor
439 regulation (TGFB1, FSTL1), cell signaling (MET, LIMS1), and immune response (B2M,
440 CLU), also processes important during peri-implantation, were enriched in response to EP
441 compared to E (**Table 2**). We also noted significant down-regulated expression of various

cytoskeletal and microtubule components in response to EP. These components include various tubulins (TUBA1A/TUBB6), tropomyosin (TPM1) and TWF2, involved in motile and morphological processes. The regulation and reorganization of the microtubule and cytoskeletal networks in this study is supported by previous studies suggesting that these elements are important for early-late stages of receptivity [59]. Further, such changes in expression correlate with plasma membrane transformation and membrane-associated cytoskeleton changes associated with uterine receptivity [60].

In the context of embryo-mediated signalling and receptivity (i.e., enriched in response to hCG), proteins associated with developmental regulation (HMGB2, SOD1, PAFAH1B3, LEFTY2), embryo development (LEFTY2, SOD1, YBX3) and angiogenesis (LEFTY2) were identified (**Table 2**). Interestingly, extracellular HMGB2, (71.5 fold increased in response to hCG, the pregnancy hormone) is secreted also by intestinal epithelial cells and has antimicrobial properties in the intestine [61], and by myeloid cells, with mitogenic and chemoattractant properties demonstrated [62]. All of these functions are highly relevant to events in the uterine microenvironment at implantation. In endometrial stromal cells, *HMGB2* knockdown promotes senescence, impairs their mesenchymal-epithelial transition to decidual cells and limits secretome changes [63]. Clearly the considerable increased secretion of the HMBG2 proteins from the endometrial epithelium, may have the reverse effects on either the trophectodermal cells of the blastocyst and/or on the luminal epithelium: changes already known as important for implantation. SOD1, an anti-oxidant enzyme (25.6 fold increased) is another protein of interest that has been detected during early phases of embryonic culture and in IVF cycles during hCG exposure [64]. A low level of SOD1 release is associated with low pregnancy outcomes and it is proposed that supplementation of SOD1 could reverse this effects [64-66]. It is thus not surprising that this

protein is also secreted by endometrial epithelial cells in response to hCG. Such enhancement of its local concentration is yet another example of how a healthy embryo can signal the endometrium to promote its own implantation. Proteins down-regulated by hCG in the hCG soluble-secreted dataset included hepatoma-derived growth factor (HDGF, -15.2 fold), and interleukin-17C (IL17C, -12.6 fold). Previously, a number of growth factors, mostly originating from the endometrium, have been identified in human uterine fluid which are proposed to support blastocyst-endometrial cross-talk important for endometrial receptivity and blastocyst implantation [20, 29]. In the bovine, HDGF is produced by both embryos and endometrium, promoting blastocyst development and increased cell counts in *in vitro*-cultured compacted morulas [67]. It was also identified as a mouse trophoblast stem cell associated gene [68]. However, HDGF-knockout mice have apparently normal development and phenotype [69]. IL-17 is one of many cytokines, chemokines, and growth factors in human endometrial fluid at the time of embryo transfer [70], but is down-regulated in response to hCG in our study. However, IL17 was found to be highly elevated in infertile women [71], thus it should be further investigated for its role in implantation and pregnancy.

Western blot and densitometric analysis of secreted proteins from hormonally-treated ECC1 cells and from pEECs validated several secreted proteins identified from GeLC-MS/MS profiling, including Y-box-binding protein 3 (YBX3), hepatocyte growth factor receptor (MET), LIM and senescent cell antigen-like-containing domain protein 1 (LIMS1), and superoxide dismutase 1 (SOD1) (**Fig. 5)**. Interestingly, there was greater agreement between results (protein expression level) from the cell line and the primary cells for secreted than for cellular proteins, reflecting that both luminal and glandular epithelium are highly secretory under EP stimulation. In accord with the profiling data, MET is elevated in both ECC-1 cells and pEECs when exposed to EP (compared to E) and hCG (compared to

EP) while YBX3 and SOD1 expression were elevated in response to hCG (compared to both E and EP). Surprisingly, significant changes in LIMS1 were only seen in the primary cells, not in the ECC1 cell line in response to hormonal treatments. This discrepancy is not necessarily surprising since the pEEC cultures comprise mostly epithelial cells of glandular origin, whereas the ECC1 cell line was selected for its similarity for luminal epithelium, which can have a somewhat different phenotype, particularly at the time of receptivity, when luminal epithelial changes for implantation are necessary [72, 73].

3.4 Correlation with previous cellular, extracellular and clinical studies

To confirm the sensitivity and specificity of our study, we compared our dataset with several key global and targeted gene and proteomic studies of receptive and non-receptive endometrium [9, 14, 19, 74] (**Table 3**). From our datasets, we identified 51 proteins that had been identified in prior endometrial studies, including 47/51 cellular and 45/51 soluble-secreted proteins. Several cellular proteins identified in our study were in common with proteins of known high abundance in the receptive phase [8, 12, 75] including: gamma-glutamyl hydrolase (GGH), 14-3-3 protein gamma (YWAG), caldesmon (CALD1), CCN family member (CYR61), protein disulfide-isomerase A3 (PDIA3), and members of the annexin family (ANXA1, A2, A4, A11). GGH, YWAG and CYR61, identified in cell lysate and soluble secretome in our study, have been shown to strongly expressed by luminal epithelium at mouse implantation sites and human endometrial epithelium [74, 76] suggesting their stability and unique roles in receptivity and implantation. These combined data emphasize the likely importance of these proteins in receptivity and highlight the need for further investigation of their roles. The annexins have been suggested to promote cellular adhesion, an essential step required for blastocysts to adhere to the endometrium. Elevated

517 ANXA4 mRNA correlates with increasing level of progesterone [5]. Similar to Hood et al
518 [9], we also identified the adapter molecule crk (CRK) as a regulated endometrial epithelial
519 protein. CRKs have been implicated in regulating cell migration, invasion, cell
520 transformation, and downstream receptor tyrosine kinase signalling [77], and integrating
521 signals for migration and invasion of highly malignant cancer cells [78]. Despite these
522 studies, the precise role of Crk adaptor proteins in endometrial receptivity and implantation is
523 poorly understood, and further investigation is warranted.
524
525 Of the secreted proteins, we identified many common proteins to those of Scotchie et al., [14]
526 who investigated uterine fluid from women in their receptive phase. Of particular interest is
527 the enzymatic protein SOD1, which we found elevated in response to hCG, confirming a
528 potentially important role in endometrial-embryo communication. Further validation that 15
529 of the proteins identified in this soluble secretome have also been identified in uterine lavage
530 (Salamonsen, unpublished). However, since uterine fluid contents are derived from a variety
531 of sources, they contain many proteins, including serum proteins in addition to those secreted
532 by the endometrial epithelium [4, 13, 79, 80]. Uterine fluid best reflects the
533 microenvironment during the early implantation period, is less complex than the endometrial
534 tissue proteome and will better reflect the endometrial epithelial secretome as examined here.
535
536 Not all proteins identified overlapped between this and previous publications: this is common
537 with genome-wide and global proteomic studies. Of note was the fact that our cellular data
538 represented 98.1% unique proteins (47 co-identified cellular proteins: 2438/2485 unique
539 proteins identified) and our soluble-secreted data represented 95.8% unique proteins (45 co-
540 identified secreted proteins: 1014/1059 unique proteins identified), previously reported in
541 receptive and non-receptive endometrium. Importantly, using a combination of targeted

ovarian steroidal and pregnancy hormones on the human endometrium, we reveal new insights into the cellular and extracellular/secretome changes associated with preparation for pregnancy. In the present case, the focus was on epithelial proteins and particularly those from a luminal epithelial cell line, whereas most previous work has used endometrial tissue samples, comprising a multiplicity of cell types, or uterine fluid. Clear but unavoidable limitations of the present study relate to the use of a cell line (ECC1) derived from an adenocarcinoma, for the proteomic analyses and of cultured primary cells for validation. Any *in vitro* or *ex vivo* studies of single cell types can never fully represent the *in vivo* situation, in which the cells are subjected to influences from the local microenvironment: these include molecular signaling between cells, signaling between cells and the extracellular matrix, and mechano-transduction, to name but a few. Furthermore, the ECC1 cells retain some features of their cancer origin, while even the primary endometrial epithelial cells used for validation, are from different women and had different hormonal histories. Both ECC1 and primary cells have been extensively utilized for research purposes in our laboratories and elsewhere and comparison of certain phenotypic similarities and differences published [81] along with responses to both progesterone and hCG [29]. Further confidence is provided by previous identification of some of the secreted proteins in uterine fluid. The use of the cell line provides a stable model, amendable to hormonal control that has not been subjected to the variability of the *in vivo* hormonal milieu inevitable with freshly isolated cells, while culture of the isolated cells enables regulation of their milieu by the hormonal stimuli under study.

Furthermore, many earlier proteomic studies used less sophisticated profiling techniques with very limited sensitivity. In these studies, many of the proteins identified were high abundance structural proteins whereas most proteins with functional roles are of lesser abundance and detected only by the newer technologies. This study highlights the need to

567 revisit analysis of clinically important samples as more sophisticated proteomic analyses with
568 a greater depth of coverage become available, providing new insights into the key events and
569 dynamic changes associated with preparation for pregnancy.
570
571 In conclusion, our understanding of embryo-maternal signalling during the peri-implantation
572 period, has been hampered by a lack of compartment-specific analyses of the receptive
573 endometrium. The present study provides a global view of the cellular and secreted
574 proteomes of the endometrial luminal endometrium (modelled by ECC1 cells), in response to
575 menstrual cycle and pregnancy hormonal treatments. Of the many novel proteins
576 differentially expressed several were validated in human primary endometrial epithelial cells,
577 thus endorsing our model. The global proteomic alterations provided here will provide the
578 basis for future mechanistic investigations characterising their functional roles in endometrial
579 biology and establishment of pregnancy.

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COMPETING INTERESTS

The authors declare no competing or financial interests.

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FIGURE LEGENDS

Figure 1 – The menstrual cycle of human endometrium. The endometrial menstrual cycle (normalised as of 28 day duration) is divided into three main phases; menses, proliferative and secretory. The dynamic changes are regulated by the changing levels of ovarian hormones estrogen and progesterone (upper panel). After menstruation, when most of the functional endometrium is shed, endometrial repair and restoration of endometrial thickness occur (driven largely by estrogen action) during the proliferative phase. Following ovulation at mid-cycle, all the cells enter a phase of differentiation, driven by progesterone in the continuing presence of estrogen. Successful embryo implantation can occur only during a brief period in the mid-secretory phase, known as the receptive phase (lower panel). In a conception cycle, when an embryo at blastocyst stage is present, the hatched blastocyst secretes human chorionic gonadotrophin (hCG), that signals to the endometrium to further promote receptivity, enabling implantation and establishment of pregnancy.

Figure 2 – Isolation and characterization of hormonally-treated endometrial cell lysate and secretome. Following hormonal treatments, ECC1 or pEEC cells were grown in serum-free medium for 24 h, cell lysates collected on ice and prepared as described. Conditioned medium (CM) was collected (secretome) and shed microvesicles (sMVs) were removed from the CM by differential centrifugation. The supernatant was further centrifuged at 100,000*g* for 1 h to remove exosomes. The supernatant was then filtered (0.1 μm), concentrated by centrifugal ultrafiltration through a 3K NMWL membrane, and transferrin (TF) depleted using IgY microbeads to provide the soluble-secretome (SS).

Figure 3 - Proteomic profiling of hormonally-treated ECC1 cells and soluble-secretome. (A) Schematic illustration of the experimental setup, proteomics workflow, and data analysis

of E-, EP-, and hCG-treated endometrial cell lysates and soluble-secretome. Stringent peptide and protein identification criteria were implemented (1% FDR protein, 5% PEP), with proteins identified in two or more biological replicates utilised for subsequent analyses. (B) Comparison of E/EP-treated and EP/hCG-treated cell lysates, showing common and unique proteins identified. Number of cellular proteins significantly differentially expressed in response to hormonal treatments, with fold change >±2, $p<0.05$ in at least 2 biological replicates. (C) Comparison of E/EP-treated and EP/hCG-treated soluble-secretomes, showing common and unique proteins identified. Number of secreted proteins significantly differentially expressed in response to hormonal treatments, with fold change >±2, $p<0.05$ in at least 2 biological replicates.

Figure 4 - Validation of differentially regulated cellular proteins in ECC1 cells and primary endometrial epithelial cells. Representative Western blots of (A) ECC1 and (B) primary endometrial epithelial cells pEEC cellular proteins under three hormonal treatments: E; controls, estrogen alone, EP; estrogen plus progesterone, hCG; human chorionic gonadotropin (in presence of EP). Proteins examined are PTBP3/ROD1, MAT2B, LGALS1 and GAPDH (loading control) (representative of n = 3 biological replicates, 15 µg cell lysate protein loaded). For clinical validation (pEEC), a total of n = 6 individual tissues from different patients were obtained and hormonally treated before cellular lysates were isolated. Densitometric analysis was performed using ImageStudio v5, with mean pixel intensity normalized to loading control (GAPDH), showing mean ± SEM with $*p<0.05$, $**p<0.01$ considered statistically significant. Combined data from all biological replicates.

Figure 5 - Validation of differentially regulated secreted proteins in ECC1 cells and primary endometrial epithelial cells. Representative Western blots of (A) ECC1 and (B)

primary endometrial epithelial cell (pEEC) secreted proteins under three hormonal treatments: E; controls, estrogen alone, EP; estrogen plus progesterone, hCG; human chorionic gonadotropin (in presence of EP). Proteins examined are MET, YBOX3, LIMS1, and SOD1 (representative of n = 3 biological replicates, 15 µg cell lysate protein loaded). For clinical validation (pEEC), a total of n = 6 individual tissues from different patients were obtained and hormonally treated before secreted samples isolated. Densitometric analysis was performed using ImageStudio v5, with mean pixel intensity normalized to background, showing mean ± SEM with *p<0.05, **p<0.01 considered statistically significant. Combined data from all biological replicates.

Figure 6 - Endometrial PTBP3 localization throughout the menstrual cycle

PTBP3 (ROD1) localized mainly to the glandular epithelium (arrowheads) during the proliferative (A) and secretory (B) phases of the menstrual cycle and during the first trimester of pregnancy (C) with some immunostaining also evident in stromal cells. The intensity of immunostaining increased from the estrogen-dominated proliferative phase (A) to the progesterone-dominated secretory phase (B) and remained high in the first trimester of pregnancy (C), with a change in localization from cytoplasmic (A & B) to nuclear during early pregnancy. No positive staining was evident in IgG matched negative control (inset, C). Scale bars = 20µm. Images are representative of n = 5 for each sample group.

TABLE LEGENDS

Table 1 - Cellular proteome changes in ECC1 cells in response to hormonal treatments: E, EP and hCG

Table 2 - Soluble-secreted proteome changes from ECC1 cells in response to hormonal treatments: E, EP and hCG

Table 3 – Proteins co-identified in comparison to endometrial gene and protein-based profiling studies

Table 1 - Cellular proteome changes in ECC1 cells in response to hormonal treatments: E, EP and hCG

Category	Protein Acc	Gene Name	Protein Description	Protein abundance ratio[a] (EP vs E)	Protein abundance ratio[a] (hCG vs EP)
metabolism	Q9UBM7	DHCR7	7-dehydrocholesterol reductase	NC	22.1*
	Q8TDQ7	GNPDA2	Glucosamine-6-phosphate isomerase 2	NC	44.9*
	Q86V21	AACS	Acetoacetyl-CoA synthetase	1.5	-2.5*
	Q99519	NEU1	Sialidase-1	8.9*	-8.5*
	Q9NZL9	MAT2B	Methionine adenosyltransferase 2 subunit beta	9.6*	NC
	P42345	MTOR	Serine/threonine-protein kinase mTOR	-2.3*	-1.4
	B3KWH9	0	Elongation of very long chain fatty acids protein	-18.1*	NC
	Q6DKJ4	NXN	Nucleoredoxin	9.6*	NC
	O95881	TXNDC12	Thioredoxin domain-containing protein 12	8.9*	1.4
	P14854	COX6B1	Cytochrome c oxidase subunit 6B1	8.1*	-2.3*
	Q9UI09	NDUFA12	NADH dehydrogenase 1 alpha subcomplex subunit 12	8.1*	-2.3*
	Q9P0J0	NDUFA13	NADH dehydrogenase 1 alpha subcomplex subunit 13	7.3*	-7.0*
	P17568	NDUFB7	NADH dehydrogenase 1 beta subcomplex subunit 7	7.3*	-2.1
transport	O15427	SLC16A3	Monocarboxylate transporter 4	20.7*	NC
	O00161	SNAP23	Synaptosomal-associated protein 23	8.9*	NC
	Q5JRA6	MIA3	Melanoma inhibitory activity protein 3	-1.8*	NC
RNA binding/ regulation	P49589	CARS	Cysteine--tRNA ligase, cytoplasmic	81.3*	NC
	Q05048	CSTF1	Cleavage stimulation factor subunit 1	11.2*	-1.9
	Q05519	SRSF11	Serine/arginine-rich splicing factor 11	8.9*	NC
	Q53G19	0	Mitochondrial ribosomal protein L11 isoform a	8.9*	NC
	Q9Y3A4	RRP7A	Ribosomal RNA-processing protein 7 homolog A	8.1*	-7.8
	Q9UKV8	AGO2	Protein argonaute-2	2.7*	NC
	Q92615	LARP4B	La-related protein 4B	2.1*	-1.6
	Q92599	SEPT8	Septin-8	-9.2	25.5*
	Q9Y3B4	SF3B6	Splicing factor 3B subunit 6	NC	17.9*
	P35249	RFC4	Replication factor C subunit 4	NC	17.1*
	Q14344	GNA13	Guanine nucleotide-binding protein subunit alpha-13	NC	8.6*
adhesion	Q14517	FAT1	Protocadherin Fat 1	-1.9*	-1.6
	Q75L80	CLDN4	Claudin-4	NC	11.2*
	O00560	SDCBP	Syntenin-1	8.9*	-2.5
	Q05397	PTK2	Focal adhesion kinase 1	-1.9	NC
cell membrane	E9PEE8	ITGB6	Integrin beta-6	-5.1*	NC
	P26006	ITGA3	Integrin alpha-3	-5.9*	NC
	Q9P2M7	CGN	Cingulin	-5.4*	NC
	P23229	ITGA6	Integrin alpha-6	NC	-1.5
	P16144	ITGB4	Integrin beta-4	-1.4*	NC
	P18084	ITGB5	Integrin beta-5	-4.3	NC
extracellular matrix	P35556	FBN2	Fibrillin-2	-2.6*	NC
basement membrane	P98160	HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein	-2.1*	-1.8
	P09382	LGALS1	Galectin-1	-10.8*	12.9*
	P55268	LAMB2	Laminin subunit beta-2	-1.7*	-1.6
	Q13751	LAMB3	Laminin subunit beta-3	-1.6*	NC
	O15230	LAMA5	Laminin subunit alpha-5	NC	-1.5*
	Q13753	LAMC2	Laminin subunit gamma-2	-1.5	-2.0
ion channel	P27105	STOM	Erythrocyte band 7 integral membrane protein	6.5*	NC
innate immunity	Q9Y3Z3	SAMHD1	Deoxynucleoside triphosphate triphosphohydrolase SAMHD1	2.1*	NC
	O95786	DDX58	Probable ATP-dependent RNA helicase DDX58	-8.3*	NC
nucleotide/RNA binding, proliferation	P11233	RALA	Ras-related protein Ral-A	-16.5*	10.3*

cell cycle	Q15058	KIF14	Kinesin-like protein KIF14	-7.5*	NC
	Q9UNS1	TIMELESS	Protein timeless homolog	-2.9*	NC
proliferation, differentiation	O95758	PTBP3	Polypyrimidine tract-binding protein 3	NC	26.4*
extracellular matrix, embryo development	P11047	LAMC1	Laminin subunit gamma-1	-1.4*	NC
other	Q9Y3Y2	CHTOP	Chromatin target of PRMT1 protein	8.9*	-8.5*
	Q2LE71	ARPC3	Actin-related protein 2/3 complex subunit 3	NC	13.7*
	P55011	SLC12A2	Solute carrier family 12 member 2	-1.6*	NC

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937 *Protein abundance ratio (ratio of spectral counts; Rsc) reveals differential protein abundance between*
938 *hormonally-treated cells. The use of zero spectra is overcome using an arbitrary correction factor (1.25). The*
939 *use of this correction factor allows relative quantitation of all proteins within both normalized datasets to be*
940 *performed, based upon Old et al. [82]. Positive Rsc values reflect increased protein abundance in* ***EP*** *relative to*
941 *E/hCG relative to EP; negative values indicate decreased abundance in* ***EP*** *relative to E/hCG relative to EP.*

942 ** Differential expression with p-values <0.05 as reported in Supplemental Table S1*

943 *NC No change in abundance*

944 **Table 2 - Soluble-secreted proteome changes from ECC1 cells in response to hormonal**

945 **treatments: E, EP and hCG**

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Category	Protein Acc	Gene Name	Protein Description	Protein abundance ratio[a] (EP vs E)	Protein abundance ratio[a] (hCG vs EP)
cell adhesion	P08572	COL4A2	Collagen alpha-2	27.1*	NC
	Q13751	LAMB3	Laminin subunit beta-3	2.3*	NC
	Q99715	COL12A1	Collagen alpha-1	2.1*	NC
	P02458	COL2A1	Collagen alpha-1	2.0*	NC
	P11047	LAMC1	Laminin subunit gamma-1	1.7*	NC
	P55268	LAMB2	Laminin subunit beta-2	1.9*	NC
	P12109	COL6A1	Collagen alpha-1	1.6*	NC
	O15230	LAMA5	Laminin subunit alpha-5	1.6*	NC
	B0YJ32	LAMA3	Laminin alpha-3 chain variant 1	1.7*	NC
	O60568	PLOD3	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	1.9*	NC
	P98160	HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein	1.5	NC
	Q96PD2	DCBLD2	Discoidin, CUB and LCCL domain-containing protein 2	3.5*	-1.4
	Q02487	DSC2	Desmocollin-2	2.3*	1.4
	P98088	MUC5AC	Mucin-5AC	2.2*	-1.4
	P50895	BCAM	Basal cell adhesion molecule	2.1*	-1.5
	Q9UBX5	FBLN5	Fibulin-5	12.2*	NC
regulation of development	P02753	RBP4	Retinol-binding protein 4	13.2*	1.5
	Q59G50	SEMA3F	Semaphorin 3F	3.1*	-1.5
	Q14563	SEMA3A	Semaphorin-3A	2.2*	NC
	P26583	HMGB2	High mobility group protein B2	NC	71.5*
	P16989	YBX3	Y-box-binding protein 3	NC	18.3*
	Q9P0M4	IL-17C	Interleukin-17C	1.9	-12.6*
	P00441	SOD1	Superoxide dismutase 1	NC	25.6*
	Q15102	PAFAH1B3	Platelet-activating factor acetylhydrolase IB subunit gamma	NC	20.5*
	O00292	LEFTY2	Left-right determination factor 2 (Endometrial bleeding-associated factor)	NC	2.0*
plasma membrane, adhesion	J3KNF6	RGMB	RGM domain family member B	11.2*	NC
angiogenesis	Q08431	MFGE8	Lactadherin	1.7	1.4
cytoskeletal/ECM remodelling	P23381	WARS	Tryptophan--tRNA ligase, cytoplasmic	-1.5*	NC
	Q71U36	TUBA1A	Tubulin alpha-1A chain	-310.2*	NC
	Q9BUF5	TUBB6	Tubulin beta-6 chain	-191.2*	99.2*
	P09493	TPM1	Tropomyosin alpha-1 chain	-25.3*	17.6*
	Q6IBS0	TWF2	Twinfilin-2	-11.1*	11.8*
transport	Q99523	SORT1	Sortilin	12.2*	NC
immune response	P61769	B2M	Beta-2-microglobulin	5.2*	-1.4
	P10909	CLU	Clusterin	4.2	-3.7
hormone (estrogen), embryo development	Q13045	FLII	Protein flightless-1 homolog	-4.0*	NC
growth factor regulation	P01137	TGFB1	Transforming growth factor beta-1	10.2*	2.0
	Q9NYQ8	FAT2	Protocadherin Fat 2	1.5*	NC
	P51858	HDGF	Hepatoma-derived growth factor	-1.4	-15.2*
	Q12841	FSTL1	Follistatin-related protein 1	2.6*	NC
signalling	P08581	MET	Hepatocyte growth factor receptor	7.2*	NC
	O15031	PLXNB2	Plexin-B2	4.2	1.5
	P48059	LIMS1	LIM and senescent cell antigen-like-containing domain protein 1	4.2*	-3.7
	Q6YHK3	CD109	CD109 antigen	2.4*	NC
	Q06481	APLP2	Amyloid-like protein 2	1.8*	NC
	P05067	APP	Amyloid beta A4 protein	1.6*	NC

	Q9UNS2	COPS3	COP9 signalosome complex subunit 3	-2.7*	NC

Protein abundance ratio (ratio of spectral counts; Rsc) reveals differential protein abundance between hormonally-treated cells. The use of zero spectra is overcome using an arbitrary correction factor (1.25). The use of this correction factor allows relative quantitation of all proteins within both normalized datasets to be performed, based upon Old et al. [82]. Positive Rsc values reflect increased protein abundance in ***EP*** *relative to E/hCG relative to EP; negative values indicate decreased abundance in* ***EP*** *relative to E/hCG relative to EP.*

** Differential expression with p-values <0.05 as reported in Supplemental Table S2*

NC No change in abundance

958 **Table 3 – Proteins co-identified in comparison to endometrial gene and protein-based**

959 **profiling studies**

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Protein Acc	Gene Name	Protein Description	Current study		Identified by other previous publications	
			Cell lysate	Soluble Secretome	Gene profiling studies	Protein profiling studies
E9PGZ1	CALD1	Caldesmon	Y	N	[74]	
P30101	PDIA3	Protein disulfide-isomerase A3	Y	Y		[9]
P04083	ANXA1	Annexin A1	Y	Y	[74]	
P08758	ANXA5	Annexin A5	Y	Y	[74]	[9, 14]
Q6LES2	ANXA4	Annexin A4	Y	N	[5]	
P50995	ANXA11	Annexin A11	Y	N		[9]
Q92820	GGH	Gamma-glutamyl hydrolase	Y	Y	[74]	
P61981	YWHA	14-3-3 protein gamma	Y	Y	[74]	[14]
O00622	CYR61	Protein CYR61 (CCN family member 1)	Y	Y	[74, 76]	
P00441	SOD1	Superoxide dismutase 1	N	Y		[14, 19]
P09382	LGALS1	Galectin-1	Y	N		[14]
P06396	GSN	Gelsolin	Y	Y		[14, 19]
P14550	AKR1A1	Alcohol dehydrogenase	Y	Y		[14]
P11766	ADH5	Alcohol dehydrogenase class-3	Y	Y		[19]
P09211	GSTP1	Glutathione S-transferase P	Y	Y		[14, 19]
P14174	MIF	Macrophage migration inhibitory factor	Y	Y		[19]
P13639	EEF2	Elongation factor 2 (EF-2)	Y	Y		[19]
P07737	PFN1	Profilin-1	Y	Y		[19]
P46108	CRK	Adapter molecule crk	Y	N		[9]
P02753	RBP4	Retinol-binding protein 4	N	Y		[14]
P10909	CLU	Clusterin	N	Y		[14]
P35527	KRT9	Keratin, type I cytoskeletal 9	Y	Y		[14]
P02792	FTL	Ferritin light chain	Y	Y		[14]
P02790	HPX	Hemopexin	N	Y		[14]
P11021	HSPA5	78 kDa glucose-regulated protein	Y	Y		[14]
P07237	P4HB	Protein disulfide-isomerase	Y	Y		[14]
P52565	ARHGDIA	Rho GDP-dissociation inhibitor 1	Y	Y		[14]
P04406	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Y	Y		[14]
Q06830	PRDX1	Peroxiredoxin-1	Y	Y		[14]
P67936	TPM4	Tropomyosin alpha-4 chain	Y	Y		[14]
P30043	BLVRB	Flavin reductase	Y	Y		[14]
P06733	ENO1	Alpha-enolase	Y	Y		[14]
P30041	PRDX6	Peroxiredoxin-6	Y	Y		[14]
P04792	HSPB1	Heat shock protein beta-1	Y	Y		[14]
P11142	HSPA8	Heat shock cognate 71 kDa protein	Y	Y		[14]
P23528	CFL1	Cofilin-1	Y	Y		[14]
P12277	CKB	Creatine kinase B-type	Y	Y		[14]
P78527	PRKDC	DNA-dependent protein kinase catalytic subunit	Y	Y		[14]
Q04760	GLO1	Lactoylglutathione lyase	Y	Y		[19]
P09493	TPM1	Tropomyosin alpha-1 chain	Y	Y		[19]
P14618	PKM	Pyruvate kinase PKM	Y	Y		[19]
P18669	PGAM1	Phosphoglycerate mutase 1	Y	Y		[19]
P40926	MDH2	Malate dehydrogenase, mitochondrial	Y	Y		[19]
P07195	LDHB	L-lactate dehydrogenase B chain	Y	Y		[19]
P10599	TXN	Thioredoxin	Y	Y		[19]
P35579	MYH9	Myosin-9	Y	Y		[19]
P07900	HSP90AA1	Heat shock protein HSP 90-alpha	Y	Y		[19]
P00338	LDHA	L-lactate dehydrogenase A chain	Y	Y		[19]
P35580	MYH10	Myosin-10	Y	N		[19]

Q13308	PTK7	Inactive tyrosine-protein kinase 7	Y	Y		[19]
P18206	VCL	Vinculin	Y	Y		[19]
Q96KP4	CNDP2	Cytosolic non-specific dipeptidase	Y	Y		
O00299	CLIC1	Chloride intracellular channel protein 1	Y	Y		
P67936	TPM4	Tropomyosin alpha-4 chain	Y	Y		
P04792	HSPB1	Heat shock protein beta-1	Y	Y		
Q14697	GANAB	Neutral alpha-glucosidase AB	Y	Y		

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