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Human Endometrial Extracellular Vesicles Functionally Prepare Human Trophectoderm Model for Implantation: Understanding Bidirectional Maternal-Embryo Communication

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In vitro human implantation model reveals a role for endometrial extracellular vesicles in embryo implantation: reprogramming the cellular and secreted proteome landscapes for bidirectional fetal-maternal communication

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Abstract

Embryo implantation into maternal endometrium is critical for initiation and establishment of pregnancy, requiring developmental synchrony between endometrium and blastocyst. However, factors regulating human endometrial-embryo crosstalk and facilitate implantation remain largely unknown. Extracellular vesicles (EVs) are emerging as important mediators of this process. Here, we used a trophectoderm spheroid-based in vitro model mimicking the pre-implantation human embryo to recapitulate important functional aspects of blastocyst implantation. Functionally, human endometrial EVs, derived from hormonally-treated cells synchronous with implantation, were readily internalised by trophectoderm cells, regulating adhesive and invasive capacity of human trophectoderm spheroids. To gain molecular insights into mechanisms underpinning endometrial-EV mediated enhancement of implantation, quantitative proteomics revealed critical alterations in trophectoderm cellular adhesion networks (cell adhesion molecule binding, cell-cell adhesion mediator activity, and cell adherens junctions), and metabolic and gene expression networks) and the soluble-secretome from human trophectodermal spheroids. Importantly, we demonstrate transfer of endometrial EV-cargo proteins to trophectoderm to mediate changes in trophectoderm function. This is highlighted by correlation between endometrial-EVs, the trophectodermal proteome following EV uptake, and EV-mediated trophectodermal cellular proteome, important for implantation. This work provides an understanding into molecular mechanisms of endometrial EV-mediated regulation of human trophectoderm functions – fundamental in understanding human endometrium-embryo signalling during implantation.

Significance statement

Implantation of an embryo into the maternal endometrium is critical for initiation and establishment of pregnancy, requiring developmental synchrony between endometrium and the blastocyst. We report, for the first time, molecular insights into the mechanisms underpinning human endometrial cross-talk, mediated by extracellular vesicles (EVs), to functionally prepare implantation. We used an in vitro spheroid model of human trophoctoderm (the outer cells of the blastocyst that first interact with the maternal endometrium), to recapitulate important functional aspects of blastocyst implantation, and demonstrated through functional interrogation, the capacity of human endometrial-derived EVs to regulate important functional attributes of human trophoctoderm cell implantation (adhesive and invasive capacity). To gain insights into the molecular mechanisms of endometrial EV-mediated regulation of trophoctoderm spheroid function during this fundamental phase of implantation, we performed comprehensive quantitative proteome profiling, revealing important cellular and soluble-secreted changes in human trophoctodermal spheroids regulated by endometrial-derived EVs during pre-implantation development. This work provides an understanding into molecular mechanisms of endometrial EV-mediated regulation of human trophoctoderm functions – fundamental in understanding human endometrium-embryo signalling during implantation. Significantly, our study shows a unique development in understanding the dynamic factors and their protein expression regulating human embryo-endometrium signaling during pre-implantation development that may be manipulated to enhance fertility or for new contraceptive strategies.

1 Introduction

Implantation of an embryo into the maternal endometrium is fundamental to viviparous mammalian life. The initial stages of human embryo implantation encompass apposition of the maternal endometrial and embryonic trophoctodermal epithelia, adhesion between these epithelial surfaces and invasion of the trophoctoderm through the endometrial luminal epithelium to embed it within the endometrium [1]. However, factors regulating embryo-endometrium signalling remain poorly understood. Developmental synchrony and cross-talk mediated by secreted factors are required between these tissues to achieve the initiation of pregnancy [2-5]. This is highlighted in IVF cycles where implantation is impossible if endometrial development is abnormal [6]. Although embryo implantation occurs in all mammalian species the molecular mechanisms by which this occurs differ significantly [7], with the most appropriate models for human implantation being menstruating primates. Importantly, data obtained from rodent and domestic animal models, conventionally used to understand the implantation process, often does not translate to the human situation, with contrast in mammalian embryos which display species-specific differences in post-implantation morphology and the lack of validated molecular markers [8]. *In vitro* cell culture models using cell lines, primary tissues and stem cells are commonly used to understand human-specific molecular mechanisms regulating embryo implantation.

Extensive evidence suggests that the release of membrane-enclosed compartments, more commonly known as extracellular vesicles (EVs), is a potent, recently identified, mechanism of cell-to-cell communication during the early phases of pregnancy [4, 9-11]. EVs can act to convey selectively-packaged molecules from one cell or tissue to another [12, 13]. Importantly, their contents (cargo) are protected from extracellular degradation or modification [14]; EVs protect miRNAs from degradation and contribute to their stability within biological fluids [15]. Further, they carry out many different supporting actions, including assisting follicle and oocyte development and maturation at the initial stages, and subsequently early embryo development and implantation as the embryo reaches the uterus as a blastocyst [4, 9]. Moreover, given that proteins are the executors of most biological programs, and the fact that the abundance of proteins cannot be accurately predicted from transcript

abundance [16], identifying the ability of endometrial-derived EVs to educate and reprogram the temporal proteome landscape in early embryos will provide direct insight into the molecular details governing key insights into the role of EVs in pre-implantation development. Importantly, identifying the capacity of endometrial EVs to modulate cellular function via alterations in the proteome landscape of pre-implantation human blastocysts will provide direct insight into the molecular details governing implantation signalling, embryonic development and their influence on functional pregnancy outcomes.

Mass spectrometry (MS) is a highly amenable option to identify and quantify a large, dynamic set of proteins, including defining fundamental insights into EV biology [17]. The proteomic analysis of pre-implantation embryos has been technologically challenging due to the scarcity of required materials. In the current study, we used an *in vitro* spheroid model of human trophoctoderm (the outer cells of the blastocyst that first interact with the maternal endometrium)[18], to recapitulate important functional aspects of blastocyst implantation, and demonstrated through functional assays the capacity of human endometrial-derived EVs to regulate the adhesive and invasive capacity of human trophoctoderm cell spheroids. To gain insights into the molecular mechanisms of endometrial EV-mediated regulation of trophoctoderm spheroid function during 'implantation', we performed comprehensive quantitative proteome profiling, revealing important cellular and soluble-secreted changes in human trophoctodermal spheroids regulated by endometrial-derived EVs during pre-implantation development. Our work provides a unique development in understanding the dynamic factors and their protein expression regulating human embryo-endometrium signalling during pre-implantation development that may be manipulated to enhance fertility or for new contraceptive strategies.

2 Experimental Procedures

Preparation of endometrial epithelial cell EVs

The human endometrial adenocarcinoma cell line, ECC-1 [19, 20] was used as the source of endometrial EVs. ECC-1 were validated by Karyotype analysis [21, 22] according to the ATCC guidelines [23], with allele match in STR profile of 100%. ECC-1 cells are known to be representative of the endometrial luminal epithelium [24]; the first point of contact between the maternal endometrium and the fetal trophoctoderm at implantation [21]. These cells confer a number of advantages over use of primary human endometrial epithelial cells obtained from human tissue biopsies. Primary human endometrial epithelial cells are obtained in small quantities only ($\sim 1 \times 10^6$ cells per tissue preparation) yielding insufficient cell numbers for preparation of EVs to be used in functional studies [25]. Further, due to the sampling method the majority of cells obtained from human endometrial tissue biopsies are glandular epithelial rather than luminal epithelial in origin, and are therefore less representative of the cell types at the maternal-fetal interface during implantation. Finally, tissues for primary endometrial cell culture are often obtained from infertile women undergoing uterine investigations, introducing uncertainty as to whether data obtained from these cells represent the 'normal' environment of implantation. Cells were cultured and maintained in a 1:1 mix of DMEM / 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% CO₂ [26]. Cells were washed twice with PBS and incubated in 0.5% charcoal stripped (cs) FBS for 16 hrs. Sub-confluent ECC-1 cells were sequentially treated with 10⁻⁸M 17β-estradiol (E) for 24 hrs followed by E plus 10⁻⁷M medroxyprogesterone acetate (P) for a further 24 hrs to mimic the receptive phase of the menstrual cycle [19]. EP primed culture medium was harvested and subjected to sequential ultracentrifugation (500g, 2000g, 10,000g) to remove cellular debris and apoptotic bodies. EVs (EP-EVs) harvested from each 100,000g pellet were validated for marker expression of Alix and Tsg101, and size distribution using nanoparticle tracking analysis and morphology using cryo-electron microscopy as previously described [19].

Protein quantification and Western blotting

EP-EVs for validation were suspended with 10 μ l of lysis buffer containing 4% w/v Sodium Dodecyl Sulfate (SDS), 0.1 mM dithiothreitol and 100 mM Tris-HCl pH 8.2, and incubated for 5 min at 95°C. The protein content of EVs (5 μ l) was quantified by Qubit fluorescence (Life Technologies), relative to known concentrations BSA using Qubit 4 Fluorometer. EV-treated trophectoderm cell lysates and soluble secretome were isolated from human TSC spheroids (4 x 10⁵ cells approx.) and quantitation performed using Qubit fluorescence or BCA protein quantification (Thermo Scientific, IL, USA) as per manufacturer instructions.

For western blotting, proteins were electrophoretically separated (XCell Surelock™ gel tank (Life Technologies) as described [19, 27], and electrotransferred onto nitrocellulose membranes using the iBlot™ 2.0 Dry Blotting System (Life Technologies). The membranes were blocked for 1 hr at RT with 5% milk powder in TTBS and probed with primary antibodies according to manufacturer's instructions overnight at 4°C, including primary mouse TSG101 (BD Biosciences; 1:1000), and mouse Alix (Cell Signaling Technology; 1:1000). Incubation with secondary antibody, IRDye 800 goat anti-mouse IgG or IRDye 680 goat anti-rabbit IgG (1:15,000, Li-COR Biosciences). Images were obtained using Odyssey system, version 3.0 (Li-COR Biosciences, Nebraska, USA).

Nanoparticle tracking analysis (NTA)

EV diameter (size) and concentration was determined by NanoSight NS300 system (NanoSight technology, Malvern, UK) equipped with a blue laser (488 nm). Briefly, EVs were diluted in ultrafiltered (0.1 μ m) PBS (~8 x 10⁸ particles/ml) and loaded into a flow-cell top plate using a syringe pump. Three separate technical replicates (60 sec/video) were recorded for each sample and analysed by NTA software (Build 3.1.45) [27].

Cryo-Transmission Electron Microscopy

Cryo-transmission electron microscopy (cryo-EM) imaging of EV preparations (EP-EVs) was performed as previously described [19]. Briefly, EVs (2 μ g protein, nonfrozen samples prepared within 2 days of analysis) were transferred to glow-discharged C-flat holey carbon grids (ProSciTech Pty Ltd). Excess liquid was removed by blotting, and the grids were plunge-frozen in liquid ethane. Grids

were mounted in a Gatan cryoholder (Gatan, Inc.) in liquid nitrogen. Images were acquired at 300 kV using a Tecnai G2 F30 (FEI) in low-dose mode. Size distribution of vesicles (range 40–150 nm) was calculated for 15 fields of view/sample (n = 2 biological replicates).

Trophectoderm (L2-TSC) cell culture

L2-TSC (trophectodermal, TSC) cells derived from trophoblast stem cells [18] were routinely maintained in a 1:1 mix of DMEM:F12 Glutamax (Gibco, Invitrogen) supplemented with 1% v/v P/S and 10% v/v FBS with addition of 10 ng/ml bovine fibroblast growth factor (bFGF, 233-FB-025, R&D Systems) and 10 μ M SB431542 (#1614, Tocris Bioscience). Cells were routinely grown in monolayer culture on a coating of 0.5% gelatin (#G1393, Sigma Aldrich).

Preparation of Trophectodermal Spheroids as Blastocyst Mimics

Methylcellulose (4000 centipoises, Sigma Aldrich) at 1.5% (w/v) was dissolved in DMEM medium by stirring at RT for 90 mins followed by stirring overnight at 4°C. Methylcellulose solution was subsequently centrifuged for 90 min at 3500 rpm to remove insoluble methylcellulose. 2500 trophoctoderm cells were seeded in 150 μ l of a 20% methylcellulose/trophectoderm media mix into a round bottomed 96-well plate. This promoted aggregation of all cells into a spheroid within 48 hrs (1 spheroid formed per well). Any mis-formed spheroids (<5%) were discarded. Spheroids were collected into 15 mL conical tubes using wide bore tips, centrifuged at 800 g for 8 mins to pellet the spheroids and washed 3 times in serum free media to remove traces of methylcellulose and trophoctoderm media before experimental use.

Uptake of EVs by TSC cells

Uptake of the EP-EVs by the TSCs was demonstrated as previously described [19] using fluorescently labelled EVs (30 μ g/mL) (DiI lipophilic dye (inVitrogen-GIBCO)) coupled with confocal microscopy. For fluorescence quantification, the deconvolved data sets were loaded in Imaris software (Bitplane AG), threshold in the WGA channel for segmentation of intercellular regions followed by fluorescence quantification of intracellular biotinylated EVs.

Adhesion and invasion assays

Adhesion and invasion of trophoblast spheroids was assessed by xCelligence real time cell behaviour analysis. Adhesion: E-plates were coated with 5 $\mu\text{g}/\text{cm}^2$ fibronectin (BD Bioscience, San Diego CA) 1 hr prior to seeding. 100 μL of medium (control, EV, EV/chlorpromazine [20 μM] or EV/dynamin inhibitor II [20 μM]) was placed into each well and a background reading performed. 15 spheroids/well then transferred to each well (in quadruplicate) and readings performed every 15 s for 5 hrs. Invasion: CIM plates were coated with 1:10 dilution of growth factor-reduced Matrigel™ prior to background reading and spheroid seeding as above. Readings were performed every 15 min for 96 hrs.

Isolation of trophoblast spheroid lysates and soluble fraction in response to endometrial epithelial cell-derived EVs

Human endometrial epithelial cell-derived EVs in response to 10^{-8}M estrogen/ 10^{-7}M progesterin for 24 hrs were isolated by sequential ultracentrifugation [19]. TSC spheroids (5×10^4 cells approx.) were then treated with or without EVs (30 $\mu\text{g}/\text{mL}$, single dose) for 3 days. To control for the quality of the spheroids with regards to their adhesive capacity, 10^{-8}M estrogen/ 10^{-7}M progesterin treated spheroids were also seeded into wells which did not contain endometrial epithelial cell-derived EVs (spheroid only). Spheroids were seeded for 6 hrs and wash steps performed as described. After 3 days, spheroid conditioned culture medium (CCM) was collected (on ice) and cells 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). Cell lysates were subjected to ultracentrifugation at 435,000g for 30 min at 4°C (TLA-100 rotor, Beckman Coulter), and supernatants aspirated, centrifuged to remove cellular debris, and stored at -80°C ($n = 3$). Isolation of soluble fraction was obtained from the CCM, where the supernatant was obtained from centrifugation (2,000g, 10 min) and concentrated using centrifugation ultrafiltration (3kDa, Millipore, MA, USA) ($n = 3$).

Proteome profiling of trophoblast spheroid lysates and soluble fractions in response to endometrial epithelial cell-derived EVs

Cell groups (20 μg) were lysed in in SDS sample buffer (4% (w/v) SDS, 20% (v/v) glycerol and 0.01% (v/v) bromophenol blue, 0.125 M Tris-HCl, pH 6.8)) with protease inhibitor cocktail (Complete, EDTA-free protease inhibitor cocktail, Roche), and proteins separated by short-range SDS-PAGE and the

entire in-gel fraction isolated for analysis [28]. Soluble fractions (20 μ g) were lysed in 8 M urea/50 mM Tris in 0.1% Rapigest (Waters, Milford, MA) [28]. Samples were reduced with 2 mM tri(2-carboxyethyl)phosphine hydrochloride (Sigma-Aldrich, C4706) at 28°C for 4 hr on gentle rotation, alkylated by treatment with 25 mM iodoacetamide (Sigma-Aldrich) for 30 min, and digested with 1 μ g bovine sequencing grade trypsin (Promega, V5111) at 37 °C for 18 hr. Subsequently, peptides were purified and extracted using reverse-phase C18 StageTips (Sep-Park cartridges, Waters, MA) in 85% (v/v) acetonitrile (ACN) in 0.5% (v/v) formic acid (FA). Peptides were lyophilised and acidified with buffer containing 0.1% FA, 2% ACN.

Proteomic experiments were performed in biological triplicate, with technical replicates (n=2), with MIAPE-compliance [19, 29]. A nanoflow UPLC instrument (Ultimate 3000 RSLCnano, Thermo Fisher Scientific) was coupled on-line to an LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) with a nanoelectrospray ion source (Thermo Fisher Scientific). Peptides were loaded (Acclaim PepMap100, 5 mm \times 300 μ m i.d., μ -Precolumn packed with 5 μ m C18 beads, Thermo Fisher Scientific) and separated (BioSphere C18 1.9 μ m 120Å, 360/75 μ m \times 400 mm, NanoSeparations) with a 120-min gradient from 2-100% (v/v) phase B (0.1% (v/v) FA in 80% (v/v) ACN) (2–100% 0.1% FA in acetonitrile (2–40% from 0–100 mins, 40–80% from 100–110 mins at a flow rate of 250 nL/min operated at 55°C).

The mass spectrometer was operated in data-dependent mode where the top 20 most abundant precursor ions in the survey scan (350–1500 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 2.0 Th and selected precursors fragmented by CID with normalized collision energies of 35 with a maximum ion injection time of 110 msec. 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. Dynamic exclusion was activated for 90 sec. Data was acquired using Xcalibur software v4.0 (Thermo Fisher Scientific). Raw mass spectrometry data is deposited in the PeptideAtlas (#PASS01122) and can be accessed at <http://www.peptideatlas.org/PASS/PASS01122>.

Database searching and protein identification

Raw data were pre-processed as described [27, 30] and processed using MaxQuant [31] (v1.6.0.16) with Andromeda (v1.5.6). For informatic processing, a human-only (UniProt #71,785 entries) sequence database (Feb-2018) was used. Data was searched as described [28] with a parent tolerance of 10 ppm, fragment tolerance of 0.5 Da and minimum peptide length 7, with false discovery rate 1% at the peptide and protein levels, tryptic digestion with up to two missed cleavages, cysteine carbamidomethylation as fixed modification, and methionine oxidation and protein N-terminal acetylation as variable modifications, identifications in at ≥ 2 biological replicates, and data analysed with label-free quantitation (maxLFQ) [32]. LFQ intensity values were normalized for protein length and fold change ratios calculated. Contaminants, and reverse identification were excluded from further data analysis. Resulting p-values were adjusted by the Benjamini-Hochberg multi-test adjustment method for a high number of comparisons [33] and statistics performed as previously described [34]. For pathway analyses, Kyoto Encyclopedia of Genes and Genomes (KEGG) and NIH Database for Annotation, Visualization and Integrated Discovery Bioinformatics Resources 6.7 (DAVID) resources were utilised using recommended analytical parameters [35]. For gene ontology enrichment and network analyses UniProt (www.uniprot.org) database resource (biological process, molecular function) was utilized. Expression analyses and enrichment tests were further performed using Perseus (v1.6.0.7), and gplots (<https://cran.r-project.org/web/packages/gplots/index.html>).

Western immunoblotting validation

Isolation of trophectoderm spheroid lysates and soluble fraction in response to endometrial epithelial cell-derived EVs or basal treatment alone were performed as described. Samples were lysed in SDS sample buffer, and electrophoretically separated as described; cell lysates (equal volume, 25 μ L), soluble fraction (equal volume, 8 μ L). SDS-PAGE gels were immunoblotted using BioRad TURBO transfer packs, with immunoblots subsequently washed in TBS-0.2% Tween followed by blocking in 10% skim milk/TBS-0.2% tween for 60 min. Excess milk was washed off with TBS-0.2% tween prior to overnight incubation at 4°C with antibodies for cell lysates (including primary mouse S100-A10 (Cell Signaling Technology; 1:1000), rabbit EPCAM (Abcam, 1:1000), rabbit PFN2 (Abcam, 1:1000) (cellular lysates)) and soluble fraction (including rabbit ITGA6 (Abcam, 1:1000)). Incubation with HRP conjugated antibodies, with immunoblots washed with TBS-0.2% tween before application of ECL

(BioRad) and visualization using a BioRad XR+ GelDoc. Cellular immunoblots were normalized against β -actin load control, while soluble fractions normalized against total protein (BCA total protein assay, Thermo Fisher, 23225).

Statistical analysis

Statistical testing of proteomic data was performed using a Poisson distribution with EdgeR software (v3.2), with $*p < 0.05$ considered statistically significant. Student's t-tests (GraphPad v5.0) were calculated, with $*p < 0.05$ and $**p < 0.01$ considered statistically significant.

3 Results

Endometrial EVs are internalised by human trophoderm cells

Endometrial epithelial cell-derived EVs (hormonally primed under the exposure of physiological levels of estrogen and progesterone (EP) to mimic the peri-implantation phase of the human menstrual cycle) were prepared using a differential ultracentrifugation strategy, and characterised based on their protein yield, marker expression, size distribution, and morphology (Fig 1A-D). Importantly, EVs were positive for Alix and Tsg101 marker proteins (biological replicates, Fig 1B), and size distribution indicating small EVs, with maximum intensity 140.2 nm, mean 168.4 nm, and total concentration of particles $3.10e+008$ particles/ml (Fig 1C). Cryo-electron microscopy reveal homogeneous, round-shaped vesicle structures of 40–150 nm in diameter (Fig 1D).

For EV's to release their cargo and mediate functional effects they must first be taken up by the target cell – in this study human trophoderm. Fluorescently labelled endometrial EP-EV's (green) with (Fig 2)[19] were shown to be progressively localised to the cell membrane and taken up by human trophoderm cells (TSC, red). At 24 hrs post EV treatment, biotinylated EV's (green) are visualized within trophoderm cells, with Z-stack demonstrating distribution (Fig 2A, compared to Biotin control)). Quantification of uptake by multiple cells at 24 hrs demonstrates a significant increase in intracellular fluorescent intensity within cells treated with biotinylated EV's (Fig 2B, $***p=0.0002$).

Endometrial EVs enhance human trophoctodermal spheroid adhesion and invasion capacity

An important step during blastocyst implantation is the ability of the trophoblast to adhere to endometrial epithelial fibronectin [36] and invade into the endometrium [37]. We thus assayed TSC monolayers and spheroids (generated to be of physiological size of a human blastocyst) for their adhesive capacity to fibronectin and invasive capacity across a MatrigelTM matrix. Whereas endometrial-derived EVs did not influence TSC cell monolayers versus control (■, Fig 3A), EVs significantly increased TSC spheroid adhesion (Fig 3B, * $p < 0.05$, ** $p < 0.01$) and TSC spheroid outgrowth on fibronectin (Fig 3C, ** $p < 0.01$, *** $p < 0.001$, each versus control). Inclusion of specific EV uptake inhibitors, chlorpromazine (an inhibitor of clathrin-dependent endocytosis) and dynamin inhibitor II (an inhibitor of dynamin-plasma membrane interaction and clathrin-independent endocytosis), partially inhibited EV-mediated TSC spheroid adhesion (Fig 3D) although this did not reach significance likely reflecting the multiple methods utilized by EV's to enter cells whereby a single inhibitor will not inhibit all uptake mechanisms [12, 13]. Moreover, EVs had no impact on TSC invasion when seeded in monolayers (Fig 3E) whereas TSC spheroid invasion was significantly increased (Fig 3F, * $p < 0.05$). Thus, TSC spheroids mimicking a blastocyst, recapitulate important functional aspects of blastocyst implantation. As endometrial hormone-primed (EP) EVs can enhance the adhesive and invasive capacity of TSC spheroids, this model can provide important molecular leads underpinning EV-mediated implantation.

Endometrial EVs temporally reprogram the dynamic cellular protein expression landscape of human trophoctodermal spheroids

As a first step towards understanding the molecular events associated with human trophoctodermal spheroid reprogramming by endometrial-derived EVs, we examined the protein profile of TSC spheroids and TSC spheroids treated with endometrial EVs using label-free quantitative mass spectrometry (Fig S1, biological triplicate). For stringency we report those proteins that were identified in at least 2/3 biological replicates (Fig S1A). A total of 1,632 and 1,702 proteins were identified in TSC spheroids and TSC spheroids treated with EVs, respectively, with 176 proteins uniquely

identified in EV-treated TSC cells (Fig. 4A) (Suppl. Table 1). Of proteins uniquely identified in TSC spheroids treated with EVs, Gene Ontology (GO) revealed various cellular compartments and biological processes associated with this unique subset.

Reported peptides identified in each dataset were of very high mass accuracy (within 10 parts per million) and have their distributions centred near zero (Fig S1B). The distribution of protein sequence coverage is also highly similar between the replicate analyses (Fig S1C). We assessed the reproducibility of the quantification by plotting the peptide intensities between biological replicates and found that the Pearson's correlation coefficient is >0.8 , indicating very consistent measurement (data not shown).

We applied differential expression comparison based on maxLFQ intensity, to reveal TSC spheroid proteins differentially regulated in response to endometrial EV's, revealing 206 proteins significantly upregulated in expression ($\log_2 >1$, $p < 0.05$) (Suppl. Table 2). DAVID-based informatic analysis of these proteins revealed biological processes associated with actin cytoskeleton regulation, cell adhesion, cell division, gene expression, and categories including oxidative stress response and T-cell receptor and Wnt signalling pathways (Fig. 4B-C); several of these processes are implicated in embryo implantation [38-47].

Additionally, we report several known regulators of implantation, including AHNAK [48], S100A10 [49], and PLAT [50], and human trophoblast markers important in early implantation including cathepsin CTSC, actin ACTA2, and phosphoglucomutase 1 (PGM1) [51] (Table 1). EpCAM (CD326) has been implicated for differentiation and survival of parietal trophoblast cells, normal development of the placental labyrinth and establishment of a competent maternal-fetal circulation [52] Further, PFN2 expression in the endometrium has been shown to be modulated by early embryo implantation and growth [53].

Overlaying our previously published analysis of the proteome of human endometrial EVs [19] used to reprogram the TSC in this study - strikingly, cargo of these EVs were intimately associated with the aforementioned biological processes (Fig. 4B), suggesting that the EV-cargo are potentially dictating changes in TSC proteome and function. Proteins identified within these biological processes are listed (Suppl. Table 3), indicating common and distinct biological processes associated with TSC proteome treated with EVs (part i; Suppl. Table 3) and endometrial EVs (part ii; Suppl. Table 3). Moreover,

16/206 proteins upregulated in TSC spheroids (treated with EVs) were also found in endometrial EVs (highlighted in Fig. 4D, Suppl. Table 2) – intriguingly, these proteins are potentially upregulated in expression, and transferred to TSC spheroids by EVs. This is consistent with the idea that EVs have the capacity as nanocarriers to constitutively transfer and reprogram cellular function (reviewed [17, 54]) – an important interface between the maternal and the embryo in pre-implantation [4, 9, 55]. Functional protein annotation of these include various intracellular trafficking and signalling components (GTPase, calcium signal regulators), regulation of actin cytoskeleton organisation, and translational initiation and RNA splicing. We demonstrate that the expression of several cellular components in TSC spheroids including S100A10 (Figure 5A), EPCAM (Figure 5B), and PFN2 (Figure 5C) are modulated by EVs by immunoblotting, highlighting that the relative abundance of these factors are regulated by endometrial EVs [19]. Importantly, each of these protein targets validated to be differentially regulated by mass spectrometry and immunoblotting in trophoctoderm, were not previously identified in EVs derived from a hormonally-regulated human endometrium [19], suggesting that the expression of these targets are regulated by endometrial EVs.

As an orthogonal approach, many of these processes enriched in TSC spheroids following EV treatment were also represented in an independent manually-annotated KEGG pathway database. These include ‘RNA transport’, ‘spliceosome’, ‘regulation of actin cytoskeleton network’, and ‘endocytosis’ (Fig. 6). KEGG database analysis additionally revealed that spheroids treated with EVs displayed enrichment in several ‘metabolic pathways’ including ‘folate biosynthesis’ (e.g., ALPG, SPR), ‘arachidonic acid metabolism’ (e.g., PTGES3), and ‘thiamine metabolism’ (e.g., ALPP) (Fig. 6). Previous functional and ‘omic studies of human and mouse blastocyst have reported that temporal regulation of metabolic pathways is important not only for the pre-implantation blastocyst [56-60] but also for embryo development [61, 62] and subsequent stages of embryogenesis [63].

Thus, the endometrial EV-cargo regulates cellular changes in TSC proteome and function. We highlight not only a correlation in proteins between EVs and the EV-treated TSC proteome, validated also by immunoblotting, but also common pathways associated with the EV-mediated TSC cellular proteome, important in blastocyst development and pre-implantation.

Human trophoctoderm secretome altered in response to endometrial EV treatment

We next questioned whether the trophectodermal spheroids alter their secreted profile in response to endometrial EV treatment. This is critical as, while endometrial signals are proven to be important in embryo implantation competency [64], so too are embryonic signals essential in enhancing endometrial receptivity [65] and in turn, the likelihood that a high quality embryo will implant into the maternal endometrium. Importantly, we focused on secreted proteins actively released from trophectoderm spheroids, limiting confounding effect of endometrial and TSC-derived EVs by removing proteins identified without predicted signal peptide sequences (SignalP) [66], in addition to requiring their annotation as human protein-coding genes and classification in Human Protein Atlas as secreted [67]. This comparison ensured that soluble-secreted proteins identified in the secretome were derived from TSC spheroids containing a signal peptide sequence, and not transferred through non-classical secretion mediated by EVs (i.e., endometrial EVs). A total of 753 and 897 proteins were identified in the secretome from TSC spheroids and TSC spheroids treated with EVs, respectively (Figure 7, FigureS3, Suppl. Table 4) (Figure S3, biological triplicate). To focus on secreted-specific proteins with a predicted signal peptide, the unique 267 proteins identified in the secretome in response to EVs were compared with SignalP and Human Protein Atlas (Figure 7, Suppl. Table 4). For this secreted subset we applied differential expression comparison based on maxLFQ intensity, to reveal 49 significantly upregulated ($\log_2 >1$, $p < 0.05$) secreted proteins in response to EV treatment (Suppl. Table 4). Functional annotation of biological processes, molecular function, and cellular localisation of these proteins, reveal association with cell and substrate adhesion, cell migration, binding capacity associated with integrins, laminins, collagens, neuregulin, chemokine, and alkaline phosphatase activity (Suppl. Table 5). These data suggest that latent pathways that are associated with embryo implantation can be discovered by constructing and analysing proteins secreted by TSC spheroids in response to endometrial EVs.

Of note, 15 proteins were uniquely identified in the TSC secretome in response to EV treatment, and classified as specific secreted proteins (Fig. 5). Importantly, several of these proteins (indicated * in Fig. 5) have previously been associated with embryo implantation and endometrial signalling, including the chemokine ligand CXCL12 [68], CXCL8 (IL-8) alkaline phosphatase ALPP [69], cysteine-rich protein CYR61 [70], tissue factor inhibitor TFPI [71], and integrin alpha 6 ITGA6. Notably, TFPI has previously been demonstrated not identified in EP-EVs [19] (Suppl. Table 4).

Further, ITGA6, in embryo has been shown to be temporally regulated and detected only in the murine ectoplacental cone (differentiating trophoblast) [72], suggesting specific roles in trophoblast adhesion and/or differentiation. Members of the integrin family have been shown essential for endometrium-embryo communication and implantation [73, 74]. We have shown previously that ITGA6 is identified in EP-EVs (exosomes) [19]. In this study we further validated ITGA6 as differentially regulated in TSC spheroid secretome by immunoblotting, indicating that the identification and expression is mediated by endometrial EV transfer (Figure 5D). This insight of ITGA6 and other such integrins (ITGAV, ITGA2, and ITGA5 were all significantly upregulated in Tsc secretome following EP-EV treatment), may be important for EV docking to recipient cells, about which very little is known, or they may be released intracellularly to relocate to the cell surface and mediate trophoblast adhesion by interacting with ligands including fibronectin and laminins [36].

Importantly, CXCL12 expression has been demonstrated in implanting/invading trophoblasts [75], and human trophoblast cells secrete CXCL12 [76]; important in regulating the functional endometrial-fetal interface [77]. A further ligand, CXCL8, has been suggested as a key secreted factor in the regulatory signalling loop between the implanting embryo and the permissive endometrium [78], and shown in an autocrine manner to stimulate trophoblast cell migration and invasion [79]. Importantly, CYR61 has been shown in microarray screen to be upregulated at the murine implantation site, specifically localized in the luminal epithelium surrounding the implanting blastocyst, but is not expressed in inter-implantation sites or under delayed implantation [70], highlighting a key role of CYR61 in mediating embryonic-uterine dialog during embryo attachment. Further, expression of TFPI in human secretory endometrium is highly specific (in contrast to several other tissue factor inhibitors) [71], suggesting an important functional role at the time of embryo implantation.

We demonstrate protein identification of integral membrane protein Sushi domain-containing protein 2, SUSD2 in TSC secretome in response to EVs. SUSD2 has been shown a cell-surface marker of naive pluripotency in the human embryo, with SUSD2 transcripts enriched in the pre-implantation epiblast of human blastocyst [80] and further spatially organized at the embryo-maternal interface to regulate distinct cytokine and chemokine profiles that potentially could direct trophoblast toward maternal vessels and govern local immune responses in pregnancy [81]. These results demonstrate that significant upregulated expression of SUSD2 may be transferred from endometrial EVs to

reprogram the TSC environment during implantation. Thus, our data show that trophoctoderm spheroids dynamically alter their secreted profile in response to endometrial EV treatment (Table 3), and provide important molecular leads underpinning maternal-embryo cross-talk and implantation.

4 Discussion

Endometrial EVs have recently been implicated as potential drivers of the embryo-maternal dialogue essential for implantation and establishment of pregnancy in a number of species [82]. However, understanding the molecular mechanisms of function of human endometrial-derived EVs – specifically in their ability to reprogram human trophoctoderm - remains unknown. The present studies support their biological role in regulating the trophoctoderm and trophoctoderm-endometrial interactions prior to implantation. Here, using an all human system, changes in the cellular and secreted proteome of trophoctodermal spheroids (representative of the blastocyst), following uptake of EVs from the endometrial epithelium, are defined and linked with simultaneous functional changes. In particular, EV-treated spheroids undergo both enhanced adhesion to and invasion of extracellular matrices representative of an endometrial epithelial monolayer (summary, Fig. 8).

Human embryo implantation, particularly the initial adhesive and invasive stage, is considered as the 'black box' of reproduction [83]. The earliest stages of human implantation involve trophoctodermal adhesion to the uterine epithelium, followed by its invasion between the epithelial cells. Some trophoctodermal cells, form a syncytium at the epithelial-stromal interface while others invade further into the decidualizing stromal compartment. Such developmental reprogramming from trophoctodermal cells provides a variety of trophoctoderm subtypes, particularly syncytio-, cyto- and invasive trophoblast, all important components of the placenta [1]. Importantly, we still do not have a comprehensive understanding of the specific factors involved in maternal-embryo cross-talk at this time, thus the reason for embryo implantation failure of genetically normal embryos is largely unknown. Many soluble factors facilitate this communication, including cytokines, chemokines, proteolytic enzymes and prostaglandins [55], released into the uterine cavity from receptive

endometrium. However, EVs have recently emerged in providing a unique intercellular transfer mechanism by which the maternal endometrium may transmit 'pro-implantation' signals to the embryo. Select EV cargo can be transferred to and regulate gene expression in target recipient cells, including various aspects of pre-implantation and reproductive biology (reviewed [4]). Such EVs, released from human-derived endometrial epithelial cells, contained distinct RNA cargo from that of their parent cells [20], were internalised by trophoblast cells, resulting in altered functional adhesion [19]. Further, the composition of endometrial-derived EVs and secreted factors was shown to be dependent on hormonal stimuli, differing between the non-receptive estrogen-dominated proliferative phase of the menstrual cycle, and the progesterone-dominated receptive secretory phase [19, 25]. Endometrial EVs have likewise been detected in the uterine cavity of sheep [84] and rats (C Murphy, L Lindsay and S Kalam, personal communication) although the mechanisms of implantation in these species differ from those in women [1, 85].

Studies of human endometrial-trophectoderm interaction at the earliest stages of implantation have been limited by paucity of material and ethical considerations in the use of human embryos for such research. However, appropriate human models are required for understanding this process: as indicated above, implantation in commonly used model species differs from that of the human. Previously, trophoblast and choriocarcinoma cell lines have been used for human implantation research [18]: these include HTR8SVneo [86], JEG-3 [87], ACIM-88 [88] and BeWo [89] (reviewed [24]): however, these cells are further along the differentiation pathway and not representative of early trophoctoderm. More recently, trophoctoderm-like cells derived from embryonic stem cells [90-93] and trophoblast biopsies from donated blastocysts [18, 94], have been described. These trophoctodermal cells can be formed into spheroids to physiologically mimic the early pre-implantation embryo. Although they do not form a blastocoele or inner-cell mass like structure, these spheroids do appear to function physiologically, differentiating between receptive versus non-receptive endometrial epithelial cells (Evans, Salamonsen, Greening, manuscript submitted).

In the present study, a gifted trophoctodermal cell derived from human embryonic stem cells was used [18]. Importantly, substantial differences in their proteome were determined, depending upon

whether the cells were grown as monolayers or as spheroids, with the latter more likely to be representative of trophoblast as the outer layer of the blastocyst *in vivo*. Nevertheless, it is acknowledged that the absence of both blastocoele and inner cell mass may further alter trophoblastic phenotype. Our recent discovery that TSC spheroids can differentiate between endometrial cells derived from fertile versus infertile women in terms of adhesion, reinforces the functional validity of this model. Moreover, Aghajanova et al, [51] have demonstrated that while there were certain differences in the transcriptomes of true human mural trophoblast cells and human trophoblast derived from human embryonic stem cells, the similarities were sufficient for the latter to be used as a viable representative of true human trophoblastic cells.

While primary human endometrial cells would perhaps have been optimal for this study, these are predominantly of glandular origin, while the human endometrial ECC1 cell line used, is defined as the best representative of luminal epithelial cells [74], with which the blastocyst interacts *in vivo*. This cell line was validated before use [25] despite a publication querying the validity of those held elsewhere, particularly in the USA [22]. The co-culture system between monolayers of EP-treated ECC1 cells and trophoblastic spheroids used here, is particularly applicable for extensive studies on human implantation and has enabled meaningful functional studies as described here and elsewhere (Evans *in preparation*). In a similar co-culture system, between Ishikawa cells (another widely used human endometrial epithelial cell line) and trophoblast stem cells, an effect of co-culture on trophoblast invasion and galectin 1 release was reported: however, whether this was due to EVs or soluble secreted factors from the Ishikawa cells, was not examined [95].

The current study demonstrates not only that EV's derived from 'receptive' endometrial cells specifically alter the function of trophoblast spheroids, and that in doing so they dynamically alter the cellular proteome, facilitating functional changes critical in implantation. Highly up-regulated cell functions regulated by treatment of trophoblast spheroids with EVs from EP- treated endometrial cells include cell-cell adhesion and cadherin binding involved in cell-cell adhesion, which are critical functions when we consider the interaction of the maternal and embryonic epithelia at implantation. Furthermore, extracellular matrix organization and cell adhesion were down-regulated functions,

potentially suggestive of disaggregation of the spheroid as the cells become invasive; a function significantly enhanced by EV's from 'receptive' endometrial epithelial cells.

Trophectodermal differentiation is species specific, and for the human this has been transcriptionally staged as early, mid and late, within a transcriptional atlas of the human pre-implantation embryo [96]. Indeed, several of these genes had been previously associated with trophoblast differentiation including CCR7 [97], CYP19A1 [98], DLX [99], ERVFRD-1 [100], GCM1 [99], GREM2 [101], MUC15 [99], and OVOL1 [102]. For all of these, translation into protein was shown in our analysis of trophoblast spheroid proteins up-regulated by endometrial EVs.

In conclusion, endometrial EV's clearly play an essential role in altering trophectoderm function to facilitate successful implantation and represent a novel communication mechanism during the establishment of pregnancy. Comprehensive studies have provided key insights into pre-implantation development precisely controlled by a series of genes and transcription factors [60, 103], however this current study provides the exciting potential of endometrial EVs as mediators of trophectoderm function of critical protein factors in human implantation. This has significant implications in the field of infertility and cell free therapeutics. Stem cell treatment has been proposed as a potential intervention to improve endometrial quality and receptivity [104, 105]. However, the field is moving towards cell free therapies to improve safety and efficacy [106] making the function of endometrial derived EV's in improving embryo implantation potential described here, an attractive future therapy to improve fertility and the success of IVF cycles in which implantation failure is a major limiting factor.

Summary

Embryonic implantation, the process by which the human embryo orientates towards and attaches to the maternal endometrial tissue, requires a receptive endometrium, a functionally normal blastocyst and an adequate cross-communication between them. Human developmental biology at these early stages is still limited by availability of material. However, advances in *in vitro* cell culture models using cell lines, primary tissues and stem cells are being used to understand human-specific molecular mechanisms regulating embryo implantation. In this study human trophectoderm cells in spheroid

form representative of human blastocysts, internalize EVs derived from human endometrial epithelial cells (hormonally treated to represent 'receptive endometrium'). Functionally, we demonstrate that these endometrial-derived EVs enhance adhesion and invasion of human trophoctodermal spheroids. To gain important insights into molecular leads important in EV-mediated cross-communication, comprehensive quantitative proteomic profiling identified cellular and soluble-secreted changes induced by the EVs in trophoctoderm, including dynamic regulated adhesion protein networks regulated by embryo-maternal communication and core chemokines and factors important for blastocyst implantation. Functionally, EV uptake resulted in increased trophoctoderm adhesion to and invasion of the endometrial epithelium, thus enhancing implantation potential. Finally, much remains to be learned concerning the communicating signals comprising the embryonic–maternal cross-talk through EVs. We provide important findings on the function and reprogramming capacity of endometrial EVs to trophoctoderm, which will help elucidate and understand the source and role of EVs in human implantation. Deepening our understanding of the mechanisms how EV cargo is controlled and sorted, in addition to recognition mechanisms associated with EV transfer will be critical in understanding the physiology of EVs in early pregnancy recognition and pre-implantation.

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Author Contributions

J.E., H.N., L.A and D.G conceived and designed the experiments. J.E., H.N., and D.G. carried out the majority of experiments. J.E. performed the TSC spheroid generation and functional experiments. K.A. performed the confocal microscopy studies. A.R. performed informatics and data interrogation. All authors analyzed the data. J.E. A.R. L.A. and D.G wrote, reviewed and edited the manuscript. All authors approved the final manuscript.

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Data and Software Availability: The accession number for the mass spectrometry data reported in this paper is PeptideAtlas Consortium via the PeptideAtlas proteomics repository: PASS01122.

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Figure 1

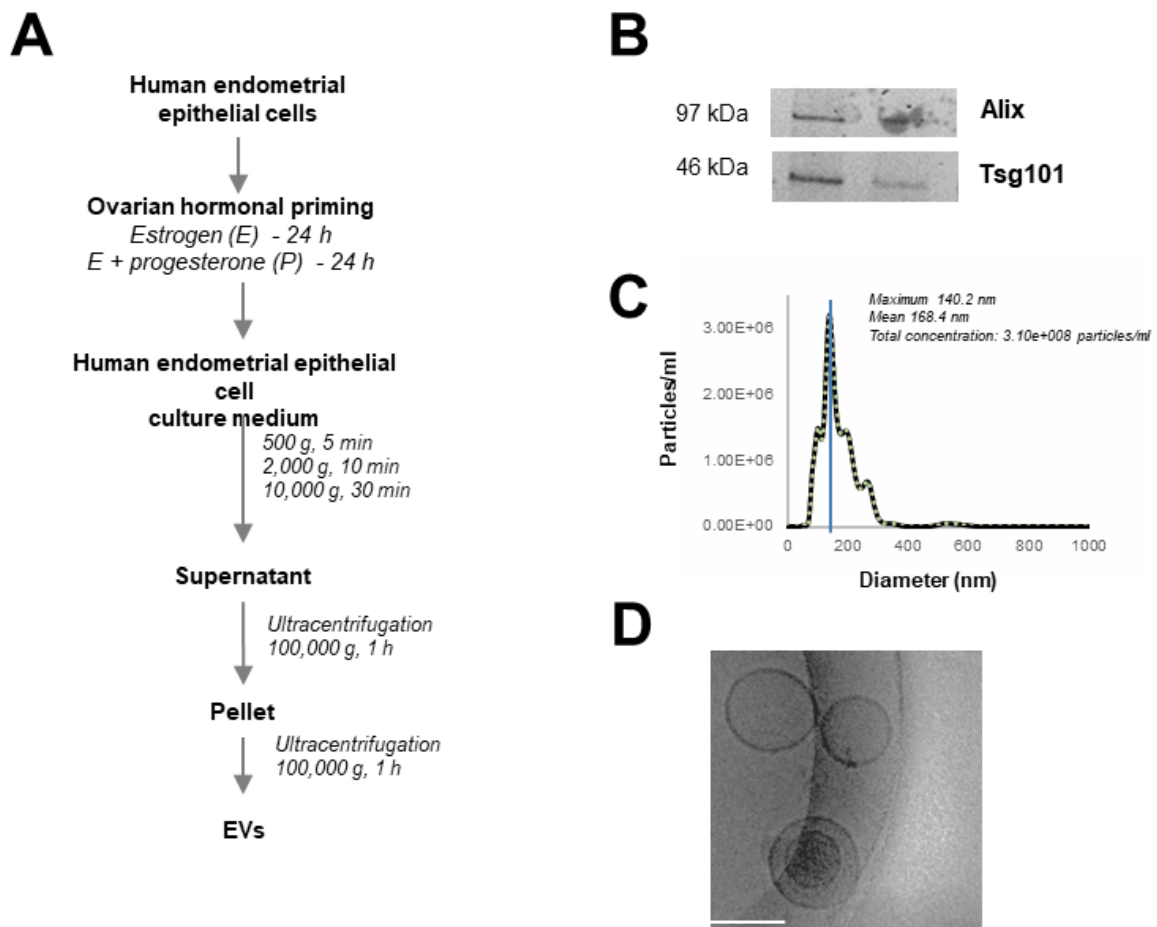


Figure 1. Isolation and characterization of endometrial epithelial cell EVs.

A - Experimental workflow for isolation and preparation of EVs from the culture medium (CM) of human ECC-1 cells. ECC-1 cells were treated with 10^{-8} M 17β -estradiol (E) for 24 hrs followed by E plus 10^{-7} M medroxyprogesterone acetate (P) for a further 24 hrs to mimic the receptive phase of the menstrual cycle. Culture medium was harvested and subjected to sequential ultracentrifugation (500g, 2000g, 10000g) to remove cellular debris and apoptotic bodies and remaining supernatant further subjected to ultracentrifugation at 100,000g, with the EV pellet washed, and resuspended for subsequent validation. B - Western blot analysis of EVs (post 100,000 g EVs) using indicated antibodies (n=2 biological replicates). C - EV diameter (size) and concentration was determined by NanoSight NS300 system. D - Cryo-electron microscopy of EVs revealing textured round vesicles (40–150 nm). Bar 100 nm.

Figure 2

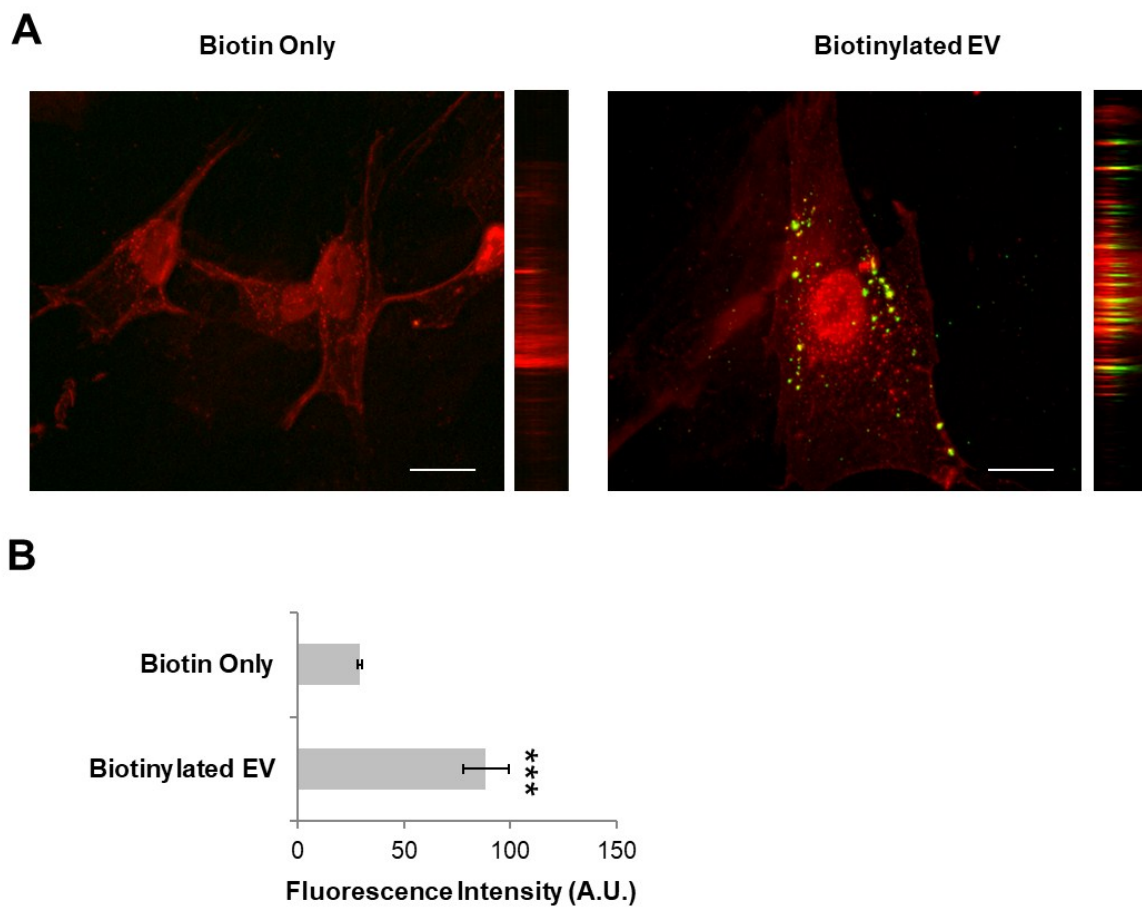


Figure 2 - Endometrial EVs are internalised by human trophoblast cells. Endometrial EVs labelled with Dil were incubated with TSC cells for 24 hrs (A) and are distributed intracellularly (z-stack) (A). Minimal staining observed in biotin control. Intracellular fluorescent intensity is significantly higher in biotinylated EV treated cells at 24 hrs (B, ***p=0.0002).

Figure 3

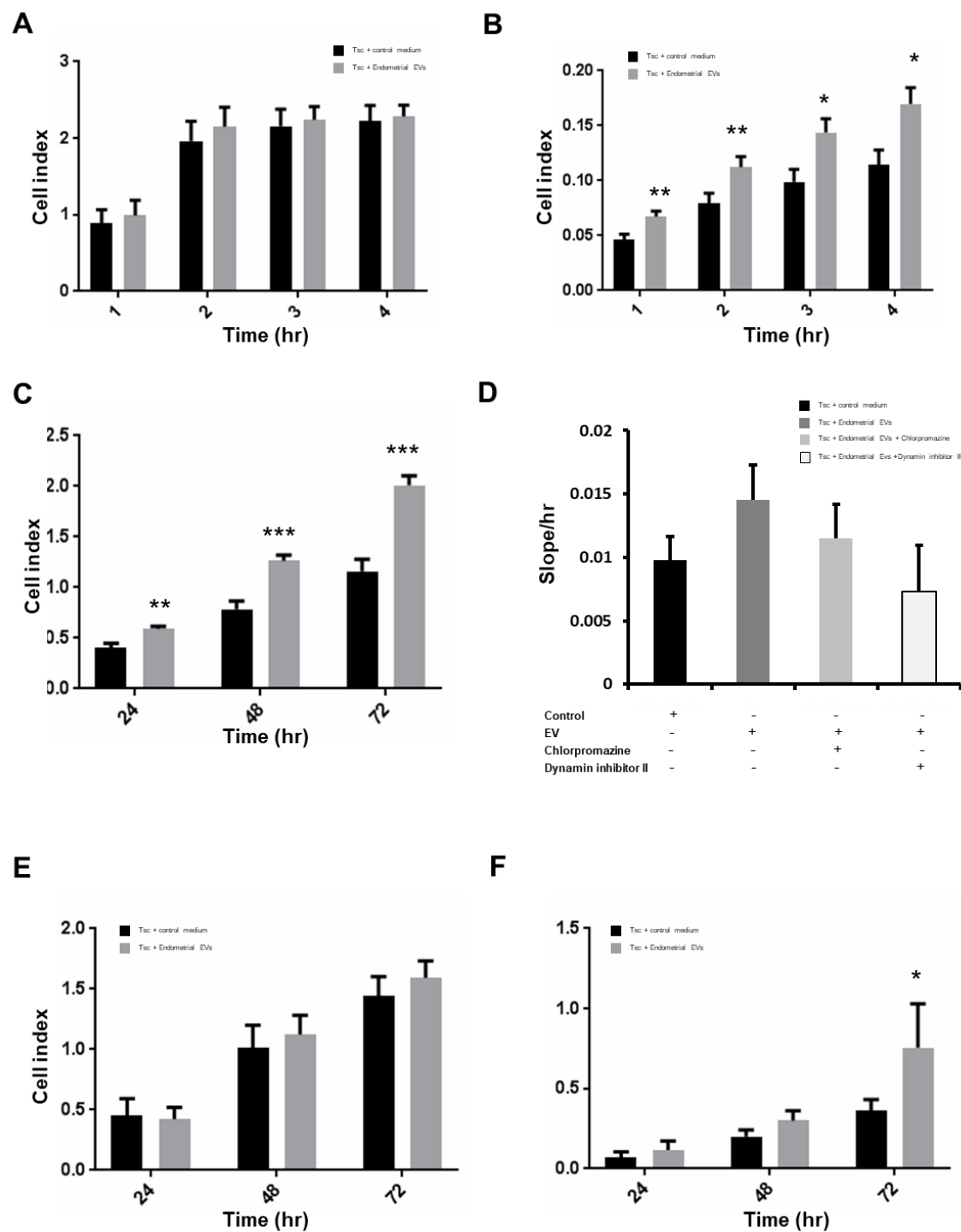
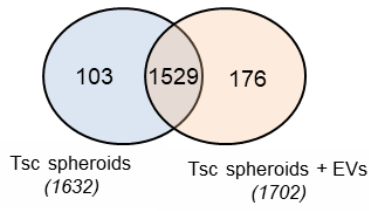


Figure 3 - Endometrial EVs enhance human trophoblastic spheroid adhesion and invasion capacity. Endometrial EVs (30 $\mu\text{g}/\text{mL}$) did not impact the adhesive capacity of TSC cells grown as monolayers on fibronectin (A), in comparison to loading control (■), although when the TSC cells were grown as spheroids, the EVs did significantly increase their adhesion (B) and outgrowth (C). Abrogating EV uptake was performed using chlorpromazine or dynamin inhibitor II, which did partially

reduce EV-mediated TSC spheroid adhesion (D). Endometrial EVs had no impact on TSC monolayer invasion (E) whereas TSC spheroid invasion was significantly increased (F). Data presented as mean \pm SEM of 4 experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4

A



B

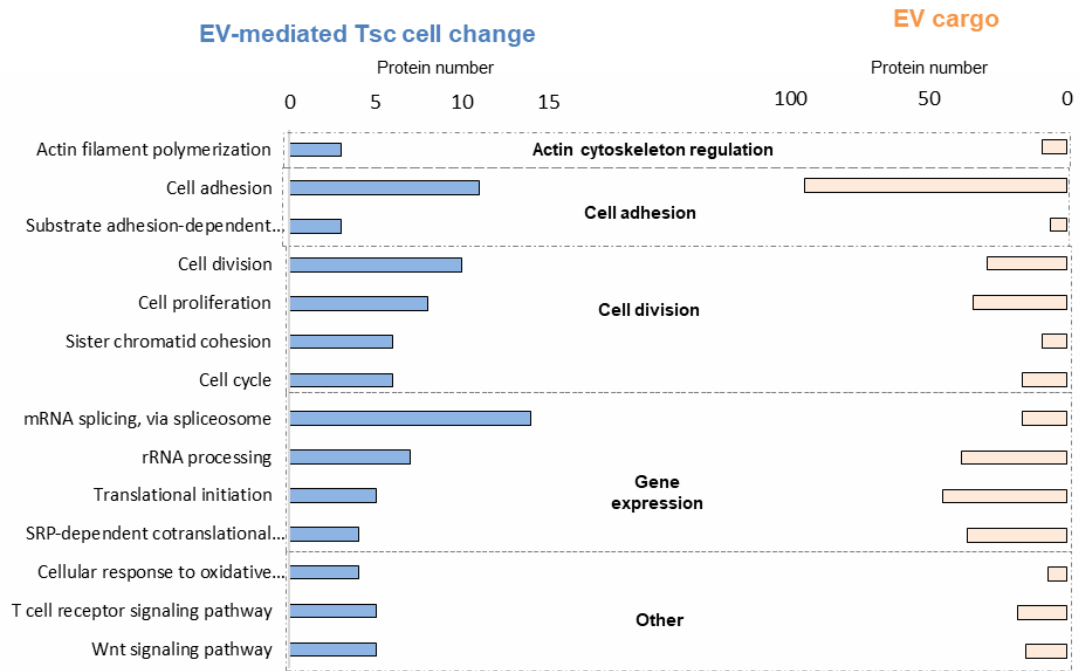


Figure 4

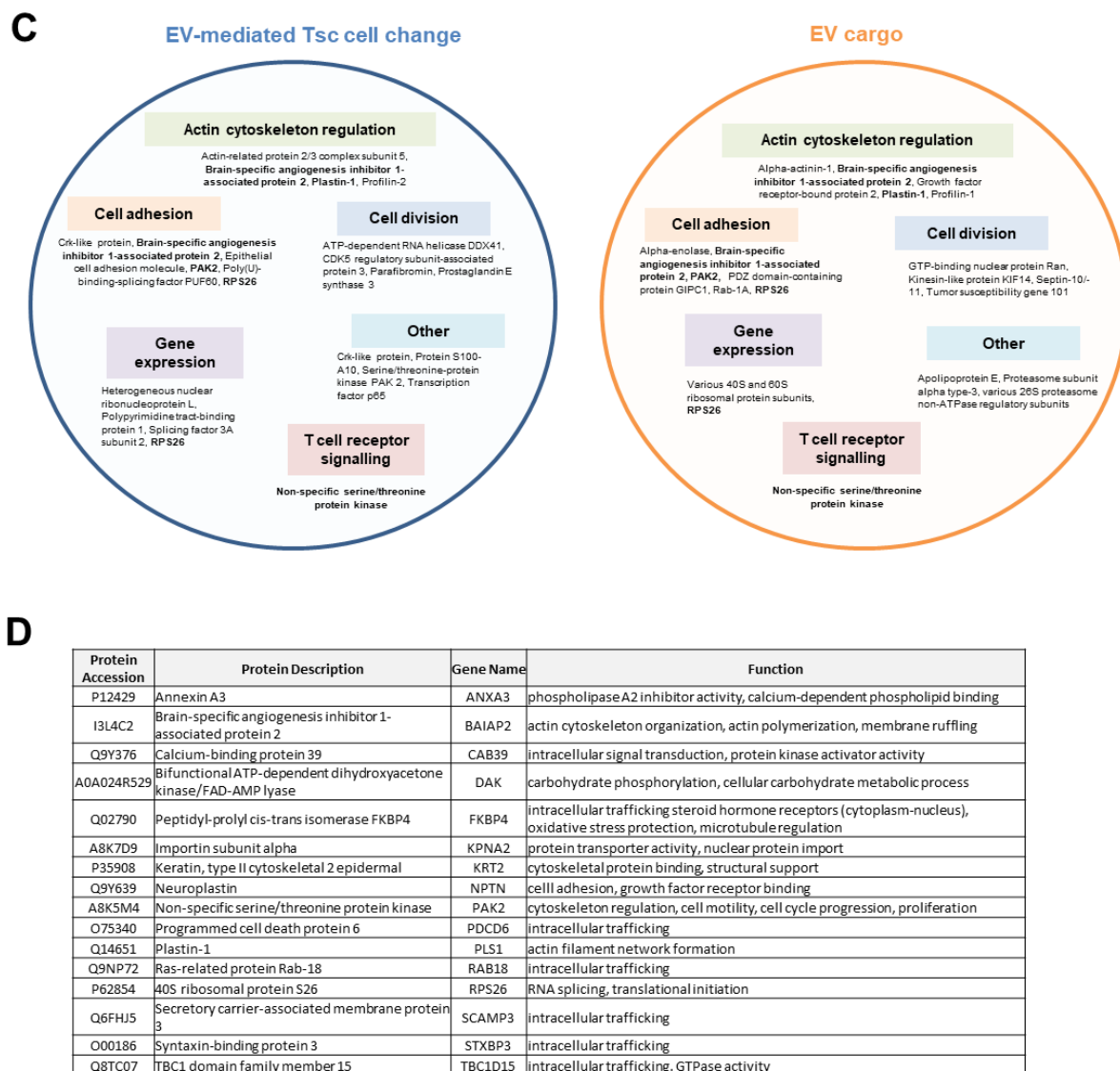


Figure 4 – Endometrial EVs reprogram the human TSC cellular proteome

Venn diagram of proteins identified in TSC spheroids and TSC spheroids following treatment with endometrial EVs (estrogen and progesterone hormone exposure), with 176 proteins uniquely identified in EV-treated TSC spheroids (A). The functional annotation of 206 proteins differentially enriched in TSC spheroids in response to endometrial EVs ($>1.0 \log_2$, P -value < 0.05) (Suppl. Table 2) were then investigated using DAVID database statistical overrepresentation test using reference human genome (B, C, left). Bar lengths are displayed as number of proteins identified for each category. Raw data and P -values for each category are shown in Supp. Table 3. The proteome of endometrial EVs was then analysed in the same format (gene ontology, biological process) to reveal

that specific EV-cargo are potentially dictating changes in TSC proteome and function (B, C, right) (Suppl. Table 3). Proteins upregulated in TSC spheroids in response to EV treatment were also found in endometrial EVs (D) (Suppl. Table 2) - these proteins are potentially transferred to or their expression upregulated in TSC spheroids by EVs. Relative abundance of xxxx in endometrial EVs, TSC cells and TSC cells in response to EV treatment based on precursor ion intensity (left panel) (E). Western blot analysis of each sample using anti-xxxx antibodies (right panel) (E).

Figure 5

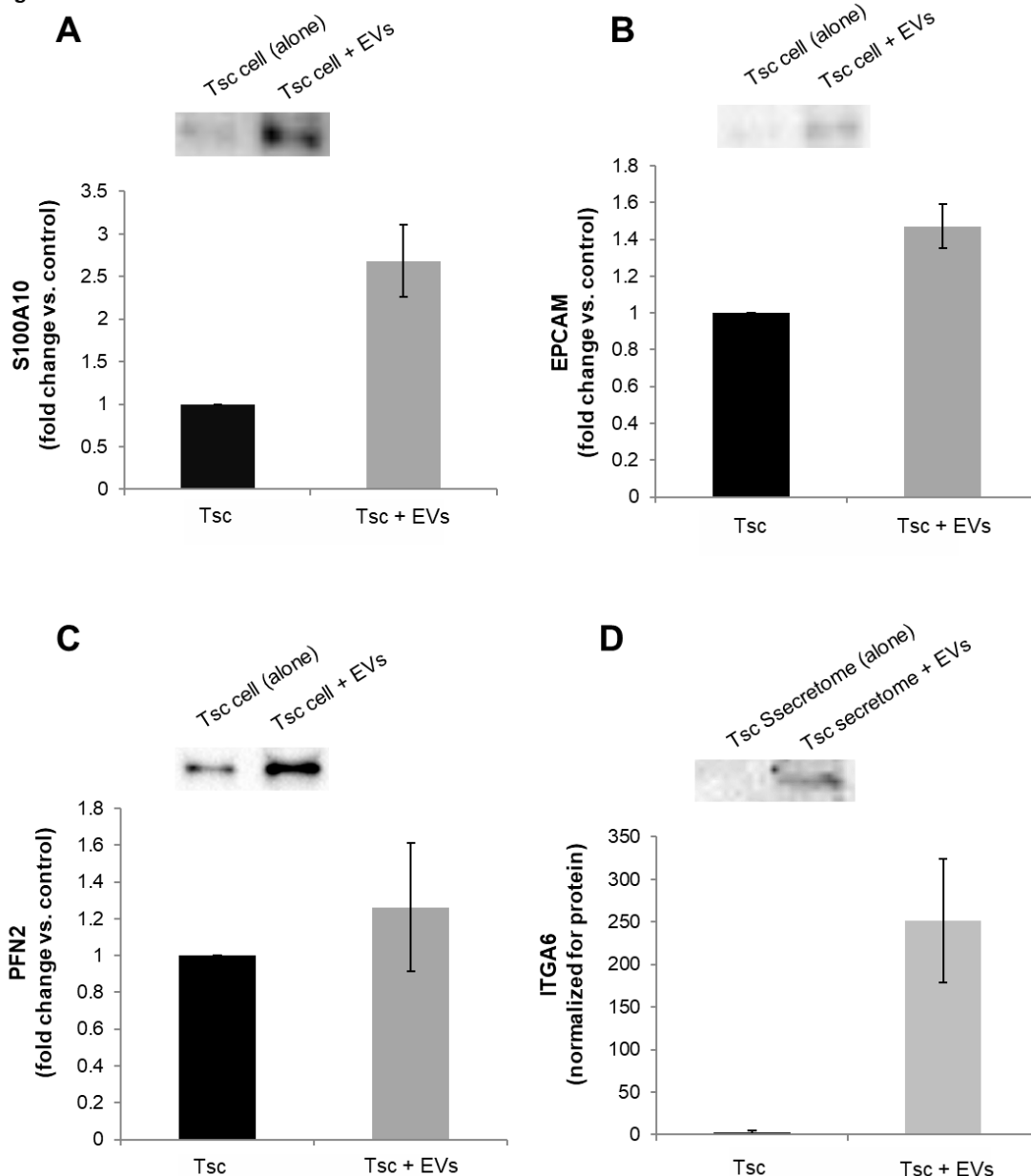


Figure 5 - Independent immunoblotting validation of cellular proteins and secretome modulated by endometrial EVs. TSC spheroids and TSC spheroids following treatment with endometrial EVs (estrogen and progesterone hormone exposure), were immunoblotted against specific protein targets of interest in cellular lysates and soluble fraction of trophectoderm spheroids. Treatment of trophectodermal spheroids (5 x 10⁴ cells approx.) with EP-EV's (30 µg/mL, single dose) mediated an increase in cellular elevation of protein S100-A10 (A), profilin-2 (B) and EpCAM (C), and integrin α6 secretion (D). Data presented as mean ± SD of 3 independent experiments with pooled technical duplicates.

Figure 6

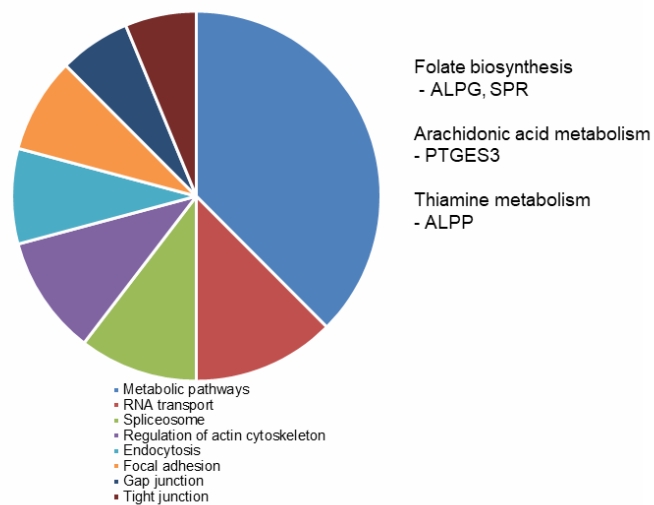


Figure 6 – Endometrial EVs facilitate changes in TSC cellular proteome pathways. Pathway annotation of proteins enriched in TSC spheroids in response to endometrial EVs ($>1.0 \log_2$, P-value < 0.05) (Suppl. Table 2) were investigated using KEGG pathway database. Metabolic pathways were further subdivided into folate biosynthesis, arachidonic acid metabolism and thiamine metabolism.

Figure 7

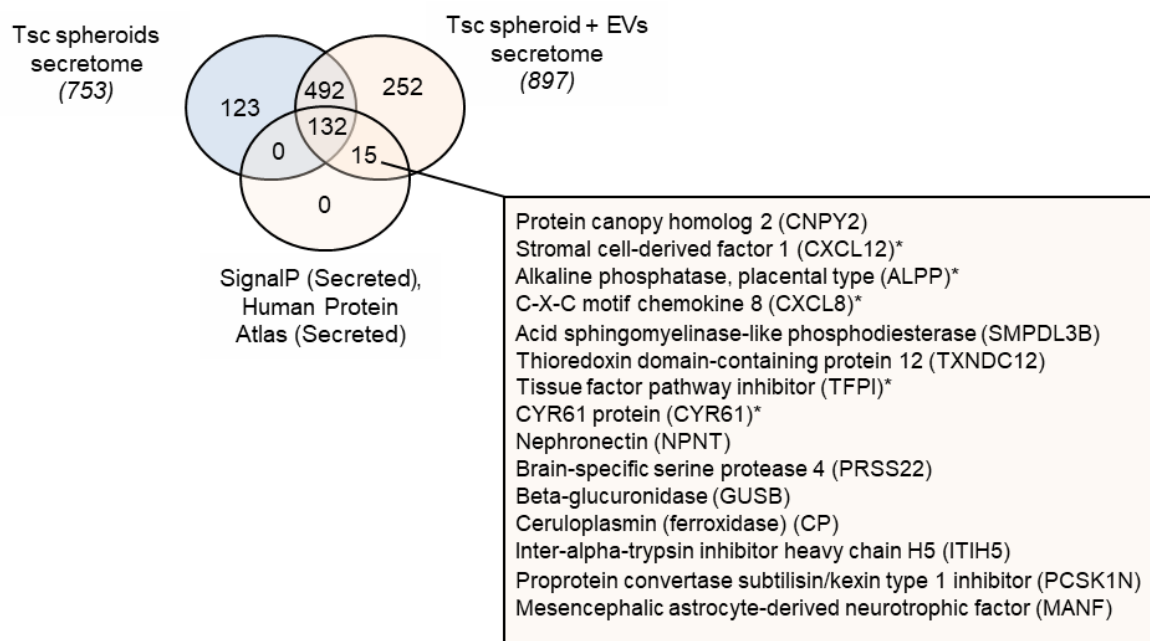


Figure 7 – Endometrial EVs reprogram the human TSC soluble-secretome. Venn diagram of proteins identified in the TSC spheroid secretome and TSC spheroid secretome following treatment with endometrial EVs (estrogen and progesterone hormone exposure). To focus on specific proteins released from TSC spheroids (soluble-secretome), proteins without a predicted signal peptide sequence (SignalP/Human Protein Atlas Secretome) were removed (Suppl. Table 4). Of these, 15 proteins were uniquely identified in the TSC secretome: functions associated with embryo development and implantation processes are highlighted (*). Relative abundance of xxxx in endometrial EVs, TSC secretome and TSC secretome in response to EV treatment based on precursor ion intensity (left panel) (E). Western blot analysis of each sample using anti-xxxx antibodies (right panel) (E).

Figure 8

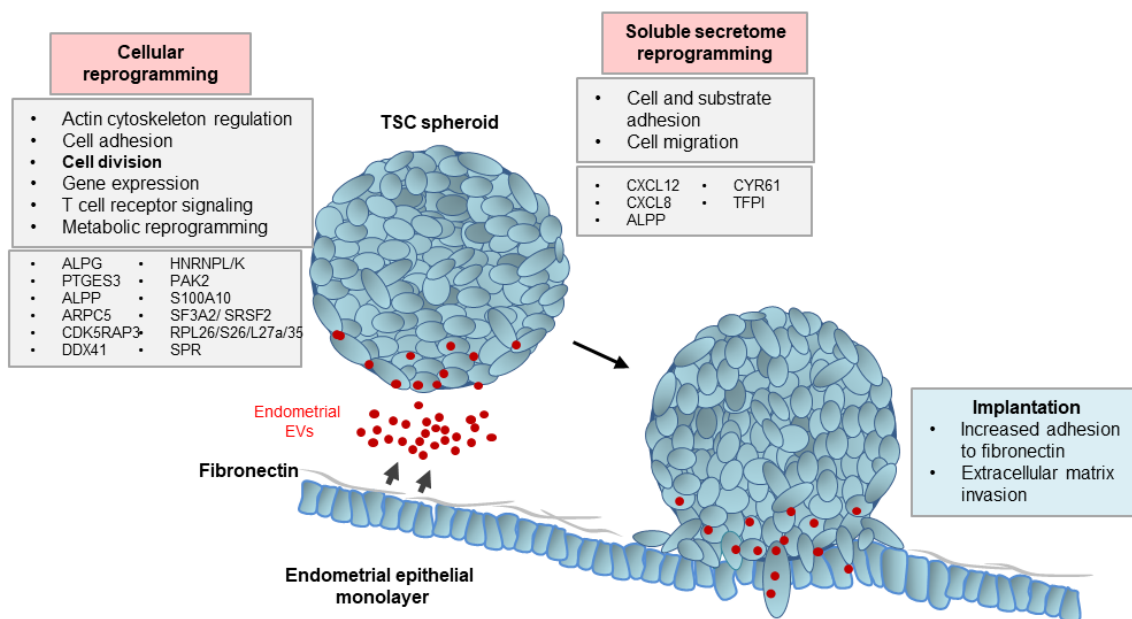


Figure 8 – Endometrial-embryo crosstalk – working model for human implantation reprogramming. A human trophoctoderm-based *in vitro* model mimicking the human embryo to recapitulate important functional aspects of blastocyst implantation (pre-implantation embryo), used to investigate molecular mechanism of how human endometrial-derived EVs facilitate intercellular signalling, functional changes associated with cell adhesion and invasion, and the proteome landscape in the trophoctoderm. Following EV treatment, trophoctoderm proteome profiling revealed distinct cellular changes associated with cytoskeleton regulation, cell adhesion, cell division, gene expression regulation and metabolic reprogramming, while for soluble-secreted changes, cell and substrate adhesion and cell migration were defined. Thus, endometrial epithelial EVs regulate both cellular and secretome mechanisms in the trophoctoderm which collectively contribute to functional changes associated with human embryo implantation.

Tables

Table 1 – Known proteins associated with implantation identified in TSC spheroids in response to endometrial EV treatment

Protein Accession	Protein Description	Gene Name	Ratio log2 (Tsc + EVs vs Tsc)	p-val
Q6FGE5	Protein S100-A10	S100A10	17.4	3.7E-02
B5MCA4	Epithelial cell adhesion molecule	EPCAM	15.3	7.9E-06
J3QL05	Serine/arginine-rich splicing factor 2	SRSF2	15.2	2.2E-02
P35080	Profilin-2	PFN2	14.9	9.4E-165
P12429	Annexin A3	ANXA3	12.8	2.1E-156
B4DJQ8	Dipeptidyl peptidase 1	CTSC	14.5	4.37E-07
Q59H46	Integrin beta-4	ITGB4	0.5	NS
B1AK53	Espin	ESPN	0.5	NS
B4DY23	Basigin	hEMMPRIN	0.4	NS
B4DJ51	Calmodulin	CALM3	0.4	NS
Q6IB11	Membrane-associated progesterone receptor component 1	PGRMC1	0.4	NS
B4DFP1	Phosphoglucomutase-1	PGM1	1.2	2.43E-03
D2JYH4	Actin, aortic smooth muscle	ACTA2	1.2	1.71E-03
Q6NVY0	Calcyclin-binding protein	CACYBP	0.3	NS
V9HW88	Calreticulin	HEL-S-99n	0.2	NS
Q09666	Neuroblast differentiation-associated protein AHNAK	AHNAK	-1.1	NS
P08133	Annexin A6	ANXA6	-0.9	NS
E7EX44	Caldesmon	CALD1	-1.0	6.3E-03
B4DRD3	Tissue-type plasminogen activator	PLAT	-1.3	8.5E-04
AOA024RDY9	Rho guanine nucleotide exchange factor 7	ARHGEF7	-12.0	1.6E-02
AOA024ROR1	Rho guanine nucleotide exchange factor 1	ARHGEF1	-12.2	7.5E-04
Q9UEI6	Nectin-2	PVRL2	-13.1	NS
K4RH61	Matrix metalloproteinase-14	MMP14	-13.4	1.0E-07
Q14118	Dystroglycan	DAG1	-13.7	5.9E-03
Q8NE26	A disintegrin and metalloproteinase with thrombospondin motifs 1	ADAMTS1	-13.9	3.7E-180
E9PH82	Protein FAM98A	FAM98A	-14.4	4.7E-170

Table 2 – Specific proteins as cargo in endometrial EVs, and their enrichment with TSC spheroids in response to EV treatment

Protein Accession	Protein Description	Gene Name
P12429	Annexin A3	ANXA3
I3L4C2	Brain-specific angiogenesis inhibitor 1-associated protein 2	BAIAP2
Q9Y376	Calcium-binding protein 39	CAB39
A0A024R529	Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase	DAK
Q02790	Peptidyl-prolyl cis-trans isomerase FKBP4	FKBP4
A8K7D9	Importin subunit alpha	KPNA2
P35908	Keratin, type II cytoskeletal 2 epidermal	KRT2
Q9Y639	Neuroplastin	NPTN
A8K5M4	Non-specific serine/threonine protein kinase	PAK2
O75340	Programmed cell death protein 6	PDCD6
Q14651	Plastin-1	PLS1
Q9NP72	Ras-related protein Rab-18	RAB18
P62854	40S ribosomal protein S26	RPS26
Q6FHJ5	Secretory carrier-associated membrane protein 3	SCAMP3
O00186	Syntaxin-binding protein 3	STXBP3
Q8TC07	TBC1 domain family member 15	TBC1D15

Table 3 – Differentially expressed proteins from TSC spheroid soluble fraction in response to endometrial EVs

Protein Accession	Protein Description	Gene Name	Ratio log2 (Tsc soluble + EVs vs Tsc soluble)	p-val
Q6FGE5	Protein S100-A10	S100A10	18.9	3.09E-196
P48061	Stromal cell-derived factor 1	CXCL12	18.7	NS
P05187	Alkaline phosphatase, placental type	ALPP	18.6	4.55E-216
C9J4T6	C-X-C motif chemokine;Interleukin-8	CXCL8	18.5	NS
P12429	Annexin A3	ANXA3	18.3	5.63E-207
P61106	Ras-related protein Rab-14	RAB14	18.2	4.12E-09
P08133	Annexin A6	ANXA6	18.2	3.76E-46
P23229	Integrin alpha-6	ITGA6	16.5	6.09E-206
K7EP40	Claudin-7	CLDN7	16.4	3.31E-177
A5YM53	Integrin alpha-V	ITGAV	16.2	2.40E-202
P15328	Folate receptor alpha	FOLR1	15.3	1.02E-175
P10646	Tissue factor pathway inhibitor	TFPI	14.6	NS
Q6FI18	Protein CYR61	CYR61	14.6	NS
P17301	Integrin alpha-2	ITGA2	14.1	9.21E-05
Q9H5V8	CUB domain-containing protein 1	CDCP1	13.8	3.05E-05
Q59H46	Integrin beta-4	ITGB4	13.8	7.89E-186
Q9UGT4	Sushi domain-containing protein 2	SUSD2	13.7	5.35E-177
P08648	Integrin alpha-5	ITGA5	13.3	3.72E-05
P35221	Catenin alpha-1	CTNNA1	3.7	1.76E-19
B4DY23	Basigin	hEMMPRIN	2.3	2.68E-10
B4DJ51	Calmodulin	CALM3	1.8	2.80E-07
P14923	Junction plakoglobin	JUP	1.5	1.58E-05
P05556	Integrin beta-1	ITGB1	1.5	5.04E-05
Q13308	Inactive tyrosine-protein kinase 7	PTK7	-0.3	NS
Q9BUD6	Spondin-2	SPON2	-1.0	7.51E-03
Q6I9S7	C-X-C motif chemokine 5	CXCL5	-1.2	1.02E-03
P09341	Growth-regulated alpha protein	CXCL1	-1.5	7.37E-05
Q8NE26	A disintegrin and metalloproteinase with thrombospondin motifs 1	ADAMTS1	-1.6	7.91E-05
Q14118	Dystroglycan	DAG1	-1.8	1.13E-05
Q9UEI6	Nectin-2	PVRL2	-15.2	1.26E-06