Human endometrial exosomes contain hormone-specific cargo modulating trophoblast adhesive capacity: insights into endometrial-embryo interactions

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ABSTRACT

Embryo implantation into receptive endometrium requires synergistic endometrial-blastocyst interactions within the uterine cavity and is essential for establishing pregnancy. We demonstrate that exosomes (40-150 nm nanovesicles) released from endometrial epithelial cells are an important component of these interactions. We defined the proteome of purified endometrial epithelial-derived exosomes (Exos) influenced by menstrual cycle hormones; estrogen (E; proliferative-phase) and estrogen+progesterone (EP; receptive-phase) and examined their potential to modify trophoblast function. E-/EP-Exos were uniquely enriched with 254 and 126 proteins, respectively, with 35% newly identified proteins not previously reported in exosome databases. Importantly, EP-Exos protein cargo was related to fundamental changes in implantation: adhesion, migration, invasion, and extracellular matrix remodeling. These findings from hormonally-treated ECC1 endometrial cancer cells were validated in human primary uterine epithelial cell-derived exosomes. Functionally, exosomes were internalized by human trophoblast cells and enhanced their adhesive capacity; a response mediated partially through active focal adhesion kinase (FAK) signaling. Thus, exosomes contribute to the endometrial-embryo interactions within the human uterine microenvironment essential for successful implantation.

INTRODUCTION

Initiation of human pregnancy requires synergistic interaction between the endometrium and the blastocyst, both immediately prior to and during implantation [1,2]. The endometrium is a complex dynamic tissue that undergoes cyclical remodeling and differentiation throughout a women's reproductive life. Each menstrual cycle consists of proliferative (non-receptive) and secretory (receptive) phases, regulated by the steroid hormones, estrogen and progesterone, respectively. The development of the endometrium that is receptive to embryo implantation occurs during the mid-secretory phase of the menstrual cycle and depends upon adequate secretory transformation of the estrogen-primed endometrium in response to progesterone [3-5]. This transformation facilitates cellular and molecular regulation of cell-cell communication, cytoskeletal/extracellular matrix (ECM) remodeling, adhesion responses, and expression of many growth factors, cytokines and their mediators [1,6,7]. Implantation takes place within the microenvironment of the uterine cavity. The embryo enters this cavity as an unhatched blastocyst where it undergoes hatching and final preparation for implantation. This involves a series of steps: apposition, adhesion and invasion (**Fig. 1**). In particular, the trophectoderm (destined to form the maternal component of the placenta), is modulated to enable endometrial attachment. Cell-cell communication between trophectoderm and the endometrial epithelium are thus essential for embryo implantation and establishment of pregnancy.

Proteomic profiling of the endometrium has revealed many proteins important during various stages of the menstrual cycle [8-18]. Importantly, in terms of the peri-implantation microenvironment, secreted proteins of endometrial epithelial origin have been identified in uterine fluid or lavage samples during receptive and non-receptive phases of the cycle [11,12], and importantly have defined functions in embryo-maternal interaction [19].

Exosomes, small extracellular vesicles (40-150 nm) of endosomal origin, are released by many cell types including endometrial epithelial cells, trophoblast cells and cancer cells [20-24]. Exosomes are known to play essential roles in cell-cell communication [25], cell transformation [26], immune regulation [27] and tumorigenesis [28]. Exosomal content has been shown to be diverse, comprising proteins [21,29], different nucleic acids and transcripts [30,31], DNA [32], lipids [33], and metabolites [34]. More recently, exosomes have been identified in the follicular fluid of ovarian follicles, where they have been shown to contain diverse cell-derived miRNAs, and exosome- and cell-type-specific proteins, therefore suggesting a role in mediating cell communication within mammalian ovarian follicles and regulation of follicular maturation [35-37]. Trophoblast cells secrete exosomes to recruit and educate monocytes to initiate the pro-inflammatory microenvironment associated with early pregnancy [38]. Importantly, exosomes have been identified in the uterine microenvironment within human uterine fluid and shown to contain select miRNAs [24].

Given their potential in modulating intercellular communication in a local microenvironment, this study investigated the contribution of exosomes during peri-implantation to regulating endometrial-embryo interactions throughout the uterine microenvironment. In particular, it focuses on the characterization of protein cargo of human endometrial epithelial-derived exosomes in response to pregnancy hormones and its potential functions in embryo-maternal interactions. The data suggests that during pre-implantation, when the endometrium becomes receptive, specific exosomal cargo is packaged by the endometrial epithelium and delivered to trophoblast cells, enabling changes in their adhesive capacity to initiate successful implantation and hence establish a pregnancy.

EXPERIMENTAL PROCEDURES

Endometrial and primary cell isolation and culture

ECC1, a human endometrial adenocarcinoma epithelial cell line [39] were cultured and maintained in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen-GIBCO, Carlsbad, USA) supplemented with 10% fetal calf serum (FCS, Invitrogen-GIBCO), 1% (v/v) Penicillin Streptomycin (Pen/Strep) (Invitrogen-GIBCO), and incubated at 37°C with 5 % CO_2 [21]. These ECC1 cells were validated by Karyotype analysis as in [39,40] according to the ATCC guidelines [41], with allele match in STR profile 100%.

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. Primary endometrial epithelial cells (pEECs) were prepared from endometrial curettage as previously described [42]. Briefly, endometrial tissue was finely minced and digested in phosphate buffered saline (PBS, Invitrogen) solution containing DNAse (25 µg/mL, Roche, Basel, Switzerland) and collagenase type III (150 µg/mL, Sigma Aldrich, USA) in a shaking water bath (130 rpm, 37 °C) for 20 minutes. Tissue digestion was stopped by adding DMEM/F12 (Invitrogen) and the tissue digest was vacuum-filtered through 45 µm and 11 µm filters. pEECs were retrieved from the filters, centrifuged at 300*g* for 5 minutes, resuspended in culture media (DMEM/F12) supplemented with 10% charcoal-stripped FCS and 1% Pen/Strep (Invitrogen-GIBCO) and plated into 24-well plates for 2 days prior to hormonal treatment.

Hormonal treatment of endometrial epithelial cells (ECC1 and pEECs)

ECC1 cells, which most accurately resemble endometrial luminal epithelium [39,43]), were grown under hormonal regimes that mimic the proliferative and secretory phases of the menstrual cycle. Limited numbers of pEEC cells were treated similarly. ECC1 cells (1×10^6 per 150 mm^3 culture dish, total of 120 dishes/replicate) (**Fig. 2**) and/or pEECs (n = 3, 80% confluence, grown in triplicate in 24-well plate) were washed three times with PBS and cultured for 24 hours in serum-free DMEM/F12 supplemented with 0.5% insulin-transferrin-selenium (ITS) solution (Invitrogen-GIBCO), and 1% (v/v) Pen/Strep (**Fig. 2A**). After 24 hours, media were removed, replenished with serum-free media, and primed with estrogen (10^{-8} M, Sigma-Aldrich) for 24 hours. Cells were divided into two groups and treated with (E) estrogen (10^{-8} M) or (EP) estrogen (10^{-8} M) and progesterone (medroxyprogesterone-17-acetate, 10^{-7} M, Sigma-Aldrich) (**Fig. 2A**). Conditioned culture media (CM) were collected at 24 and 48 hours after hormonal treatments, and used for isolating and purifying exosomes (**Fig. 2B**). The serum-free ECC1 culture conditions were initially optimized with assessment for cell morphology, proliferation and viability.

Cell morphology, proliferation and viability assays of ECC1 cells

To ensure ECC1 cells retain the same morphology upon hormonal treatments, ECC1 cells (1×10^6) were grown in DMEM/F12 supplemented with 0.5% ITS in the presence of steroid hormones E or EP (as described) and imaged on an inverted Nikon Eclipse TE300 microscope equipped with a 10x objective (Nikon Plan Fluor) in phase-contrast mode using an attached 12.6mp digital camera (Nikon DXM1200C) (Nikon Corporation, Tokyo, Japan). Images of ECC1 cells (10 independent fields of view, 3 biological replicates) were captured and processed with Nikon Elements Imaging Software (v3.0, Nikon, Japan).

Cell viability of ECC1 cells was measured using the Trypan blue assay following 24 hour culture in DMEM/F12 containing growth conditions as specified above. Viability was expressed as percentage of viable cells from total cells and presented as mean ± SEM. Cell proliferation MTT assay (Madison, WI, USA) was used to determine ECC1 metabolic activity in different cell culture conditions. Briefly, ECC1 cells were subjected to different culture media with basal DMEM/F12 media and 1% Pen/Strep with the following conditions: i) 10% FCS, ii) 1% ITS, iii) 1% ITS and 1% bovine serum albumin (BSA), iv) 0.5% ITS, v) 0.5 % ITS/BSA with either no steroid hormones or with E or EP for 24 hours. For MTT assays, measurements were performed in triplicate and expressed as relative absorbance (570 nm) for each condition.

Isolation of crude endometrial exosomes

Conditioned medium (CM) of E- and EP treated ECC1 cells was collected and centrifuged at 500*g* for 5 minutes, 2000*g* for 10 minutes to remove floating cells and cell debris respectively. Large shed microvesicles (sMVs) was subsequently removed by centrifugation at 10,000*g* for 30 minutes, followed by ultracentrifugation at 100,000*g* for 1 hour to isolate crude exosomes [44,45] (**Fig 2B**). These exosomes will be referred to as E-Exos and EP-Exos respectively.

Purification of exosomes from ECC1 cells using OptiPrep density gradient

Briefly, a discontinuous iodixanol gradient was prepared by diluting a stock solution of OptiPrep (60% (w/v) aqueous iodixanol solution with 0.25 M sucrose/10 mM Tris, pH 7.5 to generate 40% (w/v), 20% (w/v), 10% (w/v) and 5% (w/v) iodixanol solutions, which were then layered sequentially by adding 3 mL each of 40%, 20%, 10%, and 2.5 mL of 5% iodixanol solution to a 14×89 mm polyallomer tube (Beckman Coulter). Crude exosomes

(100,000*g* pellet) were resuspended in 500 μL of 0.25 M sucrose/10 mM Tris-HCl, pH 7.5, loaded on the top of the gradient and centrifuged at 100,000*g* for 18 hour at 4°C. Twelve individual 1 mL fractions were collected (starting from top of the gradient with increasing density) and each fraction was diluted with 2 mL PBS. After centrifugation at 100,000*g* for 1 hour at 4 °C, the supernatants were discarded and pellets washed with 1 mL PBS (100,000*g* for 1 hour at 4 °C) and resuspended in 50 μL of PBS. The density of each fraction was determined using a control OptiPrep gradient loaded with 500 μl of 0.25 M sucrose/10 mM Tris, pH 7.5 run in parallel. Fractions were collected as described, serially diluted 1:10,000 with dH_2O, and iodixanol concentrations were estimated by absorbance at 244 nm using a molar extinction coefficient of 320 L $g^{-1}cm^{-1}$ [46]. Purified exosomes were derived from two biological replicates for proteomic profiling and from two biological replicates for Western immunoblotting validation. Exosomes derived from primary EEC cells were not purified using density-based fractionation due to insufficient primary material.

Exosomal protein quantification and validation

Exosome pellets isolated from ECC1 and primary EEC cells were quantified using 1D-SDS-PAGE / SYPRO Ruby protein staining densitometry, as previously described [21,45]. This method is sensitive, reproducible, has a linear quantitation range over three orders of magnitude [47], and is compatible with GeLC-MS/MS [48]. Densitometry quantitation was performed using ImageQuant software (Molecular Dynamics) to determine protein concentration relative to a BenchMark Protein Ladder standard of known protein concentration (1.7 μg/μL) (Life Technologies). In addition, Western blot analysis was used initially to determine exosome-containing fractions from the density gradient and subsequently to validate proteins identified by proteomic analysis in exosomes from both ECC1 and pEECs. Briefly, membranes (10 μg protein) were probed with primary antibodies

[mouse anti-TSG101 (BD Transduction Laboratories; 1:500), mouse anti-Alix (Cell Signaling Technology; 1:1000), rabbit anti-GAPDH (Cell Signaling Technology; 1:1000), goat anti-Collagen Type XV (Santa Cruz Biotechnology; 1:200), rabbit anti-Laminin α5 (H-160) (Santa Cruz Biotechnology; 1:200), mouse anti-FAK (BD Biosciences) (1:4000), mouse anti-FAK Tyr^{397} (BD Biosciences) (1:4000), rabbit anti-EpCAM (Abcam) (1:3000), rabbit anti-Fibronectin (Abcam)] for 3 hours at room temperature (RT) in 50 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween 20 (TTBS) followed by incubation with either IRDye 800 goat anti-mouse or goat IgG or IRDye 700 goat anti-rabbit IgG (1:15000, LI-COR Biosciences) for 1 hour at RT in TTBS. Immunoblots were visualized using the Odyssey Infrared Imaging System, (v3.0, LI-COR Biosciences, Nebraska USA).

Cryo-transmission electron microscopy (cryo-EM)

Cryo-EM imaging of exosome preparations was performed as previously described [44,45], with minor modifications. Briefly, exosomes (~2 µg protein, non-frozen samples prepared within 2 days of analysis) were transferred to glow-discharged C-flat holey carbon grids (ProSciTech Pty Ltd, Kirwan, Qld, Australia). Excess liquid was removed by blotting and grids were plunge-frozen in liquid ethane. Grids were mounted in a Gatan cryoholder (Gatan, Inc., Warrendale, PA, USA) in liquid nitrogen. Images were acquired at 300 kV using a Tecnai G2 F30 (FEI, Eidhoven, NL) in low dose mode. Size distribution of vesicles (range 40-150 nm) was calculated for 15 fields of view/sample (n=2 biological replicates).

Proteomic analysis

Exosome pellets (10 µg protein) for individual biological replicates of both E-Exos and EP-Exos were lysed in SDS sample buffer, electrophoresed by 4-12% Bis-Tris SDS-PAGE (approximately 20 mm into the gel) and visualized by Imperial Protein Stain (Thermo Fisher

Scientific). Individual samples were excised and destained (50 mM ammonium bicarbonate/acetonitrile), reduced (10 mM DTT (Calbiochem) for 30 minutes), alkylated (50 mM iodoacetic acid (Fluka) for 30 minutes) and trypsinized (0.6 μg trypsin (Promega Sequencing Grade) for 16 hours at 37°C) as described [49]. For all samples, peptides were desalted using reverse-phase C18 StageTips [50], 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 5μm 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- minute 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/minute.

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. For each biological replicate of E- and EP-Exos technical replicates were performed and data acquired using Xcalibur software v2.1 (Thermo Fisher Scientific). Raw mass spectrometry

253 data is deposited in the PeptideAtlas and can be accessed at
254 http://www.peptideatlas.org/PASS/PASS00713 [51-53].
255
256 ***Database searching and protein identification***
257 Raw data were processed using Proteome Discoverer (v1.4.0.288, Thermo Fisher Scientific).
258 MS2 spectra were searched with Mascot (Matrix Science, London, UK; v 1.4.0.288), Sequest
259 (Thermo Fisher Scientific, San Jose, CA, v 1.4.0.288), and X! Tandem (v 2010.12.01.1)
260 against a database of 178,618 ORFs (UniProt Human+Bovine, 2015_06). Peptide lists were
261 generated from a tryptic digestion with up to two missed cleavages, carbamidomethylation of
262 cysteine as fixed modifications, and oxidation of methionine and protein N-terminal
263 acetylation as variable modifications. Precursor mass tolerance was 10 ppm, product ions
264 were searched at 0.6 Da tolerances, minimum peptide length defined at 6, maximum peptide
265 length 144, and max delta CN 0.05. Peptide spectral matches (PSM) were validated using
266 Percolator based on q-values at a 1% false discovery rate (FDR) [54,55]. With Proteome
267 Discoverer, peptide identifications were grouped into proteins according to the law of
268 parsimony and filtered to 1% FDR [56]. Scaffold (Proteome Software Inc., Portland, OR, v
269 4.3.4) was employed to validate MS/MS-based peptide and protein identifications from
270 database searching. Initial peptide identifications were accepted if they could be established
271 at greater than 95% probability (PEP 5%) as specified by the Peptide Prophet algorithm [57].
272 Protein probabilities were assigned by the Protein Prophet algorithm [56]. Protein
273 identifications were accepted, if they reached greater than 99% probability and contained at
274 least 2 identified unique peptides. These identification criteria typically established <1% false
275 discovery rate based on a decoy database search strategy at the protein level. Proteins that
276 contained similar peptides and could not be differentiated based on MS/MS analysis alone,
277 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. Comparisons with exosome database ExoCarta [58] were performed. This database contains curated exosomal protein information from diverse cell types and body fluids (June-2015, 146 studies).

Semi-quantitative label-free spectral counting

Significant spectral count normalized (Nsc) and fold change ratios (Rsc) were determined as previously described [21,29,44,45]. 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 were corrected for multiple testing using the Benjamini-Hochberg procedure [59] and statistics performed as previously described [44].

Exosome labelling and live cell uptake

E-Exos and EP-Exos purified from ECC1 cells (500 µg/mL) were labelled with 1 µM Vybrant DiI, or DiD lipophilic dye (Invitrogen-GIBCO) and incubated at 37 °C for 10 minutes. Excess dye was removed by washing with PBS and ultracentrifugation at 100,000*g*

for 70 minutes at 4 °C. DiI- or DiD-labelled exosomes were resuspended in 0.2 mL PBS and overlaid on a 4 mL 30% sucrose cushion (300 g/L sucrose, 24 g/L Tris base, pH 7.4) and ultracentrifuged at 100,000*g*, for 70 minutes at 4 °C. Labelled exosomes were harvested at the PBS-sucrose interface and pelleted and stored at –80°C (maximum of 4 weeks) for downstream experiments including uptake assays.

First trimester human HTR8 trophoblast cells [60] (2×10^5) were plated in 8-well Ibidi chamber slides (Ibidi, Martinsried, Germany) and cultured for 24 hours prior to live cell imaging using a DeltaVision Widefield microscope (GE Life Sciences, Uppsala, Sweden) with a 60x 1.42 NA objective, using Optical Axis Integration (OAI) scans over a z-range of 10 μm. A single plane brightfield image was recorded as the reference point. DiI-labelled exosomes (10 μg) were added immediately prior to image acquisition for 4 hours with individual images obtained every 10 minutes. Three different positions were recorded per well with three sample repeats for each biological condition. Image processing and analysis were performed using Imaris (Bitplane AG). For DiI-labelled exosome uptake quantification, mean DiI fluorescence intensity was determined per time point and field-of-view and normalized to the respective mean auto-fluorescence intensity in the green channel to compensate for cells moving in and out of the field of view during the time-course. Normalized fluorescence intensities are presented as mean ± SEM. To obtain 3D images of exosome accumulation within HTR8 cells, DiD-labelled exosomes were added to HTR8 cells and incubated at 37 °C for 4 hours, followed by addition of wheat germ agglutinin (WGA) Alexa594 as a red fluorescent membrane marker (2.5 μg/mL, Life Technologies, USA) and incubated at 37 °C for 6 minutes. Cells were replenished with new media and z-stack images were immediately recorded using a z-step size of 0.5 μm. For image visualization, 3D data sets were de-convolved using SoftWoRx (Applied Precision, GE Healthcare, UK) and

displayed as maximum intensity projections in xy/xz/yz planes. For z-stack image presentation and visualization, WGA is displayed in green and DiD in red.

Exosome functional real-time adhesion assay

Adhesion assays were performed using the xCELLigence system (ACEA Biosciences, San Diego, CA, USA). This system enables real-time measurement of cell adhesion based on cell interaction with microplate electrodes, resulting in electrical impedance, expressed as Cell Index (CI). Adhesion of cells was monitored every 15 seconds for 4 hours via the incorporated sensor electrodes, which measure the change in impedance caused by cell attachment and spreading. To initiate the experiment, a background reading of the microplate was taken as threshold CI. HTR8 cells (2×10^4) were added to each well of an E-Plate 96 microplate (ACEA Biosciences) in the presence or absence of E-Exos or EP-Exos exosomes (50 μg/mL, each in triplicate) and cell adhesion measured. Data were evaluated using RTCA-integrated software, normalizing each data point on the CI obtained at the last time point of adhesion to background CI prior to adding HTR8 cells and exosomes. Data are presented as relative cell index over a 4 hour recording as mean ± SEM (n=3 biological analyses).

Exosome co-culture and cell lysate preparation

To define the effect of ECC1-derived exosomes in response to hormonal treatment on human HTR8 trophoblasts cells (2×10^5, 6 well plate) were cultured either without exosomes (control) or with E-Exos or EP-Exos (50 μg/mL, each in triplicate) for 24 hours. Following incubation, cells were washed with ice-cold PBS and lysed on ice 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). Lysates were subjected to ultracentrifugation for 30 minutes (386,000*g*, TLA-100 rotor, Beckman Coulter), and supernatants aspirated for quantitation. Cellular lysates (15 μg) were

separated by SDS-PAGE, transferred onto NC membranes and monitored by Western blotting. Densitometric analysis was carried out using Image Studio (v5, LI-COR) and normalized by loading control (GAPDH). Independent exosome co-culture experiments were performed in biological triplicate, each with technical triplicate, and results expressed as mean ± SEM for individual experiments.

Statistical analysis

Statistical analyses were performed to assess differences in adhesion response of HTR8 cells treated with exosomes (E-Exos or EP-Exos) or without exosomes over 4 hours. Analysis of variance (ANOVA) and Tukeys 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. All analyses were calculated using GraphPad Prism (v6.05) software. Unless otherwise stated, data are presented as mean ± SEM (n = 3 biological replicates, 3 technical replicates), with $*p<0.05$ and $**p<0.01$ considered statistically significant.

RESULTS

Exosomal research has grown exponentially in recent years, and although endometrial exosomes have been identified in the uterine cavity of women [24], it is unknown whether they are hormonally regulated to influence the developing blastocyst and initiate implantation. The human ECC1 cell line, which most accurately represents luminal epithelial cells, were treated with estrogen (E) or estrogen and progesterone (EP), and the conditioned media used to isolate and purify exosomes (E-Exos and EP-Exos) and to characterize their protein cargo (**Fig. 2 A&B)**. To attest for the ECC1 cell line culture conditions prior to exosome isolation; cell morphology, viability and proliferation were examined in the presence of menstrual cycle hormones (E, 10^{-8} M, and P, 10^{-7} M). Crude exosomes of E-Exos yielded 64 μg/10^6 cells and EP-Exos yielded 58 μg/10^6 cells (representative one of three similar biological replicates) suggesting that exosomes were released in numbers independent of hormonal regulation. ECC1 cells exhibited and retained their epithelial-like morphology, despite hormonal treatment (**Fig. S1**). Additionally, cell viability and proliferation (**Fig. S2&3**) and protein yield (**Fig. S4**) were not affected by either E or EP treatments. Consequently, culture medium supplemented with 0.5% ITS was used to culture ECC1 cells in the presence of hormonal treatments, for subsequent exosome isolation (**Fig. 2A**).

Validation of exosomes isolated from ECC1 and primary EEC cells

An OptiPrep density gradient was used to further purify E-Exos and EP-Exos. This purification showed that exosomes were enriched within fraction 7/8 (buoyant density 1.09-1.11 g/mL) and were positive for exosomal markers Alix and TSG101 (**Fig. 2C**). To ensure exosomes were within the expected extracellular vesicle size range, cryo-electron microscopy (cryo-EM) was used to analyze purified E-Exos and EP-Exos. Cryo-EM revealed that both E-Exos and EP-Exos contained a relatively homogenous population of round membranous

vesicles 40-150 nm in size (representative of 15 fields of view from two biological replicates), which in accordance with the typical size reported for exosomes (**Fig. 2D**) [44,45,61]. Importantly, almost all the vesicles in both E and EP samples are less than 150 nm.

Hormonal treatment reprograms endometrial-derived exosome cargo

Proteomic profiling was performed to characterize the protein cargo of hormonally-regulated endometrial-derived purified exosomes (**Fig. 3A**). A total of 1073 protein cargos were identified, comprising 917 and 786 in E-Exos and EP-Exos respectively (**Fig. 3B** and **Table S1**). From this analysis, 254 protein cargos were uniquely packaged within E-Exos while 126 were within EP-Exos. Of the 663 proteins common to both E-Exos and EP-Exos, many are involved in exosome biogenesis (including proteins involved in the endosomal sorting complex required for transport (ESCRT) machinery such as the vacuolar protein sorting associated protein family and tetraspanin (**Table 1**). Protein cargos required for exosome sorting, trafficking, release, recognition and uptake were found in both E-Exos and EP-Exos (**Table 1**), a feature common to all exosomes in other tissues and biofluids [62]. Based on normalized spectral count ratios (Rsc), our dataset showed a range of relative differential protein expression levels that were up- and down- regulated in response to EP (**Fig. 3C**).

Further analysis and comparison of this dataset with that of publicly available exosomal database, ExoCarta [58], 71 % (473/663 proteins) of the cargo was found in common with previously reported exosome-associated proteins. Importantly, 189 of the 663 proteins from this study are unique to endometrial epithelial exosomes and have not been identified in any published exosome proteomic databases (**Table S2**). We also identified several important enzyme proteins within exosomes, including various ligases, oxidoreductases, transferases,

lyases, isomerases, phosphatases, kinases, metalloproteinases, and hydrolases (**Table S3**), representing an extensive identification of various differentially expressed enzymes (fold change ± 1.5) important for embryonic development and implantation.

Regulation of proteins associated with cytoskeletal reorganization and signaling in exosomes in response to estrogen

Estrogen is known to play a key role in endometrial restoration following menstruation. We examined exosome protein cargo in response to E, and noted key differences attributed to cytoskeletal reorganization, microtubule/actin networks, and various signaling cascades involved in regulating cytoskeletal rearrangements, cell migration, cell adhesion and cell spreading (**Table S1**). Cytoskeletal elements [63] including microtubule network (TUBA1C, TUBA4A, PAFAH1B1, DCTN1), actin network (ARPC2, CALD1), actin-myosin binding (CDM), and actin cytoskeleton reorganization (CDK5, CDK2, RAP2A) were uniquely enriched in E-Exos. Of particular interest, were proteins involved in cytoskeletal reorganization, adhesion, and cell signaling, including Abl interactor 1 (ABI1), Ras-related protein Rap-2a (RAP2A), and MAP kinase 1 (MAPK1).

Regulation of proteins associated with organization of ECM architecture in exosomes in response to estrogen plus progesterone

Proteins identified in exosomes in response to EP treatment have significantly enriched functions attributed to cell adhesion, attachment, migration and organization of ECM architecture, in accord with physiological changes in both the endometrium and the blastocyst during the secretory phase of the cycle (**Table 2**). These include the basement membrane molecules, member of the laminin family (Rsc range from 6.1 to 20.7), the collagen family (Rsc range 3.6 to 8.0) and cell adhesion and migration components (**Table 2**).

Given that members of the laminin and collagen family were prominently displayed as protein cargo, LAMA5 and COL15A1 were selected for validation. Representative immunoblotting of E-Exos and EP-Exos showed expression of both LAMA5 and COL15A1 as protein cargo (**Fig. 4A**). The intensity of the bands for laminin and collagen was stronger in EP-Exos compared to E-Exos samples, in accordance with spectral counts from proteomic profiling (**Table 2**) and reflected in densitometric analysis of the Western blots (**Fig. 4A**). To ensure the finding were true and consistent, LAMA5 and COL15A1 were also validated using exosomes derived from primary human endometrial epithelial cell (pEECs) treated with E and EP (**Fig. 4B**). Both exosomal proteins were elevated when the cells of origin had been exposed to EP. As expected, pEECs exosomes also package both LAMA5 and COL15A1, more prominently in EP-pEECs, validating the findings from the cell line used. Densitometric analysis of the bands also indicated higher level of LAMA5 in EP-Exos derived from ECC1 cells compared to pEECs.

Exosomes enriched for molecules involved in embryo implantation

In a comparative analysis of our current dataset to that of published studies on proteins essential for embryo implantation [5,10,13,24,43,64,65], we found at least 14 proteins of interest (**Table 3**). These are unique and selective for exosomal vesicles and are packaged predominantly in EP-Exos samples representing in vivo progesterone regulation during endometrial receptive phase (**Table 3**). Of interest to the developmental progress of pregnancy is that we identified complement decay-accelerating factor (CD55, Rsc 7.1), perlecan (HSPG2, Rsc 5.9) and EGFR (Rsc 5.1) as exosomal protein cargo. These molecules are highly regulated at the time of blastocyst apposition and attachment [66-73].

Insights into embryo development, view from cancer-associated proteins in exosomes

Embryo implantation and placental development use similar cellular mechanisms to that of cancer cells to migrate and invade [74]. We therefore compared our dataset with those proteins derived from quite different cancer exosomes and identified 23 common proteins (**Table S4**). These proteins included members of the Wnt pathway, cell surface receptors EGFR, extracellular matrix proteins, tenascin and matrix metalloproteinases.

Endometrial exosomes are internalized by HTR8 trophoblast cells in vitro and enhance their adhesive capacity

The functional activity of endometrial-derived exosomes was investigated using fluorescently labeled E-Exos and EP-Exos, added to HTR8 trophoblast cells and monitored using live cell imaging. Real-time recording showed accumulation of exosomes occurred over a 4 hour period with significant increase in fluorescence intensity seen in HTR8 trophoblast cells exposed to EP-Exos and E-Exos compared to mock control from 2.5 hr to 4 hr (**Fig. 5A**). Microscopy images confirmed cellular uptake and accumulation of E-Exos and EP-Exos exosomes in trophoblast cells (**Fig. 5B**), however there was no difference in the level of fluorescence intensity. 3D microscopy and visualization highlighted the co-localization of exosomes inside HTR8 cells with E-Exos (**Fig. 5C**) and EP-Exos (**Fig. 5D**).

To investigate whether exosomes transport functionally active cargo, the adhesive capacity of HTR8 cells was examined in the absence or presence of E-Exos and EP-Exos. Adhesion was monitored by the xCELLigence system and showed that EP-Exos induced a very rapid increase (by 1 hour) in the adhesive capacity of HTR8 cells reaching a maximum at 1.5 hours and then being maintained until 4 hours (experimental endpoint). This increased adhesion was significantly greater following uptake of EP-Exos ($p<0.001$), compared to E-Exos. However, both exosome preparations significantly stimulated adhesion ($p<0.001$) compared

with cells not exposed to exosomes (**Fig. 5E**). The EP-Exos are representative of the physiological condition at the time of implantation and this stimulation of HTR8 adhesion is in accord with the proteomic data on their adhesion molecule content (**Table 2**).

To determine a possible pathway leading to adhesion response in HTR8 cells following co-culture with exosomes, selected adhesion markers including focal adhesion kinase (FAK), phosphorylated FAK (Tyr^{397}), fibronectin, and EpCAM were analyzed (**Fig. 5F&G, Fig. S5**). A significant increase in levels of FAK (E-Exos: 3.2-fold, EP-Exos: 4.2-fold), FAK-Tyr^{397} (E-Exos: 1.6-fold, EP-Exos: 2-fold), and fibronectin (E-Exos: 1.2-fold, EP-Exos: 1.4-fold) (**Fig. 5G**) was clearly demonstrated. Further, EP-Exos induced a significantly greater increase in expression of FAK and FAK-Tyr^{397} in comparison to E-Exos ($p<0.01$).

DISCUSSION

This study demonstrated that the cargo contained within human endometrial epithelial exosomes is hormonally regulated in accord with the phases of the menstrual cycle and that these exosomes can be taken up and release this cargo to functionally enhance trophoblast adhesive capacity during implantation. The findings provide key insights into endometrial-embryos communication during pre-implantation events, offering new understanding to these processes.

This study has utilized a comprehensive exosome isolation, purification, and characterization strategy to address the issue of vesicle heterogeneity. Vesicle annotation is an ongoing problem that has plagued the field over the past decade with varying categories and names describing the different EV subtypes – this polemic has led to international efforts to standardize nomenclature and the quest for specific protein markers to distinguish one EV subtype from the other [75] (Journal of Extracellular Vesicles, www.journalofextracellularvesicles.net). For a position paper on standardization procedures for EV purification see Witwer et al. [76]. As such, we have characterized exosomes in this study based on their specific buoyant density, marker proteins Alix and TSG101, vesicle diameter using cryo-transmission electron microscopy, in addition to their protein cargo using proteomic profiling.

Our proteomic profiling of E-Exos and EP-Exos has identified and confirmed that exosomes contain basic machinery important to their biogenesis, trafficking and release, supporting our previous reports [21,29]. Of interest, this study presents a new set of protein cargo that is unique to human endometrial epithelial exosomes, which has not previously been reported in any studies. Enrichment of exosomes cargo was validated in both ECC1 cells and primary

endometrial epithelial cells, supporting the experimental design and appropriate selection of cell models. ECC1-derived and primary cell-derived exosome cargo are similarly regulated by estrogen and progesterone, with significant enrichment of components associated with cell-matrix and adhesion during the hormonal equivalent of the receptive-phase for implantation. It should also be noted that select protein vesicular cargo could be released by both luminal and glandular epithelial cells obtained from primary tissue. Thus further characterization of such cargo is warranted to confirm protein validity between cell line-derived and primary-derived material. However, given that endometrial exosomal research is still in its infancy, this report represents the only definitive exosome protein cargo resource to date, in the context of receptivity and embryonic implantation.

The cargo identified in this present study highlights some key proteins found higher in EP-Exos compared to E-Exos and may have specific role in embryo implantation. These are fibulin1 (FBLN1, Rsc 8.9), cysteine-rich 61 (CYR61, Rsc 7.0), complement decay-accelerating factor (CD55, 7.0), heparan sulphate proteoglycan 2 (HSPG2, Rsc 5.9). Fibulin-1 (FBLN1), a secreted glycoprotein associated with ECM remodeling, cell adhesion and migration, and basement membrane interaction [77] , is 8.9 fold higher in EP-Exos compared to E-Exos. Since fibulin-1 has been shown highly regulated during the differentiation stage of the menstrual cycle, implantation and pregnancy [78], this finding further validates our data and suggests that fibulin-1 could be a key molecule involved in embryo implantation. HSPG2 and CD55 are both essential for embryo implantation. Previous studies [79,80] demonstrated that the presence of HSPG2 in uterine epithelium facilitate trophoblast attachment and adhesion. CD55 has been shown with high gene and protein level during the window of implantation [81,82], and our data indicates that CD55 is being packaged in endometrial exosomes: this indicates its specificity and is worthy of further investigation.

The presence of cysteine-rich 61 (CYR61 Rsc 7.1) maximally in EP-Exos, suggests its importance. CYR61 is known in trophoblasts during placental development where it appears to promote uterine vessel growth toward the embryo [83]. Further, a global differential gene expression analysis of luminal epithelium at mouse implantation sites revealed that the *CYR61* gene is upregulated at the early-phase implantation site, specifically during embryo attachment but not in inter-implantation sites and or under delayed implantation, suggesting a direct role in mediating embryonic-uterine signaling [84]. Collectively, these data suggest that CYR61 is present in luminal epithelium both at the gene and protein level and released into the implantation microenvironment in exosomes, suggesting possible involvement in endometrial-embryo crosstalk by this route.

On further analysis of our dataset, we observed an increase in exosomal protein components in response to EP that are important in cell-adhesion and cell-cell signaling activity. These include the receptor proteins CELSR2 (Rsc 5.1), adhesion receptor CD47 (Rsc 4.1), CLDN3 (Rsc 4.1) and PARVA (Rsc 1.7), all of which are implicated in cell polarity. Additionally, ADAMTS15 (Rsc 6.1), ADAM10 (Rsc 1.4) have proteolytic activity and may regulate the adhesion response [85]. Further, we report enriched expression of ANPEP (Rsc 1.4), and DPP3 (Rsc 1.4) in EP-Exos. ANPEP is associated with proteolytic cleavage during angiogenesis, and suggested to modify the endometrial microenvironment [86].

Members of the integrin family are essential for endometrium-embryo communication and implantation [87-89]. Endometrial exosomes contain a number of integrins with differential sorting in response to both estrogen and progesterone. These integrins include ITGA6 (Rsc 1.5), ITGB1 (Rsc 1.4), ITGB4 (Rsc 1.3), ITGB3 (Rsc -3.4), ITGA2 (Rsc -2.8). This is line

with previous studies suggesting that integrin expression is hormone-dependent [88]. The present study provides new knowledge that several members of the integrin family are selectively packaged within endometrial exosomes. They may be important for exosome 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 [90,91]. Certainly, microvesicles, of which exosomes are the smallest, are known to contain functional membrane proteins associated with membrane lipid rafts (such as tissue factor and chemokines receptor CCR5) which are subsequently incorporated into membranes of other cells [92,93].

The common protein cargos identified between E-Exos and EP-Exos are mostly involved in vesicle trafficking, sorting, release and extracellular matrix reorganization. These are IST1, SNX6, and CDK1, where IST1 acts a key modulator of cargo protein sorting [94,95]. Sorting nexin-6 (SNX6) is involved in intracellular vesicle trafficking and plays a key transport link between cytoplasmic transport vesicles and dynactin. Cyclin-dependent kinase 1 (CDK1), is a key mitotic kinase importance for cell cycling and regeneration of proliferative phase endometrium and for embryonic survival at the pre-implantation stage [96]. ABI1 is another protein cargo involved in cytoskeletal reorganization and interacts with EGFR signaling pathway to regulate cell motility and proliferation [97,98]. Endosomal-derived RAP2A GTPase is part of several signaling cascades thought to regulate cytoskeletal rearrangements, cell migration, adhesion and invasion by up-regulating p-Akt [99,100]. MAPK1 is a serine/threonine kinase that mediates cell adhesion, proliferation, cell cycle progression, cell survival and transcription [101]. The identification of these molecules in our exosomes highlight the dynamic signaling pathways within the uterine microenvironment in preparation for embryo implantation and development.

In contrast, a number of factors with adhesion-related properties including galectins (Gal-1, Rsc -2.8) and Gal-3, Rsc -3.4) were identified at reduced concentrations in exosomes in response to EP. Galectins bind galactosides and have differing roles, including cell-adhesion and invasion of trophoblast and regulation of endometrial receptivity [102,103]. Recently Gal-1 has been shown to influence trophoblast immune evasion, regulating human leukocyte antigen G on expression on trophoblast cells [102]. Both Gal-1 and Gal-3 contribute to the organization of ECM and the regulation of cell motility in first trimester placental tissue, and in cell cultures derived from placental and decidual tissue [104].

Changes in adhesion of trophoblast have previously been functionally identified but without consideration of their control mechanisms [105,106]. It is known that exosomes may selectively transfer certain proteins and other cargo to recipient cells resulting in selective downstream functional effects [107-109]. The present study demonstrated that exosomes derived from receptive phase endometrium in the context of peri-implantation, can alter trophoblast behaviour to favor the functional adhesive response needed for implantation. In addition, we demonstrated that this is mediated at least in part via the FAK pathway, as evident by an increase of FAK protein expression and phosphorylation of FAK ($pTyr^{397}$) [110] in trophoblast cells after 4 hr co-culture with endometrial exosomes (Fig. 5). FAK is activated and localized at focal adhesions upon cell adhesion to the ECM through integrin binding, triggering phosphorylation of FAK-Tyr^{397} [111], thus implicating that integrin binding is among the actions of exosomal cargo on adhesion. However, given that these exosomes also contain specific miRNAs [24] and probably RNAs and lipids, further investigations are required to define precisely which components of the exosomal cargo are

most important and how the fate of trophectodermal cells may be dictated by these cell-specific exosomes and their cargo.

At this time, although the biological effects of exosomal transfer can be profound, the mechanisms of exosome uptake by cells and how their protein cargo is utilized by cells, are not known [112]. In addition, direct evidence for a causal relationship between specific exosomal molecular cargo and function is rare. Some studies have shown that cancer-derived exosomes have TGF-β1 at their surface and are tethered by its receptor betaglycan. However, their delivery requires an intact heparan sulphate side chain: this highlights a different form of vesicular transport to that of soluble forms [113]. TGFβ1 is also responsible for controlling immune responses in cells to which it is delivered from vesicles [114,115]. Exosomes also deliver mRNA that is subsequently translated to protein in recipient cells [31,116] and their miRNA cargo can regulate the recipient cell's transcriptome [117-119]. It is clear that the concept of 'pre-metastatic niche' formation, whereby secreted signals and extracellular vesicles from both tumour and host cells can promote cancerous growth and metastasis [120], can be applied to preparation of the blastocyst for its 'landing' on and subsequent invasion of the endometrium to establish pregnancy. Similarly, exosomes from one endometrial epithelial cell could act to prepare other endometrial cells in the vicinity for implantation, strongly highlighting the critical role of exosomes in cell-cell communications within uterine microenvironment prior to embryo implantation and placentation.

This study has therefore provided a new insight into the regulation of human embryo implantation, demonstrating a contribution of endometrial-derived exosomes to endometrial-embryo crosstalk. The protein cargo of endometrial exosomes is substantially regulated by estrogen and progesterone, as physiologically relevant to the proliferative and secretory

phases of the menstrual cycle respectively. Importantly, endometrial exosomes contain a number of unique proteins not previously identified in exosomes from any other tissue, biofluid, or indeed cell model. Significantly, our study shows that endometrial exosomes within the extracellular microenvironment during the peri-implantation period are capable of regulating trophectodermal cell adhesiveness partially through active focal adhesion kinase (FAK) signaling. Endometrial exosomes may provide a means for improving pre-implantation embryo developmental potential, and thus become targets for improving receptivity, implantation success, fertility, and pregnancy outcomes, hence reducing the considerable personal and financial cost of infertility.

COMPETING INTERESTS

The authors declare no competing or financial interests.

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FIGURE LEGENDS

Figure 1. Endometrial-derived exosome remodeling during embryo implantation. The embryo enters the human uterus approximately 4 days after fertilization, generally as an unhatched blastocyst, where it further develops until it attaches to and invades the endometrial epithelium several days later. Soluble-secreted factors and exosomes (SS/Exos) are released by the endometrium into the uterine microenvironment and influence the blastocyst/trophectoderm enabling the blastocyst to undergo apposition, attachment and invasion through the endometrial epithelium. GE (glandular epithelium) and LE (luminal epithelium).

Figure 2. Exosome isolation and purification from hormonally-treated ECC1 cells. (A) Experimental workflow for hormonal treatments of ECC1 cells and culture medium collection. (B) Differential ultracentrifugation of culture medium for exosome isolation and purification. (C) Western blotting of E-Exos and EP-Exos probed with exosome markers Alix and TSG101. (D) Cryo-electron microscopy of purified exosomes revealing textured round vesicles (40-150 nm). Scale bar, 100nm. CM: conditioned medium, ITS: insulin-transferrin-sodium selenium.

Figure 3. Proteomic profiling of hormonally-treated ECC1 cell-derived exosomes. (A) Biological replicates of E- and EP-treated ECC1 purified exosomes separated by 1D-SDS-PAGE (10 µg). Individual gel slices were excised, subjected further processing an analysis as shown. (B) Two-way Venn diagram of E- and EP-treated exosomes (C) Normalized differential protein expression (Rsc, ratio of spectral counts) ranked as maximal difference between EP and E exosomes (fold-change >2, p-value <0.05).

Figure 4. Validation of ECC1 cell- and primary endometrial epithelial cell-derived exosomes. Representative Western blots of exosomal markers Alix, TSG101 and cytoskeletal components LAMA5 and COL15A1 for (A) ECC1 cell-derived exosomes (n = 2, E-Exos and EP-Exos, 10 µg) and (B) Primary human endometrial epithelial cell-derived exosomes (n=3, E-Exos and EP-Exos). Densitometric analysis was performed using ImageJ v1.44, showing mean ± SEM with $^{**}p<0.01$ considered statistically significant.

Figure 5. *In vitro* uptake and functional effects of ECC1-derived exosomes on trophoblast cells. (A) Accumulation of fluorescence intensity of DiI-labelled E-Exos and EP-Exos in human HTR8 trophoblast recipient cells over 4 hours of live recording (B) Microscopic images of HTR8 in the absence (top panel) and presence of exosomes (middle and bottom panel). Arrow heads: DiI-labelled exosomes within HTR8 cells. Scale bar, 20 µm. (C, D) Representative 3D images (xy/yz/xz) show co-localization of exosomes (red) within HTR8 cells (green) in the presence of E-Exos (C) and EP-Exos (D). Scale bar, 5 µm. (E) Representative real-time measurements of HTR8 cell adhesion over 4 hours in absence (black) and presence of E-Exos (dark grey) and EP-Exos (grey) using xCELLigence RTCA instrument. Representative data is presented as mean ± SEM, n = 3, showing normalized fluorescence intensity (Fig. 5A) or relative cell index (Fig. 5E). $^{*}p<0.005$, $^{**}p<0.001$, $^{***}p < 0.0001$ are considered statistically significant. (F) Western blots of downstream target proteins (FAK, phosphorylated FAK, fibronectin and EpCAM) in HTR8 cells after 4 hours cultured with (50 µg/mL) or without E-Exos or EP-Exos. (G) Densitometric analyses of normalized Western blots in (F). Data is presented as relative intensity (mean ± SEM, n = 3 biological replicate) for each time point and target protein; $^{*}p<0.05$, $^{**}p<0.01$ are considered significant.

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Table 1. Relative quantification of selected exosome proteins involved in biogenesis, sorting/trafficking/release, and recognition/uptake by label-free spectral counting

	Uniprot Acc[a]	Gene Name[a]	Protein Description[a]	E-Exos[b]	EP-Exos[c]	ExoCarta[d]
Exosome Biogenesis						
ESCRT-associated	Q96QK1	VPS35	Vacuolar protein sorting-associated protein 35	27	9	Y
	O75436	VPS26A	Vacuolar protein sorting-associated protein 26A	11	4	Y
	Q99816	TSG101	Tumor susceptibility gene 101 protein	11	6	Y
	A0A024RB16	FAM62A	Family with sequence similarity 62	6	4	N
	Q9NP79	VTA1	Vacuolar protein sorting-associated protein VTA1 homolog	6	4	Y
	Q9NUQ9	FAM49B	Protein FAM49B (L1)	5	4	Y
	Q8WUM4	PDCD6IP	Programmed cell death 6-interacting protein	74	69	Y
	Q96TA1	FAM129B	Niban-like protein 1	29	28	Y
	P53990	IST1	IST1 homolog (hIST1)	5	4	N
	O75351	VPS4B	Vacuolar protein sorting-associated protein 4B	8	8	Y
	Q9UK41	VPS28	Vacuolar protein sorting-associated protein 28	3	4	Y
Tetraspanin	H7BXY6	TSPAN14	Tetraspanin 14	8	8	Y
	A6NNI4	CD9	Tetraspanin	12	12	Y
	Q9HCN3	TMEM8A	Transmembrane protein 8A	3	4	N
Sorting/Trafficking & Release						
GTPase	P61026	RAB10	Ras-related protein Rab-10	32	32	Y
	P51153	RAB13	Ras-related protein Rab-13	23	16	Y
	P61106	RAB14	Ras-related protein Rab-14	28	20	Y
	Q9NP72	RAB18	Ras-related protein Rab-18	2	8	Y
	P62820	RAB1A	Ras-related protein Rab-1A	31	31	Y
	Q6FIG4	RAB1B	RAB1B protein	29	33	Y
	Q9UL25	RAB21	Ras-related protein Rab-21	16	7	Y
	Q9UL26	RAB22A	Ras-related protein Rab-22A	4	3	Y
	P61019	RAB2A	Ras-related protein Rab-2A	15	12	Y
	Q8WUD1	RAB2B	Ras-related protein Rab-2B	14	10	N
	A8MYQ9	RAB34	Ras-related protein Rab-34	12	11	Y
	Q15286	RAB35	Ras-related protein Rab-35	25	23	Y
	Q6FI54	RAB5B	RAB5B protein	10	17	Y
	P51148	RAB5C	Ras-related protein Rab-5C	16	23	Y
	A0A024R5H8	RAB6A	RAB6A, member RAS oncogene family	18	18	Y
	P51149	RAB7A	Ras-related protein Rab-7a	22	20	Y
	P61006	RAB8A	Ras-related protein Rab-8A	28	29	Y
	Q92930	RAB8B	Ras-related protein Rab-8B	20	26	Y
Annexins	P50995	ANXA11	Annexin A11	32	34	Y
	P04083	ANXA1	Annexin A1	61	54	Y
	P20073	ANXA7	Annexin A7	14	12	Y
	P09525	ANXA4	Annexin A4	17	14	Y
	P12429	ANXA3	Annexin A3	32	26	Y
	P08133	ANXA6	Annexin A6	30	24	Y
	P08758	ANXA5	Annexin A5	54	43	Y
Sorting	O75955	FLOT1	Flotillin-1	11	4	Y
	P53621	COPA	Coatomer subunit alpha	18	9	Y
	Q00610	CLTC	Clathrin heavy chain 1	68	43	Y
	Q9Y678	COPG1	Coatomer subunit gamma-1	11	7	N
	P62330	ARF6	ADP-ribosylation factor 6	13	10	Y
	B2RAU5	0	Sorting nexin	5	4	N
	P84077	ARF1	ADP-ribosylation factor 1	14	12	Y

	P28838	LAP3	Cytosol aminopeptidase	9	8	Y
	A4D2P0	RAC1	Ras-related C3 botulinum toxin substrate 1	17	18	Y
	P53618	COPB1	Coatomer subunit beta	5	6	Y
	J3KNQ4	PARVA	Alpha-parvin	4	6	N
Trafficking	Q14204	DYNC1H1	Cytoplasmic dynein 1 heavy chain 1	37	19	Y
	Q02241	KIF23	Kinesin-like protein KIF23	49	49	N
	A4D2P0	RAC1	Ras-related C3 botulinum toxin substrate 1	17	18	Y
	Q15058	KIF14	Kinesin-like protein KIF14	10	11	N
Release	P46459	NSF	Vesicle-fusing ATPase	4	2	Y
	O00161	SNAP23	Synaptosomal-associated protein 23	14	9	Y
	O00186	STXBP3	Syntaxin-binding protein 3	15	10	Y
	Q99536	VAT1	Synaptic vesicle membrane protein VAT-1	16	14	Y
	Q15833	STXBP2	Syntaxin-binding protein 2	9	8	Y
	O95716	RAB3D	Ras-related protein Rab-3D	12	12	Y
	Q59GL1	0	Synaptotagmin binding, cytoplasmic RNA interacting protein	12	12	N
	P50570	DNM2	Dynamin-2	14	16	Y
	Q12846	STX4	Syntaxin-4	3	4	Y
	Q15836	VAMP3	Vesicle-associated membrane protein 3	2	3	Y
Recognition/Uptake	P16070	CD44	CD44 antigen	7	8	Y
	Q9P2B2	PTGFRN	Prostaglandin F2 receptor negative regulator	38	45	Y
	Q13740	ALCAM	CD166 antigen	2	2	Y
	Q08431	MFGE8	Lactadherin	115	83	Y

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a Accession number, protein description, gene name annotated from UniProt (http://www.uniprot.org/)

b SpC (spectral counts) values for combined E-treated exosomes

c SpC (spectral counts) values for combined EP-treated exosomes

d Comparison with exosomal database ExoCarta [58]

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Table 2. Protein cargo identified in E-Exos and EP-exos

	Uniprot Acc[a]	Gene Name[a]	Protein Description[a]	E-Exos (Combined)[b]	EP-Exos (Combined)[c]	Fold change - Rsc (EP-Exos/E-Exos)[d]	ExoCarta[e]
Basement membrane	P11047	LAMC1	Laminin subunit gamma-1	0	20	20.5*	Y
	O15230	LAMA5	Laminin subunit alpha-5	2	50	19.1*	Y
	P35052	GPC1	Glypican-1	0	16	16.7*	Y
	P55268	LAMB2	Laminin subunit beta-2	0	11	11.8*	Y
	Q14112	NID2	Nidogen-2	0	8	8.9	N
	O75487	GPC4	Glypican-4	0	7	8.0	Y
	G3XAI2	LAMB1	Laminin subunit beta-1	0	5	6.0	Y
	A0A024RAB6	HSPG2	Heparan sulfate proteoglycan 2 (Perlecan)	32	159	5.9*	Y
	O00468	AGRN	Agrin	5	22	4.5*	Y
Cell adhesion	Q9HCB6	SPON1	Spondin-1	0	9	9.9*	N
	O00622	CYR61	Protein CYR61	0	6	7.0	N
	P78310	CXADR	Coxsackievirus and adenovirus receptor	0	3	4.1	Y
	Q08722	CD47	Leukocyte surface antigen CD47	0	3	4.1	Y
	Q08380	LGALS3BP	Galectin-3-binding protein	2	0	-2.2	Y
	P17301	ITGA2	Integrin alpha-2	13	3	-2.8	Y
	P05362	ICAM1	Intercellular adhesion molecule 1	3	0	-2.8	Y
	P09382	LGALS1	Galectin-1	3	0	-2.8	Y
	P05106	ITGB3	Integrin beta-3	4	0	-3.5	Y
	P10586	PTPRF	Receptor-type tyrosine-protein phosphatase F	6	0	-4.8	Y
Extracellular matrix/ cytoskeleton	B1AHL2	FBLN1	Fibulin-1	0	8	8.9*	Y
	P12109	COL6A1	Collagen alpha-1(VI) chain	0	7	8.0	Y
	O14936	CASK	Peripheral plasma membrane protein CASK	0	6	7.0	N
	Q96JB6	LOXL4	Lysyl oxidase homolog 4	2	14	5.7*	Y
	P39059	COL15A1	Collagen alpha-1(XV) chain	4	22	5.3*	Y
	Q9HCU4	CELSR2	Cadherin EGF LAG seven-pass G-type receptor 2	0	4	5.1	N
	P00533	EGFR	Epidermal growth factor receptor	0	4	5.1	Y
	Q14512	FGFBP1	Fibroblast growth factor-binding protein 1	0	3	4.1	N
	P46939	UTRN	Utrophin	0	3	4.1	Y
	O15551	CLDN3	Claudin-3	0	3	4.1	Y
	P39060	COL18A1	Collagen alpha-1(XVIII) chain	5	17	3.5*	Y
	J3KNQ4	PARVA	Alpha-parvin	4	6	1.7	N
	P27797	CALR	Calreticulin	3	0	-2.8	Y
Protease/ peptidases	Q8TE58	ADAMTS15	A disintegrin and metalloproteinase with thrombospondin motifs 15	0	5	6.0	N
	P15144	ANPEP	Aminopeptidase N	11	13	1.4	Y
	Q9NY33	DPP3	Dipeptidyl peptidase 3	5	6	1.4	Y
	O14672	ADAM10	Disintegrin and metalloproteinase domain-containing protein 10	13	15	1.4	Y

a Accession number, protein description, gene name annotated from UniProt (http://www.uniprot.org/)

b SpC (spectral counts) values for combined E-treated exosomes

c SpC (spectral counts) values for combined EP-treated exosomes

d Normalized Rsc (ratio of spectral counts)(normalized between samples by total SpC) averaged between replicates of E and EP

e Comparison with exosomal database ExoCarta [58]

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

Table 3. Exosomes enriched for molecules known to be involved in embryo implantation

Uniprot Acc[a]	Gene Name[a]	Protein Description[a]	E-Exos (Combined)[b]	EP-Exos (Combined)[c]	Fold change - Rsc (EP-Exos/E-Exos)[d]	ExoCarta[e]
A0A024RAB6	HSPG2	Heparan sulfate proteoglycan 2	32	159	5.9*	Y
O00622	CYR61	Protein CYR61	0	6	7.0	N
B1AP13	CD55	Complement decay-accelerating factor	0	6	7.0	Y
Q8TE58	ADAMTS15	A disintegrin and metalloproteinase with thrombospondin motifs 15	0	5	6.0	N
Q9HCU4	CELSR2	Cadherin EGF LAG seven-pass G-type receptor 2	0	4	5.1	N
P00533	EGFR	Epidermal growth factor receptor	0	4	5.1	Y
O15551	CLDN3	Claudin-3	0	3	4.1	Y
Q08722	CD47	Leukocyte surface antigen CD47	0	3	4.1	Y
J3KNQ4	PARVA	Alpha-parvin	4	6	1.7	N
P15144	ANPEP	Aminopeptidase N	11	13	1.4	Y
Q9NY33	DPP3	Dipeptidyl peptidase 3	5	6	1.4	Y
O14672	ADAM10	Disintegrin and metalloproteinase domain-containing protein 10	13	15	1.4	Y
P09382	LGALS1	Galectin-1	3	0	-2.8	Y
P17931	LGALS3	Galectin-3	4	0	-3.5	Y

a Accession number, protein description, gene name annotated from UniProt (http://www.uniprot.org/)

b SpC (spectral counts) values for combined E-treated exosomes

c SpC (spectral counts) values for combined EP-treated exosomes

d Normalized Rsc (ratio of spectral counts)(normalized between samples by total SpC) averaged between replicates of E and EP

e Comparison with exosomal database ExoCarta [58]

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