Proteome reprogramming of endometrial epithelial cells by human trophectodermal small extracellular vesicles reveals key insights into embryo implantation

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Abstract

Embryo implantation into the receptive endometrium is critical in pregnancy establishment, initially requiring reciprocal signalling between outer layer of the blastocyst (trophectoderm cells) and endometrial epithelium; however, factors regulating this crosstalk remain poorly understood. Although endometrial extracellular vesicles (EVs) are known to signal to the embryo during implantation, the role of embryo-derived EVs remains largely unknown. Here, we provide a comprehensive proteomic characterisation of a major class of EVs, termed small EVs (sEVs), released by human trophectoderm cells (Tsc-sEVs) and their capacity to reprogram protein landscape of endometrial epithelium *in vitro*. Highly purified Tsc-sEVs (30-200 nm, $ALIX^+$, $TSG101^+$, $CD9/63/81^+$) were enriched in known players of implantation (LIFR, ICAM1, TAGLN2, WNT5A, FZD7, ROR2, PRICKLE2), antioxidant activity (SOD1, PRDX1/4/6), tissue integrity (EZR, RAC1, RHOA, TNC), and focal adhesions (FAK, ITGA2/V, ITGB1/3). Functionally, Tsc-sEVs were taken up by endometrial cells, altered transepithelial electrical resistance, and upregulated proteins implicated in embryo attachment (ITGA2/V, ITGB1/3), immune regulation (CD59, CD276, LGALS3), and antioxidant activity (GPX1/3/4, PRDX1/2/4/5/6): processes that are critical for successful implantation. Collectively, we provide critical insights into Tsc-sEV-mediated regulation of endometrial function that contributes to our understanding of the molecular basis of implantation.

Significance of the Study

Reciprocal embryo-maternal crosstalk is critical for embryo implantation, with implantation failure accounting for up to two-thirds of unsuccessful pregnancies. However, factors regulating this crosstalk during embryo implantation remain poorly understood. Results presented here provide molecular leads into how trophectodermal small extracellular vesicles (Tsc-sEVs) signal to, and reprogram, the endometrium to support embryo implantation. Through proteomic dissection, we report that Tsc-sEVs are carriers of critical players of embryo implantation. Importantly, they dynamically reprogram the endometrial cellular protein landscape to upregulate players of antioxidant activity, immune function, focal adhesion, and tissue morphogenesis, thus promoting a pro-implantation phenotype. Our study provides a unique understanding of trophectoderm sEV-mediated regulation of endometrial function during implantation. These findings will enable future studies seeking to characterise the molecular basis of implantation failure, and have implications in the design and development of therapeutics to enhance implantation success, or alternatively, as a non-steroidal contraceptive strategy.

1. Introduction

A critical and limiting step in pregnancy establishment is the process of implantation, during which the embryo, as a blastocyst attaches to the receptive endometrium in the mid-secretory phase of a menstrual cycle (window of implantation).[1, 2] Implantation success is dictated, in part, by timely and reciprocal signalling between the blastocyst and the endometrium.[3, 4] For example, endometrial-derived factors, such as calcitonin,[5] lysophosphatidic acid (LPA),[6] heparin-binding epidermal growth factor (HB-EGF),[6, 7] leukaemia inhibitory factor (LIF),[8, 9] and epidermal growth factor (EGF),[9] activate blastocysts to implantation competency. On the other hand, the endometrium undergoes local remodelling in response to signals derived from the human embryo[10, 11] (*i.e.*, hCG,[12, 13]IL-1,[13] BMP2,[14] IGF1,[14] FGF2,[15] WNT).[16] This local remodelling of endometrium encompasses altered protein expression and signalling pathways[14] (i.e., BMP2,[17, 18] FGF2,[19] Hedgehog,[20] and Wnt signalling),[16, 21] surface molecules (integrins),[5, 22, 23] immune regulation,[24] tissue morphogenesis[25-29] (polarity, cell-cell junction proteins, cell shape, pinopodes, actin cytoskeleton remodelling) and metabolism.[30] Collectively, accumulating evidence suggest that these endometrial changes potentially bookmark the pre-implantation endometrial niche that homes the embryo.[16, 21, 28] Although soluble factors including cytokines and growth factors have been shown to mediate this crosstalk at the time of implantation, the role for extracellular vesicles (EVs) is also beginning to emerge.

EVs, membranous vesicles released by cells, mediate intercellular communication through exchange of proteins, RNA and lipids between cells.[31-35] A major subset of EVs called small EVs (sEVs, exosomes) (30-200 nm)[33] have been shown to signal between endometrium and embryo. Endometrium-derived sEVs can be taken up the embryo (trophoblast) and alter their protein landscape to enhance implantation and invasive capacities.[32, 36, 37] We also recently demonstrated that these sEVs are an abundant component of the uterine fluid, and their protein composition is dysregulated between fertile and infertile women (*Rai et al., submitted*). On the other hand, accumulating evidence show that pre-implantation embryos produce EVs both *in vitro* and *in vivo*,[38] that can be taken up in an autocrine (trophoblast)[39] or paracrine (endometrium)[40] fashion. Notably, sEVs are released by trophoblast

cells[41] and transfer their cargo to endometrial epithelial cells.[31, 42-45] However, our understanding of trophoblast-derived sEVs protein composition and how they reprogram the endometrial epithelium remains unknown. Currently, purification of EVs and their subtypes is an international effort[129], with EV heterogeneity posing significant limitations to understanding their biology and function. Furthermore, technical challenges associated with sEV isolation and purification have limited their in-depth analysis in reproductive biology.

Here, we report that sEVs released by human trophectoderm stem cells (L2-TSC) contain critical regulators of implantation. Importantly, they are readily internalised by human endometrial epithelial cells (Ishikawa) to induce changes in the endometrial cellular protein landscape that are consistent with a pro-implantation state.

2. Experimental Section

2.1. Cell Culture

Human trophectodermal stem cells (L2-TSC/T3-TSC) (kind gift of Prof. Susan Fisher, UCSF) were derived from individual blastomeres of donated human embryos.[46] Cells were routinely maintained as described[32] in a 1:1 mix of DMEM/F12 (Gibco, Invitrogen) supplemented with 1% v/v Penicillin-Streptomycin (P/S) and 10% v/v foetal calf serum (FCS, Gibco, Invitrogen) with addition of 10 ng/ml bovine fibroblast growth factor (bFGF, R&D Systems) and 10 μM SB431542 (#1614, Tocris Bioscience) to maintain a "stem cell" trophectoderm-like state. Cells were grown on flasks coated with 0.5% w/v gelatin prior to experimental seeding and passaged using Trypsin-EDTA (Gibco).

Ishikawa endometrial epithelial cells were a kind gift to Prof Guiying Nie, from Prof Masato Nishida, National Hospital Organization, Kasumigaura Medical Center, Japan and used with permission. Ishikawa cells were routinely maintained in DMEM/F12 supplemented with 1% v/v P/S, and 5% v/v FCS and incubated at 37°C with 5% CO_2. Cells were routinely passaged using 0.5% v/v trypsin-EDTA (Gibco). Hormonal priming of Ishikawa cells was performed as previously described[32]. For estrogen priming, cells were cultured in DMEM/F12 supplemented with 0.6% v/v insulin transferrin selenium (ITS, Gibco) and 1% v/v P/S overnight, followed by 10^{-8}M 17β-estradiol (E) (Sigma-Aldrich) in DMEM/F12 (0.6% v/v ITS, 1% v/v P/S). For progesterone priming, cells were subsequently treated with E plus 10^{-7}M medroxyprogesterone acetate (P) (Sigma-Aldrich) in DMEM/F12 (0.6% ITS, 1% v/v P/S).

2.2. Purification of Small Extracellular Vesicles

Cells were cultured in 25-cm^2 dishes in EV-depleted FCS (obtained following by ultracentrifugation at 100 000 × *g* for 18 h) for 48 h. Conditioned media was collected and centrifuged at 500 × *g*, 2000 × *g*, and 10 000 × *g* to remove cellular debris and large EVs (also known as shed microvesicles).[35, 47] The supernatant was then ultracentrifuged at 100 000 × *g* for 1 h to pellet crude sEVs. Crude sEVs were

then subjected to density gradient separation[48] with modifications. Briefly, a discontinuous gradient of OptiPrep™ (iodixanol solution, STEMCELL Technologies) was prepared by layering 1 ml volumes of 40%, 20%, and 10% iodixanol solution, topped with a layer of 500 µl 5% iodixanol solution. Crude sEVs were resuspended in 100 µl of phosphate-buffered saline (PBS) and overlaid onto the gradient and centrifuged at 100 000 × *g* for 18 h at 4°C. Twelve 300 µl fractions were obtained, diluted to 1 ml PBS, and centrifuged at 100 000 × *g* for 1 h at 4°C. Pellets were resuspended in 100 µl PBS and subjected to total protein quantification using Micro BCA Protein Assay (Thermo Fisher Scientific) and stored at -80°C until further use. Fractions containing sEVs were determined using western blot analysis for ALIX/PDCD6IP and TSG101.

2.3. Western Blotting

Samples were lysed in sodium dodecyl sulphate (SDS) sample buffer (4% w/v SDS, 20% v/v glycerol, and 0.01% v/v bromophenol blue, 0.125 M Tris-hydrochloride (Tris-HCl), pH 6.8) with 100 mM dithiothreitol (DTT, Thermo Fisher Scientific), and whole cell lysates (WCL) or sEVs were resolved on Norvex 4–12% Bis–Tris NuPAGE gels with MES running buffer at 150 V for 1 h. Proteins on the gel were electrotransferred onto nitrocellulose membranes using iBlot™ Dry 2.0 blotting system (Life Technologies) at 12 V for 8 min. The membranes were blocked with 5% w/v skim milk powder in PBS-Tween (PBST) (0.137 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4, 0.0018 M KH_2PO_4, 0.05% w/v Tween 20) for 30 min at room temperature. The membranes were washed and probed with primary antibodies (1:1000 dilution) for 24 h at 4 °C in PBST. Primary antibodies used included mouse monoclonal against ALIX (#2171, Cell Signaling), TSG101 (#612696, BD Biosciences), LGALS3 (#ab2785, Abcam), ANXA2 (#A14020, Transduction Laboratories), MSN (#3142S, Cell Signaling), and RAC1 (#ab33186, Abcam). Secondary antibodies used included IRDye 800 goat anti-mouse IgG (#926-32210) or IRDye 680 goat anti-rabbit IgG (#926-68071) (1:15000, LI-COR Biosciences).

2.4. Biophysical Analysis of Trophectoderm sEVs

Cryo-electron microscopy imaging of trophectoderm sEVs was performed as described.[36] Particle size distribution trophectoderm sEVs was determined by nanoparticle tracking analysis (NanoSight NS300, Malvern), as previously described.[32, 49]

2.5. Lipophilic Dye Labelling of Small Extracellular Vesicles and Uptake Assay

L2-TSC cells grown to 80% confluency were labelled with fluorescent dye, DiO (Invitrogen) at 1 μM concentration for 15 min at 37°C as described.[47] Labelled L2-TSCs were washed three times with PBS. Fresh medium was added, cells incubated for 48 h, after which DiO-labelled Tsc- sEVs were collected at 100 000 × *g* (1 h). Ishikawa cells were grown to 70% confluency in 8-well glass chamber slides (Sarstedt). Cells were incubated with DiO-labelled sEVs at 37°C for 2 h. Cells were then washed twice in PBS. Nuclei were stained for 10 min with Hoechst 33342 stain (Thermo Fisher Scientific) (10 μg/ml) prior to imaging by Nikon A1R confocal microscope equipped with resonant scanner, using a 40x WI (1.15 NA); (Nikon, Tokyo, Japan). Images were sequentially acquired. The dXY image resolution was 0.21 μm and Z-interval of 0.5 μm. Cell were maintained at 37°C, 5% CO_2.

2.6. Transepithelial Electrical Resistance (TER) Assay

Ishikawa cells (2 x 10^5) were seeded onto polycarbonate bicameral chambers (6.5 mm, 0.4-mm pore; Corning). Cells were allowed to attach overnight in DMEM/F12 medium containing 5% FCS, and then incubated in DMEM/F12 with 0.6% v/v ITS (basal media) in apical and basal chambers. Cells were incubated for a further 24 h, followed by assessment of baseline TER. To assess the integrity of inter-epithelial cell-tight junctions, TER was quantified using a Millipore Millicell-Electrical Resistance System (Millipore), with measurements taken daily. Cells and media were maintained at 37°C; following removal from the incubator, cells were equilibrated on a warming plate within the culture hood for at least 30 min before each TER measurement. After basal readings, 24 h post media change to 0.6% v/v ITS DMEM/F12, cells were treated with Tsc-sEVs (50 μg/ml) for 24 h followed by re-assessment of TER. After the first treatment reading, cells were subsequently treated with Tsc-sEVs

(50 µg/ml) for a further 24 h followed by TER assessment. Changes in TER were calculated as a percentage of basal readings for each individual well (n=3). Each treatment was performed in three wells, and readings for each well were performed 4 times. The experiment was performed twice.

2.7. Proteomic Sample Preparation

Samples were solubilised in 1% (v/v) SDS, 50 mM HEPES, pH 8.0, and quantified by Micro BCA Protein Assay (Thermo Fisher Scientific). For mass spectrometry-based proteomics, samples (~10 µg) were normalized and prepared as described[50] in 50 µl of 50 mM HEPES, pH 8.0, and reduced with 10 mM DTT for 45 min at 25 °C followed by alkylation with 10 mM iodoacetamide (IAA, Fluka) for 30 min at 25°C in the dark. The reaction was quenched to a final concentration of 20 mM DTT. Sample digestion was performed according to single-pot solid-phase-enhanced sample preparation (SP3) method.[51] Briefly, 1 µl of a 50 µg/µl SP3 bead stock (Sera-Mag SpeedBead carboxylate-modified magnetic particles; hydrophobic and hydrophilic 1:1 mix, GE Healthcare Life Sciences, Freiburg, Germany) were added to 50 µl of protein extract and 60 µl absolute ethanol (final concentration of 50%) and incubated for 10 min (1000 rpm) at 24 °C. Tubes were mounted on a magnetic rack; supernatants were removed and beads were washed three times with 80% ethanol (200 ul each). Beads were resuspended in 100 µl 50 mM triethylammonium bicarbonate (TEAB, Thermo Fisher Scientific), pH 8.0 and digested overnight with trypsin (1:50 trypsin:protein ratio; Promega, V5111) at 37 °C, 1000 rpm. The peptide and bead mixture was centrifuged at 20 000 × *g* for 1 min at 24 °C, and the supernatant collected and acidified to a final concentration of 1.5% v/v formic acid, frozen at -20°C overnight, and dried by vacuum centrifugation. Peptides were resuspended in 0.07% trifluoroacetic acid (Thermo Fisher Scientific), quantified by Fluorometric Peptide Assay (Thermo Fisher Scientific) and normalized to 0.5 µg/µl.

2.8. Liquid Chromatography–Tandem Mass Spectrometry

Peptides were analysed on a Dionex UltiMate NCS-3500RS nanoUHPLC coupled to a Q-Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer equipped with a nanospray ion source in positive mode as described.[50] Peptides were loaded (Acclaim PepMap100 C18 5 µm beads with 100 Å pore-size, Thermo Fisher Scientific) and separated (1.9-µm particle size C18, 0.075 × 250 mm, Nikkyo Technos Co. Ltd) with a gradient of 2–80% acetonitrile containing 0.1% formic acid over 110 min at 300 nl min-1 at 55 °C (in-house enclosed column heater). An MS1 scan was acquired from 350–1,650 m/z (60,000 resolution, 3×10^6 automatic gain control (AGC), 128 msec injection time) followed by MS/MS data-dependent acquisition (top 25) with collision-induced dissociation and detection in the ion trap (30,000 resolution, 1×10^5 AGC, 60 msec injection time, 28% normalized collision energy, 1.3 m/z quadrupole isolation width). Unassigned precursor ions charge states and slightly charged species were rejected and peptide match disabled. Selected sequenced ions were dynamically excluded for 30 sec. Data are available via ProteomeXchange with identifier PXD022501.

2.9. Data Processing and Bioinformatics

Peptide identification and quantification were performed as described previously[32, 50] using MaxQuant (v1.6.14) with its built-in search engine Andromeda[52]. Tandem mass spectra were searched against Homo sapiens (human) reference proteome (74,823 entries, downloaded Jan-2020) supplemented with common contaminants. Search parameters included carbamidomethylated cysteine as fixed modification and oxidation of methionine and N-terminal protein acetylation as variable modifications. Data was processed using trypsin/P as the proteolytic enzyme with up to 2 missed cleavage sites allowed. The search tolerance and fragment ion mass tolerance were set to 7 ppm and 0.5 Da, respectively, at less than 1% false discovery rate on peptide spectrum match (PSM) level employing a target-decoy approach at peptide and protein levels. Label free quantification (LFQ) algorithm in MaxQuant was used to obtain quantification intensity values and processed using Perseus as described[48].

For Gene Ontology (GO) annotation, protein accession IDs were submitted to DAVID Bioinformatics Resources (https://david.ncifcrf.gov/),[53] and g:Profiler.[54] Hierarchical clustering was performed in Perseus using Euclidean distance and average linkage clustering. Proteins upregulated in Ishikawa cells treated with Tsc-sEVs include those that were identified in at least 3 biological replicates, either uniquely, or which fold change (log2) ≥1.0 compared to Ishikawa (untreated) control. Functional enrichment analyses (Gene Ontology (GO), KEGGs) were performed using DAVID[53] and g:Profiler[54]. Pathway EnrichmentMap analysis was performed using Cytoscape (v3.7.1).[55]

2.10. Statistical Analysis

Data were analysed using GraphPad Prism v8.4.3, with all data pre-tested for normality. If the data was non-parametric, a Kruskal-Wallis with a Tukey's post-hoc test or Mann-Whitney U analysis was performed. If parametric, one-way ANOVA with a Tukey's post-hoc test or unpaired t-test was performed. All data presented as mean plus/minus standard deviation (mean±SD). In all analyses, *$p<0.05$ is considered statistically significant.

3. Results

3.1. Isolation and Purification of Small Extracellular Vesicles from Trophectoderm Cells

We used trophectoderm cells (L2-TSC)[32, 46] as a source of sEVs in this study; we verify that these cells were significantly enriched ($p<0.05$) in trophectoderm markers, including ACTN1, PDLIM1, FHL2, CAST, MYOF, TAGLN2, GLIPR2, PALLD (based on CellMarker)[56] compared to human endometrial cells (Ishikawa) (**Figure S1**).

Trophectoderm sEVs were purified from L2-TSC conditioned media using differential centrifugation coupled to density gradient separation[57] (**Figure 1A**). Purified sEVs were positive for stereotypic exosome markers ALIX/PDCD6IP and TSG101 (**Figure 1B**), and display 1.08-1.12 g/ml buoyant density (**Figure 1B**), consistent with previous reports.[48, 57, 58] Cryo electron microscopy revealed that purified sEVs were intact, spherical in morphology (**Figure 1C**), and 30-200 nm in size (mean 136.7 nm) (**Figure 1D, Figure S2**). Henceforth, we will refer to L2-TSC whole cell lysate as Tsc-WCL and sEVs derived from L2-TSCs as Tsc-sEVs.

3.2. Proteome Analysis of Trophectoderm sEVs

To gain insight into protein composition and potential function of Tsc-sEVs, they were subjected to mass spectrometry-based proteomic analysis and their protein profile compared to that of (i) parental Tsc cells and (ii) sEVs derived from two endometrial cell lines (**Figure 2**).

Proteins enriched in Tsc-sEVs compared to trophectoderm cells

A total of 3056 and 2405 proteins were detected in Tsc-WCL and Tsc-sEVs respectively (**Figure 2A**), representing one of the most comprehensive proteome analyses of trophectoderm-derived sEVs. Principle component analysis (PCA) revealed that Tsc-sEVs have a molecular profile distinct from their parental cells, suggesting selective packaging of proteins into sEVs (**Figure S3**). Cursory inspection revealed that 1231 proteins (374 unique and 857 enriched (fold change log2>1, $p<0.05$) were detected

in higher abundance in Tsc-sEVs compared to Tsc-WCL, which include classical sEV/exosome marker proteins[59] (i.e., ALIX/PDCD6IP, TSG101, CD9, CD63, CD81) (**Table S1/2**) (**Figure 2B**). In contrast, 1804 proteins (985 not detected and 819 down-regulated (fold change log2<-1, p<0.05) were identified in lower abundance in Tsc-sEVs compared to Tsc-WCL (**Table S1**), which include organelle markers[60] (mitochondria (SFXN1, PHB2, CS), endoplasmic reticulum (BCAP31, LRRC59, VAPA), ribosome (IMPDH2, EIF6, RPS20, PRPF8)) (**Figure 2B**).

Gene ontology (biological process) analysis revealed that Tsc-sEVs were enriched in processes including cell polarity ('regulation of cell shape', and 'establishment or maintenance of cell polarity'), signalling pathways ('regulation of small GTPase mediated signal transduction', 'Rho protein signal transduction'), protein localisation ('protein localisation to cell periphery') and tissue integrity ('establishment of endothelial barrier', 'transport across blood-brain barrier') (**Figure S4, Table S3**). Notably, these processes are all important for embryo implantation[19, 61-67] and endometrial remodelling,[19, 26, 28, 29, 64, 66-70] suggesting that trophectoderm cells contribute to the implantation interface through the release of sEVs, potentially by signalling to, and preparing the endometrium to support implantation.

Because the function of sEVs is dictated by its proteome composition, we next interrogated the global sEV proteome for players of implantation. This resulted in identification of 42 known players of implantation and endometrial receptivity,[71] including antioxidants (PRDX1/2, SOD2, GPX3), adhesion molecules (ITGA2/V, ITGB1/3, ICAM1, VCAM1), signalling regulators, (MAPK1/3, MAP2K1, STAT3), and cytoskeletal regulators (EZR, RAC1, RHOA, TNC) (**Table 1**, **Figure 2C**), which collectively function to regulate reactive oxygen species (ROS) metabolism, cell-cell adhesion, tissue morphogenesis, signal transduction, and embryo implantation (**Figure 2D**).

Proteins enriched in Tsc-sEVs compared to sEVs from endometrial cells:

Next, to short list proteins specifically enriched in Tsc-sEVs, as opposed to sEVs in general, we compared their protein profile with that of sEVs derived from two endometrial cell lines (Ishikawa and ECC-1 cells). While 1312 proteins were commonly identified between all three sEV subsets (including

known classical sEV/exosome markers TSG101, PDCD6IP, CD63, CD81, CD82, CD9), 521 and 785 proteins were uniquely detected in endometrial sEVs and Tsc-sEVs, respectively (**Figure 2E, Table S4/5**). Of the 521 proteins uniquely identified to in endometrial sEVs, pathway enrichment included 'cadherin binding',[72, 73] 'magnesium ion binding',[74] 'signalling by ROBO receptors',[75] 'protein targeting to membrane', 'Rho GTPase binding',[64, 68] 'Rac GTPase binding',[67] 'cortical cytoskeletal organisation'[76, 77] and 'folate biosynthesis'[78] (**Figure S5, Table S6**), which have associated functions in endometrial receptivity, and embryo development and implantation.

Further, pathways specifically enriched in Tsc-EVs include regulation of MAPK activity, focal adhesion,[79] transmembrane receptor protein kinase activity, small GTPase-mediated signalling[64], response to TGF-β, regulation of actin cytoskeleton and cell polarity, PI3K signalling,[80] and platelet activation[81, 82] (**Figure 2F, Table S7**), which are all important processes for implantation.

Upon further inspection, several proteins identified in Tsc-EVs are associated with organ morphogenesis and development (e.g., FAK, PDGFC and its cell-surface receptor PGFRA, cytoskeletal protein NES[83]), embryo development (e.g., activin receptors ACVR1/2A,[84] BMPRA,[85] DAG1,[86] EDIL3,[87] WNT5A,[88] growth factor receptor FGFR1[89]), and placental development (e.g., EGFR,[90] MAPK14,[91] FAK,[92] HTRA1[93, 94]) (**Table S1/5**).

Interestingly, several factors in Tsc-sEVs were also previously identified in IVF spent media of implantation-competent human blastocysts (**Table S8**), including SOD1,[95] and EGFR, FAS, HGF, ICAM1, IGF1R, and MIF.[96] Thus, our data show that Tsc-sEVs potentially contribute to embryo health and endometrial remodelling by carrying and potentially transferring known players of embryo implantation.

3.3. Human Trophectoderm sEVs Reprogram Ishikawa Cell Proteome

We next investigated how Tsc-sEVs reprogram the Ishikawa cell proteome. We proteomically profiled human endometrial epithelial Ishikawa cells treated with Tsc-sEVs vs untreated control Ishikawa cells. A total of 2867 and 3063 proteins were detected in Tsc-sEV-treated Ishikawa cells and untreated control

Ishikawa cells, respectively (**Figure 3A**). Cursory inspection revealed that 297 proteins (11 unique and 286 enriched (fold change log2>1, p<0.05)) were detected in higher abundance in Tsc-sEV-treated Ishikawa cells compared to untreated control (**Figure 3A**). In contrast, we identified 236 proteins (53 unique and 183 downregulated (fold change log2<-1, p<0.05)) in Tsc-sEV-treated Ishikawa cells compared to untreated control (**Figure 3A**, **Table S9**). Most significantly upregulated proteins in Tsc-sEV treated Ishikawa cells include players of embryo implantation (CD44, F3, NT5E, DPP4, MFGE8) (**Figure 3B**).

DAVID-based analysis revealed that pathways enriched in Ishikawa cells following Tsc-sEV treatment (**Figure 3C, Table 2, Table S10**) include protein translation and localization[22, 23, 97, 98] (module 1 and 3) (translation initiation/elongation factors, ribosomal subunits), immune regulation[24, 99] (module 2), antioxidant activity[100, 101] (module 4, 8), cell-matrix adhesion and laminin binding[102] (module 12, 16), NAD metabolism[103] (module 18), cadherin binding (module 20);[72, 73] processes that are dynamically regulated at the site of implantation. On the other hand, pathways underrepresented in recipient cells include DNA replication and elongation (module 5),[104, 105] carbon metabolism (TCA cycle) (module 11, 17), actin cytoskeleton organisation (module 14, 15), and oxidoreductase activity (module 19) (**Figure 3D, Table S10**).

Upon manual interrogation, we also noted that several of the significantly upregulated proteins in Tsc-sEV-treated cells have previously been shown to be involved in implantation (BSG,[106] CD44,[107, 108] TAGLN2,[77] ANXA2,[109] PFN1),[110] trophoblast invasion (CSTD,[111] THBS1,[112] CDCP1,[113] LGALS3),[114] and endometrial receptivity (CLIC4,[115] EGFR).[116] Amongst the proteins found in higher abundance, 6 proteins (DPP4, ASS1, ANXA4, COTL1, MRPS2, SOD2) are also associated with endometrial receptivity.[117] In contrast, we noted that proteins significantly downregulated in endometrial cells following Tsc-sEV treatment were also absent in uterine fluid from fertile vs infertile women (AHNAK).[108]

Interestingly, 200/297 proteins uniquely or significantly upregulated in Ishikawa cells following sEV treatment were also detected in sEVs (**Figure 3D, Table S11**); whether these proteins are transferred by sEVs or upregulated in expression following sEV treatment warrants investigation. Of these 200

proteins, 9 proteins (ANXA2, CLIC1, ITGA2, ITGAV, ITGB1, PRDX1, PRDX2, RPL13, TAGLN2) are known players of embryo implantation.[71] Orthogonal validation (western blotting, pooled sEV treated cell lysate from all biological replicates) of protein expression in our mass spectrometry datasets is provided for LGALS3, MSN, and RAC1 (**Figure 3E/F**).

Thus, our data shows that trophectoderm sEVs reprogram the endometrial proteome towards a pro-implantation signature.

3.4. Trophectoderm sEVs Modulate Polarity of Endometrial Cells

Our data show that Tsc-sEVs carry polarity-associated proteins (**Figure 2F**) that potentially regulate processes associated with endometrial cell polarity through regulation of actin cytoskeleton, morphogenesis of a polarised epithelium, Wnt/planar cell polarity signalling, and extracellular matrix (ECM) binding (**Figure 3C**). Spatial and temporal regulation of endometrial cell polarity is critical for embryo homing and implantation.[26, 28, 118] Thus, we hypothesise that Tsc-sEVs regulate endometrial cell polarity, which could be measured by transepithelial electrical resistance (TER).[29, 119] Endometrial cells treated with Tsc-sEVs were analysed for changes in TER. Confocal microscopy revealed that sEVs were readily taken up by Ishikawa cells (**Figure 4A**), and significantly increased TER compared to control Ishikawa cells (**Figure 4B**). Estrogen and progesterone, which are known to regulate endometrial cell polarity,[29] were used here as controls (**Figure 4B**). Thus, our data show that Tsc-sEVs are taken up by endometrial cells and potentially modulate their polarity. However, the overall direction of polarity change will be determined by the sum of hormonal, soluble mediator, and vesicle actions at the implantation site.

4. Discussion

We have previously shown that sEVs derived from the endometrium can signal to the embryo to promote implantation.[32, 36, 37] Although recent evidence point towards sEVs from human embryo being taken up by the endometrium,[40] their composition, function, and influence on the endometrium remain unknown. This is particularly due to challenges associated with obtaining sufficient amount of highly purified sEVs from the embryo. To overcome these challenges, namely to obtain sufficient EVs to enable downstream analysis, we utilised human trophectodermal cells (derived from human embryonic stem cells)[46] which form the outer layer of the embryo, that interact with the receptive endometrium during implantation,[32, 120] as EV donor cells. Importantly, we obtained highly purified sEVs and separated them from non-EV contaminants[33] using density gradient separation;[36, 49] the gold-standard for purifying EVs (as reported by Journal of Extracellular Vesicles and international guidelines on EV research).[59]

Through proteomic characterisation, we identified critical regulators of embryo implantation in Tsc-sEVs, several of which (including SOD1,[95] and EGFR, FAS, HGF, ICAM1, IGF1R, and MIF)[96] were previously identified in human IVF spent media,[96] and proposed as a non-invasive indicator of embryo competence.[38, 121] Importantly, prominent signalling players in Tsc-sEVs include small GTPases[122, 123] RHOA, CDC42, and RAC1 and Wnt signalling players[124] (FAK, WNT5A, FZD7, ROR2, PRICKLE2, that are critical regulators of endometrial tissue integrity and function.[125, 126] We also demonstrate following exposure to Tsc-sEVs, an increase in endometrial cell TER; an indicator of increased cellular integrity based on tight junction formation.[127] In line with this, presence of the embryo can increase endometrial tight junction formation in rats,[128-130] pigs,[131] and sheep,[132] potentially to reduce paracellular permeability and prolong the effects of embryonic signals at the site of implantation. Additionally, accumulating evidence suggests that local and temporal regulation of these signalling pathways by embryos in the mouse can regulate formation of luminal epithelial evaginations known as crypts[16, 21, 28] that are sites of embryo homing in this species. In fact, EVs have been shown to transfer active Wnt loaded on the surface of EVs between cells and elicit downstream functional responses in breast cancer cells,[133] but whether trophectoderm EVs WNT5A also activate Wnt signalling in endometrial cells at the site of implantation warrants investigation.

A major class of proteins identified include antioxidant enzymes, for example glutathione peroxidases GPX1/3/4, and peroxiredoxins PRDX1/2/4/5/6, some of which (i.e., PRDX1/4) have previously identified in trophoblast-derived sEVs.[41] It is now well-known that embryos release antioxidants (including SOD1) into spent culture medium,[95] and that these play an important role in embryo development and implantation.[134-138] These EV antioxidants potentially scavenge or neutralise ROS in the immediate embryo vicinity and provide protection in an autocrine manner. Indeed, removal of these EVs from culture media impaired embryo development, that could be rescued by exosome supplementation;[139] suggesting that Tsc-sEVs act in an autocrine fashion to enhance embryo development. Alternatively, because EVs can transfer antioxidant enzymes between cells,[41, 140] it is likely that trophectoderm EVs can transfer these to the endometrium. Indeed, we observed a significant increase in SOD1/2 expression in Ishikawa cells following exposure to Tsc-sEV. Furthermore, SOD1/2 expression peaks in human endometrium in the mid-secretory phase.[141] Tight regulation of antioxidant activity protects the uterus against ROS-induced damage during endometrial reprogramming towards receptivity,[142] and contributes to their functional integrity,[66] with their dysregulation associated with aberrant embryo implantation and endometrial pathologies, including endometriosis.[101, 143, 144] We also report upregulation of NAD metabolism, and downregulation of TCA cycle, suggesting that Tsc-sEVs sustain glycolysis; a critical process for cellular antioxidant defence,[145] and ongoing production of pyruvate and NADH, which have antioxidative functions.[136, 146, 147]

Another prominent feature at the site of implantation is the precise regulation of the maternal immune system that prevents immune rejection of the embryo while still protecting the uterine cavity from infectious pathogens[148](reviewed[149]). We report that Tsc-sEVs are enriched in immune regulators previously implicated at the foetal-maternal interface,[150, 151] including embryo-expressed complement inhibitor CD59,[152, 153] trophoblast exosome-associated CD276 that inhibits antigen-stimulated lymphocyte activation,[151, 154] and LGALS3, which regulates natural killer cell degranulation[155] and T-cell receptor-mediated CD4+ T-cell activation.[156] Indeed, embryo-derived EVs presenting HLA-G can bind to T-lymphocytes[157] to prevent immune attacks to embryo, or be transferred to $CD4^+$ and $CD8^+$ T-lymphocytes to increase immunosuppressant IL-10

expression[158](reviewed[159]). Trophoblast EVs have been shown to be taken up by human T-lymphocytes and decrease Stat3 phosphorylation,[157] or transfer fibronectin to induce pro-inflammatory response in macrophages.[160, 161] In line with this, there is evidence that embryo culture media exerts immunosuppressive activity,[162] for example, by inducing expression of immunosuppressant IL-10 in peripheral lymphocytes.[163] Because these proteins are also significantly upregulated in Ishikawa cells following exposure to Tsc-sEVs, these Tsc-sEVs may modulate maternal immune responses either through direct regulation of immune cells,[164, 165] or by inducing expression of immunomodulatory molecules in the endometrium.[24] These data point towards the capacity of embryo-derived signals to modulate maternal immune response at the embryo-maternal interface, though the underlying mechanism of this signalling warrants further investigation.

We also observed a striking enrichment of biological processes (in sEVs: focal adhesion, L1CAM interaction, integrin cell surface interactions, and in sEV-treated endometrial cells: interaction with host, cytoskeleton regulation, ECM binding, cadherin binding) involving a variety of integrins, including ITGA2/V, ITGB1/3. Focal adhesions (FAs) are specialised structures that connect the actin cytoskeleton to the ECM that promote cell-matrix adhesion.[166] Previous studies have shown that integrin-rich FAs (integrin AV/B3) are upregulated apically at the site of implantation, promoting blastocyst attachment and invasion,[79, 167] with their dysregulation expression during the window of implantation associated with infertility.[168] Because the embryo can prime the endometrial epithelium towards a pro-implantation phenotype by inducing expression of integrins (ITGA1,[23] ITGA2),[22] it is conceivable that the sEVs from trophectoderm could regulate FAs at the site of implantation.[169] In addition to ITGA1/2/2B/4/11, we show that sEVs contain the master regulator of FAs, FAK. This is further supported by our previous observation, where we have shown that sEVs can transfer functional FAK between cells to promote trophoblast attachment.[36] Indeed, we observe an upregulation of FA molecules (integrins ITGA1, A2, B1, AV, and EGFR, CRK, CRKL) in endometrial cells following trophectoderm sEV treatment.

In summary, we show that human trophectoderm sEVs are enriched in proteins that regulate embryo implantation and can reprogram the endometrial cell protein landscape towards a pro-

implantation phenotype. Thus, our study provides useful insights into how trophectoderm sEVs can promote embryo implantation and potential molecular leads for future studies seeking to characterise implantation failure.

Tables

Table 1. Players of embryo implantation identified in trophectoderm sEVs

Gene name	Protein description	Function	Ref (PMIDs)
ABCC3	Canalicular multispecific organic anion transporter 2	Potential mediators of placental development through efflux of molecules, preventing fetal accumulation of drugs and environmental toxins	26545808
AMIGO2	Amphoterin-induced protein 2	Involved in the PI3K-AKT pathway for endothelial survival and angiogenesis	26553931
ANXA2	Annexin A2	Required at the embryo implantation interface to facilitate embryo attachment to endometrial cells through RHOA, ROCK, and F-actin regulation	26444699, 33010173, 22645245
ANXA4	Annexin A4	Expressed in the mid-secretory phase and window of implantation under regulation by progesterone, but mechanism of function unclear	16954445
ARHGDIA	Rho GDP-dissociation inhibitor 1	Downregulated expression is linked to repeated implantation failure	25248672
CALR	Calreticulin	Plays a critical role in embryo implantation in mice by modulating cell adhesiveness and integrin-dependent Ca2+ signalling. Inhibition of CALR resulted in decreased implantation in mice	20140306
CD55	Complement decay-accelerating factor	Expression in the endometrium regulated by embryonic signals such as hCG, may be important in complement regulation	23427180, 7544131
CLIC1	Chloride intracellular channel protein 1	Upregulated in the mid-secretory phase in humans and in uterine fluid of pregnant mares, with links to maternal recognition of pregnancy	29588480, 23043689
CTNNA2	Catenin alpha-2	Proposed to be a target gene for endometrial receptivity, and may be involved in uterine receptivity and embryo implantation	16960016, 31786540
RACGAP1	Rac GTPase-activating protein 1	May exert its GTPase-activating protein activity on RHOA and CDC42 and RAC1	18838676
EZR	Ezrin	Involved in cytoskeletal rearrangements that facilitate uterine receptivity and embryo-endometrium attachment	21120533,
GPX3	Glutathione peroxidase 3	Proposed to be a target gene for endometrial receptivity	16960016
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase	Activity changes in preimplantation mouse embryos, but function remains unclear	580953
ICAM1	Intercellular adhesion molecule 1	Expressed at the site of implantation under progesterone stimulation, and may mediate immunological functions, also identified in human IVF spent media	21448983, 8834767, 18556682
IDH1	Isocitrate dehydrogenase [NADP] cytoplasmic	Abundantly present in endometrium and uterine fluid of pregnant cattle, potentially with roles in conceptus elongation and required for a short period of time up to pregnancy recognition	24478148
ITGA2	Integrin alpha-2	Increases in the endometrium in response to embryonic preimplantation factor (PIF)	22788113
ITGAV	Integrin alpha-V	Increases in expression at the site of embryo implantation, potentially regulated by embryonic signals, possibly facilitates implantation by binding to osteopontin, functional blockage of this integrin led to decreased implantation sites	10775178, 24442579
ITGB1	Integrin beta-1	Acts as a marker for endometrial receptivity in conjunction with integrin β3, may play a role in mediating organisation of ECM proteins from embryos	7691869, 27782833, 9721591

ITGB3	Integrin beta-3	Increases in expression in the window of implantation at the site of embryo implantation, potentially regulated by embryonic signals, possibly facilitates implantation by binding to osteopontin, functional blockage of this integrin led to decreased implantation sites	9253342, 10775178, 24442579, 27782833
LIFR	Leukemia inhibitory factor receptor	Involved in endometrial transformation into a receptive state, embryo-endometrial interaction, and endometrial decidualisation, trophoblast invasion, blastocyst growth and development, and immune regulation	30056647, 8610178, 21966484
LRPPRC	Leucine-rich PPR motif-containing protein, mitochondrial	Upregulated in implantation window, and altered secretion is linked to primary unexplained infertility	12161539
MAP2K1	Dual specificity mitogen-activated protein kinase kinase 1	Involved in signalling pathways critical for embryo development and implantation, including ephrin receptor signalling pathway and PPAR signalling pathway	19880110
MAPK1	Mitogen-activated protein kinase 1	Functions in conjunction with MAPK3, involved in endometrial decidualisation and localised at the site of implantation	24086495
MAPK3	Mitogen-activated protein kinase 3	Functions in conjunction with MAPK1, involved in endometrial decidualisation and localised at the site of implantation	24086495
NFKB1	Nuclear factor NF-kappa-B p105 subunit	Involved in transcription at the window of implantation in mice, and induces transcription of PTGS2	20426870, 14725562
NNMT	Nicotinamide N-methyltransferase	Important in maintaining pluripotency of human embryonic stem cells, but role in implantation is unclear	26571212
PGRMC1	Membrane-associated progesterone receptor component 1	May serve as progesterone receptor, mRNA expression increases in pregnancy, and significantly decreased in endometrium of women with advanced endometriosis	23522067, 24065879, 23793472
PRDX1	Peroxiredoxin-1	Plays antioxidant role in embryos, previously identified in trophoblast EVs	29503398, 21276792
PRDX2	Peroxiredoxin-2	Downregulation linked to recurrent miscarriage by regulating trophoblast proliferation and apoptosis	28661480
CLU	Clusterin	Expression in the endometrium is indicative of tissue remodelling and reorganisation	7626703
PTGS2	Prostaglandin G/H synthase 2	Essential for embryo implantation and endometrial decidualisation	10385625, 9346237
RAC1	Ras-related C3 botulinum toxin substrate 1	Critical for maintaining endometrial epithelial integrity and preparation for embryo implantation in mice	26184908, 22645245
RHOA	Transforming protein RhoA	Involved in actin cytoskeleton reorganisation and co-localises with ANXA2 and F-actin in endometrial cells to facilitate embryo attachment	22645245, 15757515
RPL13	60S ribosomal protein L13	Potentially involved in cell cycle and reduces susceptibility to DNA damage	16786168
S100A10	Protein S100-A10	Expression increases during the window of implantation, and is critical for acquisition of endometrial receptivity phenotype	25327866, 26760977
SOD2	Superoxide dismutase [Mn], mitochondrial	Critical regulator of endometrial function, with low levels resulting in recurrent miscarriage in humans	8671393, 26821085
STAT3	Signal transducer and activator of transcription 3	Acts downstream of LIF to regulate endometrial junctional integrity and embryo attachment through juntional complexes, E-cadherin, α- and β-catenin, claudins	24100212
STMN1	Stathmin	Expression increases during embryo implantation and endometrial decidualisation	16245356, 12639930, 25866183
TAGLN2	Transgelin-2	Highly expressed in mice trophoblasts and critical for embryo implantation by regulating F-actin. In rabbits, highly expressed at the site of implantation upon interaction with embryo	30702937
TNC	Tenascin	Expression is induced at the implantation site, and may facilitate trophoblast invasion by discrupting uterine epithelial cell adhesion to underlying basal lamina	7512897
VCAM1	Vascular cell adhesion protein 1	Expression increased in endometrial epithelial cells when in contact with trophoblast cells	24803492

Table 2. Functional annotation of differentially abundant proteins identified in Ishikawa cells treated with Tsc-sEVs

	Description	Adjusted p-value[a]	Proteins
Upregulated in Ishikawa cells treated with Tsc-sEVs	Morphogenesis of a polarized epithelium	3.04E-04	ACTG1, PSMB5, PSMB7, PSMD11, SLC9A3R1, PSMD13, PSMA5, PSMC1, PSMD14, PSMD3, PSMD8, MSN, AP2S1, PFN1
	Wnt signaling pathway, planar cell polarity pathway	3.63E-03	PSMA5, PSMC1, PSMD14, PSMB5, PSMD3, PSMB7, PSMD8, PSMD11, PSMD13, AP2S1, PFN1
	Translation	1.22E-14	MRPS10, RPS24, RPS6, PPA1, RPS8, PPA2, RPS9, EEF1D, SRP14, SRP9, RPL23A, RPL24, RPL7A, RPS2, RPSA, MRPL27, MRPL46, MRPS36, RPL13, EEF1B2, RPL13A, RPL26, EIF3F, RPL27A, EIF3I, RPL32, EEF1E1, EIF3K, RPL4, EIF3L, RPL7, EIF4A1, RPS12, GFM1, RPS21
	Neutrophil activation involved in immune response	8.65E-07	ALDOA, ANXA2, LGALS3, ACLY, CTSC, CNN2, CD44, CD59, CSTB, CTSA, HSP90AB1, CTSD, GSTP1, IST1, GCA, IMPDH2, LAMTOR1, ITGAV, NME2, NDUFC2, SRP14, PRDX6, PSMB7, PSMD11, PSMD13, HSP90AA1, PSMA5, PSMD14, PSMD3, MME
	Ribonucleoprotein complex biogenesis	7.25E-14	RPS24, RPS6, RPS8, RPS9, BRIX1, DDX18, DDX21, EBNA1BP2, FBL, GLUL, MRTO4, NIP7, HSP90AB1, NOC2L, PES1, RPF2, RRP1B, SNRPD3, SRSF9, STRAP, WDR12, WDR3, WDR77, RPL23A, RPL24, RPL7A, RPS2, RPSA, RSL1D1, HSP90AA1, RPL13A, RPL26, EIF3F, EIF3I, EIF3K, EIF3L, RPL7, RPS21
	Antioxidant activity	6.33E-06	SOD1, GPX7, GSTO1, PRDX1, TXNDC17, GSTP1, PRDX6, PRDX2, PRDX3, PRDX5, NQO1, PARK7
	Interaction with host	5.50E-03	DPP4, SLC1A5, PHB, GRB2, CD81, RPSA, SNX3, EGFR, GAPDH, IST1, TCP1, ITGA2, ITGAV, ITGB1, P4HB
	Reactive oxygen species metabolic process	5.81E-04	SOD1, SPR, PRDX1, PRDX6, PRDX2, PRDX3, GRB2, PRDX5, AKR1C3, KHSRP, EGFR, HSP90AA1, THBS1, HSP90AB1, GSTP1, MT-CO2, NQO1, PARK7, SNCA
	Regulation of actin cytoskeleton	2.33E-02	ACTG1, ARPC3, PPP1CC, CRK, CRKL, EGFR, ITGA1, ITGA2, ITGAV, ITGB1, MYL12A, RRAS2, MSN, PFN1
	Extracellular matrix binding	1.24E-02	ANXA2, LGALS3, ITGA2, ITGAV, ITGB1, RPSA, THBS1
	NAD metabolic process	3.50E-02	ENO1, ALDOA, TPI1, PFKM, LDHA, NT5E, GAPDH
	Glycolysis / gluconeogenesis	9.93E-03	ENO1, ALDOA, TPI1, PFKM, LDHA, ALDH3A1, ALDH3A2, GAPDH
	Cadherin binding	4.68E-21	ABCF3, ALDOA, LIMA1, ANXA2, BSG, CTNNA1, BZW2, MYH9, FLNA, CLIC1, CNN2, CNN3, IQGAP1, CRKL, DBN1, EEF1D, CORO1B, EGFR, EHD1, ADD1, ENO1, HSP90AB1, ITGA6, IST1, ANXA1, HSPA1B, TMPO, CD2AP, ITGB1, FLNB, LASP1, VAPA, LDHA, ERC1, MAPRE1, BAIAP2, GOLGA3, PARK7, PCMT1, CC2D1A, PFN1, SNX9, PLIN3, PPFIBP1, SLK, PRDX1, AHNAK, PRDX6, RPL23A, RPL24, RPL7A, RPS2, RSL1D1, RTN4, SFN, SH3GL1, SLC3A2, TAGLN2, TMOD3, VASN, YWHAE
Downregulated in Ishikawa cells treated with Tsc-sEVs	Cell cycle DNA replication	3.67E-03	RTF2, POLD1, POLE2, MCM3, MCM5, RPA1, POLA1, FEN1
	DNA strand elongation	3.81E-03	POLD1, MCM3, MCM5, RPA1, POLA1, FEN1
	Small molecule catabolic process	1.85E-02	BDH1, GPD2, DLST, ACADVL, GUSB, MMUT, PCK2, ALDH7A1, HMGCL, OGDH, CPT1A, ACAA1, ETFDH, ALDH6A1, OXCT1, ALDH5A1, CRYL1, SULT1A1, APOE
	Cytoskeletal protein binding	2.92E-04	LIMA1, MYH14, ACTN4, CTNNA1, MYH9, MAP1S, FLNA, MYH10, ACTN1, IQGAP1, ADD3, HIP1, CORO1B, SYNE2, ADD1, UTRN, FLNB, VAPA, SETD3, HDAC6, FGD4, ANXA6, FKBP15, RAI14, MARCKSL1, DPYSL2, GSK3B, STIM1, APOE, LLGL1, KIF20A, DCTN2
	Actin cytoskeleton organization	3.79E-02	SNX9, LIMA1, MYH14, SETD3, ACTN4, FGD4, MYH9, FLNA, MYH10, ACTN1, IQGAP1, ADD3, HIP1, CORO1B, ADD1, PRKAR1A, NF1, ANXA1, STRIP1, LLGL1, ABR, CD2AP, FLNB, BAIAP2
	Citrate cycle (TCA cycle)	5.34E-04	DLST, IDH2, PC, PCK2, OGDH, PDHA1
	Oxidoreductase activity, acting on the aldehyde or oxo group of donors	5.53E-04	FAR1, ALDH7A1, OGDH, ALDH9A1, PDHA1, ALDH6A1, ALDH5A1

[a] Adjusted p-value from pathway analysis performed using gProfiler and EnrichmentMap

Figures and legends

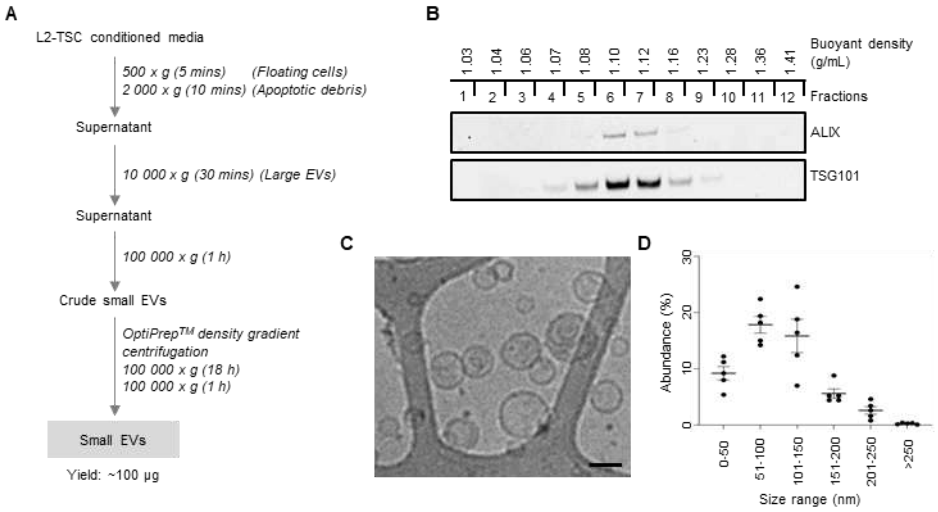

Figure 1. Purification and characterisation of trophectoderm cell-derived small extracellular vesicles. A) Workflow for isolation and purification of Tsc-sEVs from conditioned media of L2-TSC cells using differential centrifugation coupled to density gradient separation. **B)** Western blot analysis of density gradient fractions using antibodies to stereotypic EV specific markers ALIX and TSG101. **C)** Cryo-electron microscopy analysis of sEVs, scale bar = 100 nm. **D)** Size distribution of sEVs (diameter) based on cryo-electron microscopic images (n = 20 images).

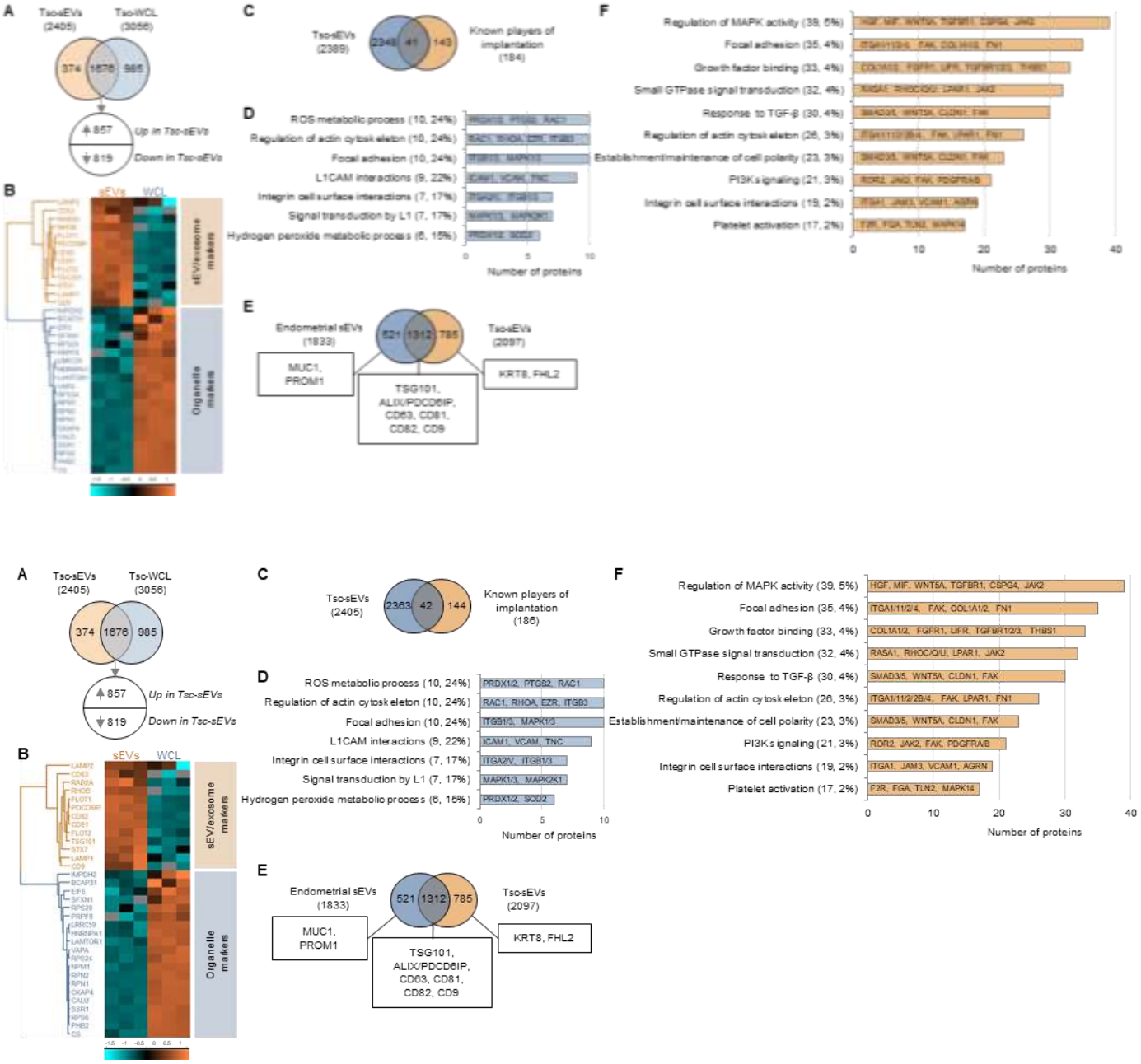

Figure 2. Quantitative mass spectrometry-based proteomics of trophectoderm small extracellular vesicles. A) Venn diagram of proteins identified in sEVs and WCL, with numbers in brackets being number of proteins identified in >2 biological replicates, and differentially expressed proteins relative to sEVs as indicated (fold change log2≥0.07/≤0.11/<-1, p<0.05, in at least 2 biological replicates). **B)** Hierarchical clustering of proteins identified in sEVs and WCL for expression of classical EV (sEV/exosome) marker proteins and cell organelle markers (i.e., ribosome, mitochondria, endoplasmic reticulum). Scale shown is the representation of label-free quantitation intensity. **C)** Comparison of Tsc-sEVs with endometrial receptivity markers from PMID: 29315421, noting 41 proteins commonly identified, and **D)** their GO terms represented as a bar plot (indicating number of proteins identified and their abundance). **E)** Comparison of Tsc-sEVs (identified in all 3 biological replicates) with sEVs from

two endometrial cell lines (Ishikawa and ECC-1 cells) (1833 proteins present in both endometrial sEVs), noting 785 proteins uniquely identified in Tsc-sEVs, and **F)** their GO terms represented as a bar plot (indicating number of proteins identified and their abundance).

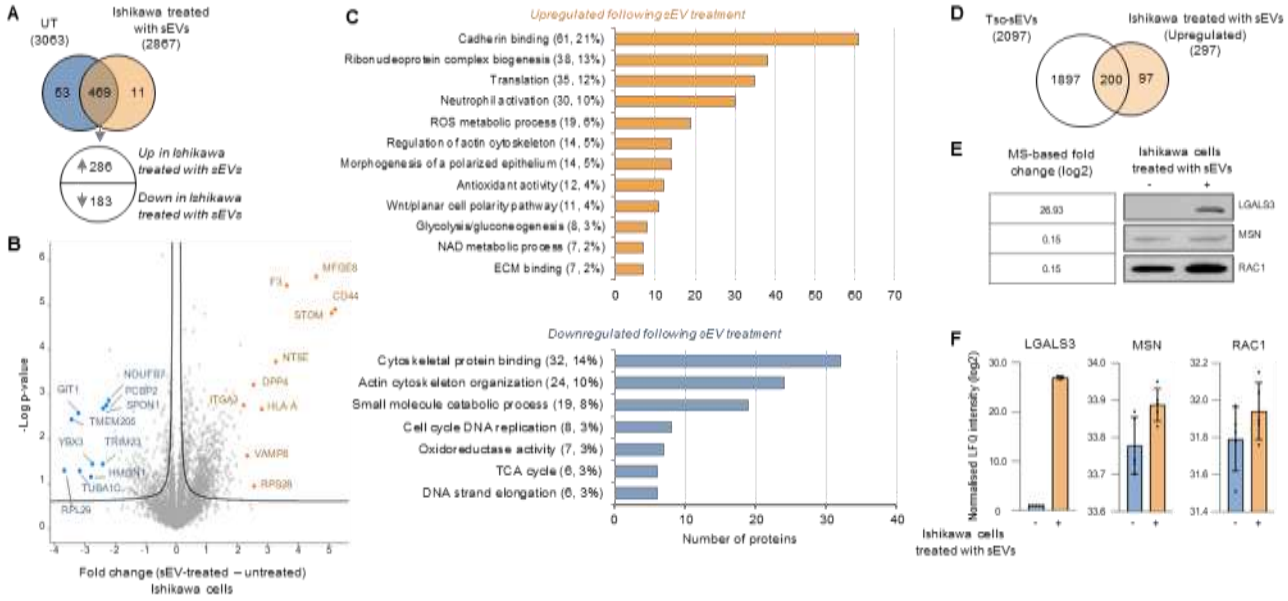

Figure 3. Tsc-sEV-mediated endometrial epithelial cellular reprogramming. A) Venn diagram of proteins identified in endometrial Ishikawa cells treated with Tsc-sEVs or untreated (PBS control) (fold change log2>1/<-1, p<0.05, ≥3 biological replicates). **B)** Volcano plot of proteins identified in Tsc-sEV treated Ishikawa cells and untreated control. Most significantly differentially abundant proteins are highlighted. **C)** Pathways and biological processes dysregulated in Tsc-sEV treated Ishikawa cells and untreated control. **D)** Venn diagram of proteins identified in Tsc-sEVs (with stringent inclusion criteria: in all biological replicates) in comparison to significantly upregulated proteins in endometrial Ishikawa cells treated with Tsc-sEVs (fold change log2>1, p<0.05, ≥3 biological replicates), with 200 proteins commonly identified. **E)** Relative quantitation (left panel, LFQ intensity log2, combined analysis of n=5 independent replicates) and Western blot analyses (right panel, validation sEV treated cell analysis, n=x) comparing cellular expression of LGALS3, ANXA2, MSN, and RAC1 following Tsc-sEV treatment. All experiments were replicated on independent samples and depicted are representative images and immunoblots. **F)** Normalised LFQ intensities (log2) of LGALS3, ANXA2, MSN, and RAC1 between Tsc-sEV-treated Ishikawa cells and untreated control.

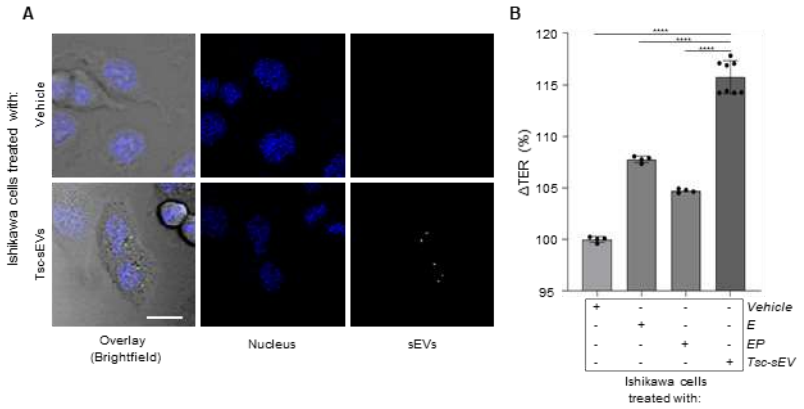

Figure 4. Trophectoderm sEVs alter Ishikawa cell transepithelial resistance. **A)** Confocal image of live uptake of Tsc-sEVs (DiO, green) or PBS vehicle control (2 h) by Ishikawa cells showing a middle slice through the nucleus, indicate that Tsc-sEVs are internalised by Ishikawa cells. Nuclei stained with Hoechst (blue). Scale bar 10 μm. **B)** Percentage change in transepithelial electrical resistance of Ishikawa cells stimulated with E or EP or Tsc-sEVs (50 μg/ml) at 24 and 48 h timepoints. Data presented as mean ± SD (error bars, ****$p<0.0001$) (biological n =3, technical n = 4).

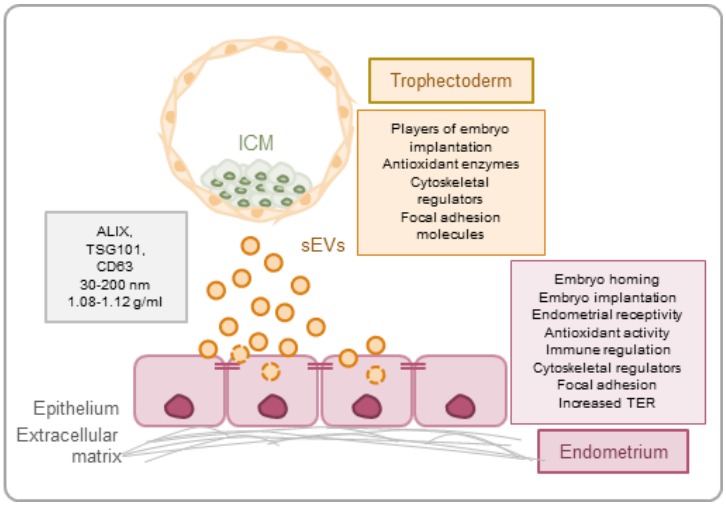

Figure 5. Human trophectoderm sEVs during implantation: working model for Tsc-sEV reprogramming at the implantation interface. Highly purified human trophectoderm stem cell-derived sEVs with biophysical properties of sEVs/exosomes, were proteomically characterised and reported to harbour players of implantation, antioxidant enzymes, cytoskeletal regulators, and focal adhesion molecules; critical factors present at the implantation interface. Functionally, Tsc-sEVs taken up by the endometrial epithelium modulated their polarity through TER changes. Importantly, they had the capacity to alter endometrial cellular proteome landscape through upregulation of players involved in embryo implantation (i.e, focal adhesion), endometrial receptivity (i.e., cytoskeletal regulators), antioxidant activity (i.e., antioxidant enzymes), and immune regulation. Thus, human trophectoderm sEVs potentially modulate changes to endometrial cellular proteome landscape that supports embryo implantation.

Supporting Information
Supporting information is available from the Wiley Online Library or from the author.

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Author Contributions

QHP, AR, and DWG conceived and designed experiments. QHP carried out the majority of experiments. IC performed experimental work (imaging). LAS helped develop project and provided critical insight. QHP, AR, and DWG wrote, reviewed and edited the manuscript. All authors approved the final manuscript.

Conflicts of interest: The authors declare no competing interests.

Data and Software Availability: The accession number for the mass spectrometry data reported in this paper is PeptideAtlas Consortium via the PeptideAtlas proteomics repository: PXD022501

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