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ORIGINAL RESEARCH

Exosomes and soluble secretome from hormone-treated endometrial epithelial cells direct embryo implantation

S. Gurung [1,*], D.W. Greening[2,3], S. Catt[4], L. Salamonsen [1,5], and J. Evans [1,6]

[1]Department of Obstetrics and Gynaecology, Monash University, Melbourne, Victoria, Australia; Centre for Reproductive Health, Hudson Institute of Medical Research, Clayton, Victoria, Australia [2]Molecular Proteomics Laboratory, Baker Heart and Diabetes Institute, Melbourne, Victoria, Australia [3]Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Bundoora, Victoria, Australia [4]EPRD, Department of Obstetrics and Gynecology, Monash University Melbourne, Victoria, Australia [5]Department of Molecular and Translational Medicine, Monash University, Clayton, Victoria, Australia [6]Department of Physiology, Monash University Clayton, Victoria, Australia

*Correspondence address. Centre for Reproductive Health, Hudson Institute of Medical Research, 27-31 Wright St., Clayton 3168, Victoria, Australia. Tel: +61-8572-2538; E-mail: shanti.gurung@hudson.org.au http://orcid.org/0000-0002-9003-652X

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ABSTRACT: A successful pregnancy requires a synchronous dialogue between endometrium and embryo within the endometrial milieu. The aim of this study was to assess the role in the implantation of mediators in the endometrial milieu. Total secretome (TS), soluble secretome (SS) and small extracellular vesicles (containing exosomes) were generated from hormonally primed human endometrial epithelial cell culture medium. Human trophectoderm stem cell-derived spheroids were cultured with TS, SS or exosomes (30 μg/ml) on hormonally primed epithelial cells, with exosomes significantly increasing cell adhesion and outgrowth. Furthermore, F1 mouse 2-cell embryos were cultured in groups for 48 h followed by culture with each secretome fraction (30 μg/ml) for 48 h. Blastocyst cell number and hatching were quantified. In addition, blastocysts were further cultured on a fibronectin matrix for 72 h or transferred to recipient mice (with corresponding secretomes) with embryo implantation assessed after 6 days. Exosomes significantly increased total cell number in mouse embryos and complete hatching from zona pellucida, with both exosomes and SS significantly enhancing mouse embryo outgrowth. Importantly, exosomes increased the embryo implantation rate in comparison to other secretome fractions (normalized based on treatment amount) from the endometrial epithelia. These data indicate that endometrial epithelial exosomes support embryo growth, development and implantation while the SS has selective involvement specifically on mouse embryo outgrowth. This finding provides new insights into the molecular differences of endometrial secretome components in implantation and early embryo development and may implicate endometrial exosomes in the pathophysiology of implantation failure in infertility.

Key word: human endometrial epithelial cells / extracellular vesicles / crude exosomes / embryo implantation / human trophectoderm spheroids / infertility / embryo development / embryo transfer in mice

Introduction

Infertility is an ongoing global reproductive issue affecting ~48.5 million reproductive-age couples worldwide (Mascarenhas *et al.*, 2012). In Australia and New Zealand alone, while ~82 000 couples sought artificial reproductive therapies in 2017, the success rate of live birth was only 18% (Newman *et al.*, 2019). The success rate also decreases with increasing number of treatment cycles (Chambers *et al.*, 2017) and age of women (Newman *et al.*, 2019). Although the causes of infertility have been reported to be equally related to male, female or both partners (Boyle *et al.*, 2004), women take the sole social burden. Recently, great improvements in *in vitro* embryo culture have generated high-quality embryos for transfer into the uterine cavity of women during an IVF cycle (reviewed in Gardner and Lane (2019)]. Despite this, failure of embryo implantation underpins the low success rate of the infertility treatment.

Successful establishment of pregnancy requires synchronous dialogue between the blastocyst and the endometrium within the uterine milieu (Salamonsen *et al.*, 2009). Uterine fluid (UF) contains a rich array of

nutrients and soluble mediators that facilitate final embryo development and implantation (Salamonsen *et al.*, 2009; Salamonsen *et al.*, 2016). Components of UF modify the endometrial epithelial cell adhesion molecules and support embryo growth, a critical step in embryo implantation (Salamonsen *et al.*, 2013; Salamonsen *et al.*, 2016). It is reported that ~40% of transcripts encode for proteins in the endometrium, of which ~15% of these proteins are secreted proteins ((Uhlen *et al.*, 2015). In other highly secretory organs such as salivary gland and pancreas, this can be as high as 60% and 70%, respectively, highlighting the specialization of tissues for the production of secreted proteins to maintain tissue homeostasis (Uhlen *et al.*, 2015). These secreted proteins can mediate paracrine/autocrine cell communication at the early stage of pregnancy, critical steps of implantation (Salamonsen *et al.*, 2009). Along with secreted de novo synthesized soluble molecules and secreted factors, the membrane-enclosed extracellular vesicles (EVs) represent an important functional mediator associated with reproductive biology (Simon *et al.*, 2018).

EVs are membrane-bound organelles, which can convey information between cells through the transfer of functional protein and genetic information to alter phenotype and function of recipient cells (Ng *et al.*, 2013; Salamonsen *et al.*, 2016; Doyle and Wang, 2019; Evans *et al.*, 2019a). Importantly, the EV membrane protects the cargo from degradation by extracellular proteases. Previously, we and others have identified small EVs (termed exosomes, Exo) based on the expression of marker proteins CD9, CD63 and CD81, and the particle size of 50–150 nm in UF, and reported their release from the endometrial epithelial cell line ECC1 (subsequently confirmed in primary endometrial epithelial cell cultures) (Ng *et al.*, 2013; Campoy *et al.*, 2016; Greening *et al.*, 2016a). Most importantly, the Exo-cargos were regulated by ovarian hormones, estradiol 17β (E) and medroxyprogesterone acetate (P). Particularly, Exo derived from EP-treated ECC1 cells (EP-ECC1) (simulating the secretory phase of the menstrual cycle) contained select protein cargo implicated in fundamental changes required for embryo implantation including adhesion [LAMA5 (laminin subunit alpha-5) and ICAM1] and migration and invasion [Fibulin-1, CD55 and heparan sulfate proteoglycan 2 (HSPG2)] (Greening *et al.*, 2016a). These exosomes were taken up by trophectodermal cells highlighting a potential role of endometrial Exo in embryo development and implantation (Greening *et al.*, 2016a). Furthermore, we reported that the soluble secretome (SS) from EP-ECC1 cells contained enriched proteins associated with metabolism but had reduced integrins essential for endometrium–embryo communication and implantation (Greening *et al.*, 2016b). Therefore, the aim of the present study was to investigate the role of different components of the secretome [total secretome (TS), SS and crude exosomes (cExo)] derived from 'receptive endometrial epithelial cells' (EP-ECC1) to mediate functional changed in embryo development and implantation.

Materials and methods

Generation of human endometrial epithelial cell secretome

The human endometrial adenocarcinoma cell line, ECC1, was used as the source of an endometrial epithelial secretome as previously described (Greening *et al.*, 2016a). ECC1 cells were validated by karyotype analysis (Mo *et al.*, 2006; Korch *et al.*, 2012) according to the American Type Culture Collection (ATCC) ASN-0002 guidelines (Sdo and Manassas, 2011). ECC1 is reported to closely represent luminal epithelial cells, the cells lining the endometrium that first interact with the blastocyst trophectoderm (Li *et al.*, 2015). ECC1 was maintained and treated according to our previously published protocol (Greening *et al.*, 2016a). Briefly, ECC1 cells were cultured in DMEM/F12 supplemented with 1% penicillin–streptomycin (v/v) and 2% EV-free fetal bovine serum (FBS, v/v) (generated by the ultracentrifugation of FBS for 18 h at 4°C). At 80% confluency, cells were cultured in serum-free media with 0.5% insulin–transferrin–selenium (v/v, Invitrogen-Gibco) and sequentially treated with 10^{-8} M 17β-estradiol (E) for 24 h followed by E plus 10^{-7} M medroxyprogesterone acetate (P) for further 48 h, to mimic the receptive phase of the menstrual cycle. Conditioned media (CM) were collected at 24 and 48 h after the EP treatment and used to isolate TS, SS and cExo. The CM were centrifuged at 500*g* for 5 min followed by 2000*g* for 10 min to remove floating cells and apoptotic bodies; the supernatant was referred to as TS. To derive SS and cExo fractions, large microvesicles were removed by centrifuging the TS at 10 000*g* for 30 min followed by ultracentrifugation at 100 000*g* for 1 h. The SS fraction was prepared by concentrating the supernatant by centrifugal ultrafiltration (3K NMWL Ultra-15; Merck-Millipore) at 3000*g*. The pelleted exosomes (cExo) were further washed with PBS, pelleted at 100 000*g* for 1 h, resuspended in PBS and stored at −80°C (Fig. 1A).

Protein quantification and western blotting

The protein concentrations of TS, SS and cExo were quantified using the bicinchoninic acid protein quantification (Thermo Scientific, IL, USA) as per manufacturer's instructions. cExo from every isolation were verified by the detection of exosome markers Alix and TSG101 using the western blotting assay (Greening *et al.*, 2016a). Briefly, cExo were lysed in 4× SDS lysis buffer for 10 min and incubated for 5 min at 95°C and proteins (10 µg protein) were electrophoretically separated and electrotransferred onto nitrocellulose membranes. The membranes were blocked with 5% skimmed milk powder (w/v) in Tris-buffered saline, 0.1% Tween® 20 Detergent (v/v), for 1 h at room temperature (RT) and probed with primary antibodies, mouse anti-Alix (1:1000; # 2171S; Cell Signaling Technology) or mouse anti-TSG101 (1:1000; # 612696; BD Biosciences) overnight at 4°C followed by horseradish peroxidase (HRP) goat anti-mouse immunoglobulin G (1:1000, # 2023-04; Dako) for 1 h at RT. Detection was performed treating each membrane for 2 min with enhanced chemiluminescence (Bio-Rad), which provided the HRP substrate, and the signal was captured by Image Lab software using ChemiDoc™ XRS+ system.

Human trophectoderm (L2-TSC) cell culture and trophectodermal spheroids as blastocyst mimics

L2-TSC cells, derived from trophectoderm stem cells (Zdravkovic *et al.*, 2015), are human trophoblast stem cells (kind gift from Prof Susan Fisher, UCSF). They were maintained in a 0.5% gelatin (v/v) (G1393, Sigma Aldrich) coated flask in media containing 10% FBS (v/v), 10 ng/ml fibroblast growth factor 2 (FGF2: # 233-FB-001mg-CF; R&D Systems) and 10 µM SB431542 (# 1614; Tocris Bioscience).

L2-TSC spheroids were generated according to our previous protocol with slight modification (Evans *et al.*, 2019a; Evans *et al.*, 2020a,b).

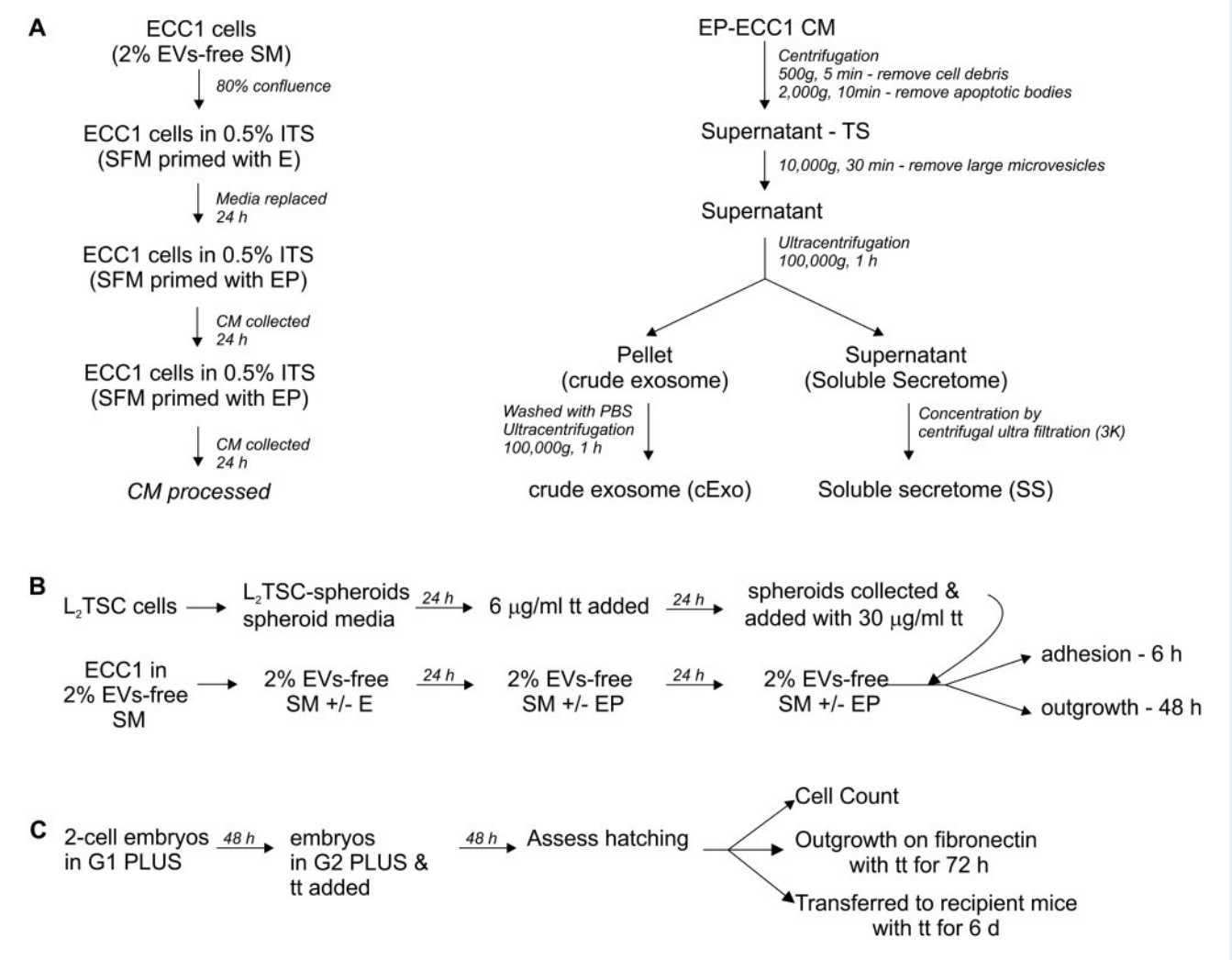

Figure 1 Endometrial epithelial cell secretome generation, isolation strategy and experimental workflows. (**A**) Experimental flowchart of ECC1 cells' treatment with estradiol 17β (E) and medroxyprogesterone acetate (P) and CM collection followed by differential centrifugation of CM to generate TS and, following ultracentrifugation, to generate SS and cExo fractions. (**B**) Experimental adhesion and outgrowth assay workflows of L2-TSC spheroid culture to assess endometrial epithelial cell secretome. (**C**) Experimental workflow of mouse embryo culture with endometrial epithelial cell secretome components *ex vivo* and *in vivo*. cExo, crude exosome; CM, culture medium; TS, total secretome; SM, extracellular vesicle-free serum media; SFM, serum-free media; tt, treatment; SS, soluble secretome.

L2-TSC cells were seeded at a density of 2500 per well in an ultra-low adhesion round bottom 96-well plate in 200 µl of trophectoderm medium with 2% EV-free FBS. This promoted aggregation of all cells into a single spheroid per well. Media alone, TS, SS and cExo were added to the spheroids 24 h following cell seeding to a final concentration of 6 µg/ml (to mimic the physiological event of the passage of the embryos through the fallopian tube, which may contain a small amount of uterine protein) and culture continued for 24 h. Spheroids were collected and washed twice in PBS before downstream analysis (Fig. 1B).

Spheroid adhesion and outgrowth assays on hormonally primed ECC1

In parallel to trophectoderm spheroid formation, ECC1 cells were seeded on a 24-well plate and primed with E and P as above (EP-ECC1). Spheroids from the four treatment groups (media alone, TS, SS and cExo) were transferred to the EP-ECC1 with corresponding secretome (30 µg/ml, protein) treatment in 2% EV-free FBS media. Twenty-four/thirty spheroids per well were used as per our previous optimization (Evans *et al.*, 2019b) with some modifications. For the adhesion assay, 6 h after spheroid seeding, the co-cultures were washed gently with PBS. Spheroid adhesion was calculated by counting the number of spheroids adhered to the ECC1 cells and dividing by the total number of spheroids seeded, expressed as percentage. For the outgrowth assay, the spheroids were allowed to grow for 48 h following the addition of spheroids to ECC1. At the end of the experiment, images were taken using a Motic AE31 inverted microscope (20× lens). The outgrowth area was measured using ImageJ-win64. The outgrowth area, arbitrary unit, was calculated by subtracting the total area covered by the spheroid to the intact spheroid area (part of the spheroid that had not outgrown).

To investigate the role of exogenously added endometrial exosomes, we first inhibited the endogenous production of exosomes from the ECC1 monolayer using an exosome inhibitor, PDDC (Kind gift from Professor Barbara Slusher, Johns Hopkins School of Medicine). PDDC is a potent inhibitor of neutral sphingomyelinase 2 (Rojas *et al.*, 2019). ECC1 monolayers were cultured as above with and without the addition of 10 μm PDDC, and spheroid outgrowth was measured. Images were analyzed as mentioned above.

Superovulation and *ex vivo* culture of mouse embryos

Animal experimental procedures were approved by the Monash Medical Centre Animal Ethics Committee (Monash University, MMCB-2018-18). F1 (C57BL\6 X CBA, 90 females and 13 males, >8 weeks) and 21 C57BL\6 female mice were used for the experiments and were housed in the animal house at Monash Animal Services facilities, Monash University in compliance with the National Health and Medical Research Council Guidelines for the care and use of laboratory animals.

Each F1 (C57BL/6XCBA) female was injected with 5 IU pregnant mare serum gonadotropin (PMSG, # HOR-272; ProSpec Protein Specialists) intraperitoneally (IP) to induce ovarian stimulation. Forty-eight hours following PMSG, they were injected with 5 IU hCG (Chorulon-MSD Animal Health) IP to induce egg release. Immediately after hCG injection, female mice were mated 1:1 to F1 males. Forty-eight hours after setting up for mating, two-cell embryos were harvested from the oviducts. All embryo culture media were equilibrated overnight at 37°C in 6% CO_2 prior to culturing the embryos.

All embryo manipulations were performed on an Olympus microscope with a heated stage (37°C). The two-cell embryos were cultured in groups (5–10) in 20 μl drops of G-1 PLUS media (Vitrolife) under Ovoil-culture oil (Vitrolife) in 6% CO_2 at 37°C for 48 h. To mimic the uterine milieu when the embryo reaches the uterine cavity, at Day 3 (D3), they were allocated into four groups and transferred to G-2 PLUS media with 30 μg/ml TS, SS, cExo or equal volume of PBS (vehicle control) for further 48 h [Day 5 (D5)]. Then, on the fifth day of *ex vivo* culture, the hatching stage of the embryos was measured and, then, they were either (i) further cultured for outgrowth measurement, (ii) assessed for total cell number or (iii) cryo-preserved by vitrification for embryo implantation experiments (Fig. 1C).

Outgrowth competence of preimplantation embryos treated with endometrial secretome

D5 *ex vivo* cultured mouse embryos were transferred into fibronectin (5 μg/cm^2, #356008; Corning)-coated wells of 96-well plate. One embryo was seeded per well with 100 μl G-2 PLUS media and 2% EV-free FBS with the corresponding treatment secretome at 30 μg/ml final concentration. The embryos were allowed to adhere and outgrow on the fibronectin matrix for 72 hours, then fixed with 4% paraformaldehyde (PFA, w/v) for 10 min, the cell surface stained with CellMask (1:1000, # C37608; ThermoFisher Scientific) at 37°C for 30 min and nuclei stained with Hoechst 33258 (1:2000, # H3569; Molecular Probes) for 5 min. Images were visualized under the Nikon C1 microscope, and the total area was analyzed using ImageJ-win64.

Embryo cell number of preimplantation embryos treated with endometrial secretome

Embryos treated with endometrial secretome (D5) were fixed with 4% PFA for 10 min and stained with Hoechst 33258 as above for 1 h. The embryos were transferred to glycerol and mounted onto a glass microscope slide in a drop of glycerol and gently flattened with a coverslip. The stained nuclei were visualized using blue illumination (460–490 nm) under the Olympus BX41 microscope (Olympus).

Embryo transfer to recipient mice and *in vivo* implantation analysis

D5 endometrial secretome pretreated, vitrified and warmed embryos were used to assess the role of endometrial secretome on embryo implantation using Day 3 pseudopregnant C57BL/6 females (a model with low implantation efficiency, ~50%) (Blake *et al.*, 2017). All embryo transfers were performed at Monash Animal Research Platform, Monash University, Clayton, Australia. Recipient C57BL\6 females were prepared by mating with vasectomized studs. Pseudopregnant D3 recipient mice were anesthetized using isoflurane, and six embryos from each treatment group were transferred into one uterine horn with 3–5 μl of the corresponding secretome at 30 μg/ml in G-2 PLUS/2% EV-free FBS media. The recipients were monitored every day until the end of the experiments. There were no complications, and the recipients recovered well from surgery. The recipients were humanely killed 6 days after the transfer, and a total number of embryos implanted were counted. The uteri were fixed in 10% formalin overnight at 4°C and followed by 70% ethanol (v/v) at room temperature and embedded in paraffin. Eight-micron sections were dewaxed and rehydrated through descending grades of alcohol (100–70%, v/v) to distilled water. Sections were assessed following hematoxylin and eosin staining.

Proteomic profiling of cExo and SS

Proteomic profiling was performed as described previously (Greening *et al.*, 2016a,b), where cExo and SS derived from EP-ECC1 were analyzed using in-gel reduction, alkylation, tryptic digestion, solid-phase extraction before proteomic liquid chromatography–tandem mass spectrometry on a Dionex 3000 nanoUHPLC coupled to an Q-Exactive Orbitrap Elite mass spectrometer in a positive mode. Detailed sample preparation, instrument parameters and informatics are described in the Supplementary Data.

Statistical analyses

Normality tests were performed to test normal distribution. Differences between two groups were analyzed with paired *t*-test with two tails, and more than two groups were analyzed using one-way ANOVA with Tukey's multiple comparisons test, using GraphPad Prism 8, and considered statistically significant at $P < 0.05$.

Results

Validation of exosomes in ECC1-EP condition media

For the functional assays for this study, as demonstrated previously, we used the same differential ultracentrifugation protocol and validated the presence of cExo isolated from EP-ECC1 CM by exosomal markers ALIX (PDC6IP) and tumor susceptibility 101 (TSG101) using western blotting (Supplementary Fig. 1A). Immuno-based validation of exosome marker proteins was performed for each isolation procedure. EP-ECC1 cExo express surface receptor proteins such as MPZL1, CD44, EGFR and PLXNB2 and ligand proteins such as integrins α 2/6, β1/4, laminins α5, β1/2 and γ1, which are reported to be responsible to facilitate their uptake by the recipient cells (Supplementary Table I).

EP-ECC1-cExo enhance human trophectodermal spheroid adhesion and outgrowth capacity

Implantation is the rate-limiting step in establishing pregnancy, and this requires interactive adherence of trophectoderm to receptive endometrial epithelial cells. EP-ECC1-soluble factors contain cExo and SS with distinct protein profile associated with adhesion, migration, proliferation and differentiation such as ITGB1, ITGA2, EPCAM, S100A11, TGFB1 and MAPK14 (Supplementary Tables II and III). Therefore, we investigated the role of components of the EP-ECC1 secretome on trophectoderm spheroid adhesion and outgrowth using a human embryo implantation model developed in our laboratory. Hormonally (EP) primed ECC1 were receptive and significantly enhanced L2-TSC spheroid adhesion compared to non-hormonally primed ECC1, highlighting the utility of this model as a functional representation of implantation (Fig. 2A). cExo significantly increased the adhesiveness of the trophectoderm spheroids compared to TS ($P < 0.03$), SS ($P < 0.002$) and control groups ($P < 0.03$) (Fig. 2B). In addition, SS-treated spheroids were significantly less adhesive than control ($P < 0.002$) and TS ($P < 0.03$) group indicating that depriving any EVs (100K-cExo and 10K-large microvesicles) completely from the additive has a negative effect on the adhesive capacity of the spheroids.

Next, we investigated the ability of the spheroids to outgrow on the ECC1-EP-primed monolayer following their exposure to the different components of secretome. Similar to the effects on adhesion, cExo significantly increased the spheroid outgrowth compared to control ($P < 0.03$) and SS ($P < 0.03$) (Fig. 2C and D). To rule out the contribution from any endogenous secretion of exosomes from the ECC1 monolayer, we inhibited endogenous endometrial epithelial exosome production using a recently discovered exosome synthesis inhibitor, PDDC (Rojas *et al.*, 2019). First, we validated that PDDC inhibited exosome production by examining the exosome marker, Alix in CM from EP-ECC1 using the western blotting assay. Indeed, 10 μm PDDC significantly reduced the endogenous secretion of exosomes by ∼40% (Supplementary Fig. 1B). Spheroid outgrowth was still significantly higher in cExo-treated group compared to the TS group in the presence of PDDC confirming the significance of exogenous exosomes in the functional outcome (Supplementary Fig. 1C). This suggests that endometrial epithelial exosomes are critical in embryo adhesion, implantation and growth of an embryo compared with the SS.

Effect of EP-ECC1-secretome on mouse embryo development

Although human trophectoderm spheroids are the best available representatives of human blastocysts, they lack the inner cell mass and blastocoel. Therefore, we investigated the functional effect of human endometrial epithelial secretome directly on mouse embryos.

Of note, 99% of the cultured mouse embryos reached blastocyst stage at D5 in all groups, suggesting no effect on the overall developmental competency of the embryos. However, more detailed assessment indicated otherwise. First, we assessed the hatching stage of these embryos across the three-treatment and control groups. In all groups, blastocysts were either in the process of hatching or fully hatched (∼96%) (Fig. 3A). On deeper analysis of the embryo hatching stage, cExo significantly increased complete embryo hatching compared to the other groups: TS (54 ± 3%), SS (60 ± 5%), cExo (75 ± 4%) and vehicle control (51 ± 3%) (Fig. 3B). Hatching of the blastocyst from the zona pellucida (ZP) is a critical event in embryo development, occurring before embryo implantation into the endometrium. Total cell number can play a role in mechanical process of hatching. Therefore, we counted the total cell numbers at D5. Embryos treated with cExo had a significantly higher number of blastomeres than the other groups ($P < 0.03$): mean cell number/± SEM in control (104 ± 5), TS (105 ± 5), SS (107 ± 6) and cExo (127 ± 5) (Fig. 3C and D). These data indicate that cExo play dual roles in embryo development and hatching.

Next, we explored the effect of treatment of embryos with endometrial secretome components on preimplantation embryonic developmental competency by an outgrowth assay on fibronectin matrix. Consistent with the blastomere number and hatching data, embryos treated with cExo showed significantly increased outgrowth over a 72-h period. Pretreatment and addition of cExo to the culture media increased embryo outgrowth by 1.7-fold (comparison to vehicle) and 1.5-fold (comparison to TS) (Figs 2F and 3E). Interestingly, although SS did not promote spheroid adhesion and outgrowth *in vitro* or hatching and blastomere number *ex vivo*, embryo outgrowth following SS treatment was increased compared to control and TS, indicating a contribution of SS in the process similar to cExo.

Endometrial-cExo-treated embryos had increased implantation rate *in vivo*

To assess the direct effect of endometrial epithelial-derived exosomes on embryo implantation, we next transferred 168 D5 embryos primed with different secretome treatments into 21 mice, with six embryos per treatment group per uterine horn. First, we assessed the decidualization status of the endometrium in all groups to confirm that the absence of embryo implantation was not due to the uterus not becoming decidualized. Figure 4A shows uteri with typical features of decidualization: reduced luminal diameter, densely packed decidual stromal cells and elongated-folded glands. Next, we analyzed the implantation rate of the embryos for control (26 ± 10%), TS (15 ± 6%), SS (22 ± 10%) and cExo (57 ± 12%) treatment groups. cExo treatment improved the implantation compared to the other groups and

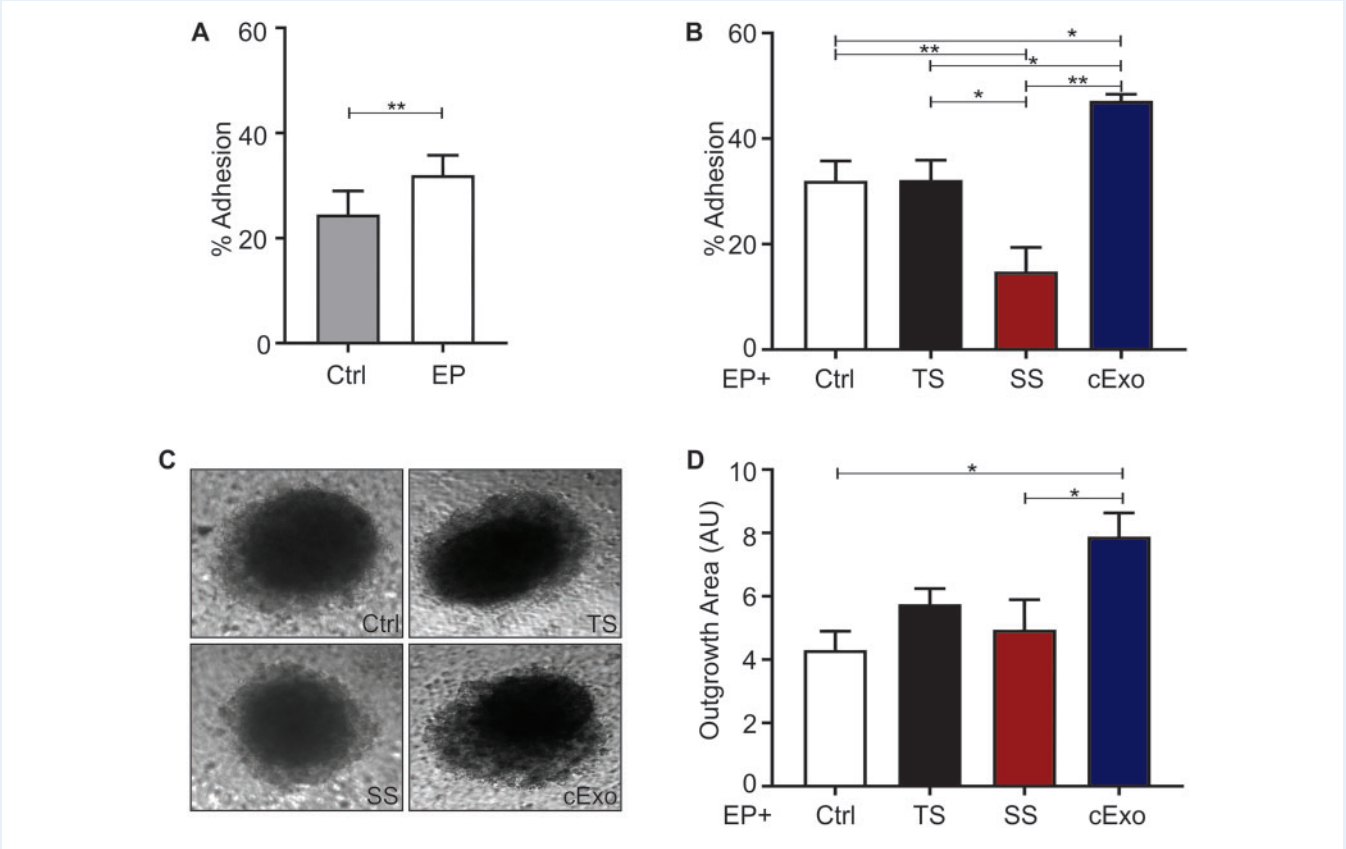

Figure 2 Effect of EP-ECC1 secretome on TSC spheroids. (**A**) Percentage of TSC spheroids' adhesion on ECC1 primed with estradiol 17β and medroxyprogesterone acetate (EP) or without (Ctrl). (**B**) Percentage of TSC spheroids' adhesion on EP-primed ECC1 with control (Ctrl), TS, SS or cExo. (**C**) Representative images of TSC spheroids' outgrowth on EP-primed ECC1 with Ctrl, TS, SS or cExo taken with ×20 lens and (**D**) quantification. Spheroids were pretreated with TS, SS or cExo (6 μg protein/ml) for 24 h before the adhesion and outgrowth assay with 30 μg protein/ml EP-ECC1 secretome components (TS, SS, cExo). Data are mean ± SEM of $n = 6$. *$P < 0.03$ and **$P < 0.002$.

was significantly higher compared to TS ($P < 0.03$) (Fig. 4B–D). Cumulatively, our data show that endometrial epithelial-derived exosomes have a significant contribution toward embryo development and enhanced implantation.

Discussion

The dialogue between endometrium and embryo is fundamental for pregnancy success. EVs have been reported as important mediators of implantation; however, understanding of their role in mediating implantation has not been demonstrated. This study used both *in vitro* (human trophectoderm stem cells) and *in vivo* (a mouse model) to demonstrate that exosomes derived from human endometrial epithelial cells act on and alter the blastocyst trophectoderm, enhancing implantation potential. We demonstrated that treating human trophectoderm spheroids with hormonally primed ECC1-derived exosomes enhanced spheroid adhesion to and outgrowth on endometrial epithelial cells, and this was supported by our findings using mouse embryos. Furthermore, exosomes augmented mouse embryo development, hatching and implantation *in utero*. On the other hand, SS from the same endometrial epithelial cells had selective involvement specifically on the mouse embryo outgrowth. This study supports the notion that each component of the endometrial epithelial cell secretome, as present in UF, can exert a selective role in the development of an embryo and embryo–endometrial interaction needed for the establishment of pregnancy. It builds on our previous observations that EP-ECC1-derived exosomes and SS protein cargos were regulated by ovarian hormones that induce receptivity in the parent cells, that exosomes are taken up by the human trophectoderm cells, which enhanced their adhesion to and invasion of ECM (Greening *et al.*, 2016a; Evans *et al.*, 2019a), and that they were essential for successful embryo adhesion and implantation (Greening *et al.*, 2016a,b).

A successful pregnancy depends on a receptive endometrium and a good quality embryo. Locally, this intricate process involves reciprocal communication between incoming embryo and the endometrium in the uterine milieu that comprise SS and EVs (Salamonsen *et al.*, 2009; Campoy *et al.*, 2016; Greening *et al.*, 2016a,b). EVs have been identified in the UF of different species including humans (Ng *et al.*, 2013; Vilella *et al.*, 2015), sheep (Burns *et al.*, 2014) and mice (Griffiths *et al.*, 2008; Lv *et al.*, 2018). They are suggested to assist early embryo development as it reaches the uterus, and in parallel prime the

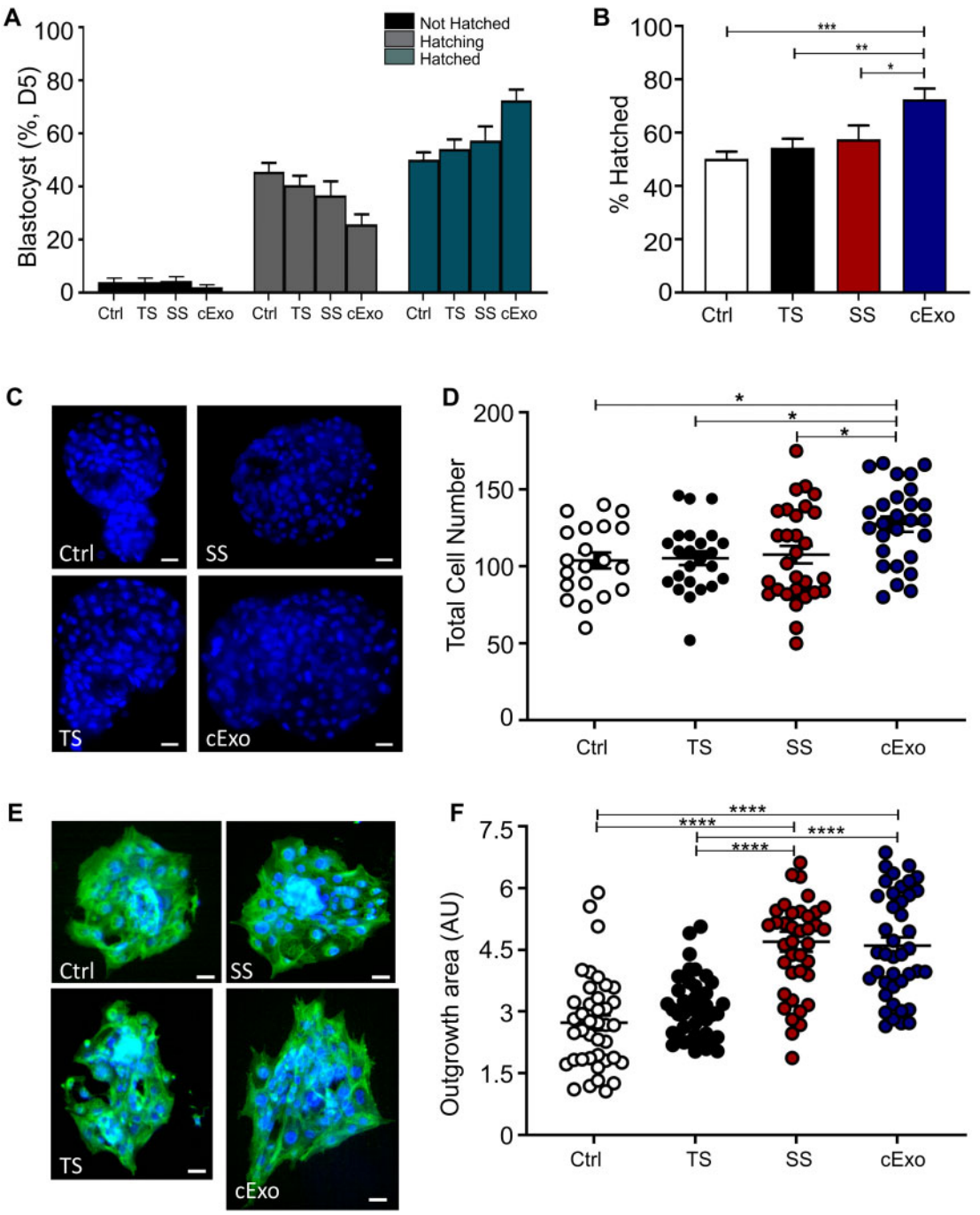

Figure 3 Effect of EP-ECC1 secretome on mouse embryo development (D5). (**A**) Percentage of mouse embryos/blastocysts (D5) of *ex vivo* culture at different stages of hatching following treatment in G-2 PLUS media with Ctrl, TS, SS or cExo. (**B**) Quantification (%) of hatched mouse embryos on D5 of treatment with 30 μg/ml of Ctrl, TS, SS and cExo from D3 to D5. Data are mean ± SEM of 19 experiments with ∼12 embryos per group per experiment. (**C**) Representative images of D5 mouse embryos following culture with 30 μg/ml of Ctrl, TS, SS and cExo from D3 to D5 and Hoechst staining. Scale bar 20 μm. (**D**) Quantification of total cell number of embryos. Each dot represents an embryo. (**E**) Representative images of mouse embryos outgrowth following culture with 30 μg/ml of Ctrl, TS, SS and cExo from D5 to D8 on fibronectin matrix and Hoechst/Cell Mask Green staining. Scale bar 100 μm. (**F**) Quantification of outgrowth surface area. Each dot represents an embryo. Data are mean ± SEM. $^{*}P < 0.03$, $^{**}P < 0.002$, $^{***}P < 0.0002$ and $^{****}P < 0.0001$.

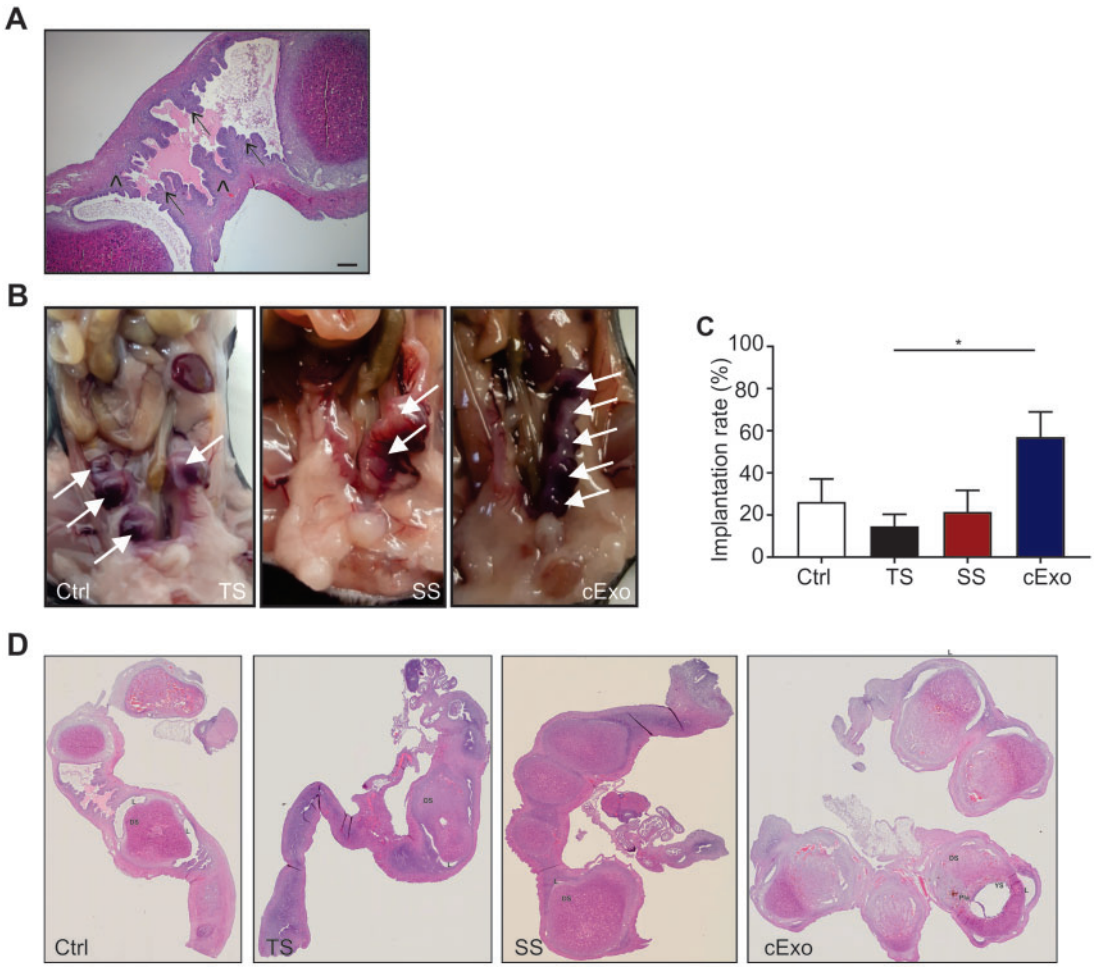

Figure 4 Effect of EP-ECC1 secretome on mouse embryo implantation. (**A**) Representative image of mouse uterus showing signs of decidualization; (*) reduced luminal diameter, (^) densely packed decidual stromal cells and (↑) elongated-folded glands. Scale bar 100 μm. (**B**) Representative images of embryo implantation (white arrow) 6 days after embryo transfer of D5 embryos pretreated with 30 μg/ml of ECC1-EP secretome. (**C**) Quantitative embryo implanted. Data are mean ± SEM, $n = 7$ mice per group with 6 embryos per mouse. (**D**) Representative hematoxylin/eosin (H/E) images of uteri harvested 6 days after the embryo transfer of D5 embryos. DS, decidualized stroma; L, lumen; Pla, placenta; YS, yolk sac.

endometrium for embryo implantation. The lipid-bilayer envelope of EVs protects the cargo from degradation, making them highly stable in the biological fluids (Simon *et al.*, 2018). In addition, they can traverse the blood–brain barrier (Chen *et al.*, 2016) as well as penetrate dense structural tissue such as cartilage (Headland *et al.*, 2015). High-throughput data analyses of human UF and endometrial epithelial cells have suggested that exosomes are enriched in proteins and RNAs involved in ECM formation and embryo implantation (Ng *et al.*, 2013; Burns *et al.*, 2016; Campoy *et al.*, 2016; Greening *et al.*, 2016a). Sheep UF-EVs contain RNA for endogenous jaagsiekte sheep retrovirus (a type of endogenous retrovirus), which are transferred to the conceptus trophectoderm (Black *et al.*, 2010) and induce trophoblast proliferation, fusion and elongation (Black *et al.*, 2010; Burns *et al.*, 2014). Similarly, human UF-EVs contain a subset of miRNAs (miR), which have target genes relevant to embryo implantation (Ng *et al.*, 2013; Vilella *et al.*, 2015), particularly, Hsa-miR-30d. Exosomes containing Hsa-miR-30d were internalized by mouse trophectoderm and increased embryo adhesion via up-regulation of adhesive molecules such as Itgb3, Itga7 and Cdh5 (Vilella *et al.*, 2015). Previous finding of EP-ECC1 exosome uptake by trophectoderm cells (Evans *et al.*, 2019a) and our finding showing the presence of uptake proteins in exosomes support their contribution in the present functional outcomes. These studies highlight the important biological roles uterine EVs/exosomes have in the establishment and maintenance of pregnancy.

Although we know that the ECC1 secretome is regulated by ovarian hormones, the relative contributions of the components (i.e. soluble and membrane vesicles) to embryo growth and implantation success or failure were previously limited. Using our recently established *in vitro* model of embryo implantation (Evans *et al.*, 2019a; Evans *et al.*, 2020b), we showed that cExo had the most significant effect on the

adhesion and outgrowth of trophectoderm spheroids compared to TS, SS and vehicle control. These exosomes contain specific proteins, distinct from their parental cell, packages fundamental to adhesion, migration, invasion and ECM remodeling including LAMA5, Collagen alpha-1(XV) chain (COL15A1), HSPG2 and glypican-1 (Greening et al., 2016a), whose expression in the trophectoderm has been correlated with the acquisition of embryo attachment competency (Carson et al., 1993; Smith et al., 1997; Crescimanno et al., 1999). Importantly, the functional capacity of exosome uptake was abrogated through inhibitors, chlorpromazine or dynamin inhibitor II, influencing how exosomes interact and are internalized in recipient human trophectoderm cells (Evans et al., 2019a). It was interesting to find that while SS had a diminishing effect on adhesion, it affected outgrowth similarly to cExo. This functional outcome also supported our proteomic findings whereby various adhesive integrins (ITG) including ITGA3 and ITGB4–6, essential components of endometrium–embryo communication and implantation, were down-regulated within the epithelial cell secretome in response to EP (the stimulus for receptivity) while proliferation and differentiation proteins such as Polypyrimidine Tract Binding Protein 3 and Hepatocyte Growth Factor Receptor were up-regulated (Greening et al., 2016b). In addition, the specific differential protein profile demonstrated that exosomes had unique factors associated with migration, adhesion and ECM while proliferation and cell differentiation were exclusive to SS. These results indicate that endometrial epithelial cells secrete pro-implantation factors that facilitate outgrowth suggesting that the intricate balance each component has in implantation.

Embryos reach the human uterine cavity at around Day 4 after fertilization at morula or early blastocyst stage and develop within the uterine cavity for another 2 days before implanting into the endometrium (Croxatto et al., 1972). During this time, the embryo is surrounded by the ZP. This consists of a specialized ECM, which provides protection from external factors until hatching and implantation (Bokhove and Jovine, 2018). The embryo must hatch from the ZP before apposing to the endometrium. Clinically, problems in hatching can contribute to failure in assisted reproductive intervention (Hammadeh et al., 2011). Our study demonstrated that cExo significantly promoted both embryo development and complete zona hatching, at least in mice. Zona hatching is reported to require both mechanical and chemical processes (Sathananthan et al., 2003; Simon and Laufer, 2012; Shafei et al., 2017). The mechanical component involves embryo development (increased cell number), which leads to ZP thinning and also mechanical pressure generated from the contraction and expansion of the embryo. The chemical process is contributed from lytic proteases, which may be present in the uterine milieu. Lytic proteases from human trophectoderm have not been identified (Shafei et al., 2017) indicating a sole contribution from the endometrium. Our exosome-proteomic study (Greening et al., 2016a) identified the presence of several proteases including hydrolases (ester hydrolase, dimethylarginine dimethylaminohydrolase 1, esterase D, bleomycin hydrolase and ectonucleoside triphosphate diphosphohydrolase 2) and metalloproteinases (ADAM metallopeptidase with thrombospondin type 1 motif 15 (ADAM-TS15), which collectively could facilitate ZP lysis and enhance embryo hatching.

We also assessed the implantation potential of embryos pretreated with endometrial epithelial cell components and using a mouse model of low implantation (Blake et al., 2017). We provided further evidence that cExo have a greater contribution to embryo implantation than the soluble components. This is the first study to demonstrate a direct role of human endometrial epithelial secretomes, especially exosomes and SS using a mouse embryo implantation model. Overall, this information has the potential to be translated in the fertility clinic, by the addition of endometrial epithelial cell-derived exosomes or nanocarriers containing their critical components, not only to further improve blastocyst development in culture but also into the uterine cavity with embryo transfer in an IVF treatment cycle. Overall, our data demonstrate that cExo contribute to successful embryo preparation for implantation. The role of exosomes in the pathophysiology of infertility and management should also be investigated further as a diagnostic tool and a therapeutic tool.

Limitations of the study

The secretomes were derived from the human endometrial epithelial cell line ECC1 rather than primary epithelial cells due to limitation in generating the large quantity of primary cells, specifically their exosomes, required to perform functional assays: indeed, we required ~800 ml of EP-ECC1 culture medium (53 T^{175} flasks) to generate 400 µg exosomes. Furthermore, this is a cross-species study assessing the roles of human secretomes using a human trophoblast cell line and mouse embryos due to strict regulation in the use of human embryos. In menstruating species such as humans, decidualization (changes in the stromal compartment of the endometrium in preparation for embryo receptivity) is a spontaneous event while in non-menstruating species like mice, it occurs only after conception. To mimic this, pseudopregnant mice were used in our study.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

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Authors' roles

S.G. – Acquisition of data, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content, final approval of the version to be published. D.G. – Concept and design, interpretation of data, revising the article critically for important intellectual content, final approval of the version to be published. S.C. – Concept and design, interpretation of data, revising the article

critically for important intellectual content, final approval of the version to be published. L.S. – Concept and study design, interpretation of data, revising the article critically for important intellectual content, final approval of the version to be published. J.E. – Concept and study design, interpretation of data, revising the article critically for important intellectual content, final approval of the version to be published.

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Conflict of interest

The authors declare no competing or financial interests.

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