# Investigating the Role of Endometrial Extracellular Vesicles in Human Embryo Implantation

By

Monique Elizabeth Fatmous

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Molecular Proteomics

Baker Heart and Diabetes Research Institute

Department of Physiology, Anatomy and Microbiology

College of Science, Health and Engineering

La Trobe University

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## Abstract

Embryo implantation is a prerequisite for establishment and maintenance of pregnancy, requiring synchronous dialogue between a hormonally-regulated endometrium and competent embryo. Extracellular vesicles (EVs) are key mediators of intercellular communication which have emerged as regulators of embryo implantation. However, how endometrial-derived EVs facilitate implantation remains poorly understood, in part, due to our current understanding being from unpurified, heterogeneous populations of EVs, thereby limiting biological and functional insights.

In my thesis, I aim to investigate the proteomic composition of purified, hormonally-regulated endometrial small EVs (sEVs) and their role in reprogramming trophectoderm (outer layer of blastocyst) cell protein composition and function, to provide insights into their regulation of embryo implantation and fertility. Here, I employed a large-scale approach to generate human endometrial epithelial cell (Ishikawa)-derived sEVs under oestrogen (E) (proliferative phase) and oestrogen/progesterone (EP) (secretory phase) regulation. sEVs were highly purified using differential ultracentrifugation and density gradient-based separation (1.06-1.11 g/cm$^3$), display ~180 nm diameter, and contain classical EV protein markers (ALIX$^+$, TSG101$^+$).

Using quantitative mass spectrometry-based proteomics, composition of purified endometrial-derived sEVs reveal enrichment of distinct phase-specific cargo, with EP-sEV cargo implicated in secretory differentiation of the endometrium to become receptive, embryo development (BMPR2, PLCG1, ADAM10) and adhesion/invasion (ITGAV, CSTB, TXN).

The trophectoderm is the first point of contact for an implanting embryo to the uterine environment. Here I show endometrial-derived sEVs are taken up by human trophectoderm cells (T3-TSC) and reprogram their proteome. Specifically, endometrial EP-sEVs promote a pro-invasive phenotype through upregulation of processes associated with cell adhesion (FN1, ITGAV/6), migration (ITGA3, PLXNB2), extracellular matrix remodelling (FN1, AGRN, DPP4) and invasion (PLOD3, DPP4, GJA1). Functionally, EP-sEVs significantly promote trophectoderm cell invasion, highlighting their critical role as extracellular modulators of embryo implantation.

This study provides a comprehensive understanding of composition and function of endometrial-derived sEVs, to develop deliverable therapeutic approaches for improved implantation and pregnancy outcomes.

## Statement of authorship

**By submitting this piece of work and signing this document, I declare that:**

Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma. No other person's work has been used without due acknowledgment in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

Monique Elizabeth Fatmous Date: 08 February 2021

# Preface

### Scholarships during candidature

This work was supported by an Australian Government Research Training Program Scholarship.

### Co-author contributions to publications during candidature*

Rai A, Poh QH, **Fatmous M**, Fang H, Gurung S, Vollenhoven B, Salamonsen LA, Greening DW. Proteomic profiling of human uterine extracellular vesicles reveal dynamic regulation of key players of embryo implantation and fertility during menstrual cycle. Proteomics. 2021 Feb 26:e2000211. doi: 10.1002/pmic.202000211. Epub ahead of print. PMID: 33634576.

Rai A, Fang H, **Fatmous M**, Claridge B, Poh QH, Simpson RJ, Greening DW. A Protocol for Isolation, Purification, Characterization, and Functional Dissection of Exosomes. Methods Mol Biol. 2021;2261:105-149. doi: 10.1007/978-1-0716-1186-9_9. PMID: 33420988.

** I contributed partly to experimental data, analysis and writing of manuscript sections to these publications during the completion of my Master of Science by Research degree.*

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## List of abbreviations

ABDs – Apoptotic bodies

AHA – Azidohomoalanine

ART – Artificial reproductive technology

ATP – Adenosine triphosphate

BMP –Bone morphogenetic protein

BSA – Bovine serum albumin

CAM – Cell adhesion molecule

CDH5 – Cadherin-5

CDK – Cyclin dependent kinase

CM – Conditioned media

CTB – Cytotrophoblast

CYCS – Cytochrome C

DAVID – Database for Annotation, Visualization and Integrated Discovery

DMEM/F-12 – Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12

DNA – Deoxyribonucleic acid

dsDNA – Double stranded deoxyribonucleic acid

DTT - Dithiothreitol

E - Oestrogen

ECM – Extracellular matrix

EDTA - Ethylenediaminetetraacetic acid

EGF – Epidermal growth factor

EGFR – Epidermal growth factor receptor

EMT – Epithelial mesenchymal transition

EP – Oestrogen/ Progesterone

EP-sEV – Oestrogen/ Progesterone treated small extracellular vesicle

ERA – Endometrial receptivity array

ERK - Extracellular signal-regulated kinase

ERα – Oestrogen receptor alpha

ESCRT - Endosomal sorting complexes required for transport

E-sEV – Oestrogen treated small extracellular vesicle

EV – Extracellular vesicle

EVT – Extravillous trophoblasts

FA – Formic acid

FAK – Focal adhesion kinase

FCS – Foetal calf serum

FDR – False discovery rate

FGF – Fibroblast growth factor

FGFR – Fibroblast growth factor receptor

FOXA2 – Forkhead box a2

FSH – Follicle stimulating hormone

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GE – Glandular epithelium

GO – Gene ontologies

HB-EGF - Heparin-binding epidermal growth factor-like growth factor

HCD - High-field collision induced dissociation

HCG – Human chorionic gonadotropin

HLA-G Human leukocyte antigen

HPL - Human placental lactogen

IAA - Iodoacetamide

ICM – Inner cell mass

IGF – Insulin-like growth factor

IGFBP-1 – Insulin-like growth factor binding protein-1

IHH – Indian hedgehog

IL-1β – Interleukin-1β

ILV – Intraluminal vesicle

ITGA2 – Integrin alpha 2

ITGAV – Integrin alpha V

ITGB1 – Integrin beta 1

ITGB3 – Integrin beta 3

ITS – Insulin-transferrin-selenium

IVF – In vitro fertilisation

JAK-STAT – Janus kinase/signal transducers and activators of transcription

JNK – c-Jun N-terminal kinases

KO - Knockout

LE – Luminal epithelium

lEV – Large extracellular vesicle

LFQ – Label-free quantification

LH – Leutinising hormone

LIF – Leukemia inhibitory factor

LIFR – Leukemia inhibitory factor receptor

LPA – Lysophosphatidic acid

MAPK – Mitogen-activated protein kinase

MMP – Matrix metalloproteinase

MMT – (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

MS – Mass spectrometry

MVB – Multivesicular body

NO – Nitric oxide

NSP – Nucleoside salvage pathway

NTA – Nanoparticle tracking analysis

P - Progesterone

PBS – Phosphate-buffered saline

Pen/Strep – Penicillin/streptomycin

PI3K-AKT – Phosphatidylinositol 3-kinase/ protein kinase B

PKA – Protein kinase A

PLA2G4A – Cytosolic phospholipase A2

PR A/B – Progesterone receptor A/B

PS – Phosphotidylserine

RBD – RNA-binding protein

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RT – Room temperature

SDC - Sodium Deoxycholate

SDS – Sodium dodecyl sulfate

SEM – Standard error of mean

sEV – Small extracellular vesicle

SILAC – stable isotope labelling by amino acids in cell culture

SP3 – Single-plot, solid-phase-enhanced sample separation

STB - Syncytiotrophoblasts

STRING – Search Tool for the Retrieval of Interacting Genes/Proteins

TEAB – Tetraethylammonium bromide

TFA - Trifluoroacetic acid

TGF-β – Transforming growth factor β

TIMP – Tissue inhibitors of metalloproteinase

TNF – Tumour necrosis factor

TPBS – Tween phosphate-buffered saline

TSC – Trophoblast stem cell

UF – Uterine fluid

uNK – Uterine natural killer cell

UT - Untreated

VEGF – Vascular endothelial growth factor

WCL – Whole cell lysate

WOI – Window of implantation

# Chapter 1: Background and literature review

## 1.1 Pregnancy

Pregnancy begins at conception and ends at birth[1]. It is a complex and highly coordinated process that requires proper development of gametes for fertilisation, and synchronous development of the endometrium (inner lining of uterine wall) and embryo[2]. Tightly regulated events including implantation – the point in which an embryo implants into the receptive endometrium, decidualisation, placentation and foetal development, dictate the establishment and maintenance of pregnancy until birth (reviewed[1]). Perturbations of these processes lead to pregnancy failure and contribute to infertility[3]. Infertility impacts ~15% of reproductive aged couples world-wide[4], and is clinically defined as the inability to conceive after 12 months of regular, unprotected sexual intercourse[5]. The success rate of natural conception in humans is low (~30%)[6], with 75% of unsuccessful pregnancies attributed to implantation failure[7]. Artificial reproductive technologies (ART), including *in vitro* fertilisation (IVF), have helped millions of couples world-wide to conceive[8], however, the success rate of ART achieving a clinical pregnancy (an initiated ART cycle resulting in live birth) remains disappointingly low at 18.1% (Australia/ New Zealand)[4]. Furthermore, human fertility rates in Australia are steadily declining from 3.55 in 1961 to 1.74 in 2017 (based on average number of children conceived) (Births, Australia, Cat. No. 3301.0.)[8,9]. Consequently, infertility remains an ever increasing emotional, physical and financial burden for couples.

## 1.2 Embryo implantation

A fundamental step for pregnancy establishment is the process of implantation, being the first point of maternal-embryonic interaction[2,10] where synchronous dialogue between the developing embryo and receptive endometrium is critical for implantation success[2,7]. However, the molecular mechanisms that underpin maternal-embryo interaction during implantation remain poorly understood[11].

Pregnancy is a dynamic process initiated with the release of an oocyte (egg) from the ovaries during ovulation, which is fertilised by a spermatozoon to form a zygote[3]. The zygote undergoes controlled cell divisions and morphogenesis as it travels through the fallopian tubes to the uterine cavity, where it transforms into a multicellular blastocyst comprised of an inner cell mass (ICM; later forming the foetus) and blastocoel cavity (fluid filled space within the blastocyst), surrounded by a specialised layer of cells called the trophectoderm (later forming the placenta)[12,13] **(Figure 1.1)**. The blastocyst is encapsulated by a protective glycoprotein layer known as the zona pellucida, from which it hatches in preparation for implantation[14]. Two critical prerequisites for successful implantation include (i) an implantation competent embryo (based on morphological grading[15]) and (ii) a receptive endometrium, which during a brief 3-4 day period termed the window of implantation (WOI), is permissive for an embryo to implant[16,17].

Implantation consists of three main stages: (i) *apposition;* where the blastocyst positions itself to the implantation site of the endometrium; (ii) *attachment;* outer (trophectoderm) cells of the blastocyst attach to the receptive endometrial epithelium; and (iii) *invasion;* where invasive trophectoderm (trophoblast) cells cross the endometrial epithelial basement membrane, rapidly invade the underlying endometrial stroma[18-20] (supportive component of endometrium that controls tissue proliferation and remodelling[21]), and differentiate into extraembryonic lineages to establish the maternal-foetal interface (placenta)[22]. The placenta is comprised of cytotrophoblasts (CTBs) extravillous trophoblasts (EVTs) and syncytiotrophoblasts (STBs)[23]. STBs connect the maternal circulation to the developing foetus[24], and secrete hormones including progesterone (P), leptin[25], human chorionic gonadotropin (hCG) and human placental lactogen (HPL), which are critical in maintaining ongoing pregnancy[26]. EVTs on the other hand, anchor the placenta to the uterine wall and remodel the maternal vasculature to maintain adequate blood supply to the growing foetus[27]. The coordinated invasion, proliferation and differentiation of trophoblast lineages is critical for successful pregnancy, with impairment of their development resulting in miscarriage, preeclampsia or intrauterine growth restriction[7,26]. These processes are both spatially and temporally regulated through reciprocal signalling between the uterine microenvironment and embryo[28,29].

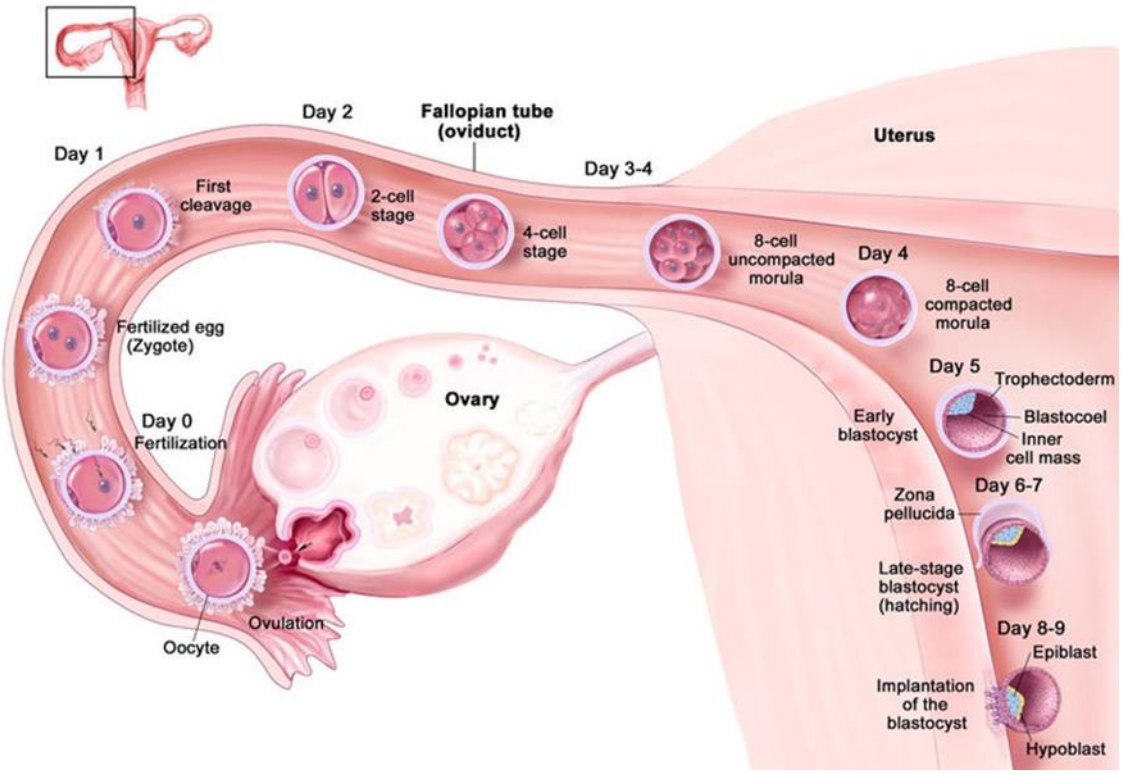



**Figure 1.1 Stages of early human embryonic development.** An oocyte released from an ovarian follicle into the fallopian tube during ovulation is fertilised by a spermatozoon to form a zygote (day 0)[30]. The zygote undergoes rapid cell divisions and morphogenesis as it transverses the fallopian tube. The developing embryo, now termed morula (day 4), enters the uterine cavity and transforms into a blastocyst (day 5) encapsulated within the zona pellucida (glycoprotein layer that protects the blastocyst)[14]. The blastocyst is comprised of an outer trophectoderm layer which surrounds the inner cell mass and blastocoel cavity. From this point (~6-10-days post-ovulation), under progesterone and oestrogen-mediated regulation, the endometrium achieves receptivity. Uterine receptivity is defined as the physiological state of the maternal endometrium in which it is permissive to embryo implantation that occurs during the brief 3-4 day WOI period[31]. Once the blastocyst hatches from the zona pellucida (~6-7 days post-ovulation), through trophectoderm interaction it undergoes apposition and attachment to the receptive endometrium, followed by invasion. *Figure obtained and used here with approval from[32].

*For the National Cancer Institute © (2001) Terese Winslow LLC, U.S. Govt. has certain right

### 1.2.1 Preparing the embryo for implantation

Dynamic morphological and molecular changes regulate embryo development from a zygote to competent blastocyst[2]. Embryos undergo blastocyst activation, a process which programs them towards an implantation-competent state[18] that possess a molecularly distinct signature from their dormant state[33]. Gene alterations in cell-cycle, cell signalling and energy metabolic pathways including steroid, cannabinoid and Wnt signalling are observed in this activated state[33]. Further, heparin-binding epidermal growth factor-like growth factor (HB-EGF) and its receptors ErbB1 and ErbB4 are upregulated in activated blastocysts[33-35], while mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/$Ca^{2+}$-signalling cascades are crucial for blastocyst development and activation[36,37]. Canonical pluripotency markers, including octamer-binding transcription factor 4 (OCT4), sex determining Region Y-box 2 (SOX2) and homeobox protein NANOG (NANOG), have been shown to maintain ICM pluripotency within the blastocyst[38,39]. These pivotal findings provide important candidates to predict blastocyst competency that may help determine embryo viability to improve IVF outcomes[2]. The current gold standard to assess embryo competency is by morphological grading of blastocyst cell number, level of multi-nucleation, and size, symmetry and percentage of fragmentation of blastomeres (individual cells from the 2-cell stage to morula)[15], which are correlated with pregnancy and live birth rates (based on a retrospective analysis)[40].

The embryo is exposed to dynamic environments as its metabolic needs evolve throughout development[41]. In the well-oxygenated oviduct, the embryo (prior to morula stage) utilises pyruvate and lactate for ATP generation[42]. Once the morula compacts and enters the hypoxic uterine cavity (now as a blastocyst), a metabolic switch is activated in which the embryo consumes high levels of glucose through anaerobic glycolysis[43]. The blastocyst undergoes blastocoel expansion (increasing blastocoel volume) which is energetically expensive due to the ionic gradient facilitated by the Sodium-Potassium-ATPase (Na/K ATPase) pump to transport fluid into the cavity[44], with proper blastocoel expansion correlated with embryo viability[40]. Production of lactate from anaerobic glycolysis influences pH in the uterine cavity[45] to promote local endometrial remodelling that facilitates trophectoderm cell invasion, by modulating endometrial vascular endothelial growth factor (VEGF) production[16].

Indeed, blastocyst secretions influence the local uterine environment to support implantation. Blastocyst hCG secretion critically promotes expression of leukaemia inhibitory factor (LIF), VEGF and matrix metalloproteinase-9 (MMP-9)[46,47], while downregulating tissue inhibitors of metalloproteinases (TIMPs)[46,48] to regulate trophoblast invasion. Soluble human leukocyte antigen G (HLA-G) secreted by EVTs[49] modulates cytokine production to induce maternal immunotolerance towards the foetal allograft[50] and is correlated with embryo quality[51]. Likewise, interleukin-1β (IL-1β) has been identified in culture media of human embryos[52,53], proposed to mediate blastocyst-endometrium communication[54]. Embryo signalling modulates maternal recognition of the embryo and local endometrial remodelling to support implantation[55,56]. Disruption of this signalling, which is equally reciprocated by the endometrium, can result in implantation failure[57]. Therefore understanding how endometrium and embryo communicate and reprogram each other towards competency/ receptivity, will provide invaluable insights into the mechanisms that potentially underpin the basis of human infertility[11].

## 1.3 Hormonal regulation of endometrial receptivity

The endometrium is a highly structured tissue comprised of luminal epithelium (LE, facing endometrium cavity) and underlying glands (glandular epithelium, GE) embedded within a supportive stroma **(Figure 1.2)**. Under the tight regulation of ovarian steroid hormones oestrogen (E) and P, the endometrium undergoes a series of well-defined, interdependent molecular and morphological changes to create a fertile environment for embryo implantation[30,58]. Hormonal remodelling of the endometrium can be divided into distinct phases: proliferative (days 0-13), ovulation (day 14) and secretory (days 15-28) which define the average 28-day human menstrual cycle[1,2] **(Figure 1.3)**. Synchronous development between the embryo and endometrium, whereby embryo competency is superimposed with the receptive state of the endometrium, is essential for implantation[1]. The endometrium is in fact hostile (non-receptive) to an embryo[17,59], and only during the WOI (~days 20-24)[60,61], will the endometrium display a receptive phenotype to allow the embryo to implant[16].

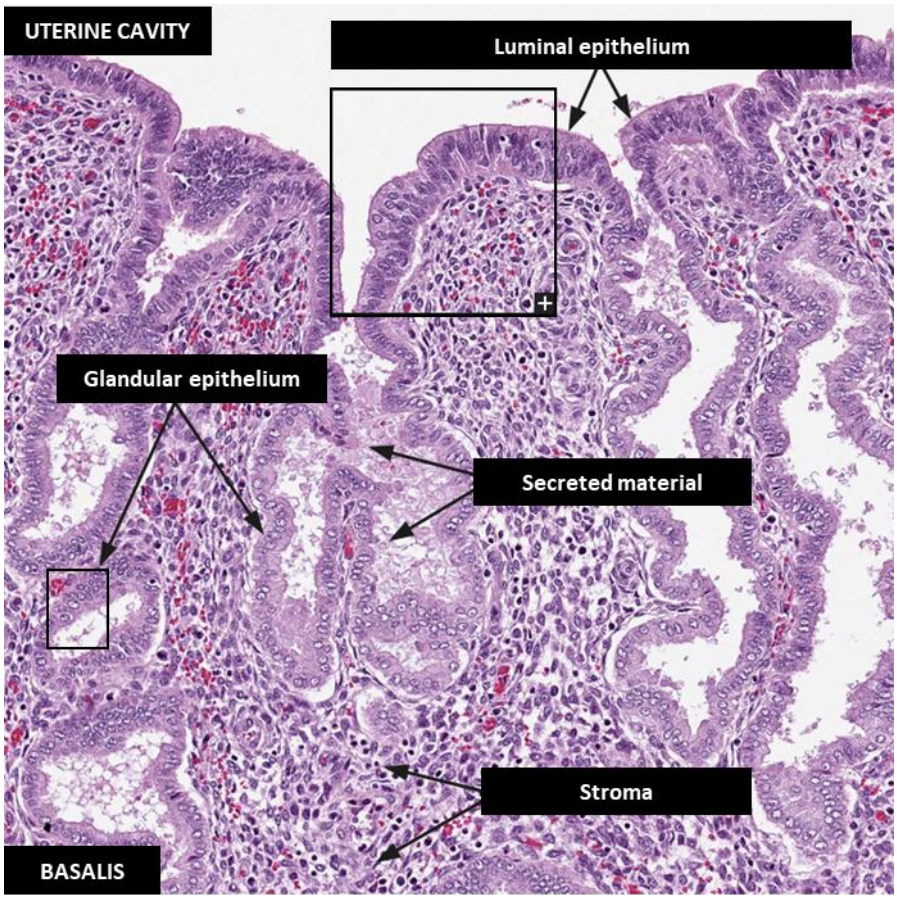
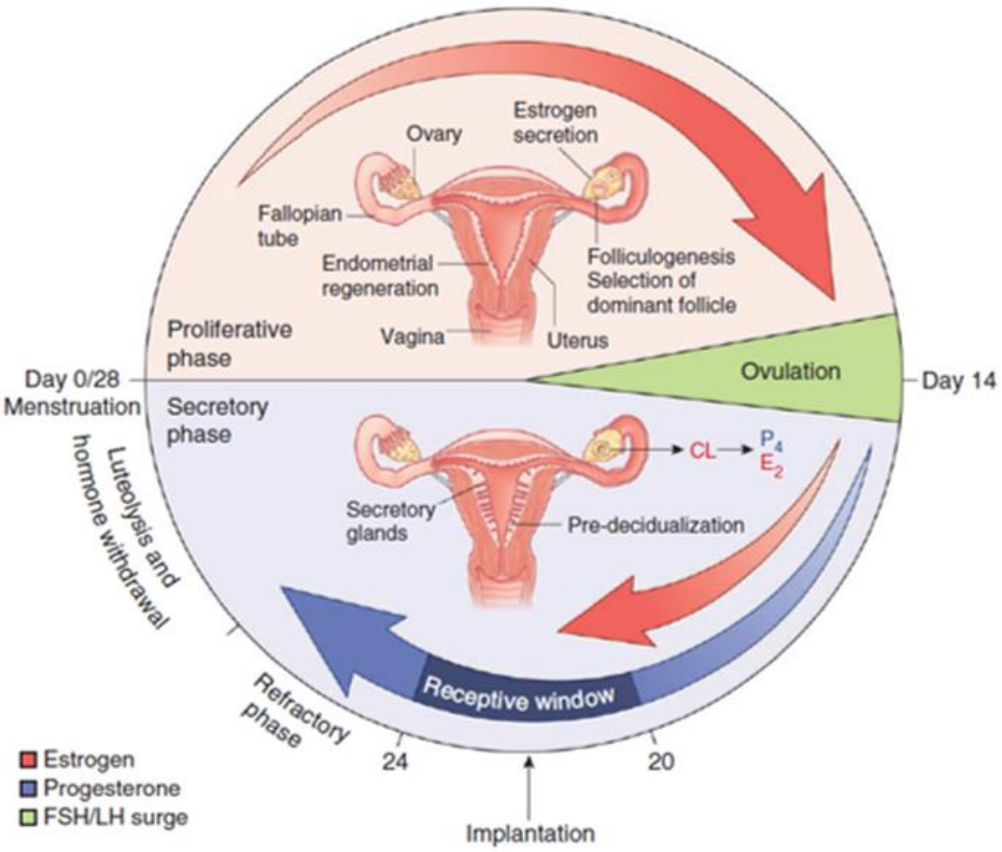
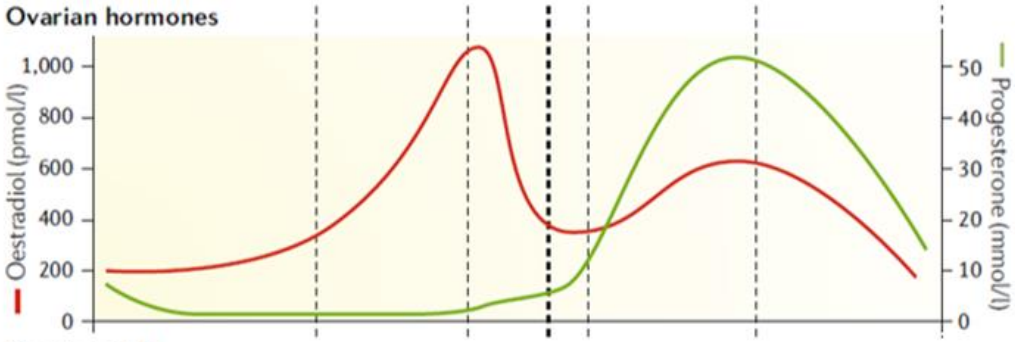



**Figure 1.2 Histology of the human endometrium during secretory phase.** Immunohistochemistry cross-section the human endometrium. The endometrium is comprised of three main cell types: the luminal epithelium (LE), glandular epithelium (GE) and stroma. The GE form uterine glands which secrete material into the uterine cavity. The LE line the apical surface of the endometrium that sheds and regenerates under ovarian hormone (E and P) regulation during the menstrual cycle. The stroma comprises the soft connective tissue layer which faces the basalis (adjacent to the myometrium). Figure adapted from[62].

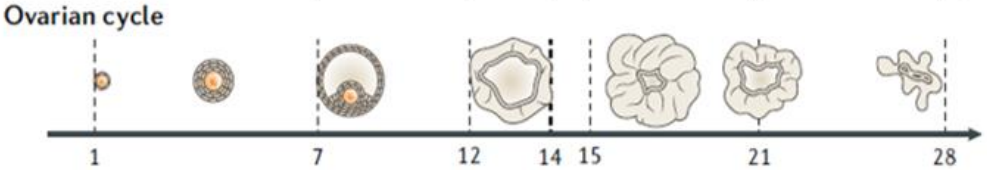
a


Ovary
Estrogen secretion
Fallopian tube
Endometrial regeneration
Folliculogenesis
Selection of dominant follicle
Proliferative phase
Vagina
Uterus
Ovulation
Day 0/28
Menstruation
Day 14
Secretory phase
CL
$P_4$
$E_2$
Secretory glands
Pre-decidualization
Luteolysis and hormone withdrawal
Receptive window
Refractory phase
24
20
Implantation
Estrogen
Progesterone
FSH/LH surge

b


Ovarian hormones
Oestradiol (pmol/l)
1,000
800
600
400
200
0
Progesterone (mmol/l)
50
40
30
20
10
0

c


Ovarian cycle
1
7
12
14
15
21
28

d

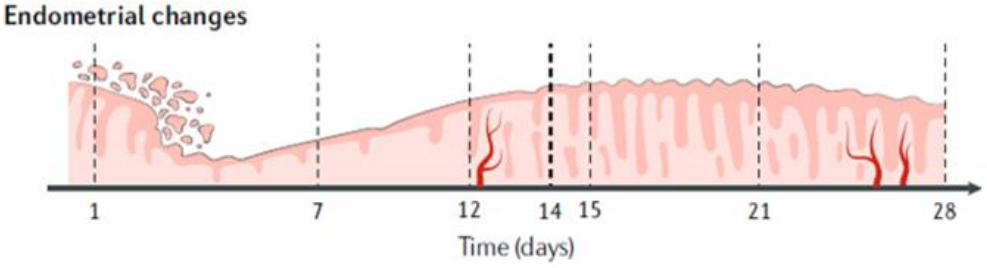

Endometrial changes
1
7
12
14
15
21
28
Time (days)

**Figure 1.3 Human menstrual and ovarian cycles. a)** The menstrual cycle is divided into proliferative (days 0-13), ovulation (day 14) and secretory phases (days 15-28), regulated by oestradiol (E) and progesterone (P)[1]. After menstruation (days 0-7), rising E levels stimulate endometrial epithelium regeneration (days 7-13)[1], and follicle stimulating hormone (FSH) and luteinising hormone (LH) production, resulting in the rupture of a mature ovarian follicle to release an oocyte (ovulation)[11]. Release of P by the ruptured follicle (corpus luteum) marks the start of the secretory phase as the endometrium thickens in response to P (days 14-20)[30]. The receptive window (~days 20-24) is the crucial phase in which the embryo implants into the endometrium[17]. Absence of an embryo shifts the receptive window into a refractory phase, resulting in E and P withdrawal to induce menstruation and reset the cycle[1]. **b)** E (red) and P (green) levels across the menstrual cycle[11]. **c)** The ovarian cycle. Follicular phase (days 1-14) involves follicular development into a dominant follicle (within the ovaries). Luteal phase (days 14-28) details the development of the corpus luteum which releases P, followed by its transformation into the corpus albicans (regressed form of corpus luteum) that is degraded in the absence of pregnancy[1]. **d)** Physical endometrial remodelling during the menstrual cycle where the endometrium lining is lost during menstruation, regenerated during the proliferative phase, and glandular and vascularised in the secretory phase[11]. Adapted from[1,11].

### 1.3.1 Proliferative phase – building of the endometrium

The proliferative phase is initiated by shedding of the endometrial LE during menstruation[30], in which increasing E (central regulator of the proliferative phase)[63] stimulates LE proliferation, accompanied by growth and development of uterine glands, stroma and blood vessels to re-establish the endometrial lining[1,64,65]. In this phase, the endometrium is non-receptive to an embryo, primarily due to the highly polarised state of the LE and its expression of anti-adhesive molecules (e.g. α-dystroglycan N-terminus (α-DG-N)[66] and mucin 1 (MUC1)[67]), which inhibit endometrial-embryo interaction[30]. The ovaries also respond to rising E levels, where generation of a dominant follicle (which encapsulates an oocyte) occurs[30]. At the same time, E surge promotes increasing levels of pituitary-derived follicle-stimulating hormone (FSH) and luteinising hormone (LH) to induce rupturing of the dominant follicle to release an oocyte (ovulation)[1] **(Figure 1.3)**.

Notably, sufficient regeneration and development of the endometrial lining during the proliferative phase provides the foundation for subsequent secretory phase activity[68]. Our current understanding of endometrial development during implantation is primarily based on animal models due to ethical limitations surrounding studies in humans. Gray *et al*.,[69] first demonstrated that ewes with defective endometrium experienced significant infertility due to failed implantation, despite implantation-competent embryos, suggesting that a functional endometrium is critical in pregnancy establishment. In line with this finding, endometrium-specific gene knockout mouse models of FOXA2[70], WNT7A[71], and WNT4[72] resulted in defective endometrial development and impaired implantation. Some of these factors are secreted by the endometrium into the uterine cavity during the secretory phase[72], which also signal to trophectoderm to facilitate attachment and motility during implantation[20,72]. Thus, these studies highlight the importance of proper endometrial development during the proliferative phase that lead to sufficient function during the secretory phase for successful implantation.

### 1.3.2 Secretory phase – maturation of the endometrium

Following ovulation, the endometrium transitions into the secretory phase, driven by the release of P from the corpus luteum (remnants of the ruptured dominant follicle)[1] (and some E[68]). During this period, the LE loses its polarity, and anti-adhesive molecules are cleaved by endometrium-derived enzymes[73,74]. On the apical surface, small microvilli protrusions called pinopodes develop, and have been shown to support embryo implantation by interdigitating with the blastocyst during adhesion[75,76]. Furthermore, cell-surface adhesion molecules (CAMs) including integrins (α1β1, α4β1, and αVβ3)[77,78] and HB-EGF[79,80] are expressed to support blastocyst adhesion. Such adhesive factors have dual roles: (i) modulating direct cell interaction (i.e., endometrial-embryo adhesion, termed adhesome[81]) and (ii) intracellular signalling (i.e., integrins extend from the endometrium and blastocyst surfaces, convey information about the extracellular environment to the nucleus[82] and modulate the network of cytoskeletal proteins and extracellular matrix (ECM)[83]).

Additionally, endometrial glands display enhanced secretory activity, releasing soluble factors[64] (e.g. LIF[84], Glycodelin-A (PAEP)[85] and VEGF[86]) which remodel the uterine microenvironment through activation of signalling pathways, including JAK-STAT[87] and PI3K-AKT[88] to modulate endometrial cell proliferation, differentiation and migration towards a receptive state[89,90]. This occurs during the mid-secretory phase (~6-10 post-ovulation)[60,61] as the endometrium becomes receptive for a very limited period to receive an implanting blastocyst[91]. Hence, timely arrival of a developed and competent blastocyst to the endometrium during the WOI is crucial for successful implantation[17], with perturbations resulting in abnormal or failed implantation[10,92,93]. The LE undergo controlled cell death (apoptosis) as a consequence of P (and E) withdrawal, or remain viable to maintain pregnancy[1,30].

## 1.4 Endometrium-derived factors for implantation

Endometrial receptivity is orchestrated by cooperation of growth and transcription factors (e.g. LIF[94], Epidermal growth factor (EGF)[95], VEGF[86]), lipid (e.g. Prostaglandins[96]) and protein (e.g. Osteopontin[97,98], Homeobox proteins[99], Wnt proteins[100]) mediators, cytokines (IL-6, IL-11[59]) and chemokines (e.g. CX3CL1, CCL7, CCL14, CCL4[101,102]), predominantly regulated by E and P **(Figure 1.4)**. E and P bind to their cognate nuclear receptors (oestrogen receptor alpha (ERα) and progesterone receptors alpha/beta (PR A/B)) to commence transcription of target genes that promote endometrial receptivity, during which stromal cells undergo functional and morphological differentiation during decidualisation[29]. Stromal cells differentiate from an elongated fibroblast-like morphology into rounded epithelial-like cells which deposit ECM to interact with, and regulate the invasion of trophoblast cells[103]. Furthermore, an influx of uterine natural killer cells (uNK) support maternal immune tolerance towards the foetal allograft[104] and secrete a range of angiogenic growth factors to regulate vascular remodelling[105] during establishment of the maternal-foetal interface[106]. It has been proposed that the decidua (decidualised stromal cells) protect the uterine tissue from inflammation and oxidative stress associated with placental formation[107,108], and protects the embryo from maternal immune rejection and stress signals[30], highlighting the complex role of the endometrium in maintaining pregnancy prior to placental formation.

A multitude of secreted signalling molecules have been identified using mouse models to regulate endometrial receptivity and endometrial-embryo interaction **(Table 1.1)**. The endometrium can signal to the embryo to coordinate implantation, while further providing nutrients for the developing foetus prior to placental establishment[109]. Endometrium-derived factors including calcitonin[110], LIF[111], HB-EGF[112], EGF[111] and lysophosphatidic acid (LPA)[113] can activate blastocysts to become implantation competent. LIF binds to the LIF receptor (LIFR) to activate JAK-STAT and PI3K-AKT signalling cascades that can modulate trophoblast invasion[114] and embryo development in mice[111,115], with LIF expression being greater in secretory phase endometrium of fertile women compared to infertile women[116]. HB-EGF has been shown to accelerate trophoblast differentiation through $Ca^{2+}$ influx to an adhesion-competent state[112], while EGF can promote blastocyst formation in trophectoderm cells by stimulating protein synthesis[117,118].

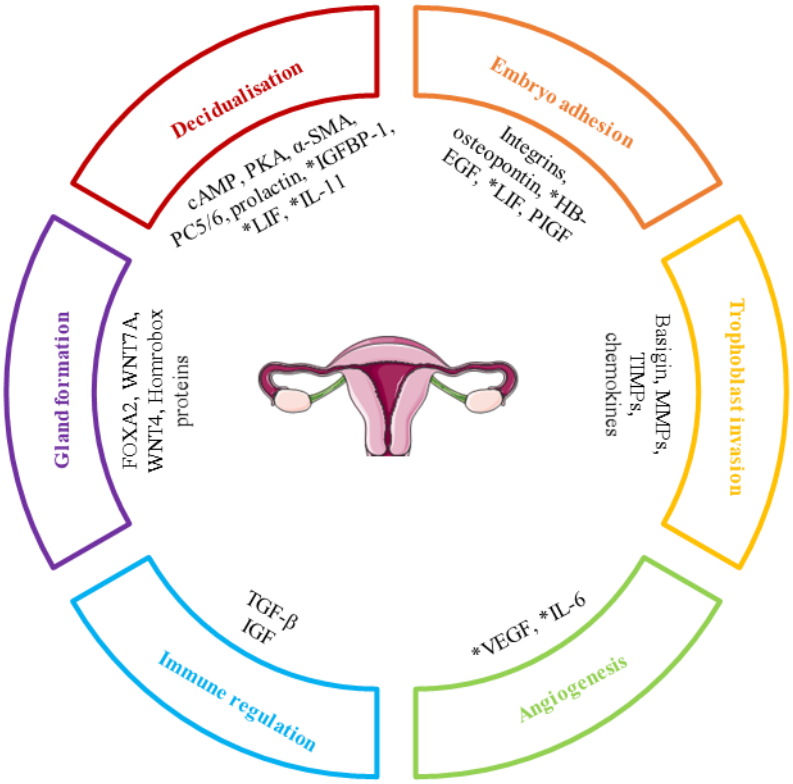



**Figure 1.4. Endometrium-derived factors for implantation.** The endometrium displays and secretes factors that regulate the uterine microenvironment, processes involved in embryo implantation (embryo adhesion and trophoblast invasion) and endometrial remodelling as it prepares for pregnancy (gland formation, decidualisation and angiogenesis factors). cAMP: Cyclic adenosine monophosphate; PKA: Protein kinase A; α-SMA: alpha-smooth muscle actin; PC5/6: Proprotein convertase 5/6; IGFBP-1: Insulin Like Growth Factor Binding Protein 1; LIF: Leukaemia inhibitory factor; IL-11, Interleukin-11; HB-EGF: Heparin-binding EGF-like growth factor; PIGF: Placental growth factor; MMPs: Matrix metalloproteinases; TIPMs: Tissue inhibitor of metalloproteinases; COL4: Collagen type 4; VEGF: Vascular endothelial growth factor; IL-6: Interleukin-6; TGF-β: Transforming growth factor β; IGF: Insulin-like growth factor; FOXA2, Forkhead box A2; WNT7A, Wnt family member 7a; WNT4, Wnt family member 4. *key regulators of embryo and endometrium development for implantation.

**Table 1.1 Molecules from the endometrium implicated in fertility: clues from mouse models**

| Factor | Study model | Putative function in fertility | Ref. |
|---|---|---|---|
| Vascular endothelial growth factor (*Vegf*) | Mouse embryos treated with human VEGF from uterine fluid, transferred to mouse to assess implantation rate. Embryo cell number, outgrowth and development assessed. | Responsible for increasing vascular permeability and angiogenesis at time of implantation and potential regulator of endometrial-embryonic interaction | 86,119 |
| Leukaemia inhibitory factor (*Lif*) | *Lif* mutation mouse model. Embryos fail to implant in *Lif*$^{-/-}$ mice, but those same embryos transferred to WT mice implant. | Signals through JAK-STAT and PI3K-AKT pathways to induce endometrial remodelling (decidualisation, proliferation, migration). Expression increases during window of implantation and modulates endometrial receptivity. | 84 |
| Indian hedgehog (*Ihh*) | *Ihh* KO mouse model in PR positive uterine cells. Embryo do not implant in Ihh$^{-/-}$ uteri. | Essential mediator of progesterone activity in the uterus, critical in mediating communication between endometrial epithelium and stroma | 120 |
| Wnt family member 4 (*Wnt4*) | *Wnt4* KO in mouse uteri. *Wnt4*$^{-/-}$ mice displayed reduced uterine gland formation and implantation failure | Critical regulator of stromal cell proliferation and differentiation (decidualisation) to support embryo implantation. | 72 |
| Heparin-binding epidermal growth factor-like factor (*Hbegf*) | *Hbegf* KO mouse model. *Hbegf*$^{-/-}$ mice displayed reduced litter size, but maintain normal ovulation and fertilisation. | Suggested to facilitate synchrony between receptive endometrium and blastocyst for timely implantation | 80 |
| Homeobox protein Hox-A10 (*Hoxa10*) | *Hoxa10* mutation induced in mouse model. *Hoxa10*$^{-/-}$ mice show significantly reduced implantation sites and suffer endometrial factor infertility. | Transcription factor upregulated during the secretory phase, suggested to have a role in endometrial receptivity | 121 |
| Homeobox protein MSX-1 (*Msx1*) | Double KO of *Msx1*$^{-/-}$*Msx2*$^{-/-}$ mice suffer implantation failure due to over-proliferation of endometrial epithelium | Transcription factor that regulates Wnt family members and downstream Wnt-β-catenin signalling. Controls epithelial cell proliferation in mid-secretory phase to maintain receptivity. | 122 |
| Cytosolic phospholipase A2 (*Pla2g4a*) | *Pla2g4a* KO mouse model compared to WT. *Pla2g4a*$^{-/-}$ mice experienced delayed implantation, smaller litter size and longer gestation period | Regulator of prostaglandin synthesis and facilitates correct timing of embryo implantation and parturition | 123 |

KO, Knockout; PR, progesterone receptor; WT, wild type

These studies highlight the role of endometrial-derived factors as key regulators of endometrial-embryo cross-talk. While much of our knowledge is derived from animal models, the use of human-derived samples or cell models overcome limitations associated with species differences. However, procedures of endometrial tissue biopsy are highly invasive[20,124,125]. Alternatively, understanding the secretory signature of the uterine microenvironment which is predominantly established by the endometrium, may provide a novel and non-invasive avenue to improve IVF embryo transfer, which would be a great advantage over endometrial biopsies.

### 1.4.1 Role of uterine fluid in pregnancy

Emerging evidence suggests uterine fluid (UF) within the uterine cavity facilitates endometrial-embryo communication[126-128], important for embryo transport, development and its implantation[20,30]. UF is a complex, protein-rich cocktail comprised of ions, growth factors, cytokines, hormones, steroids, immunomodulatory factors, proteases and their inhibitors, nutrients (amino acids, carbohydrates and lipids)[20,125], and more recently, extracellular vesicles (EVs)[128]. UF includes components possibly translocated from fallopian tube secretions, oviductal fluids, and the peritoneal cavity[30], in addition to constituents derived from activated leukocytes, blood transudate, and importantly, endometrial secretions[20]. Recent studies suggest that a developing blastocyst may also contribute to the UF milieu (reviewed[125]). The role of UF is pleiotropic and far exceeds the initial consensus that it is merely a buffering medium of the uterine microenvironment[128].

Uterine aspirate or lavage (UF derivatives) are less invasive uterine biopsies compared to endometrial tissue biopsies as they prevent disruption of the uterine microenvironment at the time of collection[124]. Molecular and proteomic studies have identified a myriad of secreted factors in UF, including VEGF[16], proprotein convertase 6[129], and LIF[130], which support various stages of the menstrual cycle and early pregnancy. A study conducted by Hannan, *et al.* [131] detected a range of such soluble factors in UF which vary between fertile and infertile women, including Antithrombin II and Alpha-2-macroglobulin, suggesting their role in embryo implantation. Apart from soluble-secreted factors[132], UF also contains EVs[20,128,133], which are emerging as important players in intercellular communication[134].

## 1.5 Extracellular vesicles – important mediators of intercellular communication

EVs are lipid membrane-encapsulated vesicles (30-1500 nm) released by cells which play a fundamental role in intercellular communication[134-136]. EVs carry selectively packaged protein[137-140], RNA[140,141], DNA[142,143] and lipid[144,145] cargo, which they can transfer to target cells at local[146] or distant sites[134] to activate diverse signalling pathways and cellular reprogramming[134]. Hence, they have emerged as important mediators of intercellular communication in pathological and physiological processes, including cancer[134,140,147], immune regulation[148,149], infectious disease[150,151], cardiopathology[152], tissue regeneration[153], and reproduction[137,138,154,155]. Major EV subtypes include large EVs (lEVs; including shed microvesicles), small EVs (sEVs; including exosomes) and apoptotic bodies (ABDs), that are differentiated by size and biogenesis[135,156], **(Table 1.2)** and perform distinct functions[157].

### 1.5.1 Small extracellular vesicles – exosomes

Exosomes are a major class of EVs (termed sEVs in this study as I have not characterised their biogenesis) of endocytic origin ranging from 30-200 nm in size[158]. They form by inward budding of the endosomal membrane to form intraluminal vesicles (ILVs) within multivesicular bodies (MVBs)[159]. MVBs can undergo lysosomal degradation (by initial fusion with autophagosome or direct fusion with lysosome)[160], or are trafficked and sorted via the endosomal pathway to fuse with the plasma membrane, and release their contents into the extracellular space as exosomes[158,161,162] **(Figure 1.5)**. Endosomal sorting complexes required for transport (ESCRT) machinery are thought to be primary regulators of sEV biogenesis, recruiting ESCRT complexes (ESCRT-0, I, II, III), tumour susceptibility gene 101 (TSG101; ESCRT-I component) and programmed cell death 6 interacting protein (PCD6IP or ALIX)[163]. As such, these proteins (i.e. ALIX and TSG101) are most commonly used as exosomal markers for biochemical characterisation. Biogenesis has been described in an ESCRT-dependent pathway involving syndecan-syntenin-ALIX pathway, which mediates protein loading and vesicle formation[164]. Alternatively, other studies have described an ESCRT-independent biogenesis pathway involving

## Table 1.2 Characteristics of extracellular vesicle subtypes

| EV Subtype | Size (nm) | Biogenesis | Protein Markers | Density ($g/cm^3$) | Method of isolation | Cargo |
|---|---|---|---|---|---|---|
| **Small EVs** | 30-200 | Inward budding of early endosomes to form ILVs within MVBs. MVB fuses with PM to release ILVS into extracellular space, now termed small EVs | Exosomal: ALIX, TSG101, tetraspanins (CD9, CD63, CD81, CD82), ESCRT components<br>Endosomal: RAB7A, EEA1, ARF6 | 1.09-1.13 | Differential ultracentrifugation at ~100,000 *g* for 1 h, buoyant density gradient-based separation, size-exclusion chromatography, membrane filtration, polymer-based precipitation and affinity capture | mRNA, microRNA, ncRNA, ss/dsDNA, mtDNA, cytoplasmic and membrane protein |
| **Large EVs** | 150-1500 | Outward budding from the PM | RACGAP1, KIF23, ARF6, HSP70 | 1.09-1.19 | Differential ultracentrifugation at ~10,000 *g* for 30 min, buoyant density gradient-based separation, size-exclusion chromatography and membrane filtration | Cytoplasmic and membrane protein, mRNA, miRNA, ncRNA, dsDNA |
| **Apoptotic bodies** | 500-5,000 | Plasma membrane blebbing in cells undergoing apoptosis | Phosphotidylserine (PS), histones, calnexin (CANX), cytochrome c (CYCS), ANXA5, TSP, C3b | 1.16-1.28 | Differential ultracentrifugation at 2,000 *g* for 10 min, flow cytometry | Cytoplasmic organelles, fragmented nuclei |

ILV, intraluminal vesicles; MVB, multivesicular body; ncRNA, non-coding RNA; PM, plasma membrane. Adapted from[136,155]

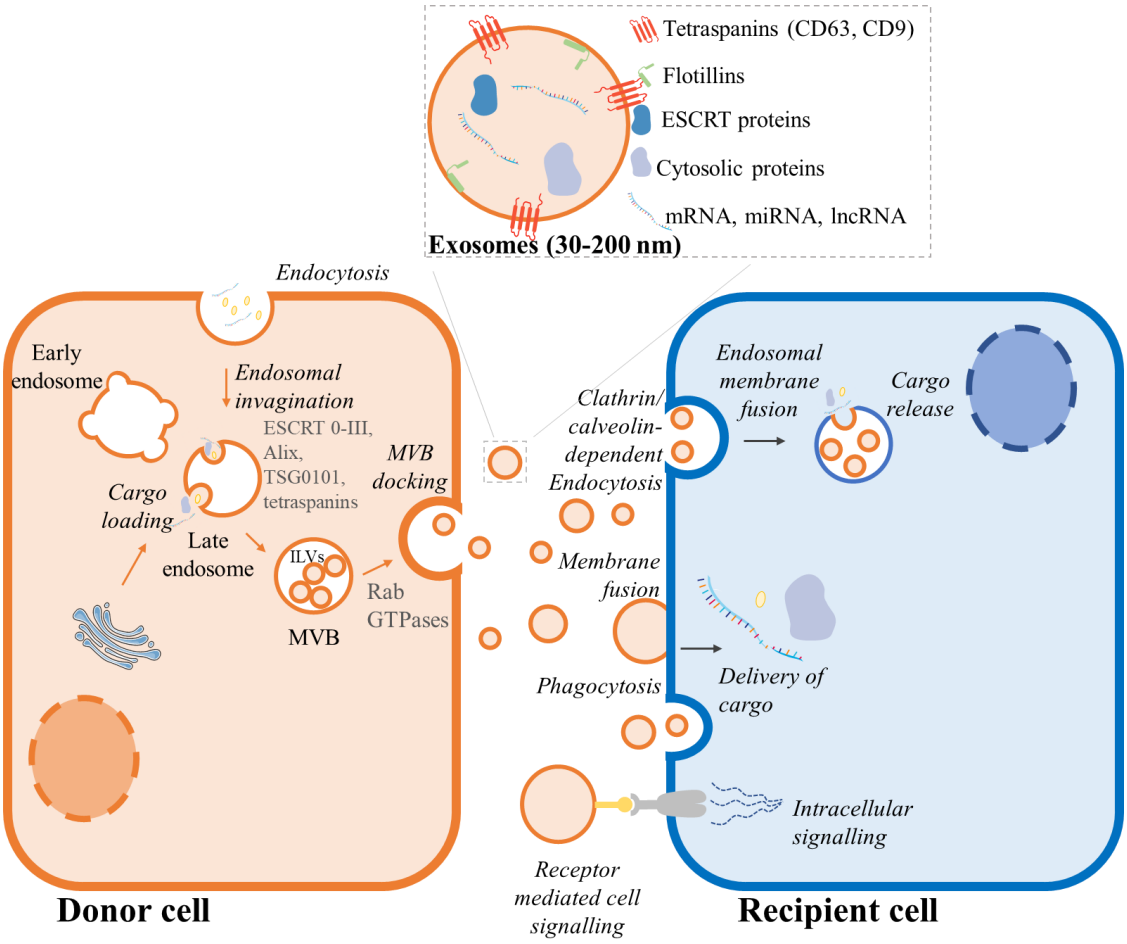

Tetraspanins (CD63, CD9)
Flotillins
ESCRT proteins
Cytosolic proteins
mRNA, miRNA, lncRNA
Exosomes (30-200 nm)
Endocytosis
Early endosome
Endosomal invagination
ESCRT 0-III, Alix, TSG0101, tetraspanins
MVB docking
Cargo loading
Late endosome
ILVs
MVB
Rab GTPases
Donor cell
Clathrin/ calveolin-dependent Endocytosis
Membrane fusion
Phagocytosis
Receptor mediated cell signalling
Endosomal membrane fusion
Cargo release
Delivery of cargo
Intracellular signalling
Recipient cell

**Figure 1.5 Exosome biogenesis.** Exosomes are formed by inward budding of early endosomal membrane as a cup-shaped structure, which comprises cell-surface/ soluble proteins, lipid and nucleic acid cargo[162,165]. Early endosomes mature into late endosomes, in which a second step of inward budding of the membrane forms intraluminal vesicles (ILVs) within multivesicular bodies (MVBs)[159]. Endosomal sorting complexes required for transport (ESCRT) machinery are thought to be the primary regulators of composition of ILVs and sEV biogenesis through the endosomal pathway. MVBs are trafficked and sorted through the endosomal pathway to dock and fuse with the plasma membrane, releasing what are now termed exosomes into the extracellular space[165]. Rab GTPase family members have been shown to regulate secretion of exosomes[166]. Exosomes are characterised by proteins, including ALIX, TSG101 and ESCRT proteins (ESCRT 0, I,II,III), which are involved in their biogenesis, and tetraspanins (CD63, CD81) displayed on their surface[163]. Exosome targeting to recipient cells are modulated by surface receptors and adhesion molecules on EVs, with interacting receptors/ surface molecules on target cells. Exosomes can be internalised via various mechanisms, including clathrin-/caveolae-dependent endocytosis, direct membrane fusion, phagocytosis or receptor-mediated binding[167]. Exosomes can be internalised entirely and undergo endosomal membrane fusion to release cargo, can directly deliver their cargo upon plasma membrane binding, or can mediate intracellular signalling via receptor-mediated binding, to elicit functional reprogramming within recipient cell[139,168].

tetraspanins[169] and ceramide[170]. sEVs can be taken up by recipient cells through receptor-mediated endocytosis[159,171], clathrin-[172] or caveolin-dependent endocytosis[173], plasma membrane fusion[174], phagocytosis or micropinocytosis[175,176].

Importantly, sEVs can selectively reprogram target cells by transfer of functional components (e.g. membrane receptors) that can be expressed on the surface of the recipient cell to activate downstream signalling pathways[139], or directly release their contents within recipient cells upon uptake (e.g. protein, mRNA content) to induce epigenetic changes for cellular reprogramming[177]. sEVs have also been shown to undergo integrin-mediated uptake to activate intracellular signalling pathways (e.g. Src phosphorylation) in recipient cells and prime them for downstream signalling events[168]. Given their role in physiology/ pathology, EV-mediated signalling offers an avenue for therapeutic intervention[178]. sEVs have been identified in biofluids and due to their composition, protect their cargo[150,151] from extracellular degradation or modification[146,147] – highlighting their potential use as a non-invasive diagnostic/ therapeutic in several diseases including infertility[178,179].

### 1.5.2 Importance of extracellular vesicle purification

Obtaining a subset of highly purified EVs separated from other EV subtypes is a major challenge[157,161,180], yet is imperative to understand their true biological role. The presence of other EV subtypes (e.g. lEVs and ABDs) may perturb interpretations of the composition and function of the EV subtype of interest (e.g. sEVs)[181]. Various isolation methods including differential ultracentrifugation, density gradient-based separation, filtration, precipitation, size-exclusion chromatography and immunoaffinity capture, which all possess varying time, cost and material requirements, have been developed to obtain EVs[182]. Differential ultracentrifugation is the most utilised technique, in which ABDs sediment at 2,000 *g*, lEVs at ~10,000 *g*, and sEVs (isolated in this thesis) at ~100,000 *g*[156,183]. However, protein aggregates and non-vesicular components have been shown to co-sediment with sEVs[147,184], which ultimately confound findings from composition and functional characterisation studies[185,186]. Density gradient-based ultracentrifugation is a purification method that exploits different buoyant densities of EVs using a sucrose or iodixanol (OptiPrep™) gradient, to separate EVs from co-sedimenting entities and avoid this contamination[161]. To date, many EV-based studies in reproductive biology utilise impure, heterogeneous EV populations, limiting our understanding of their biological role[133,137,154,187].

In my thesis, I employ a sequential differential ultracentrifugation and density gradient-based separation strategy to isolate and purify sEVs. I follow guidelines recommended by the International Society for Extracellular Vesicles (ISEV) and refer to isolated EVs as sEVs based on their size[182] (as I have not characterised their biogenesis in this study), which possess exosomal characteristics (i.e. protein markers and buoyant density). Other EV subtypes including exomeres[188], large oncosomes[189] and midbody remnants[190] exist; however, in my thesis I will focus on sEVs due to their physiologically significant representation within the uterine microenvironment. sEVs can be readily isolated from endometrial cells and fluids, and possess functional roles within the uterine microenvironment[191]. In contrast, lEVs are secreted in low amounts in endometrial cell models[184] and physiological biofluids derived from uterine lavage[192], thus, providing insufficient yield for downstream biophysical and biochemical characterisation.

### 1.5.3 Extracellular vesicles in reproductive biology

Our understanding of the functional repertoire of EVs in reproductive biology is rapidly increasing. EVs have been found in human[16,133], bovine[31,193] and ovine[194] UF, as well as follicular[195], vaginal[196] and oviductal[197] fluids. Previous studies have described the role of EVs in sperm activation and motility[198], capacitation and acrosome reaction[199], oocyte maturation[195], fertilisation,[200] embryo development[154,201] and importantly, maternal-embryo communication[137,138,154].

Ng, et al. [133] first demonstrated that the endometrial epithelium can selectively package and release EVs with distinct miRNA cargo compared to parental cells. Bioinformatic analysis identified enrichment of miRNAs (e.g. hsa04512, hsa04914 and hsa04330) related to embryo implantation processes, highlighting their suitability as biomarkers of endometrial receptivity and implantation. Accordingly, our lab[63,138] demonstrated under hormonal priming (E and EP) that human endometrial LE-derived sEVs are taken up by human trophectoderm cells to enhance their adhesive capacity to endometrial LE through focal adhesion kinase (FAK) phosphorylation. Proteomic analysis also revealed factors relating to adhesion, migration, invasion and ECM remodelling, suggesting a significant functional role of sEVs during endometrial-embryonic interaction. Recently, Gurung, et al. [154] showed endometrial sEVs internalised by mouse blastocysts can increase blastocyst cell number, enhance blastocyst hatching from zona pellucida and promote implantation *in vivo*. Menstrual cycle phase-specific cargo has also been identified within human UF-sEVs. Specifically, hsa-miR-30d was upregulated during WOI, with hsa-miR-30d-containing sEVs internalised by mouse blastocysts to regulate expression of ITGB3, ITGA7 and CDH5 in support of embryo adhesion, development and maturation[187]. These key studies demonstrate the significance of E and P regulation of endometrial secretions, and highlight growing evidence that endometrium-derived sEVs are mediators of endometrium-embryo communication within the pre-implantation environment. Defining the composition of purified endometrial sEVs and their functional role in sEV signalling within the uterine microenvironment and subsequent reprogramming of embryo, which is still in its infancy, will guide development of therapeutics to improve ART success rates to combat infertility, or alternatively, non-hormonal contraceptives.

## 1.6 Models utilised in this study

While animal models have provided significant insights into the molecular and morphological characteristics that define implantation, there are substantial differences in reproductive mechanisms[2], limiting their translation to human physiology. The role of human endometrium-derived sEVs in implantation and embryonic reprogramming is nascent, partly due to the ethical challenges associated with using clinically-derived human samples (maternal and embryonic), and technical challenges associated with sEV isolation and purification. Furthermore, in regards to physiological biofluids, UF is only present in the uterine cavity in µL volumes *in vivo*[133], hence, the use of endometrial cell lines which can be readily expanded, offer a feasible source of endometrial sEVs for biophysical and biochemical characterisation.

There remains an unmet need to obtain a purified subset of human-derived endometrial sEVs for assessment of function, with recent functional characterisation utilising an unpurified, heterogeneous population of sEVs[137] that dilute interpretation of their true biological role. Furthermore, while previous studies have catalogued transcriptomic and genomic changes throughout menstrual cycle phases, gaining an understanding of proteomic alterations will provide a representative snapshot of key functional mediators within the uterine microenvironment[125]. The power of proteomics takes into account post-translational modifications that are not identified through other -omics technologies, and expands the identified milieu, considering not all transcripts within the endometrium are translated to functional protein[62].

In this thesis I will utilise human endometrium epithelial cells, Ishikawa[202], which express functional hormone receptors (E and P receptors)[203] to allow for hormonal priming in line with the menstrual cycle. Ishikawa cells are derived from primary endometrial epithelia and are an appropriate model to study autocrine/ paracrine regulation of endometrial epithelium[203,204], as well as endometrial-embryonic interactions[205]. Ishikawa cells confer several advantages over using primary human endometrial cells obtained from tissue biopsies by overcoming challenges of tissue heterogeneity (predominantly glandular composition with stromal and luminal cell types), limited cell quantities (~1 x $10^6$ cells per tissue collection from endometrial biopsy) and sufficient sEV yield for subsequent characterisation[137]. Furthermore, primary cells are often obtained from infertile

patients, which do not sufficiently translate to normal endometrial biology[137]. To recapitulate proliferative and secretory phases of the menstrual cycle I will sequentially prime Ishikawa with E and EP as previously reported[63,138]. Importantly, molecular dissection of purified sEVs from this model will be used to validate potentially important endometrial factors in UF from fertile women during the secretory phase of the menstrual cycle.

To dissect the reprogramming capabilities of purified endometrium-derived sEVs, I will utilise human trophectodermal stem cells (T3-TSCs) derived from early stage human embryo[206,207] as an embryo surrogate. T3-TSC are representative of early trophectoderm cells, unlike other trophoblast cell lines (e.g. HTR-8/SVneo, JEG-3 and JAR cells) which are further differentiated into trophoblast lineages, or derived from choriocarcinomas[137]. Furthermore, T3-TSCs display hallmark trophectodermal characteristics[207], can differentiate into extraembryonic lineages under appropriate growth conditions[207] and can form spheroids in 3-dimensional culture[208], highlighting their multipotency and suitability in the study of embryology. Trophectoderm spheroids of this lineage can be used in functional assays, exhibiting similar physiological function of healthy implanting embryo that can differentiate between receptive and non-receptive endometrial epithelial cells[208].

## 1.7 Hypothesis and aims

Endometrial sEVs are emerging as important signalling mediators in the uterine microenvironment[137,138]. However, the role and molecular mechanisms of endometrium-derived sEV signalling to embryo (and outer layer trophectoderm) remains poorly understood. I hypothesise endometrial sEV protein cargo contain important regulators of endometrial and embryo preparation, and support embryo implantation by reprogramming the cellular proteome landscape and function of trophectoderm cells. In my thesis, I aim to investigate the proteomic composition of purified, hormonally-primed endometrial sEVs and their role in reprogramming trophectoderm cellular protein composition and function, to provide insights into their regulation of embryo implantation and fertility.

### 1.7.1 Aim 1: Isolation, purification and characterisation of small extracellular vesicles from hormonally primed endometrial epithelial cells

Specifically, I am to

i) Hormonally prime human endometrial epithelial cells (Ishikawa)

ii) Isolate and purify sEVs from endometrial cell conditioned media (CM) using differential ultracentrifugation coupled with density gradient-based separation

iii) Perform biophysical and biochemical characterisation of sEVs using nanoparticle tracking analysis and western blotting

### 1.7.2 Aim 2: Quantitative proteomic analysis of endometrial small extracellular vesicles

Specifically, I am to

i) Perform label-free mass spectrometry (MS) sample preparation and analysis of sEVs isolated from hormonally-primed endometrial epithelial cells (Ishikawa)

ii) Perform functional annotation and bioinformatic enrichment analysis

### 1.7.3 Aim 3: Investigate proteomic reprogramming of trophectoderm cells treated with endometrial small extracellular vesicles

Specifically, I am to

i) Perform uptake of labelled endometrial sEVs in recipient trophectoderm cells using confocal microscopy

ii) Perform label-free MS sample preparation and analysis of trophectoderm cells treated with endometrial-derived sEVs

iii) Perform functional annotation and bioinformatic enrichment analysis

### 1.7.4 Aim 4: Investigate the role of endometrial small extracellular vesicles in regulating trophectoderm cell invasion

Specifically, I am to

i) Generate trophectoderm spheroids as an "embryo mimetic"

ii) Assess invasive capacity of trophectoderm cells treated with endometrial sEVs into Matrigel™

iii) Perform antibody-based inhibition of candidate protein(s) to determine their functional contribution to trophectoderm cell invasion

# Chapter 2: Materials and methods

## 2.1 Cell culture

### 2.1.1 Endometrial cell culture

Human Ishikawa endometrial epithelial cells[202] were cultured and maintained in a 1:1 mix of Dulbecco's Modified Eagle's Medium / Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen-Gibco, Carlsbad, USA) supplemented with 1% (v/v) Penicillin-Streptomycin (Pen/Strep) (Life Technologies) and 5% (v/v) Foetal Calf Serum (FCS) (Life Technologies) (referred to as DMEM/F-12 complete media). Cells were maintained at 37 °C, 5% $CO_2$ and routinely passaged using 0.5% Trypsin-EDTA (Invitrogen-Gibco).

### 2.1.2 Trophectoderm cell culture

T3-TSC human trophectodermal stem cells (referred to as trophectoderm cell(s)) derived from individual blastomeres of donated human embryos[207] were maintained in a 1:1 mix of DMEM/F-12 supplemented with 1% (v/v) Pen/Strep, 10% (v/v) FCS, 10 nM fibroblast growth factor (FGF) (Tocris Bioscience) and 10 nM SB431542 (Tocris Bioscience) (referred to as T3-TSC media). Cells were cultured in T75 flasks (Corning) pre-coated with 0.5% (w/v) gelatin and maintained at 37°C, 5% $CO_2$. Cells were routinely passaged using 05% Trypsin-EDTA.

## 2.2 Large-scale continuous endometrial cell culture

For generation of large quantities of endometrial sEVs, Ishikawa cells were cultured in CELLine$^{TM}$ CL-1000 Bioreactor Classic Flasks (referred to as bioreactor flasks) (Integra Biosciences). Ishikawa cells (~$20 \times 10^6$) were initially seeded in 20 mL of DMEM/F-12 complete media into the cultivation chamber, with 500 mL of DMEM/F-12 complete media supplemented into the nutrient supply chamber. Cells were cultured for five days at 37 °C, 5% $CO_2$ to expand. Thereafter, cells in the cultivation chamber were gently washed with DMEM/F-12 to remove FCS, and media was replaced with DMEM/F-12 supplemented with 0.6% (v/v) Insulin-Transferrin-Selenium solution (ITS) (Invitrogen-Gibco) and 1% (v/v) Pen/Strep (referred

to as DMEM/F-12 serum-free media). Culture media in nutrient supply chamber was replaced weekly, while CM in cultivation chambers were collected and replaced according to routine hormonal treatment.

## 2.3 Hormonal treatment of endometrial cells

### 2.3.1 Hormonal treatment cycle in bioreactor flasks

Ishikawa-containing bioreactor flasks were cultured in a cyclic hormonal treatment regime to recapitulate proliferative and secretory phases of the menstrual cycle[63,138]. Within the cultivation chamber, cells were treated with β-oestradiol (E) ($10^{-8}$ M, E8875; Sigma-Aldrich) supplemented in DMEM/F-12 serum-free media (E-media), and cultured at 37 °C, 5% $CO_2$ for 48 h to mimic the proliferative phase. E-primed Ishikawa CM was collected and cells were subsequently treated with β-estradiol ($10^{-8}$ M) and medroxyprogesterone 17-acetate ($10^{-7}$ M, M1629; Sigma-Aldrich) (EP) supplemented in DMEM/F-12 serum-free media (EP-media), and cultured at 37 °C, 5% $CO_2$ for 48 h to mimic the secretory phase. EP-primed Ishikawa CM was collected and cells were cultured in unstimulated DMEM/F-12 serum-free media at 37 °C, 5% $CO_2$ for 72 h, to allow the cells to return to a basal condition. This hormonal priming cycle was repeated for several weeks to collect sufficient CM for sEV isolation and purification.

To minimise cell loss within cultivation chambers, harvested CM was initially centrifuged at 150 *g* for 2 min, 4 °C (Multifuge 1S-R centrifuge; Thermo Fisher Scientific) to pellet cells. Cell pellets were resuspended in appropriate treatment media and re-introduced into the cultivation chamber. The supernatant was immediately centrifuged at 500 *g*, 5 min and 2,000 *g*, 10 min at 4 °C (Multifuge 1S-R centrifuge) to remove individual floating cells and cell debris for subsequent storage at -80 °C.

Given the long-term culture of cells in bioreactor flasks, quality control measures were employed to maintain cell viability and prevent bacterial/ fungal contamination. Colour of media in the nutrient supply chamber was assessed bi-daily, indicative of pH changes. Cell viability/ morphology was assessed by re-seeding 150 *g* pellets into T75 flasks and culturing for 24-48 h at 37 °C, 5% $CO_2$. Cells were deemed viable if they were shown to re-establish in monolayer culture.

Finally, pellet size in CM collected from bioreactor flasks (500 *g* and 2,000 *g* pellets) were assessed, with small 2,000 *g* pellets indicative of minimal apoptotic body formation.

### 2.3.2 Hormonal treatment of endometrial monolayer

Ishikawa cells were cultured as monolayers and hormonally treated to recapitulate proliferative and secretory phases of the menstrual cycle[63,138]. Cells were cultured in DMEM/F-12 complete media in 6-well plates to 80% confluency. Cells were gently washed with phosphate-buffered saline (PBS) and treated with E-media for 48 h, or E-media for 24 h and subsequent EP-media for 48 h.

## 2.4 Whole cell lysate preparation

### 2.4.1 Endometrial whole cell lysate preparation

Ishikawa cell monolayers (E- or EP-treated) were washed 2 x in ice-cold PBS and scraped in 1 mL ice-cold PBS. Cells were centrifuged at 500 *g* for 5 min, 4 °C, supernatant removed and resuspended in 5x pellet volume of sodium dodecyl sulphate (SDS) lysis buffer (1% (w/v) SDS, 100 mM tetraethylammonium bromide (TEAB), pH 8) and heat treated at 95 °C for 5 min. Cell lysates (n=3) were tip probe sonicated (10 s, 23 amplitude) (Misonix – S-4000 Ultrasonic Liquid Processor) on ice and centrifuged at max speed (~16,000 *g*) for 20 min at 4 °C to pellet insoluble material. Supernatants were carefully transferred to fresh tubes and stored at -80 °C.

### 2.4.2 Trophectoderm cell lysate preparation

T3-TSC cell monolayers were washed twice in ice-cold PBS and lysed in-well with MS lysis buffer (1% SDS, 1% NP-40, 1% SDC, 1 x Ethylenediaminetetraacetic acid (EDTA), 1 x protease/ phosphatase inhibitor, 50 mM NaCl, 50 mM HEPES pH 8), transferred to low-protein binding microcentrifuge tubes and lysed at room temperature (RT) for 30 min. Samples were sonicated (23s, 1 min) and then heat treated for 5 min at 95 °C.

## 2.5 Protein quantitation

Protein quantitation was performed using the Micro BCA™ Protein Assay Kit (colorimetric-based protein assay) as per manufacturer's instructions (Thermo Fisher Scientific). Briefly, samples were diluted 1:50 or 1:100 in 1% (v/v) SDS dilution buffer and mixed with Micro BCA™ working reagent in 96-well plates. Protein (bovine serum albumin, BSA) standards and background control were assayed in SDS dilution buffer. Samples were incubated for 2 h at 37 °C and absorbance measured at 562 nm (Bio-Rad Benchmark Plate Reader). Absorbance measurements were plotted against BSA standards (0-200 µg/mL) and concentrations calculated using Microsoft Excel.

## 2.6 Endometrial small extracellular vesicle isolation and purification

Isolation of endometrial sEVs was performed as described[147]. E- and EP-CM were centrifuged at 10,000 *g* for 30 min to remove lEVs (SW28 rotor; Optima L-90K Ultracentrifuge)[134]. The resulting supernatant was centrifuged at 100,000 *g* for 1 h (SW28 rotor; Optima L-90K Ultracentrifuge) to pellet crude sEVs which were subsequently washed in 1 mL PBS at 100,000 *g* for 1 h. Crude sEVs were purified using OptiPrep™ density gradient-based separation as described[147]. Briefly, 1 mL volumes of 40%, 20%, 10%, and 0.6 mL of 5% iodixanol solution were layered sequentially in a polypropylene tube (11 x 60 mm). Dilutions of iodixanol solution were made in 0.2 M sucrose/ 1 x PBS solution. Crude sEVs (200 µL) were overlaid and centrifuged at 100,000 *g* for 18 h, 4 °C (SW60 rotor, Optima L-90K Ultracentrifuge). Twelve 300 µL fractions were collected, diluted in 1 mL PBS and subjected to a wash spin at 100,000 *g* for 1 h, 4 °C. Pellets were re-suspended in 20-100 µL PBS; fractions 6-8 were pooled (now purified sEVs, referred to as E-sEVs or EP-sEVs) and stored at -80 °C. The density of each fraction was determined using a control OptiPrep™ gradient overlaid with 200 µL PBS; each fraction was diluted 1:10,000 in MilliQ and absorbance measured at 244 nm using Nanodrop 2000c. Absorbance of fractions were converted to density using the equation: density = (Abs(244nm) + 5.7283)/5.7144, as described[209].

## 2.7 Nanoparticle tracking analysis

The size distribution and concentration for purified sEV samples was determined using nanoparticle tracking analysis (NTA) (NanoSight NS300, Malvern, UK). Purified sEVs (1 µg) were diluted 1:1,000 in PBS and analysed using an NS300 NanoSight system fitted with a NS300 flow-cell top plate and a 405 nm blue laser. Syringe pump speed was set to 100 camera detection threshold was at least 10 and temperature set to 25 °C. Three technical replicates (60 s video captures) were recorded and analysed using NTA software (3.1.45).

## 2.8 Western blot

Western blotting was performed on endometrial whole cell lysate (WCL) and purified sEV protein samples following lysis in SDS lysis buffer. Lysates were mixed 1:1 with 2 x western blot lysis buffer (4% (w/v) SDS, 20% (v/v) glycerol and 0.01% bromophenol blue, 0.125 M Tris-hydrochloride, pH 6.8) and 2 µL dithiothreitol (DTT) (1M) (Thermo Fisher Scientific). Samples were electrophoresed on a NuPAGE 4-12% Bis-Tris gel (Invitrogen) for 1 h, 150 V, alongside 0.5 µL SeeBlue™ Plus2 Pre-stained Protein Standard (Thermo Fisher Scientific). Proteins were transferred to nitrocellulose membranes using the iBlot™ 2.0 Dry Blotting System (20 V, 14 min; Life Technologies). Membranes were blocked using skim milk powder in Tween PBS (1 x PBS, 0.1% (w/v) Tween 20) (TPBS) for 30 min while shaking at RT, and rinsed three times with TPBS for 5 min. Membranes were incubated with primary mouse or rabbit antibodies against ALIX (1:1,000 dilution) (3A9; Cell Signaling Technology), TSG101 (1:1,000 dilution) (622696; BD Biosciences), GAPDH (1:1,000 dilution) (D4C6R; Cell Signaling Technology), oestrogen receptor alpha (ERα) (1:500 dilution) (D6R2W; Cell Signaling Technology) or progesterone receptor A/B (PR A/B) (1:500 dilution) (D8Q2J; Cell Signaling Technology) in TPBS, overnight at 4 °C. Membranes were rinsed with TPBS and incubated with secondary antibodies (1:15,000); IRDye 800CW goat anti-mouse antibody or IRDye 680RD goat anti-rabbit antibody (Li-cor Biosciences), for 1 h while shaking at RT. Membranes were rinsed 3 times with TPBS and imaged using Odyssey Infrared Imaging System (Li-COR Biosciences, Nebraska USA), measuring 700 nm and 800 nm wavelengths.

## 2.9 Uptake of endometrial small extracellular vesicles by trophectoderm cells

Ishikawa cells were cultured in T25 flasks to 80% confluency prior to hormonal priming (*Section 2.3.2).* After 24 h of E- or EP-treatment, confluent Ishikawa cells were washed 3x with warm PBS. Cells were labelled for 10 min at 37 °C with 5 mM DiI lipophilic dye (Invitrogen) in 2 mL DMEM/F-12 containing 1% (v/v) Pen/Strep. Cells were washed 3x with DMEM/F-12 for 10 min at 37 °C and cultured in E- or EP-media for 48 h. CM was subjected to crude sEV isolation procedure *(Section 2.6)* and pellet resuspended in 10 µL PBS. Meanwhile, T3-TSC cells (~500) were seeded in a 20 µL droplet in 8-well microscopy chambers (Sarstedt) and cultured overnight in T3-TSC media containing EV-depleted FCS (prepared by ultracentrifugation at 100,000 *g* for 18 h). Crude E- and EP-sEVs were overlaid on T3-TSC cells and incubated at 37 °C for 2 h to allow for uptake, as described[210]. Media was removed and T3-TSC cells were dyed with Hoechst 33342 nucleic acid stain (diluted 1:2,000 in DMEM/F-12 media; Thermo Fisher Scientific) for 10 min at 37 °C and washed gently 3x with warm DMEM/F-12 media. Cells were then subjected to live cell imaging using Nikon AIR with 40x magnification. Images are representative of six biological replicates repeated over two independent experiments.

## 2.10 Endometrial small extracellular vesicle treatment on trophectoderm cells

T3-TSC cells (~20,000) were seeded in 96-well plates pre-coated with 0.5% (w/v) gelatin and cultured for 24 h, 37 °C in T3-TSC media. Cells were gently washed 2 x with DMEM/F-12 and serum-starved for 24 h at 37 °C in DMEM/F-12 containing 1% (v/v) Pen/Strep, 10 nM FGF and 10 nM SB431542, before treatment with purified E- or EP-sEVs (50 µg/mL) or PBS control for 24 h at 37 °C (n=5-6). Cells were subjected to WCL preparation and protein quantitation (*Sections 2.4.2 and 2.5)* prior to MS sample preparation.

## 2.11 Mass spectrometry

I performed all mass spectrometry sample preparations, sample loading onto the Q-Exactive HF-X Orbitrap mass spectrometer and bioinformatic analysis."

### 2.11.1 Sample preparation

Purified E- and EP-sEVs (5 µg) (lysed in 1% SDS in 50 mM TEAB, pH 8), and Ishikawa and T3-TSC WCLs (20 µg) were reduced with 10 mM DTT at RT for 1 h (350 rpm) followed by alkylation with 20 mM iodoacetamide (IAA) (Sigma-Aldrich) for 20 min at RT (protected from light), and immediately quenched with 10 mM DTT. Thereafter, samples were subjected to single-plot, solid-phase-enhanced sample separation (SP3) protocol[211]. Briefly, 2 µL of a 50 µg/µL SP3 bead stock (Sera-Mag SpeedBead carboxylate-modified magnetic particles; hydrophobic and hydrophobic 1:1 mix, GE Healthcare Life Sciences, Freiburg, Germany) were added to 50 µL of protein extract, and 60 µL of 100% (v/v) ethanol (final concentration of 50% (v/v)), and incubated for 10 min (1,000 rpm) at RT. Tubes were mounted on a magnetic rack; supernatants were removed and beads were washed three times with 200 µL 80% ethanol. Beads were resuspended in 100 µL 50 mM TEAB pH 8.0, and digested overnight with trypsin (1:50 trypsin: protein ratio; Promega, V5111) at 37 °C, 1,000 rpm. The peptide and bead mixture was centrifuged at 20,000 g for 1 min at RT. Samples were placed on a magnetic rack and supernatant was collected and acidified to a final concentration of 1.5% formic acid (FA), frozen at -80 °C for 20 min, and dried by vacuum centrifugation for ~1 h. Peptides were resuspended in 0.07% trifluoroacetic acid (TFA), quantified by Fluorometric Peptide Assay (#23290, Thermo Fisher Scientific) and normalised; T3-TSC WCL (200 ng), Ishikawa WCL and sEVs (400 ng).

### 2.11.2 Mass spectrometry

Proteomic experiments were conducted in biological triplicates for Ishikawa WCL and sEVs, and six biological replicates for T3-TSC WCL. A nanoflow UPLC instrument (Ultimate 3000 RSLCnano, Thermo Fisher Scientific) was coupled on-line to a Q-Exactive HF-X Orbitrap mass spectrometer (Thermo Fisher Scientific) with a nanoelectrospray ion source (Thermo Fisher Scientific). Peptides were loaded (Acclaim PepMap100, 20 mm × 75 µm i.d., µ-Precolumn packed with 3 µm C18 beads, Thermo Fisher Scientific) and separated (Nikkyo Technos C18 3 µm 120Å, 360/75 µm × 200 mm, Nikkyo Technos Japan) with a 110-min gradient from 2-80% (v/v) phase B (0.1% (v/v) FA in 100% (v/v) acetonitrile (ACN)) (2–4% from 0-2 min, 4-28% from 2-95 min, 28–

80% from 95-98 min, 80% for 98-100 min, and 80-2% from 100-104 min at a flow rate of 250 nL/min, operated at 55 °C.

The mass spectrometer was operated in data dependent and positive ionisation mode, selecting the 25 most abundant precursor ions to be included in the survey scan (300-1650 Th) for MS/MS fragmentation. Survey scans were acquired at a resolution of 60,000 with MS/MS resolution of 30,000. Unassigned precursor ion charge states and singly/6-8 charged species were excluded, with peptide match enabled. The isolation window was set to 1.3 Th and selected precursors fragmented by high-field collision induced dissociation (HCD) with normalised collision energies of 25 with a maximum ion injection time of 60 msec. Ion target values were set to 3e6 and 2e5 for survey and MS/MS scans, respectively. Dynamic exclusion was activated for 30 sec. Data was acquired using Xcalibur v4.0 (Thermo Fisher Scientific).

### 2.11.3 Database searching and protein identification

Raw mass spectrometer files were analysed by MaxQuant software (v 1.6.6)[212] by searching against the human reference sequence database (UP000005640, 74,823 entries, downloaded Jan-2020) supplemented with common contaminants using the built-in search engine Andromeda. Cysteine carbamidomethylation was set as a fixed modification and N-terminal acetylation and methionine oxidations as variable modifications. The false discovery rate (FDR) was set to 0.01 for both protein and peptide-spectrum matches, employing a target-decoy approach using reversed protein sequences. Enzyme specificity was set as C-terminal to arginine and lysine with trypsin as the protease. A maximum of two missed cleavages were allowed in the database search. Peptide identification was performed with an allowed initial precursor mass deviation of up to 7 ppm and an allowed fragment mass deviation of up to 20 ppm. A minimum peptide length of 7 amino acids and a maximum peptide mass of 4600 Da was allowed for the searches. The label free quantification (LFQ) algorithm in MaxQuant (max label-free quantitation, maxLFQ)[213] was used to obtain quantification intensity values. Contaminants, and reverse identification were excluded from data processing and analysis. Benjamini-Hochberg multi-test adjustment method was used to adjust resulting p-values for a high number of comparisons[214] and statistics performed as described[215].

### 2.11.4 Bioinformatic analysis

Perseus (v1.6.7.0)[216] was used to quantify proteins with expression identified in at least 70% in at least one group (Ishikawa sEV and WCL comparison groups or sEV-treated T3-TSC comparison groups), at least two out of three replicates in one group (Ishikawa E-sEV and EP-sEV comparison groups), or at least 3 or 4 replicates in one group (E-sEV and EP-sEV treated T3-TSCs, and untreated T3-TSCs). Normalised intensities were $\log_2$ transformed, with statistical analyses performed using Student's T-test ($p<0.05$ considered significant). Gene Ontologies (GO) (biological processes) were obtained from Database for Annotation, Visualization and Integrated Discovery (DAVID) using recommended analytical parameters, as described[217], and gene set enrichment analysis was performed using g:Profiler[218] or Reactome[219]. Functional annotations for protein short-lists were obtained from UniProt[220].

*In silico* surfaceome analysis of trophectoderm cells was performed utilising UniProt annotations (GO: cellular compartment or cellular localisation to "plasma membrane" or "cell membrane", excluding "cytoplasmic side")[220] and TMHMM v2.0 (prediction of protein transmembrane helices >1)[221].

## 2.12 Matrigel™ invasion assay

### 2.12.1 Generation of trophectoderm spheroids

To generate trophectoderm spheroids that model an embryo mimetic, a protocol was performed as described[137], with modifications. Briefly, T3-TSC cells (~1500) in 100 µL of T3-TSC media were cultured in round bottom ultra-low attachment 96-well plates (Costar) for 48 h at 37 °C, 5% $CO_2$ (1 spheroid/ well). Spheroids were observed using bright field microscopy and any deformed spheroids were excluded from functional use.

### 2.12.2 Invasion assay

Growth factor reduced, phenol red-free Matrigel™ (Corning) diluted 1:1 with ice-cold PBS was used to coat 8-well microscopy chambers for 30 min at 37 °C. T3-TSC spheroids were

harvested after 48 h growth (~30 spheroids/ tube), allowed to settle and washed gently 2 x with DMEM/F-12 containing 1% (v/v) Pen/Strep. Media was removed and spheroids gently resuspended in 100 µL DMEM/F-12 containing 1% (v/v) Pen/Strep, 10 nM FGF, 10 nM SB431542 and either 50 µg/mL E-sEVs, EP-sEVs or PBS (untreated control). Spheroids were overlaid onto solidified Matrigel™. After 24 h media was removed, with 50 µL mixed 1:1 with Matrigel™ and gently overlaid on spheroids in wells. Matrigel™ was allowed to solidify for 30 min at 37 °C followed by addition of 200 µL of DMEM/F-12 (10% (v/v) FCS, 1% (v/v) Pen/Strep) to each well. After 72 h, spheroids were imaged using Olympus FSX100 (n= 13-20 spheroids, with four measurements (mm) taken per spheroid). The extent (%) of invasion was assessed by calculating [(outer diameter – inner diameter) / (inner diameter) x 100] using a digital ruler.

### 2.12.3 Invasion assay – inhibition of fibronectin

Matrigel™ and spheroids were prepared as described in *Section 2.12.1 and 2.12.2*. Spheroids overlaid on Matrigel™ were treated with PBS control, 50 µg/mL EP-sEVs, Anti-IgG antibody (1:50, #2729S), Anti-fibronectin antibody (1:50 dilution, ab2413), 50 µg/mL EP-sEVs + anti-IgG antibody (1:50), or 50 µg/mL EP-sEVs + anti-fibronectin (anti-FN1) antibody (1:50). After 24 h, media was removed, with 48 µL mixed with anti-IgG or anti-FN1 (1:50) for respective treatment groups, which were then mixed 1:1 with Matrigel™ and overlaid on spheroids in wells. Matrigel™ was allowed to solidify for 30 min at 37 °C followed by addition of 200 µL of DMEM/F-12 (10% (v/v) FCS, 1% (v/v) Pen/Strep) to each well. After 72 h, spheroids were imaged using Olympus FSX100 (n= 7-13 spheroids with four measurements (mm) taken per spheroid, invasion (%) calculated as described in *Section 2.12.2*).

## 2.13 Statistical analysis

Data were analysed using Perseus[216] (1.6.7.0) and GraphPad Prism (8.4.3). Two-sample Student's T.test was performed, with statistical significance defined at $p<0.05$.

# Chapter 3: Isolation, purification and characterisation of endometrial small extracellular vesicles from a hormonally primed endometrial model

## 3.1 Overview

While the role of sEVs in reproductive biology is emerging[191], majority of studies report on EV preparations that are heavily contaminated with protein aggregates and non-vesicular components[137,154,187,222]. To obtain a precise understanding of EV composition and function, it is imperative to purify them to homogeneity for downstream proteomic and functional characterisation[181,182,223]. Hence, I employed differential ultracentrifugation to separate sEVs from lEVs and ABDs, which were purified using density gradient-based separation - considered as the gold standard in purifying sEVs[182].

Endometrial secretions in particular, are crucial to support synchronous development and dialogue between endometrium and embryo for successful implantation[20,224], with sEVs emerging as regulators of these processes[133,155]. Therefore, in this chapter I obtained highly purified and well characterised sEVs from a hormonally primed endometrial epithelial cell model (Ishikawa) that recapitulates proliferative and secretory menstrual cycle phases (regulated by E and P)[208]. This chapter offers insight into cyclic receptor cycling of ovarian steroid hormone receptors in Ishikawa cells, and their suitability as an ideal model of choice. Further, large-scale isolation and purification of sEVs derived from Ishikawa cells confirmed their purity based on size, buoyant density and protein marker expression, enabling downstream proteomic and functional characterisation of endometrial-derived sEVs to elucidate their role in endometrium-embryo cross-talk.

## 3.2 Results

### 3.2.1 Endometrial cells *in vitro* are hormonally responsive

Endometrial preparation for embryo implantation is predominantly controlled by cyclic variations in ovarian steroid hormones[30]. To simulate cyclic hormonal priming **(Figure 3.1a)**, endometrial Ishikawa cells were sequentially primed with E (48 h, proliferative phase) and EP (48 h, secretory phase) at physiological levels of each hormone[63,138], followed by an unstimulated period **(Figure 3.1a,b)**.

To verify that Ishikawa cells were hormonally responsive, western blot analysis displayed the expression of ERα between hormonal treatments. Importantly, PR A/B were expressed following E stimulation (proliferative phase) **(Figure 3.1c)** which is consistent with previous reports in this cell line that E treatment is a prerequisite for PR A/B expression[225]. Further, unstimulated treatment reduced PR A/B expression. It has been established that the expression of these receptors are temporally regulated across the menstrual cycle[226], with my findings supporting sequential E priming before P stimulation, and demonstrate this temporal regulation can be recapitulated in the Ishikawa endometrial epithelial cell model.

For large-scale generation of secreted endometrial sEVs, continuous bioreactor culture using CELLine™ was established. Cell viability was not affected for Ishikawa cells grown in this condition **(Appendix figure S3.1).** Subsequently, culture medium supplemented with 0.6% (v/v) ITS (i.e., serum free cell culture chamber) was used to maintain Ishikawa cells in the presence of hormonal treatments, for subsequent sEV isolation.

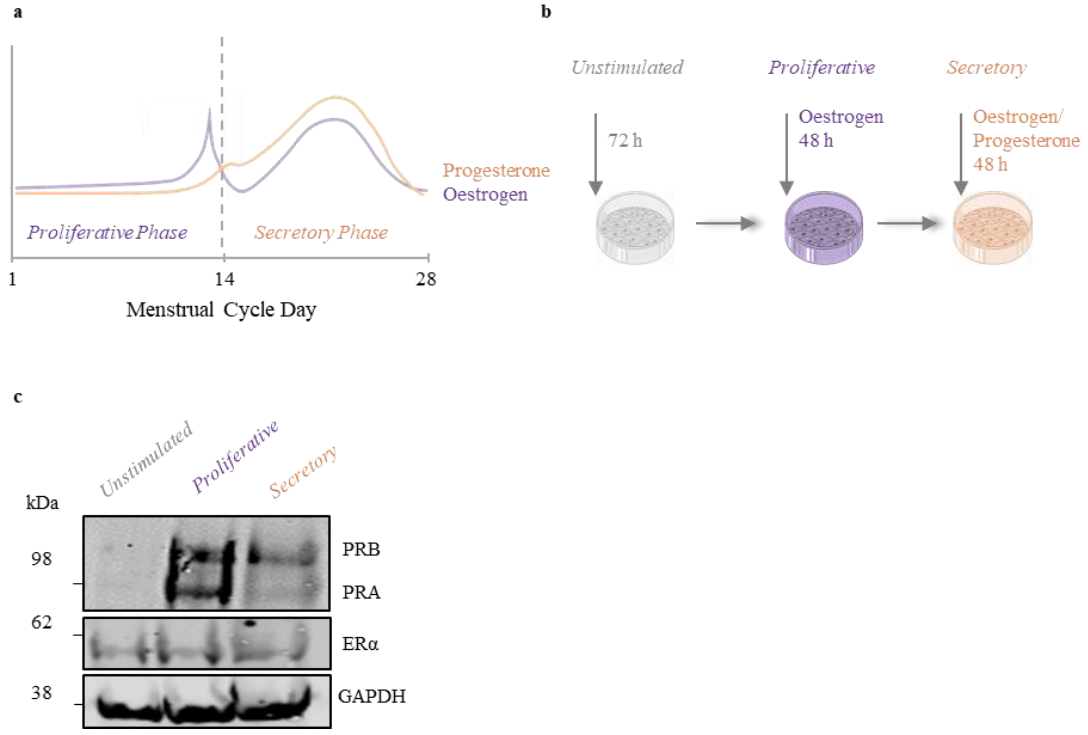



**Figure 3.1 Validation of *in vitro* hormonal treatment of endometrium. a)** Endometrium levels of oestrogen (E) and progesterone (P) according to human menstrual cycle phases. High E represents the proliferative endometrium (purple), while elevated P (and moderate E) represents the secretory endometrium (orange). **b)** *In vitro* hormonal treatment cycle workflow. Cells were sequentially treated with E ($10^{-8}$ M) (48 h) and then E and P (E, $10^{-8}$ M, and P, $10^{-7}$ M) (48 h), followed by an unstimulated (basal) treatment (72 h). **c)** Validation of cyclic hormonal priming by western blot analysis of Ishikawa cells using primary antibodies against ERα, PRA/ PRB and GAPDH (load control). Data is representative of n=3 replicates.

### 3.2.2 Small extracellular vesicle isolation, purification and characterisation

Crude sEVs were isolated from conditioned media (CM) obtained from hormonally primed Ishikawa cells **(Figure 3.2a)**. Density gradient-based separation (OptiPrep$^{TM}$) was used to purify crude sEVs based on their buoyant density **(Figure 3.2b)**. Purified sEVs were enriched within fractions 6-8 (buoyant density 1.06-1.11 g/mL) and were positive for exosomal markers ALIX and TSG101[138,147] **(Figure 3.2c)**. These purified sEV-containing fractions (6-8) were then combined and referred to as E-sEVs or EP-sEVs.

Nanoparticle tracking analysis was used to analyse the size distribution of purified sEVs, revealing that E-sEVs and EP-sEVs were 180.6 ± 1.1 nm and 185.1 ± 1.5 nm in size (diameter), respectively **(Figure 3.2d)**, consistent with previous reports[147]. No difference in particle count was observed between E-sEVs ($8.21 \times 10^8 \pm 6.73 \times 10^7$ particles/mL) and EP-sEVs ($6.62 \times 10^8 \pm 1.66 \times 10^7$ particles/mL).

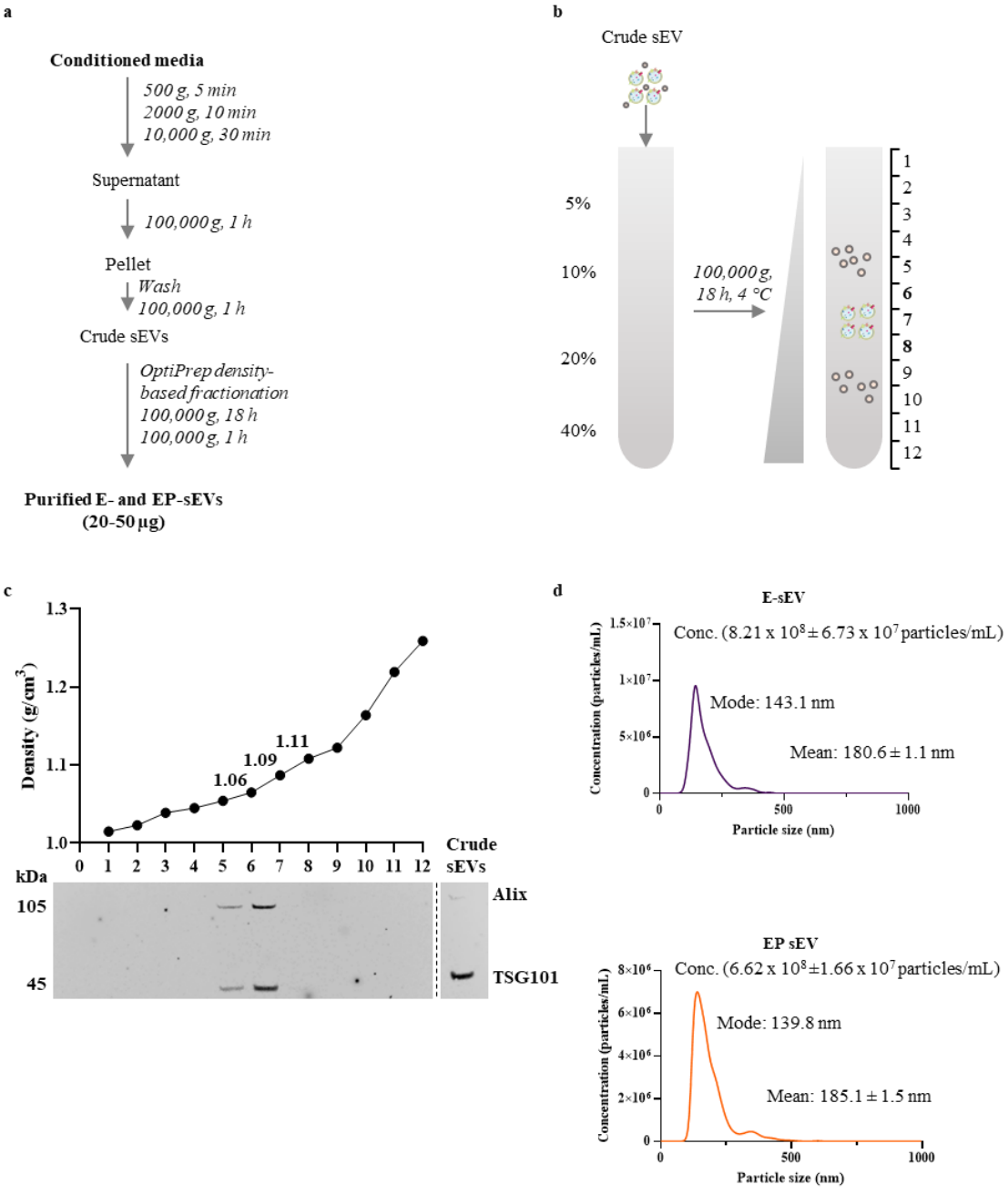

a
Conditioned media
500 g, 5 min
2000 g, 10 min
10,000 g, 30 min
Supernatant
100,000 g, 1 h
Pellet
Wash
100,000 g, 1 h
Crude sEVs
OptiPrep density-based fractionation
100,000 g, 18 h
100,000 g, 1 h
Purified E- and EP-sEVs (20-50 µg)
b
Crude sEV
5%
10%
20%
40%
100,000 g, 18 h, 4 °C
1
2
3
4
5
6
7
8
9
10
11
12
c
Density (g/cm3)
1.0
1.1
1.2
1.3
1.06
1.09
1.11
0 1 2 3 4 5 6 7 8 9 10 11 12
kDa
105
45
Crude sEVs
Alix
TSG101
d
E-sEV
Conc. (8.21 x 10^8 ± 6.73 x 10^7 particles/mL)
Mode: 143.1 nm
Mean: 180.6 ± 1.1 nm
Concentration (particles/mL)
Particle size (nm)
EP sEV
Conc. (6.62 x 10^8 ±1.66 x 10^7 particles/mL)
Mode: 139.8 nm
Mean: 185.1 ± 1.5 nm
Concentration (particles/mL)
Particle size (nm)

**Figure 3.2 Small extracellular vesicle isolation, purification and characterisation. a)** Differential ultracentrifugation of conditioned medium for sEV isolation and purification from Ishikawa cell model following hormonal priming. Crude sEVs were collected at 100,000 *g*, washed and overlaid onto density gradient-based separation workflow. **b)** OptiPrep™ iodixanol purification of crude sEVs. Twelve equal fractions were obtained following ultracentrifugation. **c)** Upper panel, density distribution of twelve fractions; density calculated from a control gradient (5-40% OptiPrep™ with 200 µL PBS overlaid). Density of fractions determined by diluting 1:10,000 and measuring absorbance at 244 nm. Absorbance converted to density using molar extinction coefficient of 320 $g^{-1}cm^{-1}$ (n= 4 technical replicates). Lower panel, western blot analysis of purified E- and EP-sEVs probed with exosome protein markers ALIX and TSG101. **d)** Nanoparticle tracking analysis of E- and EP-sEVs reveal size (diameter) distribution of 40-185 nm vesicles (maximal abundance ~140 nm) with similar concentrations (~8-7 x $10^8$ particles/mL) (n= 3).

## 3.3 Discussion

Synchronous development between the embryo and endometrium, in which embryo competency is superimposed with endometrial receptivity is essential for implantation[1]. Receptivity of the endometrium is tightly regulated by E and P through molecular and morphological changes that create a fertile environment for embryo implantation[30,58], with endometrial-derived secreted factors including EVs, also hormonally regulated[63,138] and can support implantation[138,154]. Therefore, to dissect the composition of purified endometrial-derived EVs following hormonal stimulation, this study developed a continuous culture of human endometrial epithelial cells (Ishikawa)[202-204] to recapitulate proliferative and secretory phases of the human menstrual cycle. These findings demonstrate cyclic hormonal regulation of endometrial cells, and the purification of sEVs and their subsequent biophysical characterisation.

### 3.3.1 Modelling the menstrual cycle in an endometrial cell line

Proliferative (E-mediated) and secretory (EP-mediated) phases of the menstrual cycle were recapitulated using hormonally responsive Ishikawa cells. Spatiotemporal secretion of ovarian steroid hormones critically regulate phase-specific endometrial remodelling by binding to their cognate nuclear receptors (ERα and PR A/B) in endometrial cells to establish endometrial receptivity[29]. In this study, cyclic treatment of Ishikawa cells with E and EP led to temporal expression of ERα and PR A/B. In alignment with this, E stimulation is a prerequisite for PR A/B expression[227] while P stimulation reduces PR A/B expression due to a negative feedback mechanism, in which PR ligand binding activates receptor phosphorylation for proteasome degradation[228].

E and P signalling and their receptor cycling are integral for proper endometrial function. Endometrial epithelial PR knockout in mice exhibited embryo implantation failure and defective decidualisation due to the inability to regulate expression of target genes, including Ihh[229]. Further, mouse uteri lacking ERα were incompetent for embryo implantation despite addition of exogenous hormones and healthy embryo[230]. Dysregulation of these pathways ultimately lead to uterine disorders, including endometriosis[231], and implantation failure that contribute to infertility[57]. Hence,

to understand normal endometrial physiology, cell models that express ERα and PR A/B are optimal to study E and P signalling pathways, as they can recapitulate endometrial function *in vivo*[204]. As demonstrated in this study, Ishikawa cells are a well-established endometrial model that maintain functional steroid receptors which are hormonally responsive to E and P[203]. Ishikawa cells express enzymes and structural proteins found in normal endometrium, while presenting phase-specific regulation of integrins upon hormonal stimulation[232]. Importantly, Ishikawa cells provide a consistent, polarised and receptive epithelium that are optimal for studying endometrium-trophectoderm interactions[233], indicative of an ideal model of choice.

The study of endometrial biology utilising *in vitro* cell culture systems, including the Ishikawa model, has been a significant advancement in bypassing ethical and practical limitations associated with obtaining endometrial tissue biopsies[65]. Primary cell collections are often highly heterogeneous and potentially contain other cell types (including stromal and immune cells)[137,234], compared to Ishikawa cells which are well-differentiated[204]. Furthermore, studies have raised concerns with primary cells as they are often obtained from non-receptive patients[137], display heterogeneous receptor activation[235] and cannot be maintained long-term due to potential changes of phenotype[234]. Indeed, primary cells may not retain their original characteristics beyond 3-4 passages[236], consequently providing insufficient cell numbers (~$1 \times 10^6$ cells per tissue preparation) – often a limiting factor for EV generation[137]. In this study, I demonstrate that Ishikawa endometrial cell viability could be maintained in continuous culture, which were responsive to cyclic hormonal treatment, and yielded sufficient quantity of purified sEVs for biophysical and biochemical characterisation.

### 3.3.2 Small extracellular vesicles – a matter of purity

EVs, including sEVs, have been shown to play important roles in mediating maternal-embryo communication in the pre-implantation environment[137,154]. This study has utilised an efficient culture medium generation and sEV isolation, purification, and characterisation strategy. This approach focused on obtaining sEVs for downstream proteomic and functional characterisation of their potential role in implantation, and not lEVs[223,237], predominantly due to the low protein yield of lEVs from human endometrial cell models (Ishikawa and ECC-1 cells), uterine lavage[192], and other cell models[184].

Purification of EV subtypes is currently an international effort[182], with EV heterogeneity posing significant limitations to elucidating EV composition and function[134,136]. This is primarily due to the presence of non-vesicular extracellular matter (i.e., RNA granules, RNA-binding proteins, dsDNA)[223], and co-sedimenting protein aggregates[184] that contaminate EV populations. Additionally, the presence of other EV subtypes (i.e. ABDs and lEVs) may also perturb the composition and function of the EV subtype of interest[181]. Here, I employed a sequential differential ultracentrifugation strategy to isolate endometrial sEVs from ABDs and lEVs, and subsequently, density gradient-based separation, to purify sEVs and characterise their role in signalling to trophectoderm cells to regulate implantation. Application of purification steps in EV isolation methodologies (e.g. density gradient-based separation, immunoaffinity capture) allow for more precise characterisation of the molecular composition of EV subtypes of interest[223], ultimately improving our understanding of their functional role. Studies that have assessed endometrial sEV signalling to trophectoderm cells have utilising unpurified, heterogeneous populations of EVs, severely limiting our understanding of their role in this critical cross-talk.

# Chapter 4: Quantitative proteomic analysis of hormonally reprogrammed endometrial cell-derived small extracellular vesicles

## 4.1 Overview

Accumulating evidence suggests that a central player of endometrial-derived sEVs are their protein cargo that dictate their function[138,154]. However, our understanding of how endometrial sEV protein composition is reprogrammed during proliferative and secretory phases remains limited, yet is imperative to understand their phase-specific function. Defining the protein landscape of these sEVs will provide insights into extracellular modulators of implantation – an understanding that is limited in the field.

In this chapter, I investigated the composition of purified endometrial-derived sEVs which recapitulate proliferative (E-sEVs) and secretory (EP-sEVs) phases. Quantitative MS-based proteomic analysis revealed enrichment of distinct phase-specific cargo, with EP-sEVs (compared to E-sEVs) enriched in key players of endometrial remodelling, embryo development and implantation **(Table 4.2)**. These findings highlight the significance of hormonal E and P signalling in regulating endometrial sEV composition and the uterine microenvironment to create a fertile environment for embryo implantation.

## 4.2 Results

### 4.2.1 Mass spectrometry-based analysis of purified small extracellular vesicles

Proteomic profiling was employed to gains insights into the protein composition of sEVs from hormonally regulated endometrial cells in accord with menstrual cycle phases **(Figure 4.1a)**. Based on stringent informatics (peptide/protein grouping criteria, and identifications in four or more biological replicates), I report 2,951 proteins identified in total Ishikawa WCL and sEVs **(Appendix table S4.1)**. Importantly, downstream effectors of E and P signalling, including IGFBP3[238], endometrial immune response protein SERPINB9[239], and ER co-activator SRC[240] (regulated by E), and endometrial receptivity proteins MSX1[122] and CTNNB1[241] (regulated by P), were identified within Ishikawa WCL and sEVs **(Table 4.1)**, highlighting phase-specific molecular changes in Ishikawa cells in response to hormonal treatment. Further, I report several proteins known to be highly enriched or unique to human endometrium, including MOXD1[242] (regulated by P), and the small GTPase RAP2C, based on the Human Protein Atlas database[62].

To gain insights into the composition of sEVs, I report 2554 proteins identified, comprising 1,793 and 1,999 proteins in E-sEVs and EP-sEVs, respectively **(Figure 4.1b)**. Principal component analysis revealed that E-sEVs and EP-sEVs are molecularly distinct **(Figure 4.1c)**. Correlation matrix revealed significantly greater variance between E and EP treatment (average 0.953), compared to within treatment groups (average 0.975 for E-sEVs and 0.985 for EP-sEVs) ($p<0.0001$) (based on average of Pearson's correlation) **(Figure 4.1d)**. These results demonstrate high experimental reproducibility between biological replicates, and molecular distinction between sEVs of different treatment groups. Further, I demonstrate that proteins identified in sEVs range across 4 orders of magnitude ($log_{10}$, range 4-8) for both E and EP, based on normalised LFQ intensities, indicating high sampling depth **(Figure 4.1e)**.

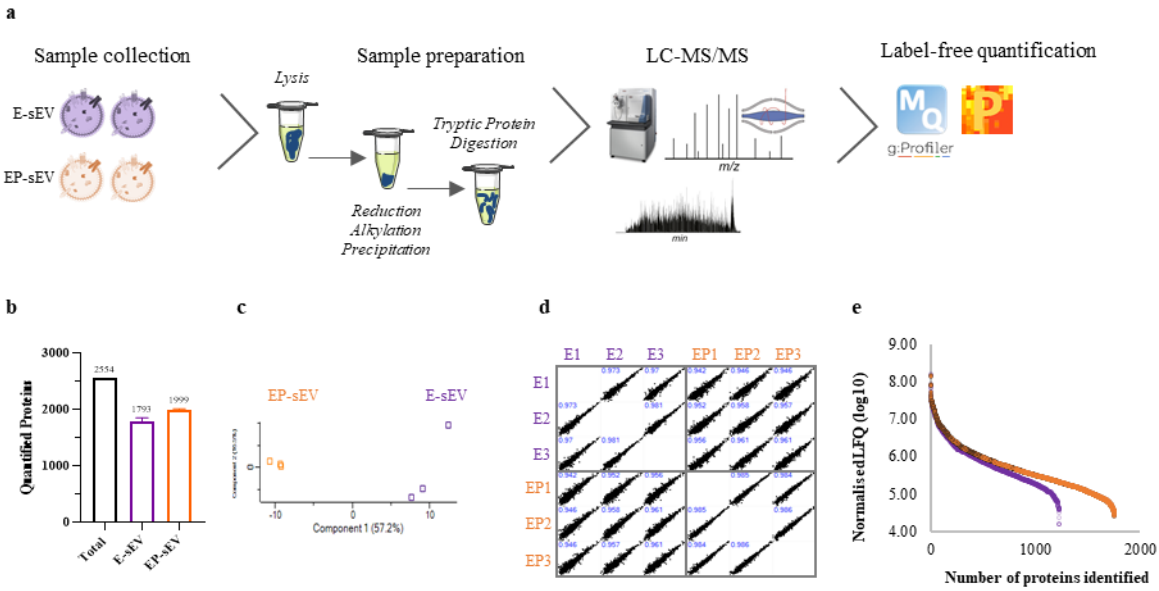



**Figure 4.1 Proteome analysis strategy of endometrial small extracellular vesicles. a)** Proteomic profiling workflow for sEVs. Purified sEVs from E- and EP-treated Ishikawa cells subjected to in-solution lysis, reduction, alkylation and enzymatic tryptic digestion. High sensitivity label-free quantitative mass spectrometry analysis and bioinformatic analyses using Perseus, g:Profiler and DAVID-based functional enrichment analyses. **b)** Quantified proteins from each sEV sample type. Error bars represent standard error of mean (SEM). **c)** Principle component analysis of E-sEV and EP-sEV, (each square indicating separate biological replicates, n=3). **d)** Correlation matrix of E-sEV and EP-sEV biological replicates (n=3). Pearson correlation displayed. **e)** Estimated abundance by LFQ intensity ($log_{10}$) of E-sEV (left, purple) and EP-sEV (right, orange), indicating sampling depth of each dataset spanning >4 orders of magnitude.

**Table 4.1 Ishikawa small extracellular vesicle and cell lysate proteins involved in oestrogen or progesterone signalling pathways***

### *Oestrogen signalling*

| Uniprot Accession | Gene name | Protein name |
|---|---|---|
| Q9Y230 | RUVBL2 | RuvB-like 2 |
| P12931 | SRC | Proto-oncogene tyrosine-protein kinase Src |
| P17936 | IGFBP3 | Insulin-like growth factor-binding protein 3 |
| Q92841 | DDX17 | Probable ATP-dependent RNA helicase DDX17 |
| Q99873 | PRMT1 | Protein arginine N-methyltransferase 1 |
| P14635 | CCNB1 | G2/mitotic-specific cyclin-B1 |
| Q92769 | HDAC2 | Histone deacetylase 2 |
| P55268 | LAMB2 | Laminin subunit beta-2 |
| P50453 | SERPINB9 | Serpin B9 |

### *Progesterone signalling*

| Uniprot Accession | Gene name | Protein name |
|---|---|---|
| Q04721 | NOTCH2 | Neurogenic locus notch homolog protein 2 |
| O15173 | PGRMC2 | Membrane-associated progesterone receptor component 2 |
| O00264 | PGRMC1 | Membrane-associated progesterone receptor component 1 |
| Q92769 | HDAC2 | Histone deacetylase 2 |
| P35222 | CTNNB1 | Catenin beta-1 |
| P17302 | GJA1 | Gap junction alpha-1 protein |
| P40763 | STAT3 | Signal transducer and activator of transcription 3 |
| P28360 | MSX1 | Homeobox protein MSX-1 |

Uniprot Accession number gene name and protein description annotated from UniProt[220]
*Information derived from UniProt (biological processes, molecular function), and literature curation[122,231,239,240,243,244]

### 4.2.2 Endometrial small extracellular vesicles are distinct from parental cells

To determine if sEVs are molecularly distinct from their parental cells, I compared the proteomic profiles of sEVs (both E and EP) to parental WCL (both E and EP). A total of 1,735 and 2,525 proteins were identified in sEVs and WCL, respectively. In sEVs, 426/1735 proteins were uniquely identified, whereas in WCL, 1216/2,525 proteins were uniquely identified (based on identifications in four or more replicates for each group) **(Figure 4.2a)**.

We identified 85 and 89 proteins in sEVs reported in EV databases ExoCarta[245] and Vesiclepedia[246], respectively **(Appendix figure S4.1; Appendix table S4.2)**. Such proteins are involved in trafficking (e.g. RAB7A, RAB8A and FLOT1), and tethering (e.g. SDCBP and ANXA1), indicative of their involvement in exosome biology and transport. Of interest, 76 proteins have not been identified in any published exosome or EV proteomic database **(Appendix table S4.3)**, which highlights selective packaging in endometrial sEVs. These proteins are comprised of enzymes (e.g. MARS1, EPRS1, NARS1 and ADSS2), and scaffold (e.g. ECPAS, SEPTIN2, and PAXX), transport (e.g. RACK1, UFD1, DOP1B and SEPTIN8) and cytoskeletal (e.g. SHTN1, SEPTIN1, and TARS1) proteins. Interestingly, Septins comprised 8/76 uniquely identified proteins (e.g. SEPTIN2, SETPIN3, SEPTIN5 and SEPTIN7-11) which are implicated at the interface of cell signalling and membrane/ cytoskeletal organisation[247], with SEPT7[248], SEPT9[249] and SEPT11[250] having associated functions in embryogenesis.

Protein expression heat map (hierarchical clustering) revealed distinct enrichment of proteins between sEVs and cells, where cluster 1 represents proteins enriched in sEVs, and clusters 2 and 3 represent proteins enriched in cells, or low abundant/ not identified in sEVs that were identified in cells, respectively **(Figure 4.2b, left panel; Appendix table S4.1)**. In line with the characteristics of sEVs, the data indicate that sEVs (cluster 1) were enriched in proteins involved in pathways of sEV/exosome biogenesis, including multivesicular body assembly (e.g. TSG101, CHMP3 and VPS28), endosomal transport (e.g. CHMP4C, VPS36 and SNX18), viral budding via ESCRT complex (e.g. MVB12A, CHMP2B and CHMP1A), intracellular trafficking (e.g. ANXA1-7 and RAB10-13) and membrane organisation (e.g. CD9/63/81) **(Figure 4.2b, yellow box)**. I also report the involvement of unique sEV proteins in cell migration (e.g. PLXNB1, GPC6, PAK4 and

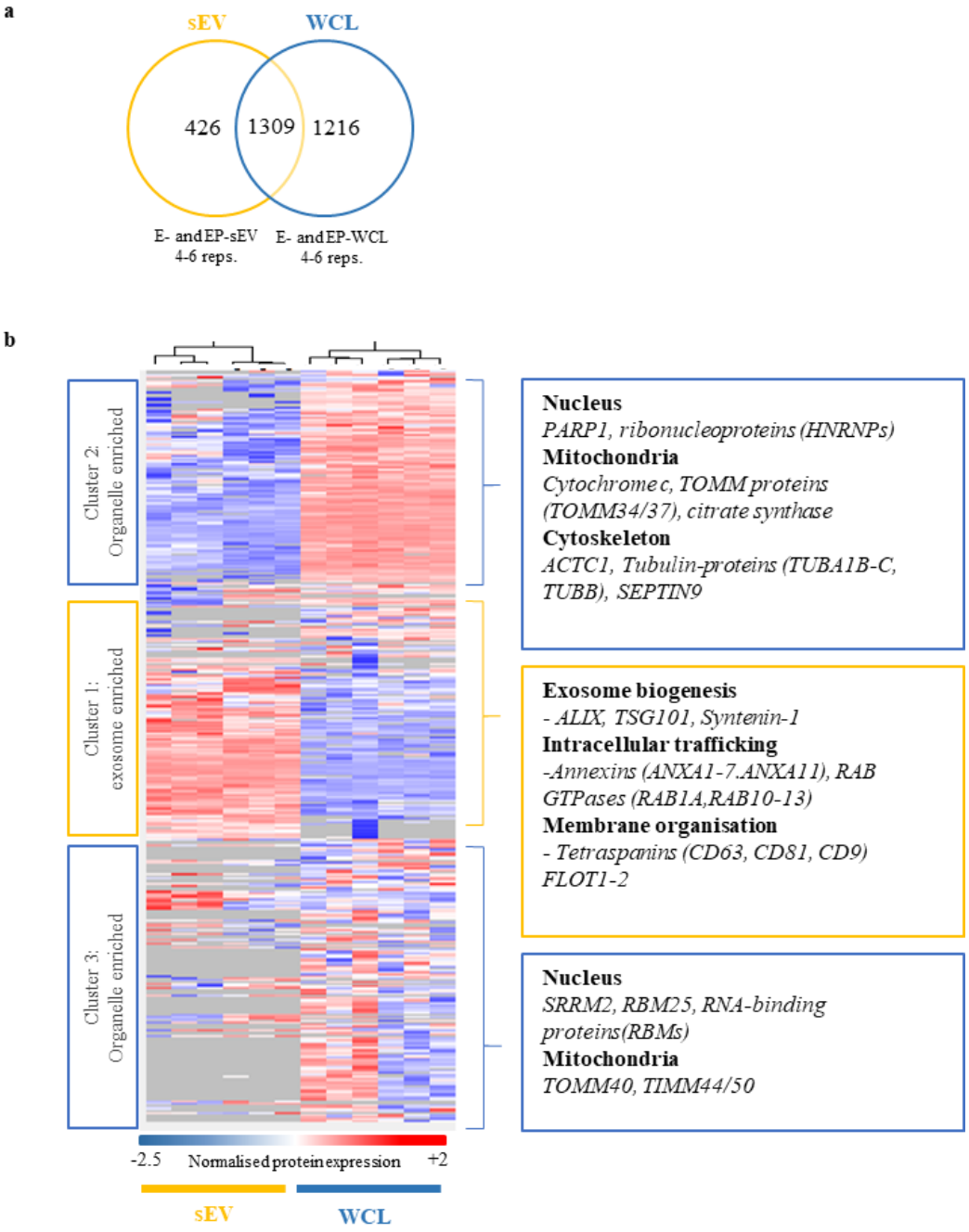



**Figure 4.2 Selective packaging and enrichment of Ishikawa small extracellular vesicles. a)** Venn diagram comparing protein identifications in Ishikawa total sEVs and whole cell lysate (WCL). n=6 replicates, with protein identification considered if identified in 4 or more replicates. **b)** Left panel, protein expression heat map of proteins identified in sEV (left) and WCL (right). Right panel, organelle protein markers identified in 3 distinct clusters from heat map. Cluster 1 (yellow box) indicative of exosomal protein markers while cluster 2-3 (blue boxes) indicative of organelle and cytosolic markers.

EPHB3) and Rho protein signal transduction (e.g. ROCK1, RHOD, CTNL1 and GNA12) processes, which are important regulators of endometrial remodelling across the menstrual cycle[251-253] **(Appendix table S4.1)**. In contrast, cells (clusters 2-3) were enriched with organelle protein markers from nucleus (e.g. HNRNPH3, HNRNPAB and other ribonucleoproteins, RMB12B, RBM3-4 and other RNA-binding proteins), mitochondria (e.g. CYCS, TOMM34/37/40 and TIMM44/50) and cytoskeleton (e.g. ACTC1, TUBA1, TUBB and other tubulin proteins)[254] **(Figure 4.2b, blue boxes)**.

### 4.2.3 Endometrial small extracellular vesicle proteome is hormonally regulated

We next examined how sEV protein cargo are regulated in response to E and EP treatment. A total of 1,617 and 1,771 proteins were identified in E- and EP-sEVs, respectively, with 1,579 proteins commonly identified **(Figure 4.3a; Appendix table S4.4)**. Differential analysis between E- and EP-sEVs identified 192 unique and 180 significantly enriched ($p<0.05$) proteins in EP-sEVs (372 proteins) **(Figure 4.3a/b; Appendix table S4.5)**, and 38 unique and 349 significantly enriched ($p<0.05$) proteins in E-sEVs (387 proteins) **(Figure 4.3a/b; Appendix table S4.6)**.

Secretory phase endometrium signals to, and interacts with an implanting embryo during WOI[224]. Therefore, to gain biological insights into the role of endometrial EP-sEVs (compared to E-sEVs) during this period, gene set enrichment analysis was performed, initially revealing 22 common biological processes associated with E and EP treatments, including "vesicle-mediated transport", "protein transport", "antigen processing and presentation of exogenous peptide", and "small GTPase signalling", consistent with the function of EV biogenesis and trafficking **(Appendix table S4.7)**. Importantly, analysis of EP-sEV protein cargo containing unique or significantly enriched proteins (372 proteins) revealed 124 unique biological processes (GO, $p<0.05$) **(Appendix table S4.8)**. Whereas analysis of E-sEV protein cargo containing unique or significantly enriched proteins (387 proteins) revealed 110 unique biological processes (GO, $p<0.05$) **(Appendix table S4.9)**.

To gain a global understanding of the repertoire of biological processes identified within EP-sEVs, GO terms were manually categorised into biological groups, represented as gene

expression, metabolic/ cellular response, trafficking/ transport, protein processing, signalling, cell growth and development, viral process, cell cycle, cell adhesion, cell motility, actin cytoskeleton reorganisation or other **(Figure 4.3c)**. EP-sEV protein cargo were most commonly implicated in metabolism/ cellular response processes, including cell-matrix adhesion (e.g. ITGAV/6 and BCAM), *in utero* embryonic development (e.g. NOTCH1, ADAM10 and COPS3), epithelial to mesenchymal transition (EMT) (e.g. CTNNB1, EPB41L5 and HNRNPAB), cell migration (e.g. RHOA/C, BMPR2 and DDR1) and nitric oxide (NO) biosynthesis (e.g. DDAH2, AK1 and SPR) **(Figure 4.3d, Appendix table S4.8)**, which are important for the endometrium to transition to a receptive state[95,241,255] and to support embryo development for proper implantation[256-258]. Whereas proteins enriched in E-sEVs were mostly implicated in cell proliferation (e.g. NAMPT, STX4 and SRGAP2), mitotic cell cycle transition (e.g. CUL1, CDK1/2, and PAK1), nucleotide biosynthesis (e.g. DCK, DCTD and PRPS2) and actin cytoskeleton organisation (e.g. DIAPH1, CAP1 and DAAM1) **(Figure 4.3d, Appendix table S4.9)**. Importantly, these processes coincide with proliferative activity under E control[1,30].

Comparative analysis of EP-sEV proteome (unique and significantly enriched protein cargo compared to E-sEVs) was performed with sEVs from the ECC-1 endometrial cell line following EP treatment[138], in which 63/372 proteins were co-identified **(Appendix table S4.10)**. These proteins were associated with embryonic development (e.g. COPS3, EGFR, ADAM10, DLG1 and AKT1), cell migration (e.g. ITGA6, ADAM10, SDCBP and ACTN4) and differentiation (e.g. ACTC1, EPCAM, ANXA7, ANPEP and CLIC4); processes implicated in the maturation and differentiation of endometrium during the secretory phase[29] **(Appendix figure S4.2)**.

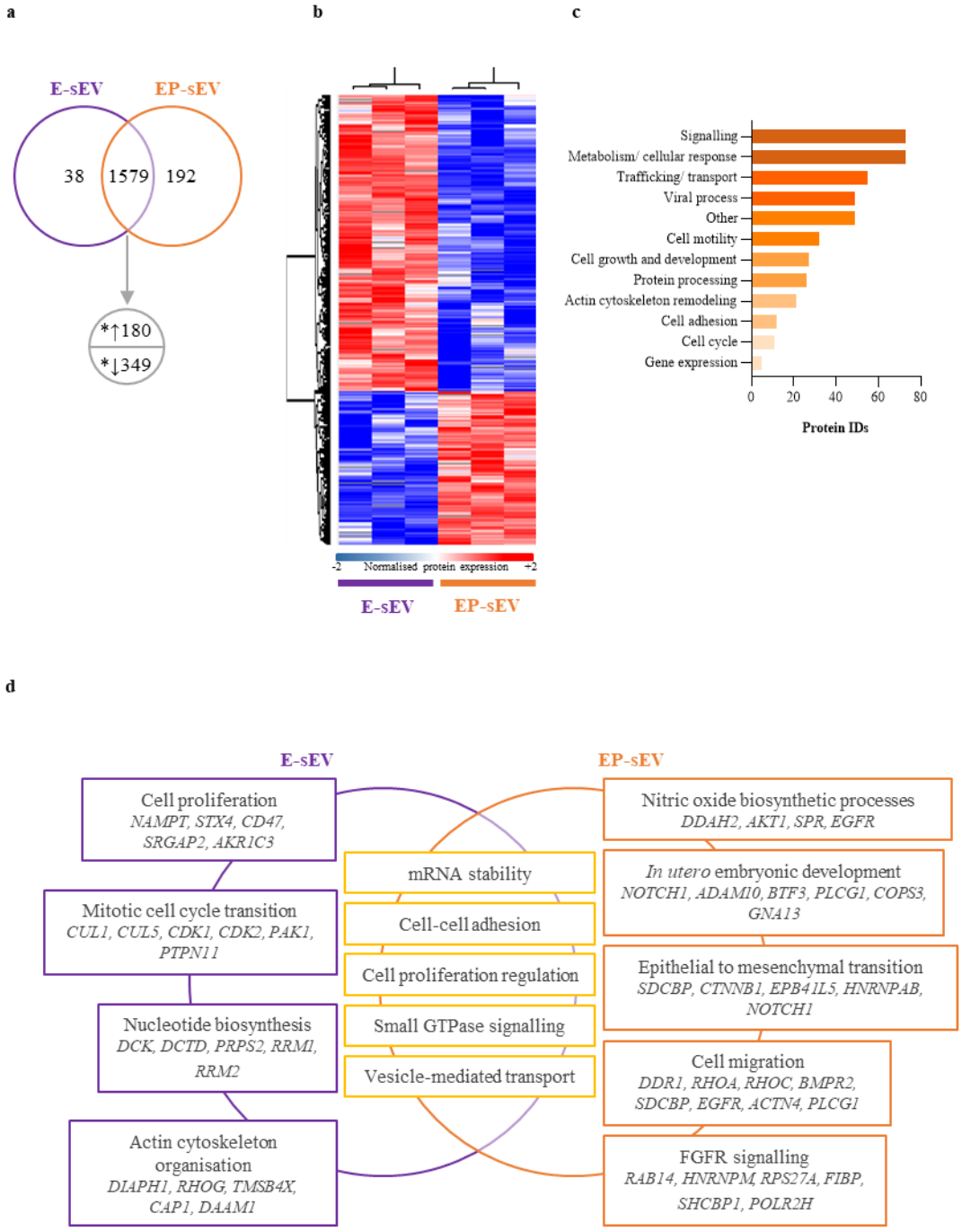



**Figure 4.3 Proteomic profiling of Ishikawa E and EP small extracellular vesicles a)** Venn diagram of protein identifications in Ishikawa E-sEV (purple) and EP-sEV (orange). n=3 replicates, protein identification in 2 or more replicates. *proteins significantly enriched in EP-sEVs or E-sEVs ($p<0.05$). **b)** Protein expression heat map of E- and EP-sEVs. **c)** Gene Ontology (GO) (biological processes) groups enriched in EP-sEVs. GO terms included with $p<0.05$. **d)** Distribution of biological processes identified in protein groups enriched (including unique) in E-sEVs and EP-sEVs.

Manual curation of our EP-sEV dataset (uniquely identified and significantly enriched, 372 proteins) revealed proteins previously shown to contribute to the developmental progress of pregnancy (i.e. endometrial receptivity, embryo development and embryo implantation). Of interest are EP-sEV proteins that potentially function in an autocrine manner to regulate endometrial receptivity and remodelling during the secretory phase (e.g. CTNNB1, EGFR, EPCAM, NOTCH1 and DDRI). Similarly, I identified proteins which may be involved in communication between endometrium and embryo to support embryo development (e.g. BMPR2, BTF3, COPS3, PLCG1 and RAB14), and adhesion/ migration of trophoblast during implantation (e.g. ADAM10, CUL3, ITGAV and AKT1). These proteins have been catalogued in **Table 4.2**. Collectively, these comparative analyses highlight key changes in protein composition of Ishikawa sEVs following E and EP treatment that contain phase-specific regulators of embryo implantation.

### 4.2.4 *In vitro* endometrial-derived small extracellular vesicles compared with human-derived uterine fluid

Endometrial secretions, including UF, play an important role in preparing the uterine microenvironment for implantation[20,224]. Our group has recently analysed uterine lavage (including total uterine lavage, sEVs and soluble secretome) obtained from proliferative and secretory phases of the menstrual cycle from fertile and infertile women[192].

To validate factors identified in EP-sEVs, I compared this dataset (significantly abundant and uniquely identified, compared to E-sEVs) with uterine lavage obtained from woman with proven fertility during the secretory phase. Of note, 52/372 EP-sEVs were co-identified **(Figure 4.4, Appendix table S4.11)**, including proteins associated with antioxidant function (PRDX1, TXN and DDAH2), signal transduction (GPS1 and YWHAE), trafficking (ANXA6), cell invasion (CSTB, TXN, TKT and PRDX1), glucose metabolism (SORD, FABP5 and PGAM1), cell migration (e.g. RHOC, YWHAE, ACTN4 and HSPB1), and developmental processes (e.g. GPS1, CALM, and SERPINH1) **(Figure 4.4)**. Several of these proteins, namely, DDAH2[259], GPS1[260], SERPINB9[239] and RHOC[261] have been demonstrated to be involved in embryonic function, and may be involved in maternal-embryo signalling.

**Table 4.2 Ishikawa small extracellular vesicle proteins enriched in EP treatment: Implications in implantation**

| [1]Uniprot accession | [1]Gene name | [1]Protein name | [2]Diff. expression $log_2$(EP/E) | [3] Implicated function | [3]Ref. |
|---|---|---|---|---|---|
| O14672 | ADAM10 | Disintegrin and metalloproteinase domain-containing protein 10 | 0.79 | Proteolytic activity which promotes cell migration. May regulate adhesion response | 258 |
| P31749 | AKT1 | RAC-alpha serine/threonine-protein kinase | 0.49 | Enables trophoblast migration in response to epidermal growth factor signalling | 262 |
| Q13873 | BMPR2 | Bone morphogenetic protein receptor type-2 | EP unique | Involved in BMP signalling for the development of extraembryonic cell lineages for the pre-implantation embryo | 263 |
| P20290 | BTF3 | Transcription factor BTF3 | EP unique | Potentially regulates ERα transcription. Required for post-implantation embryonic development | 264,265 |
| Q9UNS2 | COPS3 | COP9 signalosome complex subunit 3 | EP unique | Integral part of COP9 signalosome complex, required to maintain embryonic epiblast cell survival and development of early embryo | 260 |
| P35222 | CTNNB1 | Catenin beta-1 | 0.39 | Required for proper uterine development. Depletion results in impaired decidualisation and endometrial receptivity | 241,266 |
| Q13618 | CUL3 | Cullin-3 | EP unique | Modulation of CUL3/β-catenin pathway promotes endometrial receptivity and supports attachment of trophectoderm cells to endometrial epithelium | 267 |
| Q08345 | DDR1 | Epithelial discoidin domain-containing receptor 1 | EP unique | Regulates endometrial cell proliferation by inactivating endothelin-1, to stimulate AKT phosphorylation and DNA synthesis in stromal cells. | 268 |
| P00533 | EGFR | Epidermal growth factor receptor | 0.81 | Regulates endometrial decidualisation through BMP2 and WNT4 downstream effectors. Ablation of EGFR prevents stromal epithelial to mesenchymal transition. | 95 |
| P16422 | EPCAM | Epithelial cell adhesion molecule | 1.63 | Cell surface adhesion molecule that maintains epithelial integrity through modulation of E-cadherin. Expression of EPCAM temporally regulated to balance maintenance of epithelial integrity with endometrial receptivity | 269 |
| P06756 | ITGAV | Integrin alpha-V | 0.21 | Dimerises with integrin β3 to form an αvβ3 receptor that promotes endometrial receptivity and potentially coordinates embryo adhesion for implantation | 78 |
| P46531 | NOTCH1 | Neurogenic locus notch homolog protein 1 | EP unique | Maintains endometrial integrity during window of implantation | 270 |
| P19174 | PLCG1 | 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1 | EP unique | Signal transduction molecule in response to tyrosine kinase signalling, that is required for proliferation of embryonic cell types for normal development | 271 |
| P61106 | RAB14 | Ras-related protein Rab-14 | 1.2 | KIF16B/Rab14 complex regulates surface expression of FGFR2 and fibroblast growth factor signal transduction (essential for embryogenesis) through golgi-to-endosome trafficking of vesicles expression FGFR | 272 |

[1]Uniprot Accession number, gene name and protein description annotated from UniProt[220]
[2]Differential expression using $Log_2$(EP-sEV/E-sEV). Fold change (FC) not calculated for unique protein IDs
[3]Function of proteins annotated from cited literature.

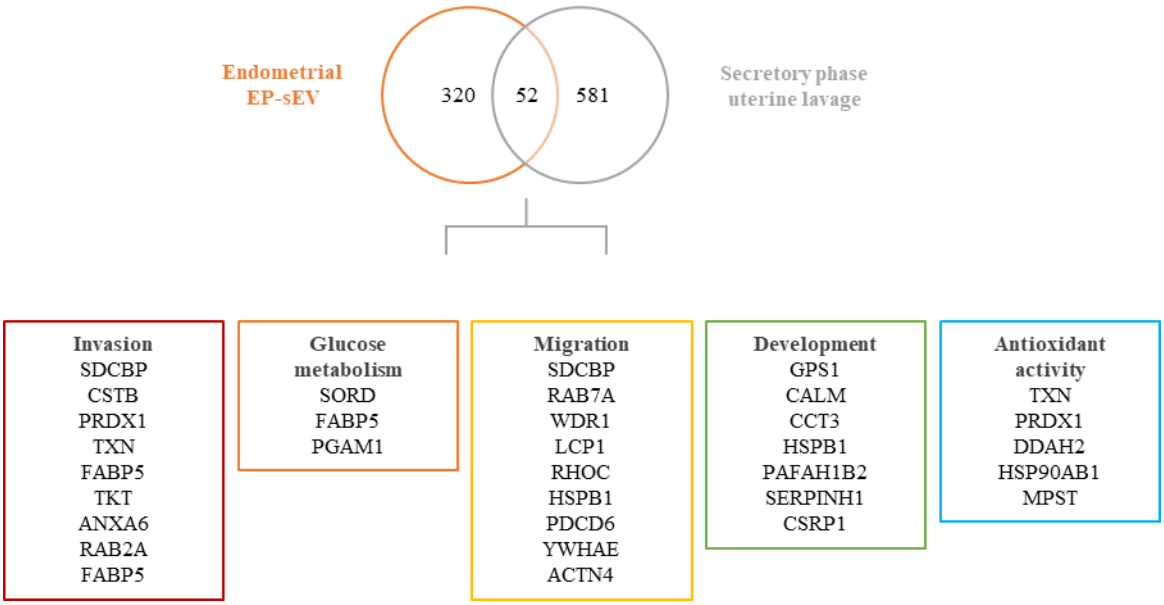



**Figure 4.4 Venn diagram comparison of Ishikawa small extracellular vesicles and human secretory phase uterine lavage.** Upper panel, Venn diagram comparison of proteins enriched in Ishikawa EP-sEVs *versus* proteins enriched in human secretory phase uterine lavage. Lower panel, sub-categories of biological functions of proteins found in common through manual literature curation.

## 4.3 Discussion

The endometrium is regulated by sequential and precise interplay of ovarian steroid hormones E and P across the menstrual cycle[273]. This regulation is critical for the endometrium to transition to its receptive phase, which synchronises with timely arrival of embryo for successful implantation[231]. To gain insights into how the endometrium is prepared for the receptive window, and how secretory phase signals, namely sEVs as important intercellular regulators of implantation process[137,138,154,187], may support implantation during this period, I defined the composition of hormonally regulated endometrial sEVs (EP-treated). Here, I provide key insights that reveal EP-sEV cargo (compared to E-sEVs) are implicated in secretory differentiation of the endometrium to become receptive (e.g. EMT, cytoskeletal migration/ remodelling and decidualisation processes). Further, I reveal EP-sEVs, distinct from their parental cells, are enriched in key regulators of embryo development and implantation (e.g. BMPR2, ADAM10, COPS3 and DDAH2). These findings provide a unique understanding of how endometrial sEV composition are hormonally regulated to recapitulate menstrual cycle phase signals and importantly, contribute to successful implantation.

### 4.3.1 Small extracellular vesicle signalling to endometrium – insights into phase-specific endometrial remodelling

Dynamic regulation of cell proliferation plays an integral role in re-establishing the endometrial lining during the proliferative phase of the menstrual cycle[1], predominantly regulated by E[231]. Through proteomic characterisation, I report proteins associated with cell proliferation, cell cycle regulation and nucleotide biosynthesis (e.g. CUL4A, CDK2, DCK, PURB and CUL5) significantly enriched in E-sEV cargo (in comparison to untreated Ishikawa sEVs; *data not shown*), which play critical roles in modulating endometrial regeneration during this period[274]. In line with these findings, CDKs have also been identified in endometrial (ECC-1) sEVs following E-treatment[138], being key targets of E signalling to mediate cell cycle transition in the endometrium[275]. Further, nucleotide biosynthetic processes are essential for DNA replication and RNA production to support this transition[276]. Knockdown studies of deoxycytidine kinase (DCK) – which maintains

cell cycling and genomic integrity in dividing cells[277] significantly attenuate cell proliferation and limits cell survival[278,279]. Indeed, proper regulation of cell proliferation ultimately maintains endometrial tissue integrity and function[280], critical for embryo implantation in the later secretory phase[281]. Dysregulation of these processes, i.e. by uncontrolled cell proliferation, may lead to endometrial pathogeneses including endometriosis – a chronic inflammatory disease characterised by growth of endometrial tissue outside of the uterus[282,283].

Proteomic characterisation of purified endometrial EP-sEVs compared to E-sEVs in this study provides an in-depth and physiologically relevant dissection of the protein repertoire enriched in the receptive phase to prepare the uterine microenvironment for embryo implantation. Factors significantly enriched in sEVs from EP treatment were predominantly involved in EMT, cell motility and cell adhesion, critical for the secretory differentiation of the endometrium to become receptive[29]. Catenin beta-1 (CTNNB1) was significantly enriched in EP-sEVs – a key finding in that this protein has not previously been identified in endometrial-derived EVs. CTNNB1 is a downstream effector of P signalling, plays a central role in the canonical Wnt signalling pathway and is critical for uterine development, decidualisation and implantation[241,266]. It has been suggested that endometrial LE undergo EMT during the secretory phase to facilitate attachment and adhesion of an implanting embryo[284], with CTNNB1 shown as a key regulator of endometrial epithelial differentiation[182]. Further, Transforming protein RhoA (RHOA), a small GTPase involved in actin cytoskeleton remodelling and cell migration[285] was significantly enriched in EP-sEVs, which has previously been identified in endometrial epithelium to direct trophoblast attachment[255]. Selective packaging of these proteins in EP-sEVs may facilitate endometrial remodelling during the secretory phase to prepare a fertile site for embryo adhesion and invasion.

Upon further inspection of the EP-sEV dataset, there was an increase in cell-matrix adhesion processes which regulate the direct interaction of endometrium and embryo. Of interest, integrin alpha V/6 (ITGAV/6) were significantly enriched in EP-sEVs. Heterodimers of integrins mediate endometrial-embryonic interaction and adhesion[77] and are suggested to be temporally regulated throughout the menstrual cycle by ovarian steroid hormones[286]. While ITGA6 was previously identified in ECC-1 sEVs, this is the first report of ITGAV selectively packaged in

endometrial sEVs, and forms part of the integrin αVβ3 receptor that mediates embryo-endometrium adhesion[77,78]. Previous studies demonstrate sEV-derived integrins assist in directing cargo to target cells to mediate uptake for reprogramming[168], while others suggest sEVs (exosomes) transfer integrins to target cells to upregulate integrin expression on their cell surface[287]. At this time, although the biological effects of sEV/exosome transfer can be profound, the mechanisms of exosome uptake by cells and how their protein cargo is utilised by cells, are not fully understood[288] due to their cell-type specific uptake mechanisms[289]. Hence, the potential role for these enriched integrins to site-direct EP-sEVs during the pre-implantation period to interact with and reprogram embryo, requires further investigation.

Collectively, the data demonstrate phase-specific reprogramming of endometrial sEVs by ovarian steroid hormones. Indeed, E-sEVs and EP-sEVs are molecularly distinct and possess implicated functions in proliferative and secretory phase activities that are temporally regulated throughout the menstrual cycle.

### 4.3.2 Endometrial small extracellular vesicle signalling to pre-implantation embryo

In this study, I identified a protein signature of EP-sEV cargo that encompasses potential regulators of embryo development and implantation, highlighting their role in mediating these processes upon local interaction with an embryo. Proteins implicated in '*in utero* embryonic development', including receptor BMPR2, signalling complex COPS3 and metalloproteinase ADAM10 **(Table 4.2)** were significantly enriched in EP-sEVs (compared to E-sEVs). Bone morphogenetic protein receptor type-2 (BMPR2) was uniquely identified within EP-sEVs, which is not only implicated in endometrial decidualisation via Wnt signalling pathways[290], but is also expressed on trophectoderm cells, reported to support development of extra-embryonic lineages of an implanting embryo[263]. Interestingly, BMPR2 (through activation of BMP signalling) has been shown to enhance cell invasion and migration[291], is important for endometrial decidualisation and trophoblast invasion/ differentiation[292], and can be transferred to recipient cells via exosomes[293]. Based on the data, I speculate endometrial sEVs may potentially mediate this signalling during the pre-implantation environment to support embryo implantation.

PLCG1 was further uniquely identified in EP-sEVs, being a mediator of fibroblast growth factor (FGF) signalling involved in signal transduction through tyrosine kinase signalling pathway[294]. Based on gene set enrichment analysis, I report enrichment of FGF signalling in EP-sEVs, which encompasses a diverse range of FGR receptors (FGFR1-4) and ligands, important for early embryogenesis, including maintenance of trophoblast lineages[295]. I also identified Ras-related protein Rab-14 (RAB14) enriched in EP-sEVs, which, in association with KIF16B, regulates FGFR2 localisation and FGF signal transduction that is essential for embryogenesis[272]. Indeed, in the uterus, components of the FGF signalling pathway can act as paracrine and/or autocrine mediators of epithelial-stromal interactions. Paracrine activation of FGFRs induces proliferation of the LE in the uterus[281,296]. In addition to FGFR membrane localisation, FGF induced cellular differentiation has been shown to be accompanied by the nuclear translocation of FGFRs[297] – a potentially important role of sEV-mediated function in target cells. This is the first report of PLCG1 in endometrial sEVs. These results highlight the expression of PLCG1 and presence of FGF signalling in EP-sEVs, indicating that an FGF signalling axis could be part of sEV signalling to blastocyst during implantation.

A number of metabolic/ cellular response processes were significantly enriched in EP-sEVs, including NO biosynthetic processes. NO is a radical molecule that has various roles in cell proliferation, vasodilation, immune response and embryogenesis[256]. Proteins including DDAH2, AKT1 and SPR were implicated in NO biosynthesis. DDAH2 in particular, is an inhibitor of NO synthase (NOS) and therefore, regulates NO biosynthesis[259]. Importantly, DDAH2 is upregulated in embryo at the blastocyst stage, shown to enhance endothelial cell proliferation and migration, and potentially support dividing embryo[259]. NO regulation is critical within the pre-implantation environment, with excessive NO suggested to inhibit embryo implantation[298]. Not only was DDAH2 uniquely identified EP-sEVs, it was also identified within secretory phase uterine lavage obtained from fertile women, highlighting its potential significance in the uterine microenvironment to regulate NO levels and stimulate embryo development and implantation.

Analysis of Ishikawa sEV protein composition has provided novel insights into regulation of endometrial remodelling to create a competent environment for implantation, as well as insights

into endometrium-embryo communication. Ishikawa sEVs demonstrated phase-specific reprogramming that are spatially and temporally regulated throughout the menstrual cycle by E and P. Significantly, cross-study comparisons have highlighted the biological relevance of endometrial sEVs in the pre-implantation environment.

# Chapter 5: Endometrial small extracellular vesicles reprogram human trophectoderm cell proteome

## 5.1 Overview

Implantation success is dictated by synchronous and reciprocal signalling between the endometrium and embryo[2]; with endometrial factors expressed in the secretory phase able to activate blastocysts towards implantation competency[110-113]. A major aspect of achieving this competency includes reprogramming of pre-implantation embryo proteome – encompassing trophectoderm/ trophoblast differentiation to acquire an invasive phenotype[233], re-localisation of surface proteins[299,300] and reprogramming of metabolic pathways[41]. However, how secretory phase endometrial-derived sEVs contribute to embryo reprogramming remains limited, and whether proliferative phase sEVs influence the embryo remains unknown.

In this chapter, I investigated how highly purified and hormonally regulated human endometrial sEVs reprogram human trophectoderm cell proteome landscape. The trophectoderm is the first point of contact for an implanting embryo to the uterine environment and the endometrium[301], with T3-TSC cells being a highly suitable model of early human trophectoderm that bypass limitations associated with animal models (which possess different modes of endometrial preparation, placental development[302] and mechanisms of embryo-endometrium interactions) and immortalised cell lines[137]. Here, I show E- and EP-sEVs can be taken up by trophectoderm cells, and using quantitative MS-based proteomic and bioinformatic analysis, demonstrate their capacity to modulate trophectoderm cell proteome. Specifically, I show EP-sEVs, which encompass secretory phase signals present during the WOI, promote expression of proteins involved in cell adhesion, migration and ECM remodelling that ultimately create a pro-invasive phenotype in trophectoderm cells in support of implantation. Further, I demonstrate for the first time that E-sEVs which encompass proliferative phase signals, can reprogram trophectoderm cells, suggesting a potential residual signalling effect from proliferative phase sEVs to signal to pre-implanting embryo. These findings demonstrate the importance of highly-purified endometrial sEVs in promoting physiological processes crucial to implantation.

## 5.2 Results

### 5.2.1 Endometrial small extracellular vesicles reprogram trophectoderm cell proteome

To monitor sEV transfer to trophectoderm cells, E- and EP-sEVs were labelled with a DiI lipophilic tracer. After 2 h incubation, confocal microscopy showed endometrial E- and EP-sEVs were readily taken up **(Figure 5.1a, Appendix figure S5.1)**.

To investigate how E- and EP-sEVs influence trophectoderm cell protein composition, I proteomically profiled human T3-TSCs treated with sEVs *versus* untreated control cells **(Figure 5.1b)**. A total of 2,318 proteins were identified, with 2,210 (E-sEV treated), 2,191 (EP-sEV treated) and 2,206 (untreated) proteins identified in each group (based on protein identifications in 3 or more replicates in E-sEV and EP-sEV treatment, or 4 or more replicates in untreated T3-TSC) **(Figure 5.1c; Appendix table S5.1)**. Of note, 24 proteins were uniquely identified in E-sEV treatment, including ETFDH, FAR1 and PCCA involved in fatty acid metabolism, NRP1, MAP7D1 and PRKCI involved in cytoskeletal organisation and GDF15 and MAPK3 involved in MAPK signalling, which provide embryo with nutrients[303] or activate signalling pathways required for pre-implantation embryo development[304] **(Table 5.1)**. Furthermore, 21 proteins were uniquely identified in EP-sEV treatment, including PPFIBP1, SCYL1 and TES involved in cell-cell adhesion, FHL1 and PSMB7 involved in cell cycle regulation and HP and SQSTM1 involved in immune regulation, being processes that are upregulated at the site of implantation[305] **(Table 5.1)**.

Notably, I observed that T3-TSC contain 21 highly enriched or unique trophectoderm markers compared to other cell types (based on CellMarker database[306]) **(Appendix table S5.2)**, including KRT18 and DAB2 which are trophectoderm lineage markers expressed by blastocyst at early pre-implantation stages[307,308]. Importantly, these trophectoderm markers were similarly expressed in T3-TSCs between all sEV treatment groups and untreated control.

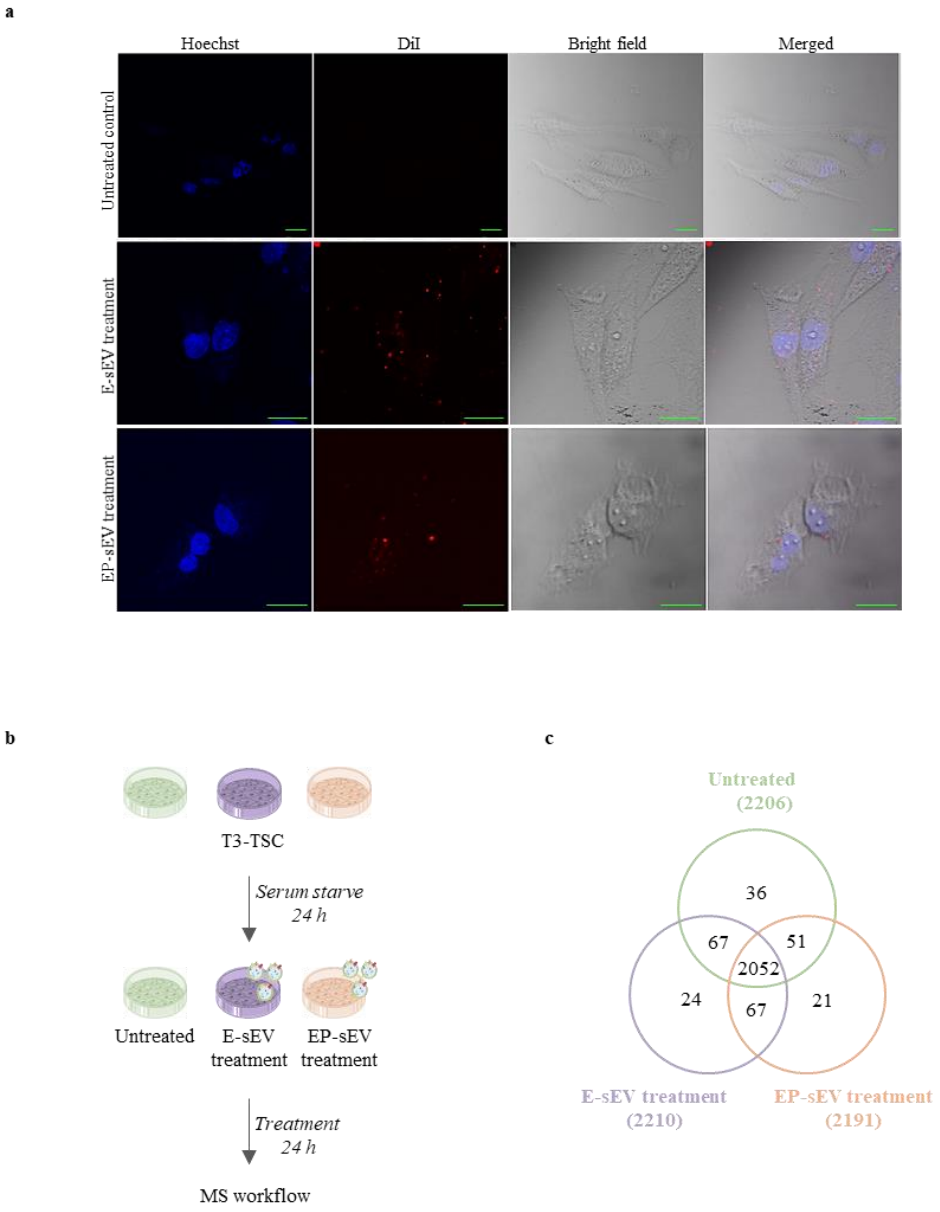



**Figure 5.1 Proteomic workflow of human trophectoderm cells treated with endometrial small extracellular vesicles. a)** Live fluorescence and bright field microscopic analysis of human trophectoderm cells (T3-TSC) incubated with Ishikawa E-sEVs, EP-sEVs or untreated (PBS) control. sEVs were stained with DiI (red) lipophilic tracer. T3-TSC cells were stained with Hoechst (blue). Scale bar = 20 µm. n= 3 biological replicates. **b)** Workflow of endometrial sEV treatment of trophectoderm cells. T3-TSC either untreated (green); treated with E-sEVs (purple) or EP-sEVs (orange), for 24 h before undergoing proteomic profiling. **c)** Protein identifications in E-sEV treatment (purple), EP-sEV treatment (orange), and untreated T3-TSC (green). n=5-6 replicates, protein identification in 3 or more replicates for sEV treatment groups, and 4 or more identifications in untreated group.

**Table 5.1 Unique proteins identified in trophectoderm cells treated with endometrial-derived small extracellular vesicles**

***Unique proteins in T3-TSCs treated with E-sEVs****

| UniProt Accession | Gene name | Protein name |
|---|---|---|
| O95400 | CD2BP2 | CD2 antigen cytoplasmic tail-binding protein 2 |
| Q16134 | ETFDH | Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial |
| Q8WVX9 | FAR1 | Fatty acyl-CoA reductase 1 |
| P34059 | GALNS | N-acetylgalactosamine-6-sulfatase |
| P32456 | GBP2 | Guanylate-binding protein 2 |
| Q99988 | GDF15 | Growth/differentiation factor 15 |
| Q3KQU3 | MAP7D1 | MAP7 domain-containing protein 1 |
| P27361 | MAPK3 | Mitogen-activated protein kinase 3 |
| Q9GZY8 | MFF | Mitochondrial fission factor |
| O75251 | NDUFS7 | NADH dehydrogenase [ubiquinone] iron-sulfur protein 7, mitochondrial |
| Q5SY16 | NOL9 | Polynucleotide 5'-hydroxyl-kinase NOL9 |
| O14786 | NRP1 | Neuropilin-1 |
| P05165 | PCCA | Propionyl-CoA carboxylase alpha chain, mitochondrial |
| Q8TD55 | PLEKHO2 | Pleckstrin homology domain-containing family O member 2 |
| Q7Z5L7 | PODN | Podocan |
| P41743 | PRKCI | Protein kinase C iota type |
| Q92530 | PSMF1 | Proteasome inhibitor PI31 subunit |
| Q9NZ71 | RTEL1 | Regulator of telomere elongation helicase 1 |
| Q9NP81 | SARS2 | Serine--tRNA ligase, mitochondrial |
| Q08AF3 | SLFN5 | Schlafen family member 5 |
| Q8WXA9 | SREK1 | Splicing regulatory glutamine/lysine-rich protein 1 |
| Q12788 | TBL3 | Transducin beta-like protein 3 |
| Q6ZXV5 | TMTC3 | Protein O-mannosyl-transferase TMTC3 |
| O60507 | TPST1 | Protein-tyrosine sulfotransferase 1 |

***Unique proteins in T3-TSCs treated with EP-sEVs****

| UniProt Accession | Gene name | Protein name |
|---|---|---|
| Q9NX62 | BPNT2 | Golgi-resident adenosine 3',5'-bisphosphate 3'-phosphatase |
| Q9NZ45 | CISD1 | CDGSH iron-sulfur domain-containing protein 1 |
| O00273 | DFFA | DNA fragmentation factor subunit alpha |
| P33316 | DUT | Deoxyuridine 5'-triphosphate nucleotidohydrolase, mitochondrial |
| Q9BQ52 | ELAC2 | Zinc phosphodiesterase ELAC protein 2 |
| Q13642 | FHL1 | Four and a half LIM domains protein 1 |
| P00738 | HP | Haptoglobin |
| Q14766 | LTBP1 | Latent-transforming growth factor beta-binding protein 1 |
| P42345 | MTOR | Serine/threonine-protein kinase mTOR |
| Q96D46 | NMD3 | 60S ribosomal export protein NMD3 |
| Q15126 | PMVK | Phosphomevalonate kinase |
| Q86W92 | PPFIBP1 | Liprin-beta-1 |
| O60256 | PRPSAP2 | Phosphoribosyl pyrophosphate synthase-associated protein 2 |
| Q99436 | PSMB7 | Proteasome subunit beta type-7 |
| P15151 | PVR | Poliovirus receptor |
| Q9ULZ3 | PYCARD | Apoptosis-associated speck-like protein containing a CARD |
| O00422 | SAP18 | Histone deacetylase complex subunit SAP18 |
| Q96KG9 | SCYL1 | N-terminal kinase-like protein |
| Q13501 | SQSTM1 | Sequestosome-1 |
| Q9UGI8 | TES | Testin |
| Q8NI36 | WDR36 | WD repeat-containing protein 36 |

* Gene name, Uniprot Accession number, and protein description annotated from UniProt[220]

### 5.2.2 Hormonally primed endometrial-derived small extracellular vesicles reprogram trophectoderm cells towards a pro-implantation phenotype

*Analysis of trophectoderm cells treated with endometrial EP-sEVs*

Here, I investigated how endometrial-derived EP-sEVs reprogram trophectoderm cell protein composition. Differential protein expression analysis between EP-sEV treated and untreated T3-TSC proteomes revealed 28 unique and 61 significantly enriched ($p<0.05$) proteins in trophectoderm treated with EP-sEVs **(Figure 5.2a; Appendix table S5.3)**. Gene set enrichment analysis of this subset identified biological processes significantly enriched, including ECM organisation (e.g. COL6A3, ITGAV, ITGA3, FN1 and AGRN), cell adhesion (e.g. ITGA3, ITGAV, VCAM1, TGFBI and IGFBP7) and cell growth (e.g. MTOR, LAMTOR and ITGAV), which are key processes indicative of ECM and cellular remodelling associated with implantation[309-312] (GO biological processes, $p<0.05$) **(Figure 5.2b; Appendix table S5.4)**. Cursory inspection of this subset further highlighted the involvement of proteins in implantation processes, including cell migration (e.g. DPP4, PLXNB2, ITGA3 and CDC42), proliferation (e.g. LIMS1, NASP, NUDC and RPS6KA3), and invasion (e.g. DPP4, ITGA3, VCAM1, GBP1 and C1QBP)[309,313-317], suggesting endometrial secretory phase signals alter trophectoderm cells towards a pro-invasive phenotype **(Figure 5.2b)**. The function of proteins within this subset are catalogued in **Table 5.2**.

In chapter 4, proteomic analysis of highly purified endometrial E- and EP-sEVs identified distinct protein cargo implicated in menstrual cycle phase-specific functions. I next questioned whether E- and EP-sEVs can differentially modulate the proteome of T3-TSC cells. Differential proteomic analysis identified 48 unique and 53 significantly enriched ($p<0.05$) proteins in EP-sEV treatment, and 100 low abundant and 68 not identified proteins in EP-sEV treatment that were more abundant/ unique in E-sEV treated cells **(Figure 5.2c; Appendix table S5.5)**. Gene set enrichment analysis of enriched or unique proteins in EP-sEV treated trophectoderm cells revealed processes associated with Wnt signalling pathway (e.g. PSMB7, AP2A2, CLTC and PSME1), TNF-mediated signalling pathway (e.g. PSMD8, PSMB7 and PYCARD) important in regulating trophoblast secretion of MMPs[318], and cell adhesion mediated by integrins (e.g. ADAM9, ITGAV and ITGA2) important to support embryo interaction/ attachment[78,319,320] (GO biological processes, $p<0.05$) **(Figure 5.2d; Appendix table S5.6)**. Furthermore, EP-sEV treated trophectoderm cells were

enriched in proteins implicated in proteolysis (e.g. DPP4, PSMD8 and GGH), complement activation (e.g. IGHG, IGKC and C1QBP) and innate immune response (e.g. IGHG, IGKC and C1QBP), which could potentially modulate the maternal immune response in the local uterine microenvironment[305]. Thus, endometrial EP-sEVs potentially prepare trophectoderm for interaction with endometrium during implantation, as well as protecting these cells from maternal immune rejection.

*Analysis of trophectoderm cells treated with endometrial E-sEVs*

In comparison, proteins that were unique to, or enriched in E-sEV treatment of trophectoderm cells (compared to EP-sEV treatment) were implicated in fatty acid beta oxidation (e.g. ACADM, ACOX1 and ETFDH), nucleotide biosynthetic processes (e.g. PUR8, PRPS2, ADK) important for cell growth, and positive regulation of EMT (e.g. COL1A1, DAB2 and HDAC2) which is important for embryogenesis[321] **(Figure 5.2d; Appendix table S5.7)**. Indeed, these findings suggest that the differences between E- and EP-sEV composition can selectively modulate trophectoderm cellular proteome landscape.

Moreover, differential proteome analysis between E-sEV treated and untreated T3-TSC cells was performed to expand our understanding of their reprogramming capacity to trophectoderm cells. This comparison revealed 44 uniquely identified and 72 significantly enriched ($p<0.05$) proteins in E-sEV treatment (compared to untreated trophectoderm cells) **(Figure 5.2e; Appendix table S5.8)**, with protein cargo implicated in leukocyte migration (e.g. BSG, ITGA3, SLC3A2, ATP1B1 and COL1A2), oxidation-reduction processes (e.g. PLOD1, FAR1, ALDH16A1, QSOX1 and PXDN) and inflammatory response (e.g. VCAM1, THBS1 and GJA1) (GO terms, $p<0.05$), which collectively function to modulate cell migration, protect embryo from reactive oxygen species (ROS) and modulate the immune response, processes which are fundamental for implantation[305,322] **(Figure 5.2f; Appendix table S5.9)**. These findings suggest that E-sEVs, while they may not be abundant during the secretory phase, may potentially signal to embryo to support its development and survival in the uterine microenvironment.

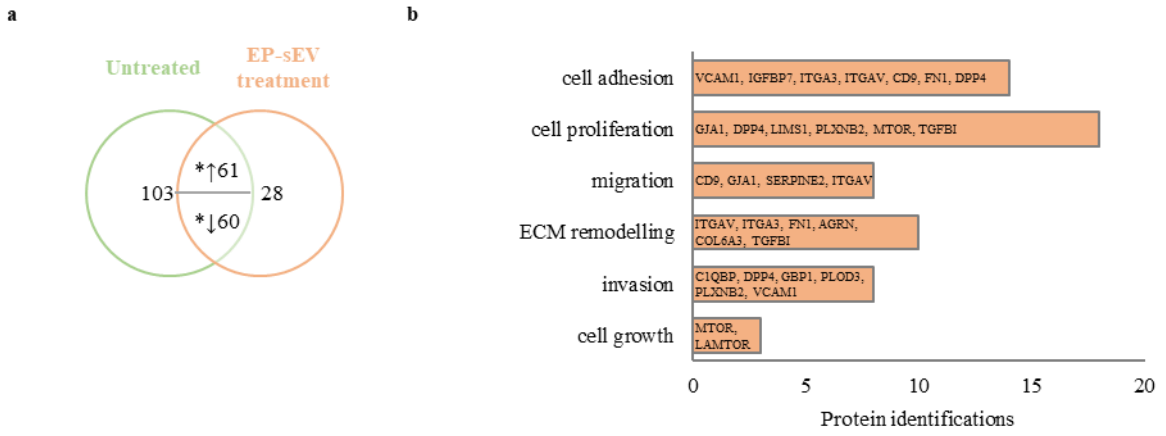

a
Untreated
EP-sEV treatment
*↑61
103
28
*↓60
b
cell adhesion
VCAM1, IGFBP7, ITGA3, ITGAV, CD9, FN1, DPP4
cell proliferation
GJA1, DPP4, LIMS1, PLXNB2, MTOR, TGFBI
migration
CD9, GJA1, SERPINE2, ITGAV
ECM remodelling
ITGAV, ITGA3, FN1, AGRN, COL6A3, TGFBI
invasion
C1QBP, DPP4, GBP1, PLOD3, PLXNB2, VCAM1
cell growth
MTOR, LAMTOR
0
5
10
15
20
Protein identifications
c
E-sEV treatment
EP-sEV treatment
*↑53
68
48
*↓100
d
Wnt signaling pathway
AP2A2, CLTC, PSME1, PSMB7
proteolysis
DPP4, GGH, PSMD8, IGKC
innate immune response
IGHG, IGKC, C1QBP
TNF-mediated signaling pathway
PYCARD, PSMD8, PSMB7
cell adhesion mediated by integrin
ADAM9, ITGAV, ITGA2
complement activation
IGHG, IGKC, C1QBP
regulation of EMT
COL1A1, DAB2, HDAC2
nucleotide biosynthetic processes
PUR8, PRPS2, ADK
fatty acid beta oxidation
ACADM, ACOX1, ETFDH
0
2
4
6
Protein identifications
e
Untreated
E-sEV treatment
*↑72
87
44
*↓23
f
oxidation-reduction...
PLOD1, FAR1, ALDH16A1, QSOX1, PXDN, NDUFS7
cell-cell adhesion
THBS1, LAMC1, CTNNB1, MFGE8
gene expression
STAT3, RBX1, LDLR, SERPINB9
leukocyte migration
BSG, SLC3A2, ATP1B1, ITGA3
inflammatory response
VCAM1, THBS1, GJA1
0
2
4
6
8
10
Protein identifications

**Figure 5.2 Proteomic profiling of human trophectoderm cells treated with endometrial small extracellular vesicles. a)** Venn diagram of protein identifications in untreated T3-TSC (green) *versus* EP-sEV treated T3-TSC (orange) cells. **b)** DAVID analysis-based Gene Ontology (GO) (biological process) terms and UniProt functional annotations identified in proteins enriched in EP-sEV treated T3-TSC (compared to untreated). **c)** Venn diagram of protein identifications in E-sEV treated T3-TSC (purple) *versus* EP-sEV treated T3-TSC (orange) cells. **d)** DAVID analysis-based GO (biological process) terms and UniProt functional annotations identified in proteins enriched in EP-sEV treated T3-TSC (orange) and in E-sEV treated T3-TSC (purple). **e)** Venn diagram of proteins identified in untreated T3-TSC (green) *versus* E-sEV treated T3-TSC (purple). **f)** DAVID analysis-based GO (biological process) terms and UniProt functional annotations identified in proteins enriched in E-sEV treated T3-TSC (compared to untreated). *significant differential expression based on student's T.test ($p<0.05$). Genes listed in bar graphs based on GO terms (biological processes with $p<0.05$) or manual curation of literature for proteins involved in functional annotations obtained from Uniprot[220].

## Table 5.2 Proteins enriched in trophectoderm cells upon EP-sEV treatment: Implications in implantation

| [1]UniProt accession | [1]Gene name | [1]Protein name | [2]Diff. expression $log_2$(EP-sEV/UT) | [3] Implicated function | [3]Ref. |
|---|---|---|---|---|---|
| O00468 | AGRN | Agrin | 0.40 | ECM proteoglycan sensor that regulates cell proliferation, motility and invasiveness. Potential role in implantation with spatiotemporal regulation in embryonic and maternal tissues | 309,323 |
| P27487 | DPP4 | Dipeptidyl peptidase 4 | 0.26 | Cell surface enzyme acts as potential adhesion mechanism for embryo-endometrium with FN1. DPP4/FAP heteromeric complex promotes invasion and migration | 317,324 |
| P02751 | FN1 | Fibronectin | 0.16 | Extracellular glycoprotein that acts as a ligand for integrin αvβ3, proposed to mediate embryo-endometrium attachment. Shown to induce MMP9 expression to support invasion. | 89,316 |
| P32455 | GBP1 | Guanylate-binding protein 1 | 1.21 | Pro-invasive protein activated by EGFR, which stimulate MMP1 *in vitro* and *in vivo* | 325 |
| P17302 | GJA1 | Gap junction alpha-1 protein | 0.31 | Forms gap junctions between cells for intercellular communication. Contributes to embryonic development and trophoblast differentiation upon TGFB1 activation, upregulating GJA1 expression in trophoblast | 314 |
| P48059 | LIMS1 | LIM and senescent cell antigen-like-containing domain protein 1 | 0.37 | Involved in integrin-mediated adhesion. Adaptor protein involved in regulation of cell survival, proliferation and cell differentiation. Regulates embryo cell polarity and survival in pre-implantation period. | 315,326 |
| O60568 | PLOD3 | Multifunctional procollagen lysine hydroxylase and glycosyltransferase LH3 | 0.24 | Enzyme required for synthesis and secretion of type IV collagens required for basement membrane formation. Interacts with STAT3 to induce MMP2/9 expression for cell invasion | 313 |
| Q92626 | PXDN | Peroxidasin homolog | 0.15 | Required for tissue development and maintenance of cell integrity. Increased expression in uterus during embryo attachment | 327 |
| Q15582 | TGFBI | Transforming growth factor-beta-induced protein ig-h3 | 0.16 | ECM protein with potential role in regulation trophoblast cell invasion during implantation and placentation. | 328 |

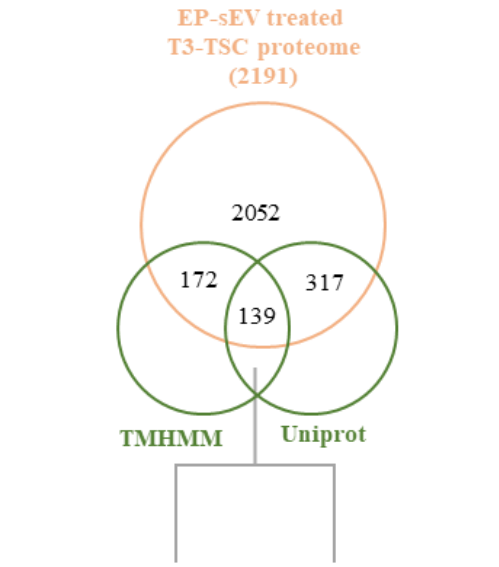
[1]Gene name, Uniprot Accession number, and protein description annotated from UniProt[220]
[2]Differential expression using $Log_2$(FC) of EP/UT
[3]Function of proteins annotated from cited literature. Abbreviations: FC, fold change; UT, untreated T3-TSC;

Interestingly, 36 proteins enriched in trophectoderm cells following EP-sEV treatment were also detected in Ishikawa EP-sEVs (**Appendix figure S5.2a; Appendix table S5.10)**. These proteins are associated with innate immune response (e.g. GGH, STUB1, LAMTOR and HSP80B1), vesicle-mediated transport (e.g. GJA1, RAB8A, RAB5C and AP2A2), β-catenin WNT signalling (e.g. AP2A2, PSMA5 and PSMB3) and PIP3/AKT signalling (e.g. STUB1, PSMA5, LAMTOR and PSMB3). **(Appendix figure S5.2a)**. Further, 43 proteins enriched in trophectoderm cells following E-sEV treatment were detected in Ishikawa E-sEVs (**Appendix figure S5.2b; Appendix table S5.11)**. These proteins have been implicated in processes associated with nucleotide biosynthesis (e.g. PRPS2, NT5C2, ADK, PURB and ME1), cell cycle regulation (e.g. USP9X, SUGT1 and PPP2R2A), cytoskeletal organisation/ remodelling (e.g. EML2, CTTN, CYRIB and CORO1B), and metabolic biosynthetic processes (e.g. EDF1, MAT2A, LANCL1, HSD17B4 and PRKAG1) **(Appendix figure S5.2b)**. These findings are consistent with the understanding that sEVs function as signalling modulators[134,146] in the endometrium-embryo interface[155]. Targeted functional studies are required to determine the contribution of specific cargo to biological functions of the trophectoderm/ pre-implantation embryo and implantation.

Collectively, these comparative analyses present key changes in protein composition of trophectoderm cells following transfer and reprogramming by endometrial sEVs, highlighting how hormonal regulation influences the reprogramming of trophectoderm and embryo implantation.

### 5.2.3 *In silico* surfaceome analysis of trophectoderm cells treated with endometrial extracellular vesicles

Understanding how trophectoderm cell membrane can interact with endometrium is an important area of research in embryo implantation[137,208]. Here, an *in silico* approach was performed to understand how the endometrium (in the secretory phase) can signal to trophectoderm and modulate protein expression on its membrane surface (termed surfaceome). Both Uniprot functional annotation (311 proteins annotated in trophectoderm cell membrane surfaceome as "plasma membrane" or "cell membrane" (excluding "cytoplasmic side")) **(Figure 5.3, upper panel)** and TMHMM analysis (456 predicted transmembrane proteins) **(Figure 5.3, upper panel)** were performed on the protein signature of trophectoderm cells treated with EP-sEVs. Comparative analysis between these database subsets revealed 139 proteins predicted to be localised, and transverse the plasma membrane **(Appendix table S5.12)**. Of this subset, 10 cell surface proteins were significantly enriched ($p<0.05$) in trophectoderm cells upon EP-sEV treatment (compared to untreated cells) **(Figure 5.3, lower panel);** including proteins involved in integrin-mediated adhesion and signalling (e.g. ITGA3 and ITGAV), ECM organisation (e.g. TGFBI and VCAM1) and cell migration (e.g. CD9, GJA1 and DPP4), which are important processes for endometrium-embryo interaction during implantation[309-312] **(Figure 5.3, lower panel)**.




Significantly upregulated/ unique to EP-sEV treated T3-TSC

| | | | | |
|---|---|---|---|---|
| ANTXR2 | ITGAV | GJA1 | TRPA1 | DPP4 |
| CD9 | SLC2A1 | ITGA3 | VCAM1 | TGFBI |

**Figure 5.3 *In silico* surfaceome of trophectoderm cells treated with endometrial EP- small extracellular vesicles.** Upper panel, Venn diagram of cell surface protein identifications found in total EP-sEV treated T3-TSC proteome, and TMHMM and UniProt databases. Lower panel, cell surface proteins significantly enriched ($p<0.05$) in trophectoderm cells with EP-sEV treatment.

## 5.3 Discussion

Although endometrial sEVs have recently been shown to alter trophectoderm cell proteome[137], a heterogeneous EV preparation was utilised, being contaminated with non-vesicular, functionally active particles (e.g. protein aggregates) that co-sediment[184,223]. Hence, interpretation of such data can be confounding and warrants EV purification to discern their reprogramming capacity. Here, I show through quantitative proteomic analysis that purified endometrial E- and EP-sEVs can modulate trophectoderm cell protein composition to support processes associated with implantation. Specifically, I show EP-sEVs internalised by T3-TSCs promote expression of proteins involved in adhesion, migration and ECM remodelling to create a pro-invasive phenotype – a key step during implantation. Interestingly, I demonstrate E-sEVs regulate fatty acid metabolism (ACADM, ACOX1 and ETFDH), suggesting a potential role that warrants further investigation. These findings provide an understanding of how hormonally regulated endometrial-derived sEVs signal to trophectoderm and contribute to embryo implantation.

### 5.3.1 Endometrial secretory phase signals reprogram trophectoderm cells towards a pro-invasive phenotype

The molecular mechanisms in which trophectoderm acquire an invasive phenotype are not fully understood[329], yet disruption of trophectoderm to display invasive potential may lead to implantation failure and placental dysfunction[330,331]. The data demonstrate purified EP-sEVs reprogram trophectoderm cell proteome (in comparison to untreated cells) to display a pro-invasive phenotype in support of implantation by promoting enrichment of key proteins involved in ECM remodelling, cell proliferation, migration and adhesion **(Table 5.2)** that potentially drive a functional invasive response. Indeed in line with these findings, our group and others have demonstrated endometrial sEVs can functionally reprogram human trophectoderm cells to enhance adhesion and invasion to endometrial epithelial cells[154], adhesion to microplate[138] and invasion into Matrigel™[137]. I propose that invasion and adhesion related proteins, including DPP4, PLOD3 and FN1, which were significantly enriched in trophectoderm cells following EP-sEV treatment may potentially modulate these functional changes. DPP4 is a membrane-bound extracellular

glycoprotein involved in immune regulation, signal transduction and cell adhesion[324]. Shimomura, et al. [317] showed human embryos displayed greater attachment to endometrial cells which overexpressed DPP4, proposing a cell adhesion mechanism in which embryonic FN1 binds to endometrial DPP4 to facilitate this attachment. Whether endometrial sEVs modulate this signalling axis between trophectoderm and endometrium to promote adhesion should be further explored.

Similarly, PLOD3 is implicated in ECM modulation and invasion, with an essential role in type IV collagen synthesis[332]. PLOD3$^{-/-}$ mice suffer basement membrane instability during early embryonic development, causing embryonic lethality[332]. Moreover, PLOD3 has a proposed role in invasion and migration by regulating phosphorylation of STAT3 to activate MMP2/9 for ECM degradation[313]. I co-identified STAT3 in Ishikawa E- and EP-sEV cargo and T3-TSCs treated with sEVs. Furthermore, upstream regulators of STAT3 including CSK[333], PI4K2A[333] and EGFR[334] were identified within Ishikawa sEVs (Chapter 4), with EGFR being significantly enriched in EP-sEVs. Indeed, phosphorylated STAT3 has been shown to be transferred by exosomes/sEVs to induce cellular reprogramming[335], therefore, endometrial sEVs may potentially transfer these signalling molecules to modulate trophectoderm invasive potential.

Promisingly, endometrial-trophoblast co-cultures have provided functional insights into the ability for endometrium to support trophectoderm in acquiring their invasive potential[336]. Ishikawa cells cultured with trophoblast stem cells (TSCs) enhanced Galectin-1 expression, thereby promoting TSC invasion[331]. However, whether endometrial EVs contributed to this was not examined. Moreover, analysis of a co-culture of human receptive endometrium with trophectoderm cells, with focus on their direct interface (termed adhesome), reported a milieu of proteins including NQO1 and STOM[81] co-identified within EP-sEV treated trophectoderm cells and Ishikawa sEV data, that have been associated with endometrial receptivity and embryo implantation. In the current study, proteins co-identified between Ishikawa EP-sEVs and trophectoderm cells treated with these sEVs include LIMS1[315] and GJA1[314], which may be mediators of such functional responses. Taken together, our findings in combination with other studies, indicate endometrium-derived signals via sEVs can modulate trophectoderm invasive potential at the endometrium-trophectoderm signalling interface to potentially promote implantation.

### 5.3.2 Endometrial small extracellular vesicle signalling – implications in embryo development

Further inspection of trophectoderm cell proteomic data identified processes implicated in embryo development, including ubiquitin system and lipid metabolic processes, under temporal regulation of hormonally primed endometrial sEVs. EP-sEV treatment enriched trophectoderm cells in ubiquitin system proteins that comprise the 26S proteasome (e.g. PSMD8, PSMB3, PSMB7, PSMA5 and PSME1), which regulate proliferative processes through controlled degradation of mitotic cell cycle proteins (e.g. cyclins, CDKs and their inhibitors)[337]. Importantly, proteasome regulation of cell cycle proteins is implicated in oocyte maturation and early embryonic development[337], with proteomic analysis of human blastocoel fluid revealing downregulation of PSMD8 and other ubiquitin-system proteins were identified in embryos with poor IVF outcome[338]. These studies emphasise the importance of ubiquitin accumulation and regulation, as well as proteasome function in implanting trophoblast, potentially regulated by endometrial EP-sEVs.

Importantly, in this study I demonstrate the capacity for endometrial E-sEVs to reprogram trophectoderm cell proteome. To our knowledge, this is the first study to assess differences in menstrual cycle phase-specific sEV reprogramming of trophectoderm cells, offering novel insights into endometrium-embryo communication. While EP-sEVs are released by secretory phase endometrium[138], signals from E-sEVs may carry across to this phase to also signal to an arriving embryo. In the current study, trophectoderm cells treated with endometrial-derived sEVs share a high degree of similarity in their proteome of ~90% (based on protein identifications). Indeed, the proteome of human uterine fluid-derived sEVs from different menstrual cycle phases (including proliferative and secretory phases) also display high similarity in protein cargo, sharing ~80% of their proteome[192], suggesting proliferative phase signals may carry over into the secretory phase to signal to embryo.

During the pre-implantation period, embryos are highly sensitive to their environment – comprised of hormonally regulated endometrial secretions that can modulate embryo growth to influence their developmental potential[20,339]. Interestingly, I observed enrichment of proteins associated with lipid metabolic processes in trophectoderm cells treated with E-sEVs (e.g. ACADM, ACOX1 and ETFDH). Fatty acid metabolism within the uterine microenvironment

provides adequate ATP levels for oocyte and early embryo development, with fatty acid supply influencing oocyte and embryo quality[303]. The uterine cavity is a hypoxic environment, therefore after the morula stage, the pre-implantation embryo alters its metabolism from low to high glucose consumption utilising anaerobic glycolysis and pentose phosphate pathway to generate ATP[41]. Previous studies have reported the importance of temporal regulation of different metabolic pathways for early embryo development[340-343] and subsequent embryogenesis stages[137,344]. Endometrial sEVs may therefore influence this regulation, with the potential residual role of E-sEVs warranting further investigation.

### 5.3.3 Altering the trophectoderm cell membrane – clues for endometrial interaction

Given the interaction between endometrium and embryo is initiated upon adhesion at the trophectoderm-endometrium interface, I applied an *in silico* bioinformatic approach to investigate regulation of cell surface protein (surfaceome) expression upon EP-sEV treatment. A number of integrins were significantly enriched in trophectoderm cell surfaceome, including ITGAV, which forms the integrin αVβ3 heterodimer with ITGB3 – a key regulator of endometrium-embryo attachment (by binding ECM or cell surface ligands)[77,78]. Significantly, I found ITGAV enriched upon EP treatment of Ishikawa sEVs, suggesting an important role during the secretory phase. Integral membrane and adhesion proteins, including integrins, ECM proteins, proteoglycans, lectins or glycolipids expressed on EV surfaces are important mediators of EV cell targeting, recognition and docking for uptake[345,346]. Distinct sEV integrin signatures, including integrins $\alpha_6\beta_4$ and $\alpha_6\beta_1$ preferentially hone to lung cells rich in laminin, whereas $\alpha_V\beta_5$ hones to the liver[168], indicating integrin profiles of sEV subtypes mediate selective cell targeting[191]. The local release and uptake of endometrial sEVs to trophectoderm during the pre-implantation period may in fact be mediated by specific integrin/ligand interaction. Desrochers, et al. [222] showed embryonic stem cells within blastocyst ICM, release EVs that dock onto recipient trophectoderm cells, mediated by EV-FN1 and laminin binding to trophoblast integrin and laminin receptors, to promote cell migration – a key step during invasion. Indeed, this emphasises the role of integrins and their ligands expressed in endometrial sEVs and trophectoderm surfaceome, to site-direct cargo, mediate uptake and docking

for cellular reprogramming[168], or activate signalling pathways that regulate expression of CAMs on the cell surface, to potentially promote trophectoderm adhesion for successful implantation[287].

Similarly, GJA1 – a connexin which forms gap junctions between adjacent cells to mediate intercellular communication[314] – was enriched in EP-sEV treated T3-TSC surfaceome. GJA1 expression on trophoblast has a proposed role in activating their development and differentiation[314]. Interestingly, GJA1 can directly and laterally associate with integrin αVβ3[347], with ligand-induced activation of integrin αVβ3 able to open the GJA1/pannexin-1 hemichannel (pore channel formed by connexin hexamers[348]) to induce intracellular signalling[349]. Specifically, Alvarez, et al. [349] determined that integrin αVβ3-induced PI3K-PLCγ-IP3R signalling opens the GJA1 hemichannel to direct cell migration. Hence, activation of these receptors and pores in trophectoderm surfaceome, potentially mediated by endometrial sEVs, could be a mechanism which regulates trophectoderm migration and interaction with the endometrium for invasion. I also identified GJA1 within Ishikawa sEVs, and has a proposed role in directing EV interaction with target cells[348]. Soares, *et al.* [350] demonstrated an exosomal GJA1 hemichannel can dock onto a recipient cell hemichannel to create a pseudo gap-junction, thereby allowing transfer of cargo from exosomes to target cells. Importantly, inhibition or constitutive activation of the exosomal GJA1 hemichannel either limited or enhanced cargo uptake, respectively, supporting this mechanism. Indeed, this proposed GJA1 channel-mediated transfer of cargo may be a potential mechanism in which endometrial sEVs signal to trophectoderm to prepare for implantation.

Collectively, the data demonstrate endometrial signals that are directed to trophectoderm cells via sEVs, alter their composition to support key processes associated with implantation – including cell adhesion, migration and invasion. These insights provide leads to target such proteins and assess their role in site-directed endometrial sEV delivery and uptake, as well as reprogramming within trophectoderm cells to prepare for implantation. The application of an inhibitory antibody approach, which targets extracellular membrane regions of a protein, will guide functional understanding of these proposed mechanisms. Indeed, such insights may guide future development of novel therapeutics that utilise EVs as nano-carriers to improve implantation outcomes in IVF.

# Chapter 6: Investigating the role of endometrial small extracellular vesicles in modulating trophectoderm cell function

## 6.1 Overview

Trophectoderm cell invasion into the receptive endometrium is a fundamental step in implantation[7,28,114,331]. Whether endometrial sEVs and their protein cargo contribute to trophectoderm cell invasion remains limited. Previous chapters revealed endometrial sEVs contain modulators of implantation and cell invasion (Chapter 4) and can signal to trophectoderm cells to reprogram their protein landscape towards a pro-invasive phenotype (Chapter 5). Here, I provide functional insight into endometrial sEV modulation of trophectoderm cell invasion. I employed human stem cell-derived trophectoderm cells grown as spheroids to mimic the early pre-implantation embryo[137,208]. Importantly, these spheroids have been shown to function physiologically[137] by differentiating between receptive *versus* non-receptive endometrial epithelial cells[81]. I show that secretory-phase endometrial sEVs can significantly increase trophectoderm cell invasion into Matrigel™, with these findings providing important functional insight into the cross-talk between endometrium and trophectoderm.

## 6.2 Results

### 6.2.1 Endometrial small extracellular vesicles increase trophectoderm cell confluency

To gain functional insights into endometrial sEV treatment of trophectoderm cells, I initially treated T3-TSC with E- or EP-sEVs (Chapter 5.2.1). Preliminary inspection using bright field microscopy revealed that both endometrium-derived sEVs increase cell confluency over a 48 h period compared to untreated control trophectoderm cells **(Figure 6.1a)**.

### 6.2.2 Endometrial small extracellular vesicles promote trophectoderm cell invasion into Matrigel™

Our data show that endometrial EP-sEVs carry pro-invasive proteins **(Figure 4.4)** that potentially regulate processes associated with trophectoderm cell invasion, namely ECM remodelling, cell migration and adhesion **(Figure 5.2; Table 5.2)**. Temporal regulation of trophectoderm cell invasion is critical for establishing and maintaining embryo implantation[7,28,351]. Thus, I hypothesise that endometrial sEVs modulate trophectoderm cell invasion. Trophectoderm spheroids were treated with endometrial E- or EP-sEVs, and analysed for changes in cell invasion through Matrigel™ **(Figure 6.1b)**. Bright field microscopy revealed that EP-sEVs promoted significantly greater trophectoderm cell invasion compared to E-sEV treated and control trophectoderm spheroids ($p<0.001$, n=13-20 biological replicates), highlighting their ability to modulate trophectoderm cell function to support implantation processes **(Figure 6.1c; Appendix table S6.1)**.

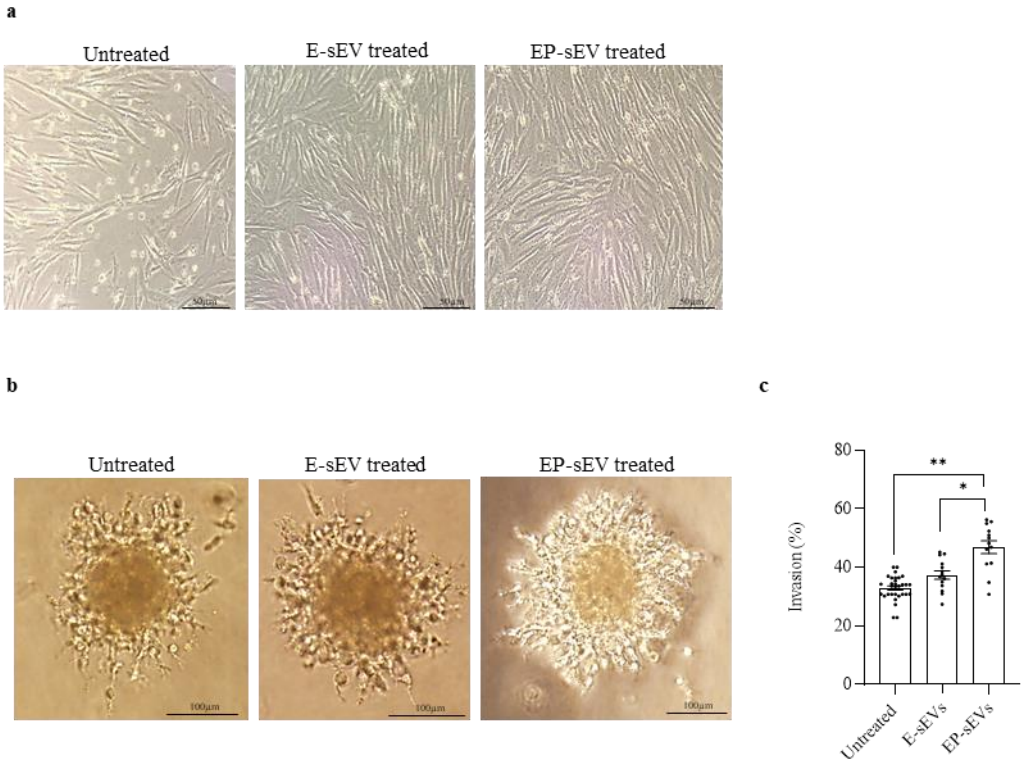



**Figure 6.1 Functional reprogramming of trophectoderm cells by endometrial small extracellular vesicles. a)** Cell confluency analysis using bright field microscopy. T3-TSC treated with Ishikawa E-sEV (50 µg/mL) or EP-sEVs (50 µg/mL) or untreated control cells. Scale bar = 50 µm. **b)** Trophectoderm cell invasion assay. Bright field microscopy images of T3-TSC spheroids treated with E-sEVs or EP-sEVs, invading into Matrigel™. Control cells include untreated T3-TSC cells. Scale bar = 100 µm. Spheroids overlaid on Matrigel™ to invade for 72 h. **c)** Bar plot quantitation representing invasive outgrowth of T3-TSC spheroids from treatment groups (n= 13-20 biological replicates with each data-point representing the mean invasion (%) of a single spheroid. Mean ± SEM *$p \leq 0.001$, **$p \leq 0.0001$).

### 6.2.3 Inhibition of trophectodermal fibronectin in an invasion assay

To gain insights into modulators of trophectoderm cell invasion by endometrial secretory phase (EP) sEVs, I analysed the role of FN1 – a specific glycoform produced by trophectoderm cells[317] – in cell invasion. Of note, I observed significant enrichment of FN1 in T3-TSCs following EP-sEV treatment **(Table 5.2)**. Previously, FN1 has been implicated in the surface of developing mouse peri-implantation blastocysts[320,352] and human blastocyst[317] to coordinate embryo implantation. Moreover, ECM proteins (including FN1) have been shown to promote acquisition of the adhesive and degradative properties required by human embryos for successful implantation[353]. In this study, to gain insights into EV-mediated contribution of FN1 in trophectoderm cell invasion, I applied an antibody-based inhibitory approach targeting FN1 **(Figure 6.2a; Appendix table S6.2)**. I show that EP-sEV treatment and EP-sEV treatment + IgG antibody control displayed significantly greater trophectoderm cell invasion into Matrigel™ in comparison to untreated and IgG antibody control groups ($p<0.0005$) **(Figure 6.2b)**. Interestingly, anti-FN1 treated trophectoderm spheroids displayed greater invasion compared to IgG antibody treated and untreated controls ($p<0.001$). FN1 antibody + EP-sEV treatment did not show significant change in trophectoderm cell invasion compared to EP-sEV treatment alone.

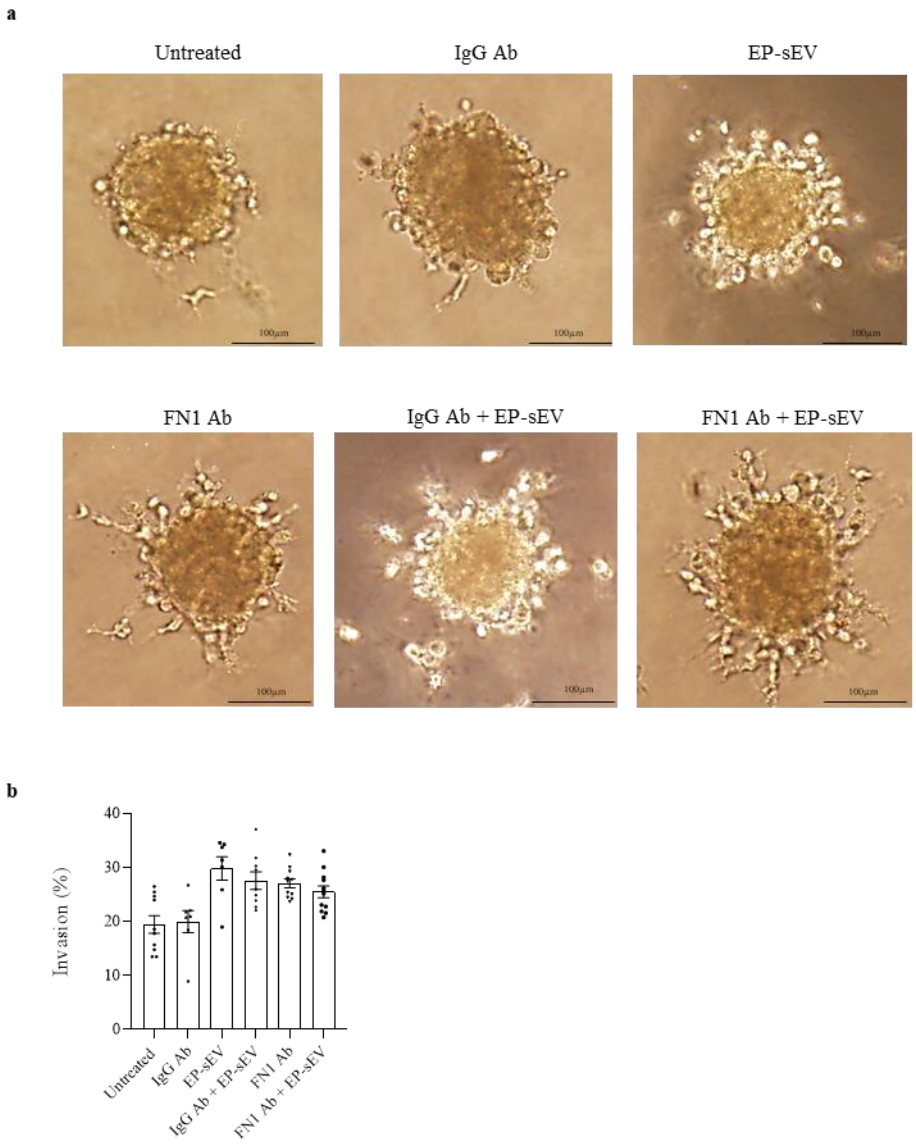



**Figure 6.2 Inhibition of fibronectin on trophectoderm cell invasion. a)** Trophectoderm cell invasion assay. Bright field microscopy images of T3-TSC spheroids untreated, IgG antibody- (1:50), EP-sEV- (50 µg/mL), FN1 antibody- (1:50), IgG antibody- + EP-sEV- (50 µg/mL), or FN1 antibody + EP-sEV- (50 µg/mL) treated groups overlaid on Matrigel™. Scale bar = 100 µm. Spheroids overlaid on Matrigel™ to invade for 72 h. **b)** Bar plot quantitation representing invasive outgrowth of trophectoderm spheroids from treatment groups (n= 7-13 biological replicates with each data-point representing the mean invasion (%) of a single spheroid. Mean ± SEM).

## 6.3 Discussion

Embryo invasion into endometrium is a critical step of implantation to establish pregnancy. Quantitative proteomic analysis has enabled the characterisation of trophectoderm cell proteome landscape treated with endometrial sEVs, to reveal their dynamic reprogramming upon phase-specific endometrial signals – specifically that EP-sEVs enrich trophectoderm cells in proteins implicated in migration, adhesion and ECM remodelling that promote a pro-invasive phenotype (Chapter 5). Here, I provide insights into the functional capacity for endometrium-derived sEVs to modulate trophectoderm cell function. EP-sEVs significantly promote trophectoderm cell invasion through Matrigel$^{TM}$, highlighting their ability to enhance their implantation potential.

Preliminary functional analysis of endometrial sEVs revealed their capacity to increase trophectoderm cell confluency, further supporting the notion that sEVs contain cargo that can functionally contribute to cell proliferation (Chapter 4). It is possible that they are able to modulate this process in the pre-implantation embryo as well. For example, endometrial-derived sEVs from ewes contain miRNAs that modulate pro-proliferative pathways including PI3-AKT and BMP pathways[354]. In fact, embryos are responsive to such signals as EVs isolated from ovine uterine fluid were shown to increase proliferation of ovine trophectoderm cells[355], whereas Gurung, et al. [154] demonstrated human endometrial sEVs can contribution to mouse blastocyst cell proliferation upon sEV treatment. Hence, the ability of endometrial sEVs to transfer pro-proliferative signals to embryo may facilitate its development and preparations towards implantation competency.

### 6.3.1 Role of endometrial small extracellular vesicles in trophectoderm cell invasion during implantation

Our understanding of the mechanisms which regulate trophectoderm acquisition of invasive phenotype are limited[329]. Based on present findings, how this phenotype is spatially and temporally regulated may be contributed by endometrial sEVs. During the pre-implantation period, the blastocyst undergoes extensive remodelling and differentiation, with multipotent trophectoderm cells differentiating to subsequent trophoblast lineages that also acquire invasive phenotypes to establish the maternal-foetal interface[22,24]. Endometrial signals including enzymes, cytokines and

growth factors, regulate the extent of trophoblast invasion through endometrial tissue and surrounding ECM, predominantly by regulating trophoblast MMP expression and function[318,356]. In this study, endometrial EP-sEVs were selectively packaged with these regulators, including TXN[318] and CSTB[357], which upon treatment with trophectoderm cells, potentially mediate ECM degradation to promote invasion. Indeed, Ruane, et al. [233] demonstrated that direct interaction between endometrial epithelium and trophectoderm induces its subsequent differentiation to invasive trophoblast lineages to breach the endometrial barrier, highlighting the significance of local endometrial-derived signalling to regulate implantation processes. Whether endometrial-derived EVs were involved in these processes in that study was not investigated.

Importantly, these intricate processes require reciprocal dialogue between endometrium and implanting embryo, regulated by ovarian hormones[63] and secreted factors in the uterine microenvironment, including sEVs[183]. The continued release of P from the corpus luteum is crucial for the maintenance of implantation processes, which in fact, is rescued by trophoblast secretion of hCG to prevent corpus luteum degradation and subsequent pregnancy failure[358]. Supplementation of P in embryo culture media has been shown to promote embryo survival and development, potentially by regulating cytokine expression to allow embryos to become competent for implantation[359,360]. Hence, secretory phase signals predominantly regulated by maternal P, are fundamental for implantation to support embryo competency and differentiation, and align with my findings that EP-sEVs signal to trophectoderm cells to stimulate a pro-implantation phenotype.

In this study, I found significant enrichment of invasion related proteins in endometrial EP-sEVs (e.g. BMPR2, PRDX6 and TKT) and their subsequent treatment of trophectoderm cells (e.g. DPP4, PLOD3, AGRN and TGFBI), with expression of PLOD3[313] and TGFBI[328] in trophectoderm previously correlated with acquisition of embryo implantation competency. Previous studies have reported similar findings that endometrial secretory phase sEVs can transfer pro-implantation signals to trophectoderm cells to modulate cell invasion[137] and adhesion[138] *in vitro*, and to embryo to regulate development and implantation *in vivo*[124]. Here, I demonstrated functional differences between sEVs from proliferative (E-sEVs) and secretory (EP-sEVs) phases in their ability to reprogram invasive phenotype of trophectoderm cells, with EP-sEV treated trophectoderm cells

displaying significantly greater invasive capacity compared to E-sEVs. Deciphering whether sEVs can also modulate differentiated trophoblast lineages in invasion and migration should be explored.

### 6.3.2 Inhibition of fibronectin alters trophectoderm cell invasion

FN1 is an ECM protein secreted by, and expressed on trophoblast cells[317] implicated in cell adhesion[317], migration[351] and invasion[361]. FN1 is temporally regulated in the developing embryo as its expression is detected in blastocyst but not morula stage, with FN1 shown to act as a ligand for endometrial DPP4 to facilitate adhesion during implantation[317]. The data suggest antibody inhibition of FN1 in trophectoderm spheroids significantly promote their invasion through Matrigel™, contrary to previous findings in the literature regarding the role of FN1 in cell invasion. FN1 inhibition has been shown to significantly attenuate cell invasion by disrupting integrin-fibronectin binding for intracellular signalling in fibroblasts[362,363], or limiting cell migration in trophoblast[351]. Moreover, FN1 expression has been shown to promote trophoblast EVT invasion through FN1-integrin α5β1 binding to activate FAK[361].

Interestingly, EP-sEVs did not rescue trophectoderm cell invasion upon FN1 inhibition as hypothesised. Numerous integrin family members, including integrin α5β3, α5β1,and α4β1[364] are receptors that bind FN1 and are essential for endometrium-embryo communication and implantation[77,352]. Further, integrin-mediated EV uptake is an important pathway for EV internalisation by target cells[168], however the precise trophectoderm cell-dependent mechanisms of this uptake and the specific integrins involved in sEV internalisation remain limited. The inability for endometrial sEVs to rescue trophectoderm cell invasion upon FN1 inhibition may in part be due to this perturbed interaction between endometrial sEV integrin receptors and trophectodermal-FN1, limiting sEV uptake and cellular regulation[139]. Indeed, inhibition of integrin α5β1 disrupted binding with FN1 in trophoblast cells to limit invasion[361], with data from my study confirming ITGA5 and ITGB1 are expressed in Ishikawa sEVs. These findings provide a preliminary assessment of FN1 function during trophectoderm cell invasion, with further validation required to determine which players, both endometrial and trophectoderm-derived, are involved in FN1-mediated invasion.

# Chapter 7: Summary and future directions

Embryo implantation requires synchronous dialogue between a receptive endometrium and competent blastocyst to establish pregnancy[1,3,10], with EVs emerging as critical regulators of this cross-talk[20,133,155]. In this study, I isolated a highly purified and homogenous subset of hormonally regulated endometrial-derived sEVs, through a comprehensive isolation and purification strategy to characterise their protein composition, and reprogramming capacity on trophectoderm cellular protein landscape and function. Endometrial-derived sEVs were obtained from the endometrial epithelial cell model (Ishikawa) that recapitulates proliferative and secretory menstrual cycle phases, to gain insights into how ovarian steroid hormones (E and P) regulate endometrial sEV phase-specific composition and function. Quantitative proteomic profiling of purified endometrial E- and EP-sEVs revealed unique insights in their contribution to endometrium-embryo cross-talk, particularly during the receptive phase. I show EP-sEVs are enriched in protein cargo associated with endometrial remodelling to become receptive, and key regulators of embryo development and implantation that may signal to embryo in preparation for implantation.

Regulation of trophectoderm cell composition and function is critical to prepare embryo for implantation, with the contribution of endometrial-derived sEVs limited in the field. Here, I reveal that purified endometrial-derived sEVs are internalised by human trophectoderm cells and regulate their proteome, with secretory phase EP-sEVs reprogramming trophectoderm cells towards a pro-invasive phenotype (through upregulation of cell adhesion, migration and invasion-related proteins and pathways). Importantly, this is the first study to characterise the proteomic and functional reprogramming of trophectoderm cells treated with purified endometrial sEVs. This is a key advancement to understand endometrial-derived sEV contribution to implantation – marked as the "black box" of reproduction[329]. In support of this proteomic data, EP-sEVs significantly promote trophectoderm cell invasion into Matrigel™, highlighting their critical role as extracellular signalling mediators of implantation processes.

## 7.1 The need to purify – understanding endometrial small extracellular vesicles within the uterine microenvironment

In chapter 3, I employed differential ultracentrifugation coupled with buoyant density gradient-based separation to obtain a highly purified subset of hormonally regulated sEVs (enriched in exosomes) which were selectively packaged with distinct phase-specific cargo (Chapter 4). Purification of sEVs, which are functional mediators of implantation processes[137,138,154,187,365], is fundamental to provide insights into their regulation of endometrial and embryo remodelling to establish pregnancy. Indeed, EV heterogeneity and the presence of non-vesicular components severely impedes our understanding of the composition and functional properties of distinct EV subtypes[161,181,223]. Contaminating components may dilute or mimic important functional aspects attributed to the EV population of interest[181] - a major hurdle which can be overcome by employing purification strategies.

Other EV subtypes including lEVs may have a functional role in endometrium-embryo cross-talk[133,312,366]. However, our lab has demonstrated the yield of such EVs from human UF[192] and endometrial epithelial cells (ECC-1 and Ishikawa; *data not shown*) are low, suggesting lEVs may not have a significant physiological representation in the uterine microenvironment. Although, within this environment are soluble signalling players which comprise an important aspect of the endometrial secretome[16,63], and can be isolated using differential ultracentrifugation at 100,000 *g* (supernatant)[147]. Further characterisation of these factors in trophectoderm/ embryo reprogramming may enhance our understanding of endometrial signalling during the pre-implantation period.

Importantly, application of a systematic multi-omics approach to characterise other signalling components within purified endometrial-derived sEVs (e.g. transcriptomic[187] and lipidomic[367] components) may further elucidate how sEVs modulate endometrial differentiation and trophectoderm preparation. Indeed, I observed differential regulation of RNA-binding proteins (RBDs) in E- and EP-sEVs, with RBDs known to regulate RNA processing that influences gene expression[368]. This suggests the transcriptomic signature of endometrial sEVs from menstrual cycle phases may also be distinct, and potentially act as transcriptomic regulators of endometrial function. EVs also selectively package lipid cargo[144,145], which may relay important metabolic pathways that exist in the uterine microenvironment[369,370]. Lipid molecules including LPA and prostaglandins

have been reported as critical mediators of embryo implantation[369], therefore, whether endometrial sEVs contribute similar lipid mediators warrants investigation.

## 7.2 Hormonally regulated endometrial small extracellular vesicles carry phase-specific protein cargo

Given the role of EVs in modulating intercellular communication in a local microenvironment[133], in Chapter 4 I investigated the contribution of endometrial sEVs and their protein cargo in regulating endometrial and embryo preparation for implantation. I performed proteomic dissection of purified endometrial sEV composition, providing novel and supporting evidence of sEV protein cargo and their functional implications. E-sEVs, were enriched in cell proliferation, cell cycle regulation and nucleotide biosynthetic processes (e.g. CUL4A, CDK2, DCK, PURB and CUL5), whereas EP-sEV cargo were associated with secretory differentiation of the endometrium, enriched in proteins that function in EMT (CTNNB1) to establish endometrial receptivity, and embryo development/implantation processes (DDAH2, PLCG1 and BMPR2) **(Figure 7.1)**.

Previously, our group were the first to report an in-depth proteomic characterisation of endometrial epithelial sEVs that contained hormonally regulated cargo[138]. Here, my findings build on previous work by reporting a number of proteins that have not been identified in any other biological fluid-, tissue- or cell model-derived EV **(Appendix table S4.3)**, expanding current knowledge of the protein repertoire of endometrial sEVs. The data suggests that in the pre-implantation environment, when the endometrium becomes receptive, selectively packaged EP-sEV cargo may signal in an autocrine manner to prepare other endometrial cell types (e.g. stroma) for implantation (i.e. by promoting decidualisation)[29,284], or immune cells to modulate the maternal immune response to the foetal allograft[50]. Importantly, the insights provided here reveal Ishikawa sEVs are molecularly distinct between hormonal treatments under the influence of ovarian hormones, supporting our previous findings[63,138] and suggesting endometrial-derived EP-sEVs may indeed reflect the receptive state of the endometrium.

Such findings provide a foundation for future opportunities to utilise EVs as potential markers of endometrial receptivity and fertility[191] to develop personalised embryo transfer therapies. Certainly, development of a non-invasive method to monitor WOI, utilising UF liquid biopsies (containing EVs), will be more suitable than existing invasive endometrial biopsies[371,372] to correctly time embryo transfer. The endometrial receptivity array (ERA) was developed to diagnose WOI in patients to correct embryo transfer time[373] without needing to perform endometrial histological dating which is invasive, outdated and potentially inaccurate[374]. While this array has been a significant advancement in improving embryo transfer outcomes in women with recurrent implantation failure (50% success, n=8)[375], its requirement for a tissue biopsy is still invasive and alters the uterine microenvironment at time of collection[20,124,125], meaning embryo transfer cannot occur during the same cycle of collection[20,125]. Promisingly, efforts are underway to develop an endometrial liquid biopsy[376] to overcome these limitations. Further, EVs are currently utilised as suitable biomarkers in other pathologies that represent a 'snapshot' of the content of the secreting cell[140], which indeed, is reflected in UF sEVs obtained from proliferative and secretory phases[192], highlighting their clinical utility as non-invasive WOI biomarkers to reflect distinct menstrual cycle phases.

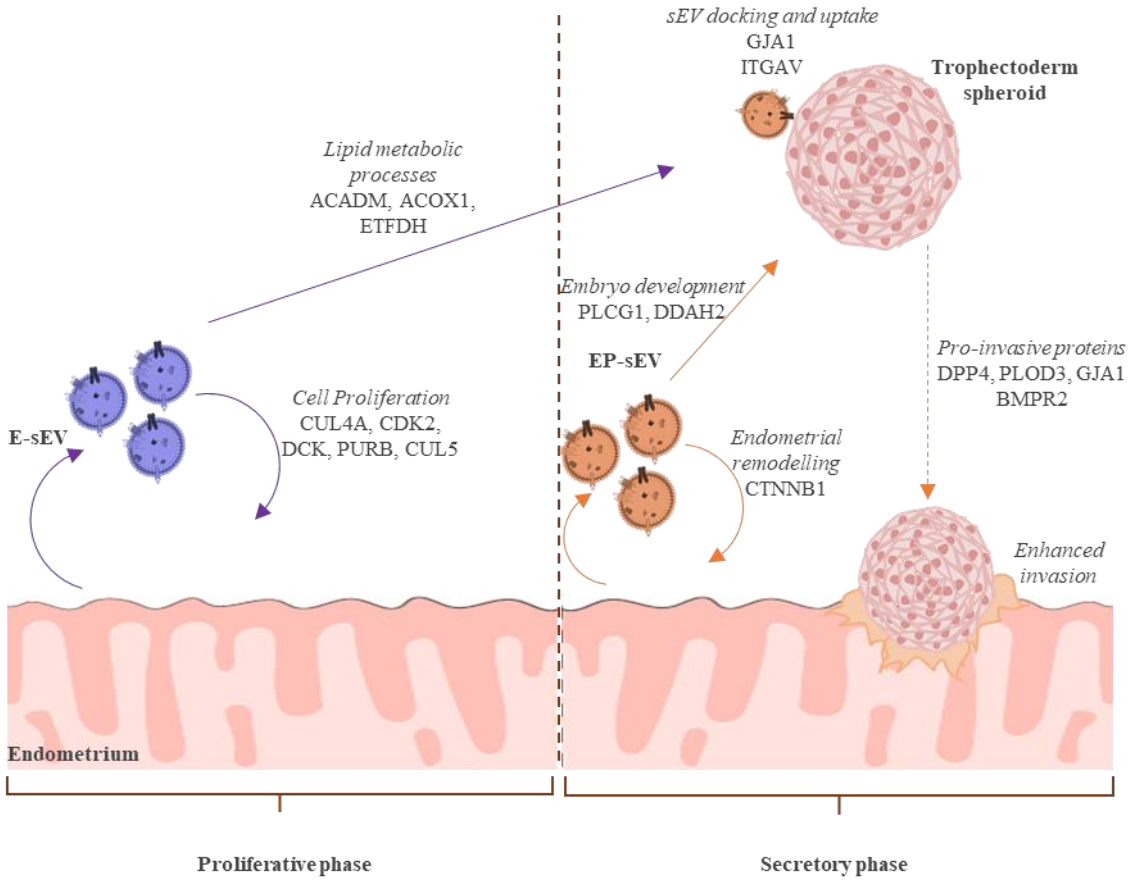



**Figure 7.1 Summary of key findings involved in endometrial-trophectoderm cell signalling.** Hormonally regulated endometrial epithelium secretes sEVs with phase-specific cargo. Purple arrows indicate E-sEV (proliferative phase) signalling, orange arrows indicate EP-sEV (secretory phase) signalling. *Left panel*, E-sEVs are implicated in autocrine signalling of endometrium to regulate cell proliferation and regeneration of endometrial lining. When treated onto trophectoderm cells, they promote lipid metabolic processes including fatty acid beta oxidation. *Right panel*, EP-sEVs contribute to endometrial remodelling by promoting epithelial to mesenchymal transition, potentially stimulating decidualisation processes to create a receptive endometrium. When treated onto trophectoderm spheroids, endometrial sEVs potentially interact with trophectoderm cell surface ligands via integrin and connexin surface proteins to mediate uptake. Once internalised, EP-sEVs promote expression of invasion-related proteins which functionally promote trophectoderm cell invasion. Hence, endometrial sEVs regulate endometrial and trophectoderm cellular reprogramming and changes associated with embryo implantation.

E-sEV, Oestrogen-primed small extracellular vesicles; EP-sEV, Oestrogen and progesterone-primed small extracellular vesicles.

## 7.3 Endometrial small extracellular vesicles modulate the cellular proteome landscape of trophectoderm

Protein cargo contained in EVs can potentially mediate implantation and fertility outcomes. In Chapter 5 and 6, I build on previous observations[137,138,154] by providing insights in the ability for endometrial sEVs to reprogram trophectoderm cellular proteome towards a pro-invasive phenotype, which was functionally supported by invasion assay data. EVs can transfer cargo to recipient cells to elicit selective functional effects[377,378] and indeed, endometrial EVs are shown to transfer cargo to trophectoderm cells to enhance their invasive and adhesive capacity *in vitro* and *in vivo*[137,138,154,187]. My findings extend insights from Evans, et al. [137] who demonstrated trophectoderm cells display enhanced adhesive properties following endometrial EP-sEV treatment, by providing potential molecular players that may facilitate trophectoderm cell invasion – the subsequent implantation step, upon secretory phase EP-sEV treatment (e.g. GJA1, DPP4 and PLOD3) **(Figure 7.1)**. To determine the contribution of these proteins in trophectoderm cell invasion, inhibitory approaches utilising silencing RNAs (siRNAs)[313], inhibitory antibodies (suitable for surface-proteins) or pharmaceutical inhibitors[379], or alternatively, overexpression approaches including plasmid transfection[317], could be employed to limit/ promote expression and subsequent function of targeted proteins in trophectoderm cells that can be monitored through an invasion assay.

Due to time constraints, I was unable to employ other functional assays to assess trophectoderm cell invasion, migration or adhesion, which could provide further insights into the functional reprogramming capacity of endometrial sEVs (and their protein cargo). Previous studies have employed trans-well invasion assays to determine paracrine signalling effects of EVs on trophectoderm cells/ spheroids to measure migration/ invasion[380-382], or a co-culture system, utilising endometrial cells overlaid with trophectoderm spheroids to determine their adhesive and invasive properties upon sEV treatment[154]. A co-culture system would provide biological insights into the significant endometrial-embryo interface during implantation. Ishikawa cells in particular, have been shown to provide a consistent, polarised and receptive epithelial model for studies of endometrium-trophectoderm interactions[233]. By harnessing the utility of a multi-omics approach to define this “interactome”, we may provide potential targets to improve fertility outcomes in the

clinic, as previously reported by Evans, et al. [81] who characterised the protein landscape of a co-culture between receptive/ non-receptive endometrium and trophectoderm spheroids.

Indeed, defining molecular drivers that assist trophectoderm/ trophoblast invasion may guide the development of therapeutic opportunities to improve implantation outcomes. Such insights could allow for designer deliverables to modulate endometrial microenvironment and secretions that may promote embryo transfer outcomes in IVF[66] for successful pregnancy. In fact, Gurung, et al. [154] demonstrated delivery of endometrial EVs to mouse blastocyst *in vivo* significantly promoted their implantation success. The unique characteristics of EVs to protect their cargo from extracellular degradation[146,147], be transferred to local[146] or distant sites[134], make them promising vehicles for delivery of bioactive therapeutic cargo[191]. In future, we could employ intercellular transfer of protein cargo targets or media supplements[322,339], mediated by modified EVs or nanoparticles in the uterine microenvironment to establish a fertile ground primed for successful implantation. Nanoparticles have already been utilised within this environment by being loaded onto sperm which could transfer genetic material to an oocyte during fertilisation[383]. Alternatively, these EVs could be utilised in the development of targeted delivery strategies to selectively transport non-hormonal contraceptives that can act directly onto the endometrium to reprogram it towards a non-receptive state[30].

EVs can transfer their cargo to elicit functional reprogramming of target cells[134,146], with my proteomic data highlighting novel proteins including ITGAV, selectively enriched in endometrial EP-sEVs and subsequent trophectoderm cells treated with EP-sEVs. To determine whether these proteins are indeed transferred from endometrium to trophectoderm cells, labelling of sEV cargo utilising fluorescent tags/probes specific for internal proteins (e.g. ExoGlow™ which diffuses through EV membranes[384]), or membrane proteins utilising antibody-based fluorescent labels[385] – both of which can be performed in real time, can be applied. Further, metabolic labelling of EV proteins, utilising Stable Isotope Labelling by Amino Acids in Cell Culture (SILAC)[386], can be employed to monitor transfer of EV cargo (heavy isotopic labelled proteins) to recipient trophectoderm cells (light labelled). Kalra, *et al.* [387] utilised SILAC labelling to demonstrate transfer of exosomal β-catenin to recipient cells to activate Wnt signalling pathway. Hence, this tool may

provide insights into the contribution of endometrial sEV signals in mediating changes in trophectoderm cell protein composition.

Currently, temporal molecular and signalling changes in human embryo prior to, and during implantation remain limited. Phosphorylation is a key regulator of pathways associated with implantation, including PI3K-AKT[36,37], MAPK[36] and JAK-STAT pathways[114], which modulate trophectoderm/trophoblast proliferation, differentiation and invasion. For example, mice with mutations in gp130 (LIFR component) suffer abolished JAK-STAT signalling and subsequent implantation failure[388]. Future work could utilise phosphoproteomics, which combines MS-based proteomics coupled with titanium dioxide phosphopeptide enrichment[389], to characterise temporal activation of signalling pathways in human trophectoderm cells and other trophoblast lineages *in vitro*, or mouse embryo *in vivo*, that may be regulated by endometrial sEVs. Gao, et al. [342] have initiated this characterisation by employing an *in silico* phosphoproteomic workflow on mouse embryos to reveal key regulators of pre-implantation embryo development. Importantly, understanding how endometrial signals influence diverse signalling pathways in embryo that lead to a pro-invasive/implantation phenotype, may guide engineering of therapeutics that can be delivered to embryo prior to IVF transfer, to inhibit or modulate these signalling pathways and subsequent rate of implantation.

## 7.4 Interaction between endometrial small extracellular vesicles with trophectoderm cells at the surface interface

Understanding how endometrial sEVs interact with trophectoderm cells will provide insights into molecular mechanisms that regulate EV delivery and uptake; which vary based on recipient cell types and their physiological states[390]. In chapter 5, *in silico* surfaceome data revealed integrins, including ITGAV, and the connexin GJA1, are expressed on endometrial sEVs and recipient trophectoderm cells, providing preliminary data that they may be mediators of sEV-trophectoderm cell interaction for internalisation **(Figure 7.1)**. To determine the role of integrins in mediating this interaction, antibody-based or commercially available integrin inhibitors, including Cilengitide[391] or RGD (Arg-Gly-Asp) inhibitory peptides (integrin-mediated uptake inhibitor)[392]

could be utilised to perturb the endometrial sEV-trophectoderm interface. Inhibition of the GJA1 hemichannel can be performed utilising the gap26 mimetic peptide, which has been shown to block hemichannels and inhibit gap junction-mediated communication[350]. Whether blocking these surfaceome proteins also limits proteomic and functional reprogramming of trophectoderm cells should also be investigated.

To assess trophectoderm cell-specific EV uptake, future studies could also utilise inhibitory agents, including chlorpromazine (clathrin-dependent uptake inhibitor)[393], dynamin inhibitor II (clathrin-independent endocytosis inhibitor)[137] and cytochalasin (clathrin-dependent micropinocytosis inhibitor)[393], which may influence how endometrial sEVs interact with, and are internalised by trophectoderm cells. However, with multiple known pathways of EV internalisation, inhibition of one pathway (e.g. clathrin-dependent) may be compensated by another (e.g. clathrin-independent)[394]. Utilising cryo-electron microscopy (cryo-EM) coupled with immunogold labelling, which label and preserve EVs in their native hydrated state[395], can further determine this interaction[289] to guide development of trophectoderm cell-specific delivery of EVs containing therapeutic cargo.

Further, to definitively characterise the contribution of target proteins identified in this study in mediating EV-trophectoderm interactions, and trophectoderm-endometrium interactions, performing surface protein capture utilising a thiol-cleavable amine-reactive biotinylation reagent, can define specific surface components[396] on EV or recipient trophectoderm cells. Indeed, our understanding of the surface repertoire of these entities within the microenvironment is poorly understood. Data for capture of trophectoderm cell surface proteins upon endometrial sEV treatment has been generated, however, due to time constraints, is not included in this thesis.

Insights from these potential avenues will allow us to better understand EV-trophectoderm interactions that can be used to modulate trophectoderm surfaceome composition for a targeted EV delivery approach of therapeutics. Such advancements highlight the clinical utility of EVs with their unique properties to facilitate transfer of bioactive EV cargo or surface ligands into therapeutic applications in infertility.

## 7.5 Conclusions

Endometrial-embryo communication within the uterine microenvironment is crucial for implantation and subsequent pregnancy success, mediated by highly-regulated endometrial secretions, including EVs. In my thesis, I demonstrate that hormonal regulation of endometrial epithelial cells also regulates their sEV protein cargo, which are implicated in menstrual cycle-phase specific functions involved in autocrine (to endometrium) and paracrine (to trophectoderm cells) signalling of the uterine microenvironment (chapter 4). I investigated how endometrial-derived sEVs modulate trophectoderm cell proteome, to reveal secretory phase EP-sEVs alter trophectoderm composition and function towards a pro-invasive phenotype (chapter 5 and 6) in support of implantation.

In summary, my thesis provides novel insights for the ability of endometrial sEVs to mediate reprogramming of the uterine microenvironment to provide potential regulators of embryo implantation and fertility. This current study provides key targets for engineered EVs or mimetic nanovesicles as a nanoparticle therapy that can be loaded with modified cargo (either by engineering parental cells or exogenous cargo loading)[397] to deliver and elicit functional reprogramming in recipient cells[398]. These approaches hold key therapeutic opportunities to directly modulate the implantation rate of embryo to improve IVF outcomes for successful pregnancy.

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# Appendix

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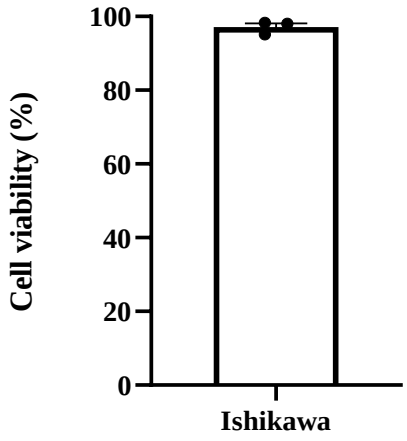
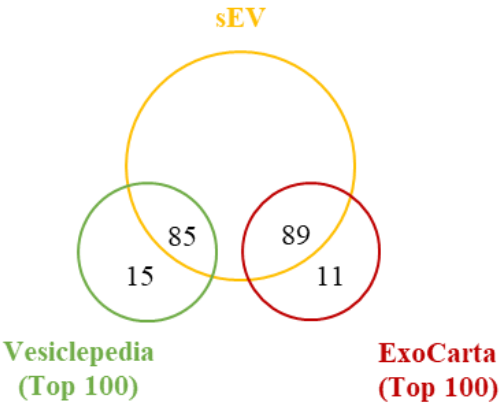
## Chapter 6 appendix tables

## Appendix figures

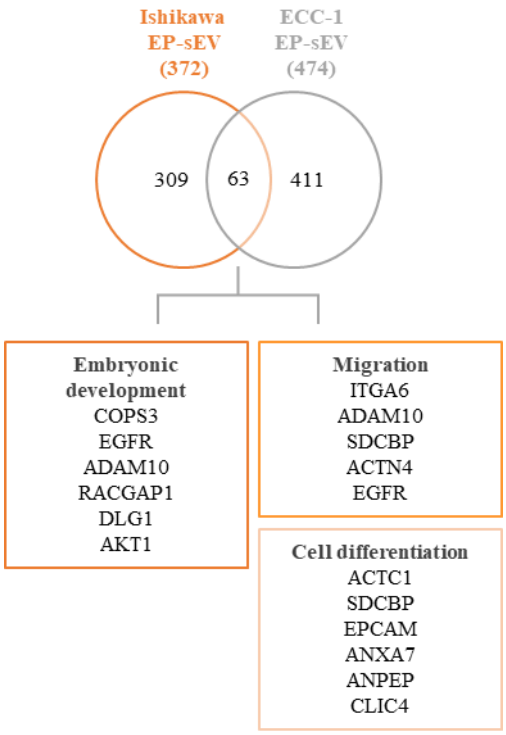



**Appendix figure S3.1 Trypan blue cell viability assay.** Cells grown to 100% confluency in DMEM/F-12 bioreactor flask media (5% (v/v) FCS, 1% (v/v) Pen/Strep, 0.6% (v/v) ITS), harvested and stained with trypan blue to assess cell viability. Stained (non-viable) and unstained (viable) cells were counted and calculated as a percentage of total. Data is representative of the mean of N=3 biological and n=3 technical replicates, error bar ± SEM.

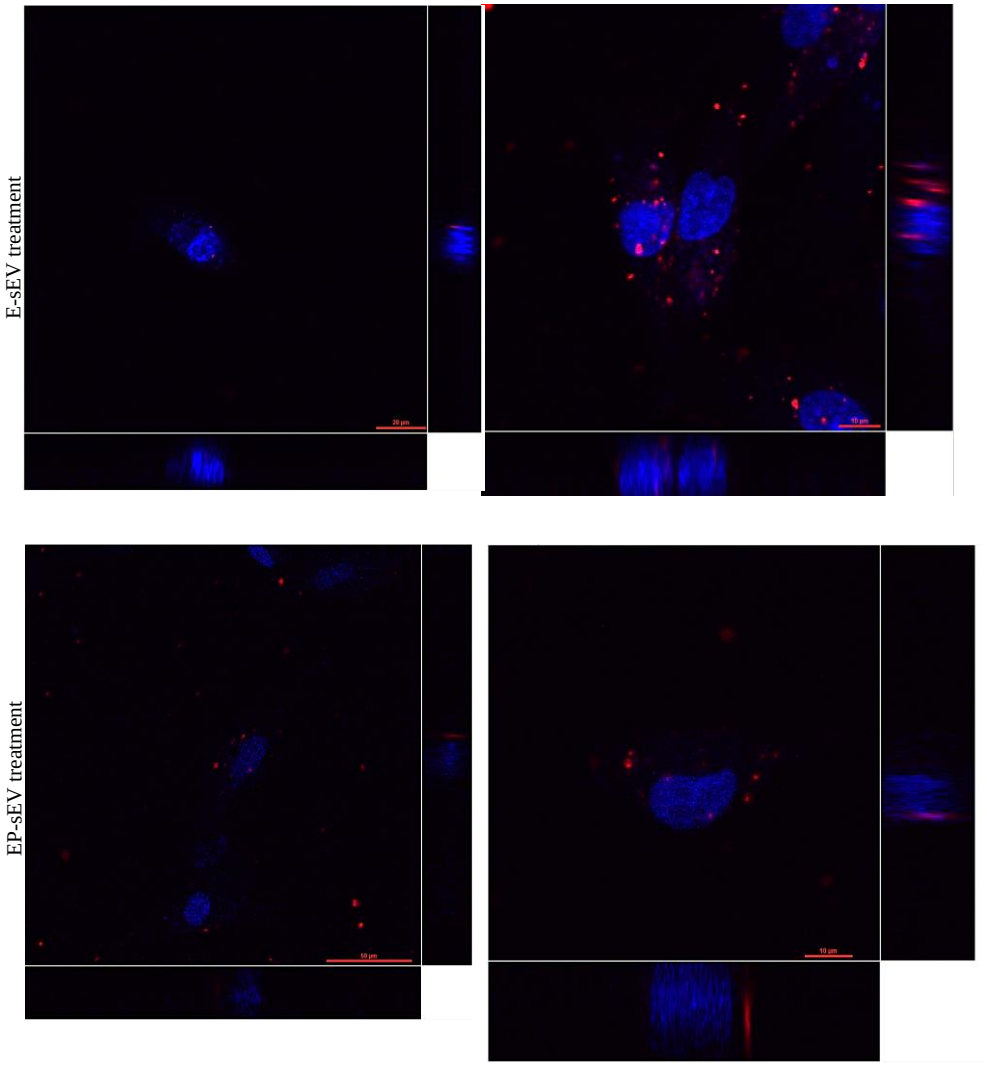



**Appendix figure S4.1 Ishikawa small extracellular vesicles are enriched in exosomal proteins.**

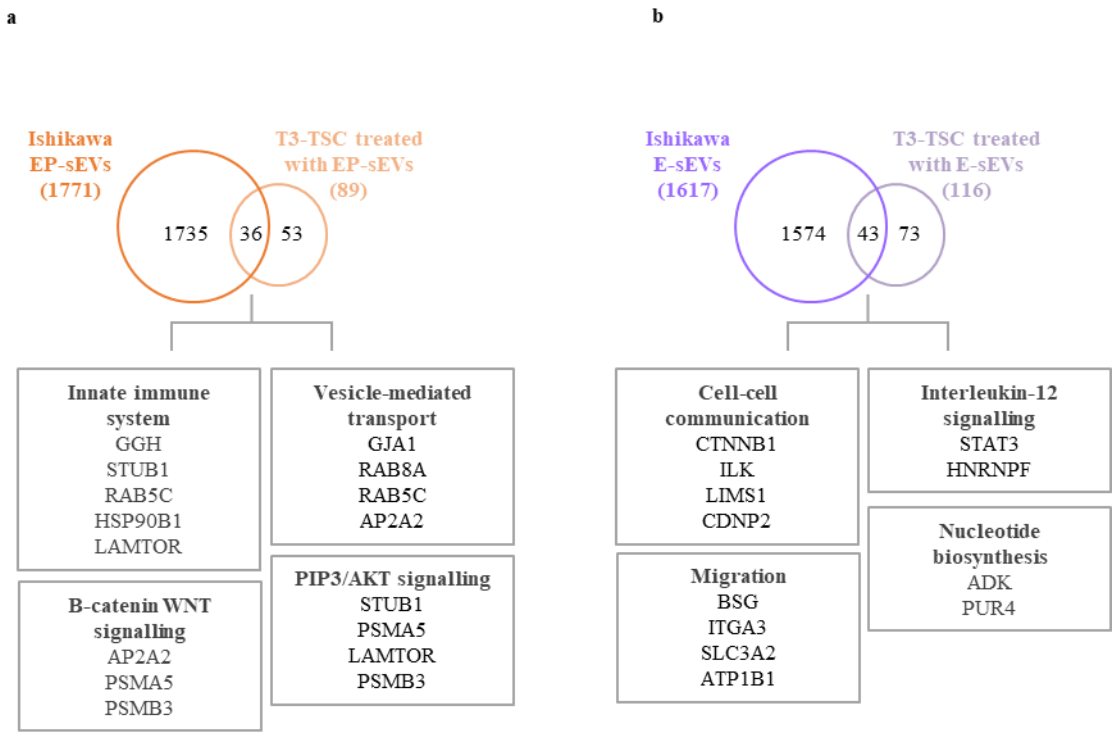
Venn diagram comparison of Ishikawa sEV protein profiles compared to ExoCarta[245] and Vesiclepedia[246] databases, which list 100 highly abundant proteins reported in exosomes.




**Appendix figure S4.2 Venn diagram comparing Ishikawa and ECC-1 small extracellular vesicles.** Ishikawa EP-primed sEV protein signature (unique and significantly enriched proteins) was compared and validated with ECC-1 EP-sEV protein signature.




**Appendix figure S5.1 Live fluorescence z-stack images of human trophectoderm cells (T3-TSC) treated with endometrial small extracellular vesicles.** T3-TSCs were treated with E-sEVs. (top panel) and EP-sEVs (lower panel) for 2 h to assess uptake. sEVs are stained with lipophilic tracer DiI (red), and nuclei stained with Hoechst (blue). Scale bar (10-50 µm) represented on images.




**Appendix figure S5.2 Venn diagram comparing Ishikawa small extracellular vesicle cargo with trophectoderm cells treated with E- and EP- small extracellular vesicles**. **a)** Ishikawa EP-primed sEV proteome compared with EP-sEV treated T3-TSC proteome **b)** Ishikawa E-primed sEV proteome compared with E-sEV treated T3-TSC proteome.

## Appendix methods

### Cell viability

Cell viability was determined using the trypan blue viability assay. Briefly, cells were cultured in DMEM/F-12 supplemented with 5% (v/v) FCS, 0.6% (v/v) ITS and 1% (v/v) Pen/Strep (DMEM/F-12 bioreactor flask media) to replicate growth conditions within bioreactor flasks. Cells were grown in 6-well plates (N=3) and harvested using 0.5% Trypsin-EDTA. CM, wash media (DMEM/F-12 supplemented with 1% (v/v) Pen/Strep) and harvested cells were combined and centrifuged at 500 *g* for 5 min, 4 °C to pellet cells. Pellets were resuspended in 5 mL DMEM/F-12 bioreactor flask media and a 10 µL aliquot was combined 1:1 with 0.4% (w/v) trypan blue stain (Sigma-Aldrich). Aliquots (10 µL) were transferred to a haemocytometer to count total, viable (non-stained), and non-viable (stained) cells (n=3 technical replicates). Data was analysed using GraphPad Prism (8.4.3).