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Publication 3. Contributed to developing the concept. Contributed to planning experiments. Initializing and optimizing methodology (Cell culture, EV preparation, EV supplementation, RNA extraction, RNA quality control, Primer design, qCPR protocol). Contributed to carrying out experiments (Cell culture, EV preparation, EV supplementation, RNA extraction, RNA quality control, Primer design, cDNA preparation, qPCR). Contributed to data analysis. Contributed to writing the manuscript.

ABBREVIATIONS

5-bromouridine (BrU)
5-ethynyluradine (5-EU)
5-fluorouridine (FU)
5-iodouridine (IU)
Arginine-glycine-aspartic acid tripeptide sequence (RGD sequence)
Biological nanoparticles (BNP)
Bovine oviductal epithelial cells (BOECS)
Counts per million (CPM)
Differential expression (DE)
Differentially expressed genes (DEG)
Elective dual embryo transfer (eDET)
Elective single embryo transfer (eSET)
Endosomal sorting complex required for transport (ESCRT)
Epidermal growth factor (EGF)
Extracellular matrix (ECM)
Extracellular vesicles (EV)
False-discovery rate (FDR)
Foetal bovine serum (FBS)
Gene set enrichment analysis (GSEA)
Generalized linear model (GLM)
G-protein coupled receptors (GPCR)
Heparin-binding epidermal growth factor-like growth factor (HB-EGF)
Human leukocyte antigen-g (HLA-G)
In vitro fertilization (IVF)
Interferon tau (IFN-T)
Interferon-stimulated genes (ISGS)
International committee for monitoring assisted reproductive technology (ICMART)
International society of extracellular vesicles (ISEV)
Intracytoplasmic sperm injection (ISCI)
Intraluminal vesicles (ILV)
Intrauterine insemination (IUI)
Leukaemia inhibitory factor (LIF)
LncRNA and long intergenic/intervening RNA (LincRNA)
Log fold change (LOGFC)
Microvesicles (MV)
Multivesicular body (MVB)
Nanoparticle Tracking Analysis (NTA)
Noncoding-RNA (ncRNA)
Normalized enrichment score (NES)
Principal component analysis (PCA)
Principal components (PC)
Recurrent implantation failure (RIF)
RNA interference (RNAi)
Transmission electron microscopy (TEM)
Unique molecular identifiers (UMI)
Uterine natural killer (UNK)
World health organization (WHO)

1. INTRODUCTION

One of the most critical steps in mammalian reproduction is Implantation. During the process, the embryo first adheres to luminal epithelium of the uterus then invades the underlying stroma. Implantation is also considered as the bottleneck step in human assisted reproduction where 2/3 of the failed attempts are caused by failure to implant (Jarvis, 2016). Despite tremendous advances in the field of assisted reproduction technologies (ART), the success rate of embryo transfer still remains less than 50% mostly due to the embryo's inability to implant (Apter *et al.*, 2017). Deciphering the causes for this unique situation is a subject of vigorous scientific inquiry given that the morphological quality of the embryo does not correlate completely with the rate of implantation or a successful birth even in cases where the uterine environment is optimal for implantation (Wilcox, Baird and Weinberg, 1999).

The endometrium undergoes multiple physiological and morphological changes in order to be receptive to an incoming implantation during the short period known as the window of implantation (WOI) (Ashary, Tiwari and Modi, 2018). These changes extend to a complete re-organisation of the uterine immune micro-environment since the embryo is technically a non-self-entity due to the paternal half of the genome and the unique antigens that result from paternal proteins. The maternal immune system should, in normal course of events, identify the embryo as a possible foreign entity and reject the embryo to protect the endometrium. However, it has been long observed that the unique endometrial immune system is somehow induced to not only ignore the embryo, but to protect the implantation site. This process is termed induced immune-ignorance and the mechanisms of its inducement is not clearly understood (Warning, McCracken and Morris, 2011).

The conventional wisdom on the matter was that the endometrium and embryo are both passive or "quite" and all the events leading up to and during the implantation and invasion of endometrium by the embryo are regulated by external factors such as hormones and cytokines. This is in fact proven scientific theory and supported by multitudinous reports. However, in recent decades evidence started to point to another component of the regulatory mechanism of embryo implantation, the embryo-maternal communication (Modi and Bhartiya, 2015).

The new hypothesis posits that embryo and the endometrium are not passively following the directives of the endocrine system but are actively participating in the process by communicating with each other using one or more signalling methods (Bhagwat *et al.*, 2013). Very little is known about the nature of the embryo-maternal communication. cells in close proximity are able to communicate via different types of signals such as soluble secretory factors like cytokines and hormones, secreted proteins and other biomolecules (Nakamura *et al.*, 2019). In fact, several agents, secreted into the uterine environment by the endometrium, are reported to regulate implantation by affecting blastocyst

development directly and/or by controlling adhesion molecule expression (Ashary, Tiwari and Modi, 2018). There are also reports of biomolecules of embryonic origin targeted towards the maternal tissues (endometrium and oviduct) to induce specific changes in their physiological makeup to bring about the WOI.

These biochemical messengers are required to traverse the intercellular space in the uterine lumen to reach their destination. The environment in the intercellular space is not conducive to the existence of most of the delicate biomolecules such as RNA species in their native state. Extracellular vesicles (EV) has been proposed as a transport mechanism along with transfer proteins to overcome the issue of cargo protection and added target specificity (Nakamura, Kusama, Suda, *et al.*, 2020; O'Neil *et al.*, 2020). Specific biomolecules are hypothesised to be loaded into EVs in the cell of origin and released to the intercellular space to directly interact with the target cell to induce desired alterations (Zaborowski *et al.*, 2015). EVs are known to transport a wide variety of cargo from DNA to nucleases. However, studies are especially indicating the mutual interchange of noncoding-RNA (ncRNA) between embryo and endometrium as a method of communication inducing transcriptomic and epigenomic alterations of the maternal system (Cuman *et al.*, 2015). Particularly, different species of ncRNA such as microRNA (miRNAs), long noncoding RNA (lncRNA and lincRNA) are reported to be packaged into EVs and transported between the embryo and the endometrium contributing to embryo-maternal interaction (Cuman *et al.*, 2015). Different ncRNA species have been shown to be involved in regulating embryo implantation gene expression, with studies showing their role in blastocyst implantation and uterine receptivity regulation (Zheng *et al.*, 2017). In the recent decades, EVs were discovered in almost all the body fluids including the uterine and oviductal fluids of human, sheep, mice, bovine and porcine species. The population of EVs were identified to be a mix of embryonic EVs and maternal EVs(Ng *et al.*, 2013; Burns, Brooks and Spencer, 2016).

If reliable evidence of EV-mediated embryo-maternal communication could be established, the resulting effects on the maternal cells can be used as a biological sensor to quantify the degree of embryo-maternal communication. There are multiple methods of evaluating the quality of embryos for transfer. Embryo grading is crucial in elective single embryo transfer where only the best embryo is transferred to the uterus in hopes of avoiding the risks associated with multiple births. Most used methods of embryo evaluation are based on morphology and morphokinetics. However, embryos selected from these methods have only achieved a success rate of around 35%. Since quantified embryo-maternal communication can measure a functionally important characteristic of the embryo it can be theoretically used as a non-invasive method of embryo grading independent of morphological characteristics.

Within the scope of this thesis, experiments were designed to enrich and characterise the EVs of embryo-maternal microenvironment in an attempt to decipher the mechanisms and effects of EV-mediated embryo-maternal

communication in order to understand whether such communications are critical to the process of implantation. Once evidence of EV-mediated embryo-maternal communication were established, the gained knowledge was applied to develop a method of non-invasive embryo grading to be used in mammalian assisted reproduction.

2. LITERATURE REVIEW

2.1. The process of mammalian embryo development

In eutherians (placental mammals), reproduction typically occurs following the same general steps. The male gametes (spermatozoa) are introduced to the female reproductive tract during sexual intercourse. The spermatozoa swim through the cervix and uterus towards the fallopian tube while maturing to a certain degree depending on the species. In the fallopian tube, the male and female gametes fuse and fertilization occurs. The fertilized embryo is then transported to the uterus through the oviduct while developing into a blastocyst. Typically, the blastocyst then implants in the endometrial epithelium. The mechanisms of implantation and the timeline of embryo development varies widely with the species.

Implantation, when the embryo and the maternal system comes into permanent physical contact, is one of the most critical stages in the process. The mechanisms used to facilitate and regulate this vital step are under rigorous scientific investigation, even though the complete picture is still elusive.

2.2. Embryo implantation and its regulation

Implantation is a complex process that involves a competent embryo and a receptive endometrium. In placental mammals, implantation is understood as the irreversible adherence of the embryo to the uterine wall. In human implantation, there are three distinct stages in the process. First, the trophoblast establishes physical contact with the site of implantation (apposition). Then the trophoblast and the endometrium attach to each other (adhesion) and finally the trophoblast invades the endometrial stroma through the endometrial basement membrane (invasion) (Kim and Kim, 2017). These events are possible only during a short period of time known as the WOI which is generally considered days 16–22 in a 28-day menstrual cycle (Ma *et al.*, 2003). Under normal circumstances, the success rate of an embryo implantation is approximately 30% due to the complex and exacting nature of preconditions that should be met for a successful implantation.

Both the embryo and the endometrium undergo significant functional and structural remodelling in readiness for implantation (Murphy, 2004). In the endometrium, the major regulators of these alterations are estrogen and progesterone (Greening, Nguyen, Evans, *et al.*, 2016; Aplin and Ruane, 2017). Rising estrogen levels in the proliferative phase stimulates the proliferation of epithelium, stroma and vascular endothelium thus regenerating the endometrium (Rao *et al.*, 2007; Cha, Sun and Dey, 2012). A subsequent reduced level of estrogen is inducive to an extended uterine receptivity (Ma *et al.*, 2003). Progesterone, binding to both nuclear and membrane receptors, induces structural alterations in the endometrial epithelium. Epithelial cells loose polarity and the microvilli on the apical surface developing a flattened surface with

numerous protrusions named pinopodes (Thie *et al.*, 1995). The expression of cell adhesion inhibitor, mucin 1 is significantly downregulated increasing the potential of embryo attachment (Gipson *et al.*, 2008). The morphological and physiological changes occurring during these early stages of pregnancy, termed the "plasma membrane transformation" or the "attachment reaction", are a part of the more elaborate process of decidualization (Murphy, 2004).

During apposition and adhesion, the vascular permeability of endometrium is drastically increased, suggesting that apposition is a pro-inflammatory process. The vascular permeability is reported to be regulated by cyclooxygenase (Cox)-derived prostaglandin E2, marking it as one of the major regulators of trophoblast invasion (Wang *et al.*, 2004; Niringiyumukiza, Cai and Xiang, 2018). Vascular permeability is also critical for the process of decidualization, where decidual leukocytes such as the specialized uterine natural killer(uNK) cells and dendritic cells accumulate around the implantation site and the endometrial stromal cells undergo a stromal to epithelial transformation requiring major changes in the extracellular matrix (Okada, Tsuzuki and Murata, 2018).

Another aspect of the regulation of implantation is the action of cytokines which function in autocrine or paracrine manner during the process of implantation (McEwan *et al.*, 2009). The leukaemia inhibitory factor (LIF) is a mediator of estrogen activity and a member of the interlukin-6 family (Massimiani *et al.*, 2020). LIF is reported to stimulate and regulate the stromal proliferation via epidermal growth factor (EGF) signalling pathway(Hantak, Bagchi and Bagchi, 2014). Interleukin-6 (IL-6) is highly expressed in endometrial epithelium and being transferred to blastocysts. Viable blastocysts' uptake of the IL-6 was significantly higher compared to that of degenerated blastocysts, implying a potential role of IL-6 in embryo viability(Dominguez *et al.*, 2010, 2015). The Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is another crucial molecule in the implantation process (Cha, Sun and Dey, 2012). Functioning in a juxtracrine manner, HB-EGF is a transmembrane protein that releases a soluble growth factor which reacts with the EGF family receptors in the trophoblast surface to regulate adhesion (Raab *et al.*, 1996; Paria *et al.*, 1999; Stavreus-Evers *et al.*, 2002).

Cell adhesion molecules such as integrins, cadherins, immunoglobulin superfamilies, CD44 proteins and selectins mediate the adhesion of the trophoblast to the luminal epithelium of the uterus (Merviel *et al.*, 2001; McEwan *et al.*, 2009). The adhesion molecules are expressed on the trophoblast surface and attach to their specific ligands expressed in the extracellular matrix (ECM) of the endometrium(Lyall, 2006). Integrins are a family of transmembrane glycoproteins. In the mid luteal phase, the upregulation of integrins in the endometrium is considered as a classic marker of WOI (Lessey *et al.*, 1992). The maternal integrin subunits α and β contains a divalent cation binding site that recognizes an arginine-glycine-aspartic acid tripeptide sequence (RGD sequence) that can be found in many ECM proteins such as fibronectin and collagen I. the trophoblast integrins bind to the endometrial RGD sequences during the adhesion process (Merviel *et al.*, 2001). Cadherins are a family of

glycoproteins involved in the Ca^{2+}-dependent cell-cell adhesion mechanism (Zhou, Santos and Dimitriadis, 2020). The classical cadherin members (E-cadherin, N-cadherin and P-cadherin) are significantly enriched in the secretary phase of the endometrial epithelium (Van der Linden *et al.*, 1995; Carson *et al.*, 2002). Cadherins can facilitate mechanical direct binding to the trophoblast. In fact, endometrium that has been artificially overexpressed with E-cadherin has shown significantly increased binding to BeWo trophoblast-like spheroids (Rahnama *et al.*, 2009). The carbohydrate binding protein, L-selectin is expressed in the trophoblast and its ligands, the oligosaccharides, are expressed in the pinopodes of the plasma-membrane-transformed endometrial epithelium (Wang *et al.*, 2008). The attachment of trophoblastic carbohydrate L-selectin and its oligosaccharide ligands in the endometrial epithelium is one of the initial steps in embryo adhesion in humans. Blocking L-selectin with antibodies prior to implantation has led to impaired adhesion (Genbacev *et al.*, 2003; Nejatbakhsh *et al.*, 2012).

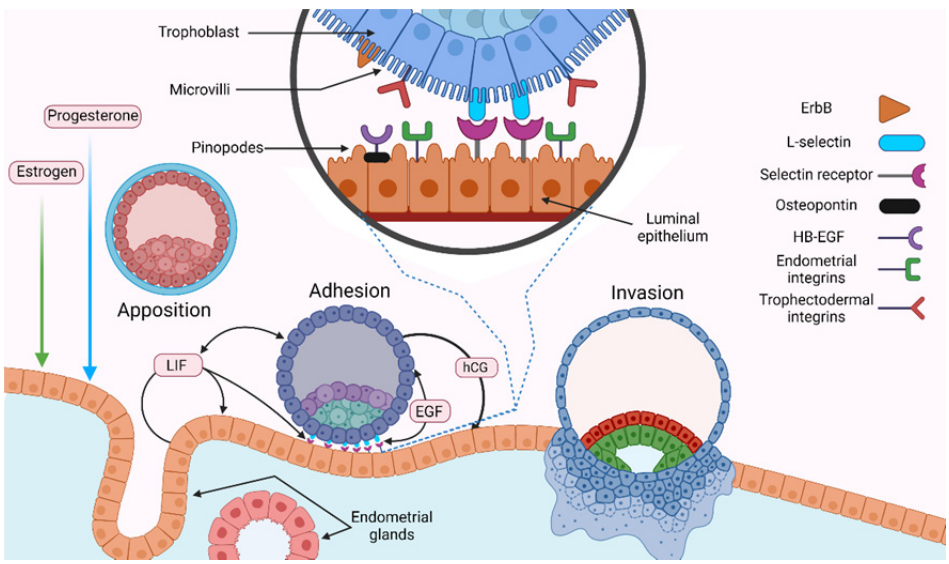


Figure 1. Events of embryo implantation and its regulation
The three major events of implantation process (apposition, adhesion and invasion) is depicted with their major regulators. Estrogen and progesterone are the main regulators of implantation, altering the endometrial physiology to increase receptivity. Human chorionic gonadotropin (hCG), leukaemia inhibitory factor (LIF) signalling and epidermal growth factor (EGF) based signalling are among the many signalling mechanisms regulating the implantation process. In the embryo-maternal interface (zoomed view) many adhesion molecules such as L-selectin, trophectodermal and endometrial integrins, cadherins etc. ensure a tight physical connection between embryo and the luminal epithelium. The Heparin-binding epidermal growth factor-like growth factor (HB-EGF), its receptor ErbB and osteopontin are highly upregulated in receptive endometrium facilitate adhesion and paracrine signalling between embryo and the endometrium.

2.3. The theory of embryo-maternal communication

All these regulatory mechanisms endeavour to explain the process of embryo implantation. However, the overall image that can be envisioned by the current knowledge is incomplete. This insufficiency of explanation is more apparent when considering the immune aspects of the process of implantation. Since the pre-implantation embryo is essentially an individual entity in the sense of immunity, the maternal immune system, if functioning properly, should be rejecting the implantation as an invasion from a foreign entity. However, in a unique instance of acquired immune ignorance, the maternal system not only ignores the implantation and the subsequent invasion, it accommodates the invading conceptus. The mechanism of this phenomenon is yet to be described adequately.

Multiple hypothesis has been proposed to fill in the gaps of knowledge in an attempt of a deeper understanding of the events that takes place in the embryo-maternal and feto-maternal interfaces. One such hypothesis that has gained considerable traction in the recent decade is embryo-maternal communication-based regulation of implantation.

Since the initial production of gametes, to the time of birth, cells in the reproductive system are hypothesized to be engaged in continuous and bilateral communication with the cells and structure in their immediate environment (Hung *et al.*, 2015; MATSUNO *et al.*, 2017; de Almeida Monteiro Melo Ferraz *et al.*, 2020). This communication has been understood to be conducted and regulated by the endocrine system in general. Every milestone of the developing embryo is theorized to be carefully controlled and stimulated by specific hormones and cytokines following a complex rhythm differing from species to species. These hypothesis and theories are backed by decades or even centuries of rigorous scientific investigations. However, as in many a biological process, the explanation is neither perfect nor complete. Since both the pre-implantation embryo and the recipient endometrium both produce EVs and EVs are known to be potent agents of intercellular communications, it has been hypothesized that both embryonic and endometrial EVs take part in embryo-maternal communications (Saadeldin *et al.*, 2015). Recent mounting evidence expose direct intercellular communications such as EV-mediated communication as another facet of the complex narrative that is the regulation of the reproductive process (Chen *et al.*, 2009; Tannetta *et al.*, 2014; Almiñana *et al.*, 2018; Evans *et al.*, 2019). Hence, the scope of this thesis is not to refute the overwhelming evidence of regulation of reproduction via endocrine system, but to elucidate the contribution of EV-mediated portion of the whole intricate mechanism.

2.4. EVs: their biogenesis and nature

EVs are defined as membrane bound nano-sized particles produced by living cells and released into the extracellular space. All the known cell types across biological kingdoms are reported to produce EVs (Ng *et al.*, 2013). Many of the biological fluids such as serum, cerebrospinal fluid and seminal plasma are known to contain EVs (Smijs *et al.*, 2017). In the earlier days of EV research, these biological nanoparticles were considered to be of little importance to physiology or pathology, being considered a method of waste elimination via membrane budding (Crawford, 1971; Trams *et al.*, 1981). Active EV secretion, the process of EV biogenesis and release via exocytosis, was described in reticulocytes in 1983 (Pan and Johnstone, 1983; Harding, Heuser and Stahl, 2013).

Nomenclature for biological nanoparticles (BNP) such as EVs has not been systematic during the formative years of the field of EV related biological studies. A wide range of classifications were introduced by various researchers to describe the subjects of their studies based on the cell of origin. For example, BNPs in the field of reproduction were termed epididymosomes, prostasomes, vaginosomes, uterosomes and oviductosomes (Al-Dossary, Strehler and Martin-DeLeon, 2013; Belleannée *et al.*, 2013; Sullivan and Saez, 2013; Fereshteh *et al.*, 2019) based on the cell or tissue of origin. Depending on the biogenesis, BNPs are generally divided into three categories. Exosomes are produced as a result of inward budding of the multivesicular body (MVB) membrane. Vesicles within the large MVBs are termed intraluminal vesicles (ILV) and most of the ILVs are released to the extracellular space when the MVB is fused with the plasma membrane. The released ILVs are named exosomes. Formation and regulation of the ILVs are known to require the endosomal sorting complex required for transport (ESCRT) machinery (Zhang *et al.*, 2019) among others (Villarroya-Beltri *et al.*, 2014). Microvesicles (MV) are another type of BNP originating as a result of outward budding of the plasma membrane (Zaborowski *et al.*, 2015). Regulation of the MV biogenesis is controlled via acidic sphingomyelinase and AKT activation (Bianco *et al.*, 2009), flux variations in intracellular calcium, and enzymes implicate in upholding the asymmetry of the membrane phospholipid (Piccin, Murphy and Smith, 2007) among other molecular cascades. Apoptotic bodies are released by cell death and are the largest group of generalized BNPs (Zaborowski *et al.*, 2015). There has been a tendency to categorize BNPs according to their sizes. For example, exosomes, being the smallest vesicles, are said to be around 30 nm to 120 nm in diameter and apoptotic bodies can be as big as several micrometres. However, since there is no clear demarcation point between each type of vesicle, using size as a criterion for BNP categorization would be inaccurate. Since most of the BNP populations are highly heterogenous in both size and origin, it is not possible to study just one group using the currently available preparation methods. Therefore, the International Society of Extracellular Vesicles (ISEV) suggests an umbrella term “extracellular vesicles” to encompass all the BNPs. The term “EVs” will be used throughout this thesis to avoid confusion.

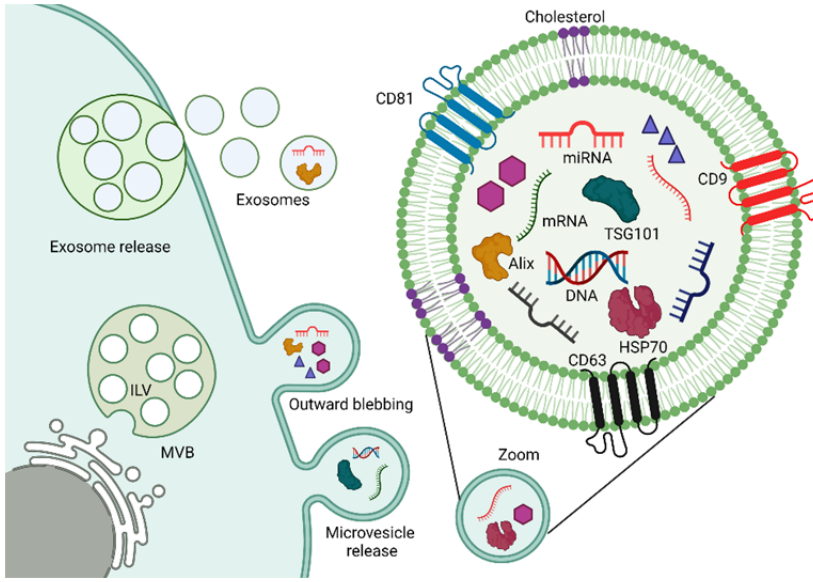


Figure 2. Biogenesis and morphology of EV
Exosomes are formed by the inward blebbing of the multivesicular body (MVB) membrane. The vesicles, while inside the MVB are intraluminal vesicles (ILV). When the MVB is fused with the cell membrane, ILVs are released to the intercellular space and termed exosomes. Microvesicles are formed by outward blebbing of the cell membrane. Zoomed view shows a generalized depiction of an EV. The phospholipid bilayer is interspersed with tetraspanins such as CD81 and cholesterol. The cargo of the vesicle is highly diverse. Usually it contains, miRNA, mRNA fragments and DNA fragments. Numerous proteins such as HSP70, HSP90, ALIX and TSG101 are also usually present.

2.5. Function of EVs

One of the primary functions of EVs is to transport biologically significant cargo between cells. The transfer is known to occur between cells of the same individual, between individuals and even cross-kingdom. The cargo encompassed in EVs are extremely varied and heterogenous even among EVs extracted from purified cell cultures. Thousands of proteins, lipids, all species of RNA (miRNA, mRNA, siRNA, lncRNA, lincRNA etc) are reported to be packaged into EVs as cargo. Communication via EV surface membrane molecules (such as membrane proteins) is another potential method of EV based communication. The term "cargo" is used within this thesis to refer to any biomolecule carried in or on EVs.

The proteomic cargo of EVs are extensively studied as potential signalling agents. There are reports of EV proteins being transported between cells and inducing alterations in the activity of the target cells. Tetraspanins such as CD63, CD81 and CD9 are known to be enriched in EVs. In fact, they are considered classic EV markers. Tetraspanins are regulators of cellular signalling,

especially in adhesion mediated signalling such as integrin pathways (Vlassov *et al.*, 2012; Termini and Gillette, 2017). Other EV markers recommended by ISEV such as the cytosolic proteins ALIX and TSG101 are both components of ESCRT pathway and could potentially be enriched in exosomes compared to other BNPs. There are multiple reports of EVs containing any number of physiologically critical proteins and cytokines.

The main group of EV cargo of interest during the experimentation in the context of this thesis is RNA. EVs are known to carry a wide variety of RNA species ranging from intact mRNA (according to some reports, termed exRNA) to fragments of long non-coding RNA and small RNA species such as miRNA, siRNA and tRNA fragments which all have significant biological roles that has been reported to be induced in the target cells (O'Brien *et al.*, 2020).

The exchange of ncRNA has been reported to be a major part of intercellular communication (Ratajczak *et al.*, 2006; Valadi *et al.*, 2007). These exchanges seems to be partly mediated through EV (Raposo and Stoorvogel, 2013). One species of RNA that has not been examined in great detail in this context is lncRNA. Defined as autonomously transcribed RNA with more than 200 nucleotides, lncRNA and long intergenic/intervening RNA (lincRNA) (Ransohoff, Wei and Khavari, 2018) reported to have multiple gene expression regulatory functions. More than 30,000 lncRNAs haves been annotated, half of which are lincRNA (Dragomir, Chen and Calin, 2018). lincRNAs modify gene expression by directly affecting nuclear architecture (Ransohoff, Wei and Khavari, 2018).

MicroRNAs are indubitably the best studied type of EV bound RNA since they are generally found to be significantly enriched in EVs compared to the origin cell transcriptomes. It is hypothesized that miRNA transported via EVs can regulate the gene expression in target cells (Capalbo *et al.*, 2016). In the initial phases of the studies into ncRNA facilitated embryo-maternal communication, there was concrete proof of embryonic miRNA being transferred to the endometrial cells (Cuman *et al.*, 2015) and *vice versa* (F. Vilella *et al.*, 2015). Embryonic miRNAs have been extracted from embryo conditioned media of trophoblasts used in ART and had putative targets in critical functions such as cellular adhesion and extracellular matrix remodelling (Gross, Kropp and Khatib, 2017). However, the hypothesis of miRNA-based embryo-maternal communication should be carefully subjected to rigid experimental scrutiny to calculate the efficiency of such communications, since some reports suggest that the actual quantity of miRNA carried in EVs might not be sufficient to influence the target transcriptome significantly (Chevillet *et al.*, 2014; Zhang *et al.*, 2015; Albanese *et al.*, 2020).

The cargo of the EVs are conventionally considered to be packaged inside the lipid bilayer for protection in the intercellular space. However, EVs are capable of using surface bound molecules in intercellular communication also. There are multiple reports of EVs carrying transmembrane ligands for membrane bound receptors of the recipient cells (Sobo-Vujanovic, Munich and Vujanovic, 2014; Gurung *et al.*, 2021). The term "direct interaction" is used to

describe this method of signalling. Since the implantation is a relatively rapid event, it can be hypothesized that a similar mechanism could be utilized in EV based embryo-maternal communication.

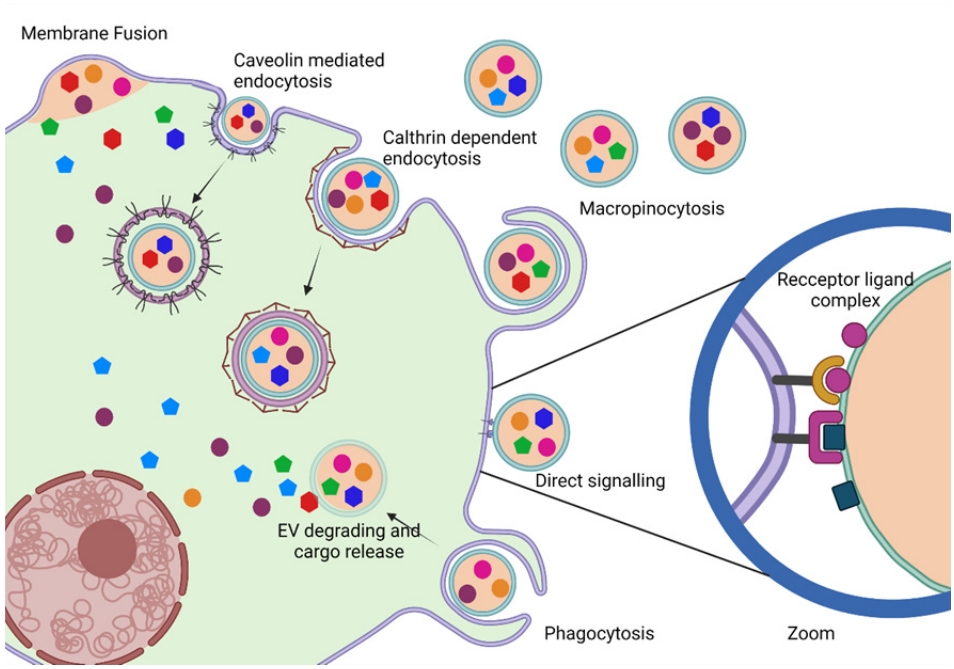


Figure 3. Mechanisms of EV-mediated signalling
EVs are known to carry messages between cells using various different mechanisms. They are hypothesized of being able to fuse directly with the recipient cell membrane and release the cargo. The recipient cell can actively uptake the EV using endocytosis, phagocytosis or macropinocytosis. Inside the recipient cell, the EVs are hypothesized to be degraded and cargo is released to the cytoplasm. EVs can transfer messages without physically being uptaken by directly binding to the receptors of the recipient cell membrane and activating a signalling cascade.

2.6. Probable evidence of EV cargo release to the cytoplasm

For any external RNA to be functional within the target cells, it should first reach the cytosol. Throughout the ever-increasing body of research work concerning EV based RNA delivery and overall EV based cargo delivery, the mechanisms of how or even if EV cargo is released into to the cytoplasm of the target cells has rarely been discussed. It has been proposed that EVs can directly bind to the plasma membrane, transiently bind to the ER membrane (the so-called kiss and run exchange), bind to endosomal membrane after endocytosis and even escaping via endosomal rupture (Joshi *et al.*, 2020). In most studies,

the endosomal escape of EV cargo has been inferred by the apparent functionality of the cargo molecules such as miRNA carried in EVs inducing gene silencing at the target cells. When endosomal escape is induced by chloroquine & UNC10217938A, EVs were able to release their cargo with significantly higher success (Heath *et al.*, 2019), further confirming the requirement of endosomal escape for delivery of functional cargo.

However, the functionality of EV cargo released into the cytoplasm is also debatable. For example, even with successful uptake, the amount of functional proteins translated by the mRNA carried in EVs has been vanishingly small (Hung and Leonard, 2016), suggesting that EV cargo might not be released to the cytoplasm efficiently and instead are degraded by the lysosomes (Iversen, Skotland and Sandvig, 2011). There is a possibility that EVs have hitherto unknown mechanisms of cargo delivery post-uptake. However, the evidence is sparse on the subject (O'Brien *et al.*, 2020).

The difficulties in elucidating mechanisms of EV cargo delivery within recipient cells are partially due to the lack of specific markers that can be used to track the cargo molecules to their ultimate destiny once uptaken. Technologies developed to track viral genome release within the host cells are being used to investigate the possible mechanisms of EV cargo escaping the endosome to the cytosol.

In a study conducted in 2020, Joshi and colleagues experimentally demonstrated that EVs uptaken via endocytosis can indeed expose their cargo to the cytosol. Interestingly, they have also been able to demonstrate that endosomes containing EVs do not get ruptured, rather the cargo release is achieved via membrane fusion. The mechanism of which could possibly be related to the pH of the endosome/lysosome and the cholesterol accumulation (Joshi *et al.*, 2020). There has been reports of EV cargo escape using interactions with ER and nucleoplasm reticulum membranes (Heusermann *et al.*, 2016; Santos *et al.*, 2018). In dendritic cells, both EVs and retroviruses such as HIV-1 are shown to be captured in a CD81+ intracellular compartment and the cargo is transmitted to T cells thus bypassing degradation by lysosomes (Izquierdo-Useros *et al.*, 2009). Similar to enveloped viruses, EVs are thought to fuse with the late endosome and release cargo into cytosol using anionic compounds such as the lipid bisoleoyl-lysobisphosphatidic acid (LBPA) (Hessvik and Llorente, 2017; Staring, Raaben and Brummelkamp, 2018), however, experimental evidence supporting this hypothesis is still lacking.

Current knowledge on the phenomenon of EV cargo release within the target cells is incomplete. It has been posited that a significant portion of the uptaken EVs are indeed degraded by the lysosomes (Gurung *et al.*, 2021). EVs could be using multiple methods to release their cargo to the cytoplasm. With mounting evidence, it can be expected that a more complete picture of the phenomenon would be visible in the future.

2.7. Involvement of EVs in embryo-maternal communication in the embryo-oviduct microenvironment

EVs are known to influence the production and maturation of both ova and spermatozoa and to take a critical role in embryo fertilization. However, the minutiae of intercellular communications prior to embryo development are outside the scope of the experimentations that make up this thesis. The site of initial development of embryo is the oviduct during the embryo's movement through the ampulla and the isthmus. During this period, the embryo is immersed in the oviductal fluid containing a potent mixture of many soluble factors, including proteins, growth factors, and metabolites (Avilés, Gutiérrez-Adán and Coy, 2010) which influence the development and the phenotype of the embryo while the embryo affects the cells in the oviduct as well (Lee *et al.*, 2002). Bovine oviductal epithelial cells (BOECs) when co-cultured with pre-implantation embryos are reported to improve the quality of the embryos (Cordova *et al.*, 2014) implying positive paracrine effects of maternal tract on embryonic development. Considering the embryo to maternal portion of the cross-talk, there are multiple reports of bovine embryo conditioned media altering the transcriptome of the BOECs *in vitro* (Schmaltz-Panneau *et al.*, 2014; García *et al.*, 2017; Hamdi *et al.*, 2019) and *in vivo* (Maillo *et al.*, 2015).

Evidence of EVs involvement in embryo-maternal communication in oviducts of any species are rare. However, oviduct does contain EVs (Lopera-Vasquez *et al.*, 2017) and could potentially be involved in the intercellular cross-talk reported in the aforementioned studies. The cargo of the EVs derived from cultured BOECs are reported to have different cargo depending on the stage of the oestrous cycle (Almiñana *et al.*, 2018; Gatien *et al.*, 2019) implying that EVs might be carrying different messages during different phases of the oestrous cycle. Oviductal EVs are readily uptaken by embryos (Almiñana *et al.*, 2017) and the uptaken EVs are reported to alter the genetic makeup of the embryos increasing the embryo quality (Lopera-Vasquez *et al.*, 2016, 2017; Almiñana *et al.*, 2017; Qu *et al.*, 2019, 2020). The mechanisms of such actions are not clearly understood but there are some preliminary evidences that could shed some light on the matter. For example, in mouse embryos, oviductal EVs are known to suppress the expression of apoptotic activators, thus, protecting the embryo (Qu *et al.*, 2019). In bovine embryos, BOEC derived EVs can be used to provide additional protection to embryos during vitrification by up-regulating the water channel gene aquaporin-3 (*AQP3*). Aquaporins are proteins that selectively conduct water through the plasma membrane. They are very crucial to cell survival during cryopreservation. (Lopera-Vasquez *et al.*, 2017). Although the evidence is not overwhelming and concrete, these reports suggests that EVs could be playing a role in the embryo-maternal communication that occurs during the embryo development in the oviduct.

2.8. Involvement of EVs in embryo-maternal communication in the trophoblast-endometrium microenvironment

Similar to other cells, embryos are also known to produce EVs in different concentrations in the different stages of development (Giacomini *et al.*, 2017; Mellisho *et al.*, 2017; Dissanayake *et al.*, 2020). Current understanding of embryonic EVs posits a significant increase of EV production as the embryo attains further developmental milestones (Giacomini *et al.*, 2017). In human, embryo is exhibited by the difference of EV production between day 3 and day 5 embryos (Giacomini *et al.*, 2017). A fact that could be explained by the number of cells available to produce EVs between the two stages. Interestingly, it was observed that in bovine embryos, the quality of the embryo itself is reflected by the EV production (Dissanayake *et al.*, 2020). It has been reported that embryos with high potential of implantation produce a lower EV amounts compared to embryos that failed to implant (Pallinger *et al.*, 2017). Concentration and size of the embryonic EVs could also vary between male and female embryos, and depend on the growth conditions such as the O_2 percentage in the incubator (Abu-Halima *et al.*, 2017; Andrade *et al.*, 2019).

Embryos produce EVs that are uptaken by the endometrial cells. Multiple reports show labelled EVs produced by embryos cultured *in vitro* are readily uptaken by both epithelial and stromal cells of the endometrium (Saadeldin, Oh and Lee, 2015; Battaglia *et al.*, 2017, 2019; Czernek and Düchler, 2020). However, most of the dyes used in these studies, for example dyes that target lipid bilayers, are not specific to EVs and therefore the results generated might not be conclusive. Most commercial embryo culture media contain EVs in a lower concentration than conditioned culture media. Interestingly, at least in one study, endometrial cells did not uptake unconditioned culture media EVs. Human serum albumin and serum substitute supplements were the source of EVs in the unconditioned media (Giacomini *et al.*, 2017). In *in vivo* studies, the elongating conceptus of sheep embryo is reported to produce EVs that are uptaken by the uterine epithelium(Burns, Brooks and Spencer, 2016).

Compared to embryo to maternal communications, there have been rather more studies conducted on the maternal to embryonic aspect of the embryo-maternal communications in the context of endometrial to trophoblast communications. Some of the studies are concerned with EV-based communication. EVs were extracted from endometria of human (Ng *et al.*, 2013), murine (Tan *et al.*, 2020), bovine (Nakamura, Kusama, Ideta, *et al.*, 2020), porcine (Bidarimath *et al.*, 2017), ovine (Burns *et al.*, 2018) and equine (Almiñana *et al.*, 2021) species in both tissue samples such as mucus and cultured primary and immortalized cells. Uterine EVs are generally extracted from the apical surface of the uterine luminal epithelium via uterine aspirates (Ng *et al.*, 2013). Endometrium releases significantly increased amounts of EVs during the WOI in mice and humans. The human endometrial transcriptome alters significantly during the

WOI. There are reports that suggests a connection between these alterations, EV production and embryo receptivity. For example, the EV marker and ESCRT component CD63 is upregulated in the WOI (Ng *et al.*, 2013; Felipe Vilella *et al.*, 2015; Altmae *et al.*, 2017). Released EVs could target the embryo, regulating the embryo development and preparing the embryo for implantation. There are studies substantiating this hypothesis by supplementing embryos with EVs derived from endometria in different physiological and pathological stages and measuring the embryo responses. Endometrial cells isolated from patients with recurrent implantation failure (RIF) produced EVs that drastically reduced the quality of embryos (C. Liu *et al.*, 2020). EVs extracted from human endometrial cell lines (with or without priming with oestradiol and progesterone) were able to increase the invasiveness and adhesiveness of embryo analogues such as HTR8/SVneo and L2-TSC (Greening, Nguyen, Elgass, *et al.*, 2016; Evans *et al.*, 2019; Gurung *et al.*, 2020; M. Liu *et al.*, 2020) while altering their transcriptomic and proteomic profiles. In bovine endometrium epithelium, the preimplantation proteomic cargo of EVs are significantly different from the post-implantation proteomic cargo. Proteins involved in cell adhesion associated proteins were upregulated in pre-implantation EVs while apoptosis associated proteins were upregulated in post-implantation EVs(Kusama *et al.*, 2018). EVs extracted from estrogen and progesterone primed endometrial cell lines contained proteins that were critical in embryo implantation process such as components of the extracellular matrix and adhesion molecules (Greening, Nguyen, Elgass, *et al.*, 2016) implying a proteomic involvement in endometrial-embryonic communications.

Being an important and well-studied component of EV cargo, miRNA-based communication between endometrium and the embryo is prominently reported compared to other cargo molecule-based communications. Uniquely endometrial miRNA were enriched from the endometrial cell line ECC1 derived EVs along with other ncRNA that could have a role in the implantation process (Ng *et al.*, 2013). The miRNA has-miR-30d, uniquely identified in endometrial epithelial cells derived EVs, is repeatedly studied for its possible involvement during the WOI (Altmäe *et al.*, 2013; Felipe Vilella *et al.*, 2015). When treated with enriched miR-30d, mouse embryos showed upregulation of adhesion molecules implying a clear targeted regulation of the embryo by the endometrial miRNA. The data is still limited concerning EV-mediated RNA transfer between maternal tract and the embryos and *vice versa*.

2.9. The specificity of EV based intercellular communication

A communication is most effective when it is specific to its intended target. This fact is also applicable in intercellular communications. In the context of embryo-maternal communications, endocrine and paracrine mediated signalling is specific to the recipient cells to a remarkable degree because only the

intended recipient cells express the necessary receptors for the chemical signals used in these modes of communication. Considering the EV based intercellular communication, the scientific community disagrees whether such communication is specific or non-specific to recipient cells. Two levels of specificity can be considered when discussing EV-mediated intercellular communications:

1. EV uptake specificity
2. Functional specify of the communication

Uptake specificity of EVs has been studied extensively using various methods of EV labelling and detection of uptake with varied results ranging from definitely specific uptake and EVs being uptaken wholesale without concern of origin of recipient cell type (Mathieu *et al.*, 2019a; Bonsergent *et al.*, 2021).

An increasing number of reports support the argument of functional specificity in EVs. The theory of functional specificity posits that the effects certain population of EVs have on certain population of recipient cells are specific to those populations alone (Arslan *et al.*, 2013; Singh *et al.*, 2014; Théry *et al.*, 2018). However, the underlying mechanisms of functional specificity in EV-mediated intercellular communications are not well understood. The cargo of each population of EV could be significantly different from other populations, thus, inducing significantly different effects on recipient cells. On the other hand, the recipient cells could have selective mechanisms, such as membrane receptors that would selectively bind to EVs expressing specific ligands that enables them to produce a significantly different reaction to each type of EVs.

An assay developed using the theory of functional specificity in EV-mediated intercellular communications could potentially be implemented as a method of non-invasive cell sorting. If certain recipient cell reacts differently to one EV type (for example EVs from one type of cells) compared to another EV type, the cellular reaction can be used as a means of identifying the different cell populations without physically sampling each cell type. Applications of this methodology is best used in sorting cells that are too delicate and precious for direct physical handling, a quality perfectly suited for non-invasive embryo sorting/grading in ART.

2.10. Assisted reproduction and the requirement for embryo grading

Infertility is defined as “a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse” by the International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) (WHO, 2014). Global fecundability rate indicates that 85% of women would conceive within 12 months of unprotected sexual intercourse. An identifiable cause could be attributed to 85% of the infertility cases. The leading causes are ovulatory dysfunction, male factor infertility and tubal disease (Carson and Kallen, 2021). Infertility assessment should be done after attempts

of conceiving for 12 months. Treatment of infertility could be any of the multitudinous methods of surgical and/or medical interventions depending on the cause of infertility and the age of the patients in both instances of male and female infertility. In cases where the cause of infertility cannot be corrected by more conventional medical or surgical interventions, ART should be attempted.

In vitro fertilization (IVF) and intracytoplasmic sperm injection (ISCI) are the most common forms of ART used in the clinical setup. Intrauterine insemination (IUI) is also used widely but not considered an ART according to the definition by ICMART, "all treatments or procedures that include the *in vitro* handling of both human oocytes and sperm or of embryos for the purpose of establishing a pregnancy"(WHO, 2014). In almost all ARTs, the ova and sperm are fused outside the female reproductive tract and transferring the resulting embryo/s to the uterus, thus the "*in-vitro*" moniker.

In the infancy of ARTs, multiple embryo transfer was the norm. The success rate of ARTs was very low (less than 10% in patients aged less than 35 years) and multiple embryo transfers were seen as a method of raising the odds of success (HFEA, 2020). However, even though multiple embryo transfers indubitably increased the success rate of embryo transfer, the rate of multiple births was also high. For example, in 2017, a delivery rate of 33.3% was observed in patients who underwent elective single embryo transfer (eSET) compared to 34.8% delivery rate of patients who underwent elective dual embryo transfer (eDET). However, the rate of multiple births was 2.2% in eSET group and 26.2% in eDET group, indicating a tenfold risk of multiple births in multiple embryo transfer (*Reports & Publications | ICMART*, 2021). Multiple births are riskier to the mother and the foetuses since it often leads to preterm deliveries and all the associated health risks such as low birth weight, cerebral palsy, long-term lung and gastrointestinal problems. Reports indicate that more and more embryo transfers are done in eSET configuration and the multiple birth rate has been declining as a result (HFEA, 2020). Thus, eSET is seen as a method of giving the pregnancy the best chance of success while circumventing the high possibility of multiple pregnancies introduced by the more traditional multiple transfer of embryos (Pandian *et al.*, 2004; Klitzman, 2016).

With the increased popularity of single embryo transfer, where only the "best" available embryo is transferred per cycle, the need for embryo grading has also increased. To most of the practitioners of ARTs, the term "best" embryo for transfer is synonymous with the "morphologically best" embryo from the batch of embryos produced after IVF or ICSI. Embryo morphology has been used as the main criteria for embryo selection since the beginning of ARTs (Nasiri and Eftekhari-Yazdi, 2015). There are multiple embryo scoring methods developed to assess the morphological quality of the gametes (Scott and Smith, 1998; Tesarik *et al.*, 2000), zygotes (Scott, 2003; Brezinova *et al.*, 2009), morulae and the blastocysts (Gardner *et al.*, 2000). Morphological evaluation, at its basic format, is a relatively simple method that does not require any of the more expensive equipment but the trained eye of the embryologist. However, there are some major drawbacks in the system; mainly the subjectivity of the scoring

and the low success rate of the selected embryos (Nasiri and Eftekhari-Yazdi, 2015). Morphokinetic methods of embryo evaluation such as time lapse imaging endeavors to overcome the subjectivity of the observations by introducing algorithms based on the major milestones of embryo development while reducing the stress of constant out-of-the-incubator observations (Zaninovic, Irani and Meseguer, 2017; Gallego, Remohí and Meseguer, 2019; Babayev and Feinberg, 2020). The success rate of time-lapse based morphokinetic evaluations such as the EmbryoScope system, is still a matter of scientific inquiry (Rubio *et al.*, 2014; Holschbach *et al.*, 2017).

A number of non-morphological and non-morphokinetic methods have been developed to evaluate embryos for transfer. They can be broadly classified into two categories. One type of techniques aims to categorize embryos by their metabolic parameters such as oxygen and glucose consumption (Ferrick, Lee and Gardner, 2020), the amino acid turnover in the culture media (Houghton *et al.*, 2002), existence and the quantity of various biomarkers such as hGC (Xiao-Yan *et al.*, 2013), soluble HLA-G (Sher *et al.*, 2005), leptin (González *et al.*, 2000), ubiquitin (Katz-Jaffe, Schoolcraft and Gardner, 2006; Wiener-Megnazi *et al.*, 2011) and glutamate (Seli *et al.*, 2008) *etc*. in the culture media. Most of these techniques are recommended to be used in conjunction with morphological parameters and despite being theoretically viable, are not in widespread commercial use.

The other type of non-morphological embryo evaluation techniques involves embryo biopsy to quantify the genomic (A. Y. Wang *et al.*, 2018) and transcriptomic (Groff *et al.*, 2019) characteristics of the embryo. Embryo biopsy is extensively utilized to evaluate the genomic status of the embryo in terms of genetic defects and ploidy (Zacchini *et al.*, 2017). Embryo biopsies are performed at various stages of embryo development and there are reliable reports that suggest its effectiveness in detecting genomic defects of the pre-implantation embryo (Cimadomo *et al.*, 2016). However, a biopsy is always an invasive procedure and the subsequent development of the embryo and its potential of implantation (Zhang *et al.*, 2016) may be affected by the biopsy procedure.

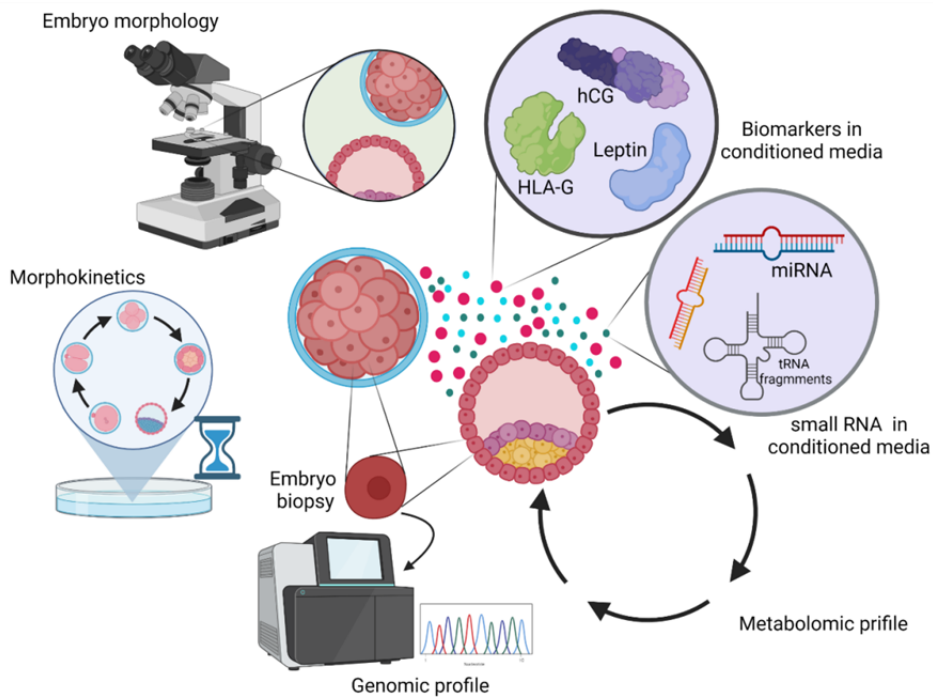


Figure 4. Methods of embryo quality evaluation
Embryo quality is conventionally evaluated by microscopic examination. A modified approach to microscopy is time lapse microscopy or morphokinetics where not only the embryo morphology is evaluated, but also the development of the embryo is quantified using the time taken to reach developmental milestones. There have been efforts to evaluate embryo quality by examining biomarkers like human leukocyte antigen-G (HLA-G) and small RNA in the embryo conditioned media. Metabolomic qualities such as oxygen consumption and amino acid turnover has also been utilized as a marker for embryo quality. Invasive embryo biopsies are used to profile the genomics and transcriptomics of the embryo to identify possible disorders.

2.11. The need of evaluating the functional competency of embryos

Neither morphological nor known non-morphological approaches measure any functional characteristics of the embryo that is significantly important in the process of embryo implantation. Implantation failure accounts for 75% of the failed attempts of assisted reproduction when morphologically “good” embryos are used (Bashiri, Halper and Orvieto, 2018). However, there are reports that refute the impact of implantation failure on the low rate of success in assisted reproduction (Pirtea *et al.*, 2021). The scope of this thesis deals with applying the earlier hypothesis that changes in recipient cells induced by EV-mediated intercellular communications can be used as a method of identifying subtle

differences in the cells that originated the communication (cells that produced the EVs) to the embryo-maternal communication.

The success rate of embryo transfer remains less than 50% in ARTs despite using the most technologically complicated and expensive methods to sort embryos (HFEA, 2020). The apparent inadequacy might be stemming from the criteria used to grade the embryos. Many morphologically superior embryos fail to result in a pregnancy while some morphologically inferior embryos find success. Some functional factor independent of embryo morphology, metabolic state (measured by many of the non-invasive systems of grading) or genetic makeup (measured in embryo biopsies) could be a key factor in implantation success. It can be hypothesized that embryos which communicate correctly with the maternal tissue would be more successful in resulting in positive pregnancies. In other words, an embryo grading system based on embryo functionality would result in a higher success rate of ART.

2.12. Models for studying embryo-maternal communication

Studying the communication between the embryo and the maternal tissue is experimentally challenging because not only of the associated ethical and legal issues but also of the extremely localized nature of the communication. The effects usually occur only in the site of communication, and it is difficult to isolate the messenger molecules/ EVs from the biological fluids involved. Therefore, many models have been developed over the years to mimic the embryo-maternal microenvironment.

Animal models are used in hopes to provide a more holistic picture of the complicated events in embryo implantation. Many different animal models (mouse and rat, Guinea pig, rabbit, pig, sheep, cow and primate) have been used to gather significant information about embryo implantation (Lee and DeMayo, 2004). However, murine models are thought to resemble the human implantation the most (Ban, Knöspel and Schneider, 2020). There are definite advantages in using animal models for embryo-maternal cross talk studies such as the reduced ethical and legal constrains governing the use of animals, the ability of develop distinct models with gene deletions, relative ease of handling and relative abundance of material. On the other hand, since the known mechanisms of human implantation are unique to humans, it is difficult to develop an *in vivo* system that can reasonably mimic the events in human implantation. Even murine models, which resemble the implantation process to a degree, have differences in the gross morphological sense in stages such as epithelial invasion (Weimar *et al.*, 2013; Ban, Knöspel and Schneider, 2020). The underlying signaling mechanisms could be even more drastically different. While using an animal model allows the researchers to investigate the mechanisms of implantation in a holistic manner, it is difficult to isolate and study individual signaling mechanisms involved in the embryo-maternal communication aspect of the

implantation process. Therefore, for the purposes of this thesis, *in vitro* models that endeavor to resemble the pre-implantation embryos and the maternal tissue were used to better elucidate the individual mechanisms that govern the events embryo development and implantation.

In vitro models have the advantage of convenience and ease of handling compare to any animal model. The results are more scalable and easier to standardize by using standard cell lines. Primary cells representing the maternal tissue such as endometrial epithelial cells, oviductal epithelial cells and endometrial stromal cells have been used in studies (Petersen *et al.*, 2005; Gellersen *et al.*, 2013). These cells best represent the characteristics of endometrial epithelium, the oviductal epithelium and the endometrial stroma. However, due to the cyclical nature of the female reproductive tract, the cells need to be harvested at the right time and terminally differentiated cells such as endometrial epithelial cells perform poorly in *in vitro* environments. The benefits of primary cell culture should be calculated along with the disadvantages of availability, difficulties in handling and variability of results.

Considering the embryos, there has been studies conducted using donor human embryos and experimental embryos in animal assisted reproduction situations, however, the likelihood of obtaining such precious material is slim in most cases (González *et al.*, 2000; Caballero-Campo *et al.*, 2002).

Established cell lines can be used as an alternative for primary cells. There are a number of cell lines available to represent both the embryo and the maternal tissue. Spheroids made out of embryonic cell lines have been used as an embryo surrogate. The spheroid configuration is intended to better represent the trophoblast/ pre-implantation embryo. The human choriocarcinoma cell line JAr is the most commonly used cell line in a spheroid configuration. First described in 1968 by Pattillo and Gey, JAr cells have both villous and extravilluos characteristics and express hCG and progesterone (Weimar *et al.*, 2013). The cell line has been used extensively in embryo attachment and invasion models. Apart from JAr, Jeg-3, Sw-1 and BeWo cell lines can also be used as analogues for the trophoblast (Weimar *et al.*, 2013). The JAr cell line was used in the studies described within the thesis because of the large pool of existing data and protocols of being used in a spheroid formation to represent the trophoblast.

There are multiple cell lines that can be used as a surrogate for receptive endometrium. Ishikawa, ECC-1 and RL95-2 cell lines are the most commonly used options for studies of this nature. Other cell lines such as HEC-1A exist but are not considered as receptive. Both RL95-2 and ECC-1 are of epithelial carcinoma origin and Ishikawa cell line is of adenocarcinoma origin. There are reports that consider Ishikawa cells as less receptive compared to RL95-2 cell line (Vergaro *et al.*, 2019). There are speculations that ECC1 cell line is a derivative of Ishikawa (Korch *et al.*, 2012). RL95-2 cell line has been used in conjunction with JAr derived spheroids to study embryo attachment. The cell line is limited by the fact that the original donor was 69 years old when the cells were harvested and already in menopause (Ban, Knöspel and Schneider, 2020).

Despite the obvious advantages of convenience, using cell lines in embryo-maternal communication models can introduce some limitations to the experimental rigor. Since they are of cancerous origin, it can be argued that the models do not represent correct physiological conditions of the uterine micro-environment. The well characterized attributes of cell lines could be altered by constant passaging, contamination by pathogens like mycoplasma or contamination my other cell lines (Korch *et al.*, 2012).

2.13. Summary of literature review

Embryos and the maternal system communicate constantly in the peri-implantation period using numerous biomolecules as signal carriers. This communication is reported to be crucial in embryo development, implantation and a successful pregnancy. Endocrine, paracrine and juxtracrine signalling methods are all used in embryo-maternal cross talk. The physiological outcomes of these communications are thought to alter the physiology of the maternal tissue and the nature of the embryo to facilitate a successful pregnancy.

Embryo maternal communication could be a method of measuring the functional competency of an embryo. In assisted reproduction, the main reason for low rates of success is failure of the transferred embryo to implant in the endometrium. Some experts posit that the failure to implant may be partially due to the failure of the embryo to alter the endometrium sufficiently for a successful implantation. Today, selection of embryos for transfer is carried out largely based on embryo morphology. However, a method of embryo selection based on the functional competency of each embryo would be more efficient and accurate.

A novel method described in the context of embryo maternal communication is extracellular vesicles. Observed in all biofluids, EVs are nanometre sized membrane bound carriers of biologically active compounds utilized in intercellular communication. There are multiple reports of EVs being involved in embryo-maternal communication throughout the embryo development process.

The mechanisms of EV-mediated embryo maternal communication are not clearly understood. Observed evidence has led to several theories into how EVs are used in intercellular signalling. It has been hypothesized that the cargo of EVs is released to the cytoplasm of the target cell where the uptaken biomolecules, especially RNA, can alter the transcriptome of the target cells using known gene expression regulation mechanisms. There is also evidence of EVs acting as cell membrane signalling ligands, activating receptors on the cell membrane and inducing significant effects on the target cells.

3. AIMS OF THE DOCTORAL PROJECT

The overall aim of the doctoral project was to investigate the non-coding RNA based embryo-maternal communication utilizing EVs as molecule carriers in order to elucidate the mechanisms that embryo employs to alter the endometrial physiology.

Specific aims of separate sub-studies

Study 1: To identify RNA transferred from human trophoblast analogue (JAr spheroid) to human mid-secretary endometrial epithelial analogue (RL95-2 cells).

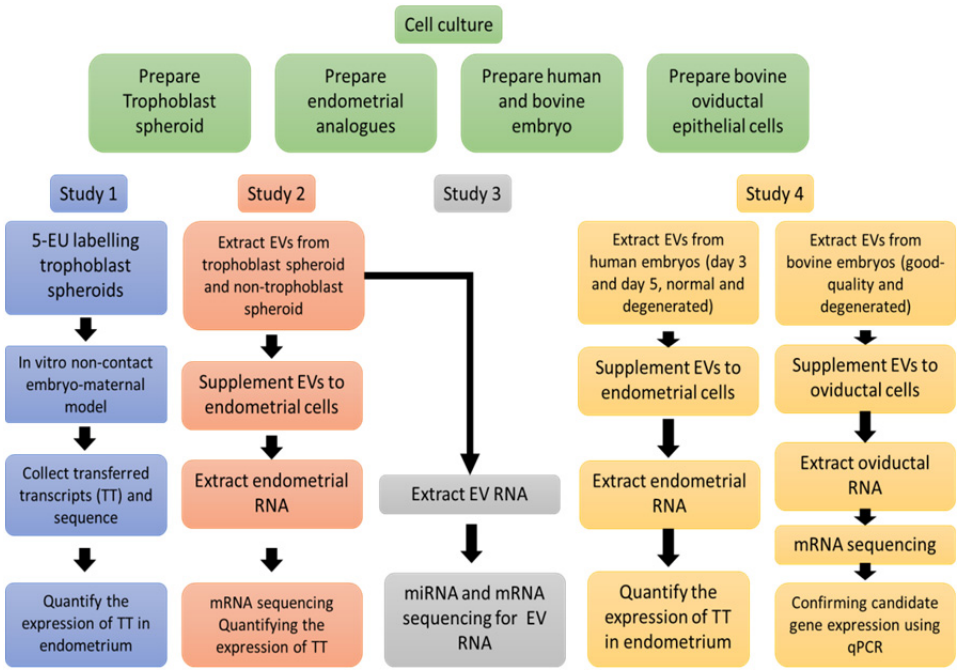
Study 2: To evaluate the overall effects and specificity of trophoblast spheroid-derived EVs on the transcriptome of the endometrial analogue.

Study 3: To investigate the RNA cargo of trophoblast spheroid-derived EVs and correlate the biologically active ncRNA to the effects observed in study 2 in an attempt to elucidate the mechanism of EV induced effects on endometrial analogue.

Study 4: To investigate the maternal tissue effects of morphologically good quality embryo-derived EVs compared to morphologically bad quality embryo-derived EVs in both human and bovine systems to identify a non-invasive embryo grading method.

4. METHODS

4.1. Overall Experimental Plan



4.2. Cell culture

4.2.1. Culturing trophoblast and endometrial analogues in the in vitro embryo-maternal communication model

All the immortalized cell lines were purchased from the American Type Culture Collection in Teddington, United Kingdom. All the cell lines were cultured in 37°C and 5% CO_2 environment. The endometrial analogue cell line was prepared using RL 95-2 cells (human adenosquamous carcinoma, ATCC CRL-1671). The culture medium for the RL95-2 cells consisted of Dulbecco's modified eagle medium (DMEM 12-604F, Lonza, Verviers, Belgium), 5 µg/ml human recombinant insulin (Gibco, Invitrogen, Denmark), 1% Penicillin-Streptomycin (P/S, Gibco™ 15140122, Bleiswijk, Netherlands), 1% glutamine (Sigma, 59202C, Saint Louis, USA) and 10% foetal bovine serum (FBS, Gibco™, 10500064). Cells were cultured in 37°C and 5% CO_2.

The trophoblast analogue, human first trimester choriocarcinoma cell line JAr (HTB-144 ™, Teddington, UK) cells were cultured in the following culture media. RPMI 1640 (Gibco, Scotland), 1% L-glutamine, 10 % FBS, 1% penicillin/ streptomycin. When the cells reached 80% confluency, the cells were collected

using the trypsin-EDTA method and re-suspended in 5ml of complete medium (1×10^6 cells/ml concentration) and shaken overnight using a gyratory shaker (295 rpm, BioSan, PSU-2, 37°C, 5% CO_2). The resulting spheroids were used as the analogues for the hatched trophoblast.

The well-established embryonic kidney cell line HEK293 was used as a negative control. The cells were cultured in DMEM high glucose medium containing 1% L-glutamine, 1% penicillin/streptomycin and 10% FBS. The culture medium was changed every other day until the required confluency was reached. Then the cells were subjected to the spheroid formation method mentioned earlier in the context of JAr spheroids.

4.2.2. Labelling spheroid RNA using 5-ethynyluradine (5-EU)

Trophoblast spheroids were incubated in complete medium supplemented with 0.2 mM 5-EU solution for 18h under 295 rpm gyratory shaking. After the incubation, the spheroids were washed three times in un-supplemented RPMI 1640 medium by letting the spheroids sediment momentarily and removing the supernatant. At the end of washing, the spheroids were immediately used for co-culture experiments.

4.2.3. Trophoblast-endometrium non-contact co-culture system

Endometrial cells (RL 95-2) were cultured in 6 well plates and maintained until 80% confluency. When the endometrial cells reach confluency, translucent cell culture inserts with 0.4 μm pores were introduced to each well (Falcon). The distance between the porous surface and the endometrial cell layer was about 1 mm trophoblast spheroids were placed in the insert facilitating a close proximity to the endometrial cells without physical connection. Approximately 2000 spheroids were used per insert. The co-culture was incubated for 24 hours in a starvation medium prepared using DMEM (DMEM/F12, Verviers, Belgium v/v 1:1) supplemented with 1% L-glutamine, 1% P/S, transferrin (10 mg/ml; BioReagent, T8158), selenium (25 mg/L; Sigma, 229865), bovine serum albumin (1 mg/ml; HyClone™, SH30574), linoleic acid (4.7 mg/ml; Sigma, L1012) and insulin (5 mg/ml).

4.2.4. Imaging for 5-EU labelled RNA

After co-incubation, the inserts containing the trophoblast spheroids were removed and the starvation medium was replaced with pre-warmed PBS for washing to remove any unbound 5-EU. Washing step was repeated three times and then the endometrial cells were incubated in pre-warmed cytoplasmic dye solution (Cell Tracker™ Deep Red dye; Life Technologies, C34565) for 30 in the dark incubator. Cytoplasmic dye was used as a contrasting material to the 5-EU labelled RNA and to detect and confirm EV uptake. After the incubation with the dye, the cells were washed three times with warm PBS, fixed using 4% formaldehyde and permeabilized using Triton X-100 (0.1 % solution). 5-EU

labelled RNA were identified using the Click-iT nascent RNA tracking kit (Invitrogen, C10329) following the manufacturer recommended immunofluorescent protocol. The green (Alexa Flour 488) 5-EU labelled RNA were identified in the contrasting (Cell tracker deep red) cytoplasm using LSM510 Laser Scanning Confocal Microscope (LSM 510 Duo; Carl Zeiss Microscopy GmbH, Jena, Germany).

4.2.5. Primary bovine oviductal epithelial cell (BOEC) culture

Slaughterhouse sourced bovine oviducts attached to ovaries were transported to the cell culture facility in 37°C normal saline within 4 hours of slaughter. Oviducts were washed with 1% penicillin/streptomycin and 1% Amphotericin B in PBS solution to remove bacterial and fungal contaminants. Isthmus and ampulla were separated and the oviductal mucosa was squeezed out between two sterile glass slides. Then, cells were collected in a sterile conical tube containing 1% penicillin/streptomycin, 1% Amphotericin B and 5% FBS in PBS. Cells were washed twice in this medium by centrifuging at 180 × g for 2 min in 4°C. The final cell pellet was resuspended in DMEM/F12 medium supplemented with 10% FBS, 1% Penicillin/Streptomycin and 1% Amphotericin B and cultured for 72 hours in 5% CO_2 and 38°C conditions. When cells reached confluency, they were trypsinized and stored in liquid nitrogen until the experiment. All experiments were conducted with the second passage.

4.2.6. Supplementing embryo derived EVs to BOEC culture

Frozen BOEC cells were thawed and seeded into 100 mm cell culture dishes in DMEM/F12 medium supplemented with 10% FBS and 1% penicillin/ streptomycin. Once he cells reach confluence, they were trypsinized and seeded into 24 well plates with 5×10^4 cells per well. When the cells reached 80% confluency, the medium was removed and the residual medium was washed away with PBS. PBS was replaced with 400 µl of synthetic oviductal fluid medium supplemented with 0.8% BSA and 100 µl of extracted EVs. The cells were incubated in this supplemented medium for 8h at 5% CO_2 and 90% N_2 in 38.8°C.

4.2.7. Depleting EVs from FBS using ultrafiltration

In 2018, Kornilov *et al.* described a method of depleting EVs from FBS without compromising the material makeup of the reagent. The ultrafiltration method was used with modifications to deplete EVs from FBS that would later be used in the culture media. FBS was centrifuges in a 100 kDa cut-off ultrafiltration unit (100 kDa, MERCK KGAA, Darmstadt, Germany) for 55 minutes in 3000x g. The filtrate was found to be up to 95% EV depleted(Kornilov *et al.*, 2018).

4.3. Embryo culture

4.3.1. Human embryo culture

All the experimentation carried out using human embryo conditioned media were sanctioned by the approval no. 2567/T2 of the research ethics committee of University of Tartu. The embryos were fertilized using either IVF or ICSI and cultured for 5 days using the Origio's Sequential series culture system. The first 17–21 hours of embryos culture was carried out in sequential fertilization medium (Sequential Fert™, Origio, Måløv, Denmark). The embryos were grown in sequential cleavage stage medium (Sequential Cleav™, Origio) for the next 48 hours and sequential blastocyst medium (Sequential Blast™, Origio) was used in the final 48 hours. Embryos were morphologically evaluated every day and separated into two groups depending on the results. At day 3, "normal" embryos presented with equal sized blastomeres with no fragmentation. At day 5, "normal" embryos were selected by identifiable blastocyst cavity, inner cell mass and the trophoblast. Embryos that did not exhibit the desired level of morphological character were considered "degraded".

4.3.2. Bovine embryo preparation

In vitro bovine embryo production protocol described by Nõmm et al was followed with modifications (Nõmm *et al.*, 2019). Bovine ovaries were sourced from the regional slaughterhouse and delivered to the embryo laboratory within 4h of collection. Samples were washed with twice in 0.9% NaCl and cumulus oocyte complexes (COCs) were aspirated from the follicles with the diameter of 2-8mm using a vacuum pump (Minitüb GmbH, Germany). *In vitro* maturation (IVM) was performed on selected quality code 1 COCs in groups of 50 in IVM medium (0.8% fatty acid free BSA fraction V supplementation) for 22h to 24h.

Frozen semen samples were used for IVF of matured COCs in concentrations of 1×10^6 spermatozoa per ml. For fertilization, the COCs were co-incubated with spermatozoa in groups of 50 in FERT-TALP medium (500 ul) in 4 well plates at 38.5^0C.

Presumptive zygotes were separated from cumulus cells by vertexing and the separated zygotes were individually cultured for 8 days in 60 ul droplets of modified synthetic oviductal fluid (myo-inositol and amino acid supplemented). Developmental stages of embryos were morphologically characterized on days 2,5 and 8 post-fertilization. Developmental stages were identified as cleavage, morula and blastocyst (Bó and Mapletoft, 2013). Culture media without zygotes were incubated the same duration of time to prepare the "day 5 control" samples.

4.4. EV preparation

4.4.1. Collecting and processing conditioned media from *in vitro* embryo-maternal communication model

Conditioned media of cells grown for 24h in EV depleted medium were used as the source for EVs. Conditioned media were removed from the cells in aseptic conditions and transferred to sterile 50 ml falcon tubes for further processing. The media were then sequentially centrifuged in 400 × g for 10 min to remove cells, 4,000 × g for 10 min to remove cell debris and 20,000 × g for 15 min to remove apoptotic bodies. The conditioned media were then concentrated to 500 µl using 10 kDa ultrafiltration units (Amicon® Ultra-15, Merck Millipore, Burlington, Massachusetts, United States). Concentrated media were used for size exclusion chromatography.

4.4.2. Collecting and processing human embryo conditioned media

Embryo conditioned media were sequentially centrifuged at 400 × g for 10 min and 2,000 × g for 10 min to remove cells and debris. Then the media were concentrated using 10 kDa cut-off centrifugal filter units (Amicon® Ultra-15). Nanoparticles were prepared using size exclusion chromatography.

4.4.3. Bovine embryo conditioned media collection and processing

Embryo conditioned media were collected on the 5^{th} day after fertilization. The embryo was further cultured in the remaining medium for three further days to determine the final morphological quality. Collected media samples were grouped according to the morphological character of each embryo the 2^{nd}, 5^{th} and 8^{th} day of culture. Embryos that exhibited normal growth on day 5 and subsequently developed into blastocysts by the 8^{th} day were termed “good quality embryo media”. Embryos that initially achieved the cleavage stage on day 2 but degenerated thereafter were termed “bad quality embryo media”.

Forty (n=40) samples from each group (“good quality embryo media”, “bad quality embryo media” and “control media”) were pooled and subjected to sequential centrifugation to remove any single cells, cell debris or apoptotic bodies. Conditioned media were centrifuged 400 × g for 10 minutes and 2000 × g for 10 minutes and then concentrated to 150 µl using 10 kDa ultrafiltration units. Concentrated media were subjected to SEC using commercial columns to extract EVs.

4.4.4. Size exclusion chromatography

Size exclusion chromatography method used in the three studies were different implementations of the same scientific principle. For studies 1, 2 and 3, self-

packed columns were used. Sepharose beads (cross linked 4% agarose matrix of 90 µm beads) were used in both cases (Sepharose 4 fast flow™, GE HealthCare Bio-Sciences AB, Uppsala, Sweden). The column packed for the study 1 was 30 cm and the column for second and third study was 10 cm. In both cases, a 500 µl sample was added to the top of the column and fractions 7 to 10 of 1 ml fractions were collected in DPBS. Collected fractions were then further concentrated using 10 kDa ultra filtration units.

Pre-packed columns purchased from Izon Sciences were used to extract EVs from Bovine embryo conditioned media (study 4) because of the smaller volume of the initial sample (qEVsingle/70nm by Izon Sciences, UK, product code SP2). qEVsingle/70 nm columns were used with fresh filtered DPBS as the elution buffer. The 150 µl samples were loaded to the top of the columns and fractions 6–9 of 200 µl fractions were collected. Collected fractions were further concentrated using 10 kDa ultrafiltration units

4.4.5. Nano particle tracking analysis (NTA) for EV size and concentration

The ZetaView nanoparticle analyser was used to measure the size and the concentration of collected EVs (ZetaView, Particle Metrix GmbH, Inning am Ammersee, Germany). The NTA was calibrated using 100nm NanoSphere size standards prior to every measurement (Thermo Scientific, USA. 3100). The quality of the calibration was also measured daily by running the daily performance, trueness and precision evaluations. Before every measurement, the elution buffer used (DPBS) was loaded in to the analyser to confirm that the elution buffer did not contain a significant number of nanoparticles. Samples were measured using 75 (sensitivity), 100 (shutter) and 30 (frame rate). Each sample was measured 3 times across 11 focal planes. The sample planes were washed Milli-Q water and DPBS between sample measurements.

4.5. Western blot analysis for human EVs

Purified EV proteins were subjected to western blotting to confirm their EV origin. Following the guidelines set forth by the minimal information for studies of EVs (Théry *et al.*, 2018), three positive markers (CD9, CD81 and CD63) and one negative maker (Apo-A-I) was investigated. EV proteins were precipitated using methanol and chloroform. 30 µg of protein was used either in reducing (Apo A-I) or non-reducing (CD63, CD9 and CD81) Laemmli buffer. After resolving in 12.5% SDS-PAGE, proteins were transferred to polyvinylidene difluoride membrane. The primary antibodies, anti-CD63 (Santa Cruz), anti-CD9 (Thermo Fisher Scientific), anti-Apo A-I (Santa Cruz), and anti-CD81 (BD Biosciences), were then introduced in 5% non-fat milk for 24h on 4^{o}C. The secondary antibody, horse radish peroxidase conjugated goat anti-mouse secondary antibody (Santa cruz) was the introduced for 1h in RT. After washing, the

protein bands were visualized using detection reagent (ECL Select™, GE healthcare) and ECL imager (ImageQuant™, GE healthcare). Western blot protocol was initiated, optimized and performed by Dr. Janeli Vill.

4.6. Electron microscopy

Transmission electron microscopy (TEM) was performed to confirm the shape of nanoparticles using the protocol published by Théry and the group in 2006 (Théry *et al.*, 2006). Briefly, Particles were fixed with 1% glutaraldehyde (Sigma) and 2% paraformaldehyde (Sigma). Uranyl oxalate was used for contrasting and the samples were visualized using JEM 1400 TEM (JEOL, Japan) at 80 kV and imaged using a numeric camera (Morada, Germany). TEM protocol was initiated, optimized and performed by Prof. Aneta Andronowska of the Polish Academy of Sciences, Olsztyn, Poland.

4.7. RNA extraction

4.7.1. RNA extraction and quality control

RNA was extracted using phenol-chloroform method with TRIzol reagent acting as the chaotropic agent (TRIzol® reagent; Invitrogen). Concentrated EVs or adhered cells were used as the RNA source. In case of EVs, the TRIzol reagent was added directly to the solution. In case of adherent cells, the conditioned medium was removed and then the TRIzol reagent was added directly to the cell layer. The samples were then left in room temperature for 10 minutes to completely dissociate the nucleoprotein complexes and 300 µl of chloroform for 1 ml of TRIzol was added. The samples were then vigorously shaken for 15 seconds and centrifuges at 12000 × g for 15 min in 4˚C. When the phases were separated, the aqueous phase containing the RNA was removed and the RNA was precipitated using 500 ul of isopropanol in room temperature for 20 minutes. Glycogen (10 µg, UltraPure™ Glycogen, 10814-010, Thermo Fisher Scientific, Bleiswijk, Netherlands) was added to the mixture to increase the efficiency of RNA extraction. Once the precipitation was done, the samples were centrifuged at 18000 × g for 30 min at RT to pellet the RNA. The RNA pellets were washed three times in 70% ethanol and eluted in 20 ul of nuclease free water. Quantity and the quality of RNA were measured using the Qubit HS RNA kit and the Agilent Pico 6000 kits (Agilent technologies, Santa Clara, CA).

4.7.2. Affinity precipitation of EU-labelled RNA (Study 1)

The Click-It nascent RNA capture kit was used to selectively precipitate 5-EU labelled RNA from the collected pool of RNA (Thermo Fisher Scientific, Waltham, MA; C10365). First the whole RNA samples were incubated with

1 mM biotin azide (PEG4 carboxamide-6-azidohexanyl biotin) solution from the click-it reaction mixture for 30 min at RT in a gyratory shaker to attach biotin to the alkyne group of the 5-EU. The Biotinylated RNA were selectively enriched using streptavidin T1 magnetic beads (MyOne™ Streptavidin T1 magnetic Dynabeads®) by incubating with 12 µl of bead solution in the dark for 40 min using a gyratory shaker. After the incubation, the non-specifically bound RNA was washed away using the buffers included in the kit while subjecting the samples to 700 rpm vigorous shaking. After the final wash, the beads were immobilized in a magnetic rack (DynaMag™-2) and the wash buffers were removed. Isolated beads were re-suspended in nuclease free water and used for library prep and/or qPCR. Affinity precipitation protocol was optimized by Dr. Masoumeh Es-Haghi.

4.8. RNA sequencing

4.8.1. Library preparation for affinity precipitated RNA fragments on beads (Study 1)

cDNA library preparation was carried out using the Ovation RNA-seq system V2 (NuGEN technologies, San Carlos, CA, 7102-32) with slight modifications to accommodate RNA fragments immobilized on beads. First strand primer mix was added to the RNA fragments immobilized on beads and incubated at 65°C followed by an ice bath. Then, first strand buffer and first strand primer mix were also added to the mixture. The mixture was kept at 43. 5°C for 1h while shaking at 800 RPM to avoid the bead settling. The reaction mixture was heat shocked at 85°C and DNA was separated from the beads by rapid magnetic immobilization of beads. Collected cDNA was used for double strand DNA synthesis. Prepared cDNA was quantified using Agilent high sensitivity DNA 1000 kit. Libraries were prepared using the AB library builder system (Thermo Fisher, 4477598) and Io Ion Xpress™ Plus Fragment Library Kit (Thermo Fisher), according to the manufacturer's instructions. The barcoded libraries were sequenced on two Ion 540™ Chips (ThermoFisher Scientific Inc, CA, USA, Cat. No. A27766) with four libraries per chip using the Ion S5 XL sequencer (Thermo Fisher Scientific Inc). IonTorrent Library prep and sequencing was performed by Ms. Annika Haling of the genomics core facility.

4.8.2. Library preparation for mRNA sequencing (Studies 2, 3 and 4)

RNA sequencing libraries were generated using multiplexing capacity of Smart-seq2 methodology with slight modifications (Picelli *et al.*, 2014). Total RNA (20 ng) was used in pre-amplification step using Phusion High-Fidelity DNA Polymerase (Thermo Scientific). Illumina Nextera XT DNA Sample Preparation Kit was used to prepare the dual-index libraries. Size selection (200–

700 bp) was performed using AMPure XP beads (Beckman Coulter). Pooled libraries were sequenced using Illumina NextSeq500 using High Output Flow Cell v2.5 (single-end, 75 bp). mRNA library prep was performed by Dr. Kersti Jääger.

4.8.3. Differential expression analysis of affinity precipitated RNA-seq data (Study 1)

Torrent mapping alignment program, a specifically optimized program to map ion-torrent data, was used to align the reads to the human reference genome hg19 utilizing the map4 algorithm with default parameters. read counts for 55,766 annotated coding and non-coding elements were used for differential expression (DE) analysis using the generalized linear model (GLM) pipeline of edgeR package in R (van de Lavoir *et al.*, 2006; Robinson, McCarthy and Smyth, 2009). Elements that counted more than 0.7 counts per million (CPM), a value translates into 10 aligned reads per element, in at least three of the four technical replicates were used for DE analysis. The differentially expressed transcripts were considered significant if the false-discovery rate (FDR) reported by edgeR was less than or equal to 0.05 (FDR $\leq$ 0.05). Integrative Genomics Viewer (IGV) was used to inspect the coverage of differentially expressed (enriched) transcripts.

4.8.4. Processing, alignment, and quantification of RNA sequencing (RNAseq) reads (Studies 2, 3 and 4)

The quality of raw reads was assessed using FASTQC v0.11.8 (Brown, Pirrung and Mccue, 2017). Trimmomatic v0.39 was used for read trimming and adaptor sequences removal (LEADING:20, SLIDINGWINDOW:4:15, ILLUMINACLIP: *:1:30:15 and MINLEN:25). Reads were the aligned to hg38 human genome (studies 2 and 3) or ARS-UCD1.2 *B. Taurus* genome assembly (study 4) using HISAT2 (Kim *et al.*, 2019) with default parameter. Splice site information was derived from Ensembl H. sapiens annotation file (GRCh38.97) for studies 2 and 3 and genome assembly annotation file version 1.2.97 (Kim *et al.*, 2019) for study 4. Read counts in the gene level were obtained by using featureCounts (Liao, Smyth and Shi, 2014). Genes with at least 10 counts for all the samples in at least one of the experimental groups were retained in the analysis for subsequent differential expression testing.

4.8.5. Differential gene expression analysis (Studies 2, 3 and 4)

edgeR package v 3.26.8 (Robinson, McCarthy and Smyth, 2009) running on R environment v 3.6.1 was used for DE analysis. Tagwise dispersion estimates were obtained based on the trended dispersions, and statistical comparisons were performed using a generalized linear model followed by likelihood ratio

tests, also accounting for the experiment batch. Differential expression was considered significant when FDR <0.05.

Gene set enrichment analysis (GSEA), and pathway over-representation analysis was conducted using the ReactomePA package (Yu and He, 2016)and Reactome Pathway database annotations (Yu and He, 2016). GSEA was used for full gene lists obtained from DE analysis that were ranked by -log10p × log2FC, where p denotes unadjusted p-values and FC the fold-change.

Principal components were calculated using prcomp function from the Stats package and visualized using the ggplot2 package (H. Wickham, 2016). The pheatmap package (Kolde, 2019) was used for heatmap visualization with hierarchical clustering based on Euclidean distance.

4.9. Quantitative real time PCR (qPCR)

To confirm and quantify the observations from RNAseq, qPCR was performed on identified transferred transcripts (Study 1) and significantly altered genes in the target cells (Study 4). The primers were designed using Beacon designer 8 (PREMIER Biosoft International, Palo Alto, CA) for optimal signals in a SYBR green based qPCR (Primer sequences are presented in the appendix). cDNA synthesis was carried out using a mixture of random primers and oligo dT and the reverse transcriptase (SuperScript® VILO™ cDNA synthesis kit, 11754 050) for study 1 and FIRESript RT cDNA Synthesis mix ™ with Oligo (dT) and random primers (06-20-00100, SolisBiodyne, Tartu, Estonia) for study 4. cDNA products were then amplified in EvaGreen assay system (Solis BioDyne, Tartu, Estonia). Melting curves were analysed using the fluorescence signals collected continuously from 65°C to 95°C at 0.05°C per second. qPCR data were analysed either with absolute quantification (Study 1) or relative quantification using ddCt method.

4.9.1. Spike-in and absolute quantification of qPCR data

Isopenicillin N-CoA synthetase gene (100 bp fragment) was used (Biomer.net company, Ulm/Donau, Germany, molecular weight: 32239 g/mol, 100 pmol/µl) was used for spike-in and absolute quantitation normalization. The synthetic RNA was diluted serially 20 times to generate a 4-x dilution gradient. A serial dilution of 4x was performed on the synthetic RNA. For the first serial dilution, 1 µl of synthetic RNA was added to 39 µl RNase-free water to final concentration of 2.5 µM. The diluted RNA was converted into cDNA using the same random primers, oligos and RT enzyme that was used in experimental qPCR. The cDNA samples were the amplified using unique primers designed on the template of the synthetic RNA. The Threshold values (Ct) were plotted against the number of RNA copies in each dilution and an exponential calibration curve was fitted. To normalize for the cDNA prep and RNA extraction processes, 1 µg of synthetic RNA was added to samples during the RNA extraction pro-

cess and the resulting cDNA were also measured for the expression of the synthetic RNA. A normalizing factor was added to the final calculation of the RNA copy number according to the ratio of the expected and observed expressions of synthetic RNA (Wang *et al.*, 2015). Spike-in normalization protocol was optimized by Dr. Masoumeh Es-Haghi.

4.10. miRNA sequencing

4.10.1. RNA extraction for miRNA sequencing

Conditioned medium from one 100 mm plate was used for the EV extraction for miRNA seq. The collected particles (approximately 1×10^8) were mixed with 100 ul of lysis buffer (RLT buffer, Qiagen). Then, 2 ul of pellet paint was added to the mixture, vortexed and mixed with 19 Ul of sodium acetate (3M, pH 5.5) and 300 ul of absolute ethanol. The mixture was vortexed and incubated overnight in +4°C. After incubation, the samples were centrifuged at 16000 × g for 15 min at 4°C and the pellet was collected washed twice in 80% ethanol and eluted in 10 ul of RNAse free water.

4.10.2. miRNA library construction and data analysis

The miRNA library was constructed for JAr and HEK293 EVs using 3 µl of extracted RNA. Methods described by Faridani et.al. were used with minor modifications (Picelli *et al.*, 2014; Hagemann-Jensen *et al.*, 2018). The amplified libraries were then purified using AMPure XP beads and eluted in RNase free water. Pooled libraries were sequenced using Illumina NovaSeq platform (National Genomics Infrastructure, SciLifeLab, Sweden). miRNA library prep and sequencing were initiated and performed by Dr. Omid Faridani and Dr. Nageswara Rao Boggavarapu.

Initially, the data analysis was carried out using the proprietary software PARTEK Flow by removing the mitochondrial and ribosomal contaminants. Unique molecular identifiers (UMI) were removed from the sequences and subsequently appended to the read names. The adapters and poly (A) sequences were removed and the trimmed reads were aligned to the human genome Hg38 using the Bowtie 2 aligner. After the alignment, the UMIs were deduplicated and the reads were quantified using the Hg39 miRBase version 22.

Three small RNA libraries from each EV type (JAr and HEK293) were examined for unique miRNA. The dataset was first filtered to retain miRNA that were present in 2 out of the three libraries of either JAr or HEK293 EV derived RNA and the further filtered for count thresholds of 1,3,5, and 10. A miRNA was considered specific to a library when it was observed with at least 5 counts in 2/3 of a dataset and not present at all in the other dataset.

A potential list of JAr miRNA specific targets were filtered from the predicted target list miRDB with the highest confidence of target (target score >90) (Chen and Wang, 2019). A list of putative targets were generated by converting

REFSEQ transcript IDs to ENSEMBL gene IDs using the R package AnnotationDbi (Pages, H., Carlson, M., Falcon, S. Li, 2008). The expression of putative targets in endometrial cells were selected using the ENSEMBL IDs of each transcript. The number of putative targets that were upregulated, downregulated or non-DE in RL95-2 cells treated with EVs were counted.

The abundance of each miRNA, expressed in mean CPM was correlated to the mean expression of downregulated targets (log2FC) in endometrial cells. Each miRNA was weighted according to the number of downregulated targets using the R package weights (*CRAN - Package weights*, 2021).

5. RESULTS

5.1. Experimemtal design for Study 1

5.1.1. Characterization of transcripts transferred from trophoblast to endometrial cells

Trophoblast spheroids were prepared using the trophoblast analogue human choriocarcinoma JAr cell line. RNA in the trophoblast spheroids were bio-orthogonally labelled using the uracil analogue 5 ethynyl uridine. Controls were prepared using unlabelled spheroids. The spheroids were co-incubated with the endometrial analogue RL95-2 cells in a non-contact *in vitro* embryo-maternal communication model. After 24h of co-incubation, the whole RNA from the RL95-2 cells were collected and 5-EU labelled RNA were affinity precipitated. The collected RNA samples were then used to prepare cDNA libraries and sequenced. There were (n=4) 5-EU labelled samples and (n=4) unlabelled control samples. Differentially expressed transcripts were bioinformatically identified. Any significantly differentially expressed transcripts were considered as transferred transcripts. Transfer of 5-EU labelled transcripts were confirmed using confocal microscopy and quantitative real time PCR.

5.1.2. Investigating the mode of RNA transfer between trophoblast and endometrial epithelium

A non-contact co-culture of 5-EU labelled and unlabelled trophoblast spheroids were prepared and the conditioned medium of each group was collected after 24h of incubation. Each sample of conditioned medium was separated into two equal parts in volume. One part of each sample was used to extract EVs using size exclusion chromatography. Then both parts (EVs and conditioned media) were subjected to RNA enrichment and qPCR was performed to quantify expression of identified transferred transcripts in each sample.

5.1.3. Transcriptomic effects induced by trophoblast /endometrial epithelial cell co-culture

A non-contact co-culture of trophoblast spheroids and RL95-2 cells was prepared using 12-well cell culture plates. After 24h of incubation, the whole RNA was collected from the RL95-2 cells an analysed for the expression of identified putatively transferred transcripts against a negative control of RL95-2 cells cultured in a co-culture system without trophoblast spheroids and a co-culture with HEK293 spheroids.

5.1.4. Effects induced by trophoblast EVs on specific endometrial transcripts

EVs were extracted from JAr cells cultured in EV depleted medium for 24h. The EVs were co-incubated for 24h with a monolayer of RL95-2 cells. The ratio between the particles and cells were 50:1. After the incubation, the RL95-2 cells were collected and the cellular RNA was analysed for the expression of identified transcripts. Expression of control genes (beta actin and beta-2-microglobulin) were used as an indicator of overall effects on cellular physiology. Control samples were prepared using untreated RL 95-2 cells and RL 95-2 cells treated with a similar amount of HEK293 cell derived EVs.

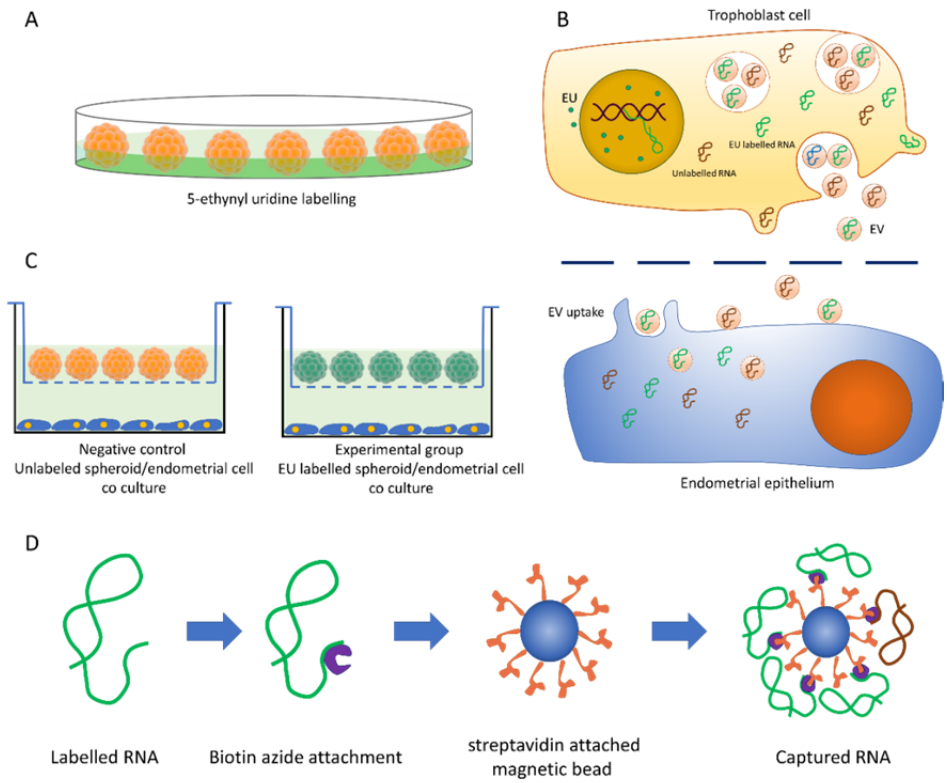


Figure 5. Experimental design for Study 1.
(A) Trophoblast analogues (JAr spheroids) were prepared using gyratory shaking and incubated with 5-EU for bio-orthogonal nucleotide labelling. **(B)** Hypothesis: The 5-EU is incorporated into new RNA and loaded into EVs in the MVB and cell membrane. EVs containing the labelled RNA is transported through the permeable membrane and uptaken by the recipient (RL95-2 endometrial analogue) cell. **(C)** Experimental groups for study 1: Negative control was prepared using a non-contact culture between unlabelled trophoblast spheroids and recipient endometrial cells separated by a translucent permeable membrane (0.4 µm pores). The experimental group was prepared with the same setup and 5-EU labelled trophoblast spheroids. **(D)** Capturing transferred trans-

cripts: Transferred transcripts were conjugated with biotin azide and captured using magnetic beads coated with streptavidin. Captured RNA was then sequenced using IonTorrent platform.

5.2. Characterization of trophoblast spheroid derived nanoparticles as EVs

Trophoblast derived EVs were extracted using the SEC method. Prepared nanoparticles were analysed for size and concentration using the NTA, electron microscopy was used to visualize the particles and the presence of EV specific protein markers were confirmed using western blot according to the minimum requirements for EV publications set up by the International Society of Extracellular Vesicles. Particles were of the typical cup shape and the size when visualized with TEM (Fig 6B) and NTA showed a population of particles ranging from 75-135 nm (Fig 6A). The specific EV markers CD63, CD81 and CD9 were all enriched in EV samples compared to the conditioned media while the negative marker apolipoprotein A1 was not enriched (Fig 6C), confirming the particles as EVs in all required parameters.

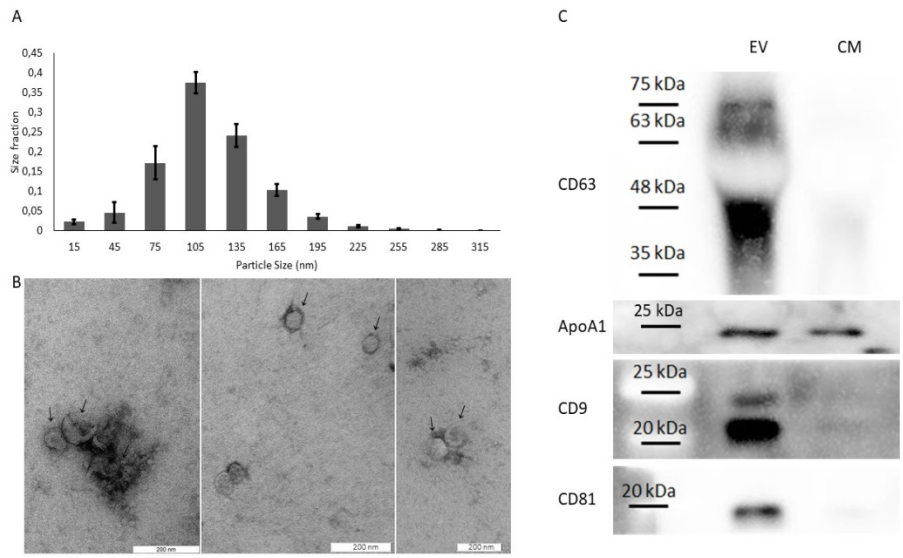


Figure 6. Characterization of trophoblast spheroid derived nanoparticles for EV characteristics
(A) The nanoparticles show the typical size profile for EVs in NTA. Most particles are less than 200 nm. Data is presented mean ± SEM **(B)** Nanoparticles have the typical "cupped" shape and clear lipid bilayers of EVs under TEM. Scale bar = 200 nm. **(C)** Nanoparticles (EV) are enriched with tetraspanins markers of EVs (CD9, CD81 and CD63) while not enriched in Apo A-1 compared to the conditioned media (CM).

5.3. Visualizing the 5-EU labelled transcripts by confocal microscopy

The labelled transcripts were visualized using Alexa 488 fluorescence and confocal microscopy. In trophoblast spheroids, labelled RNA was visible in nuclei and nucleoli (Fig 7A). In endometrial cells incubated with labelled trophoblast spheroids, 5-EU was observed in the cytoplasm (Fig 7B and 7C). Neither the possibility of RNA escaping into the cytosol nor subcellular localization was investigated. No signals were observed in unlabelled trophoblast spheroids or endometrial cells co-incubated with unlabelled spheroids (Fig 7-A1, 7-B1), confirming that 5-EU labelled RNA was transported between the two types of cells without physical contact. However, there is no evidence that once uptaken, the labelled RNA is exposed to the cytosol of the target cell.

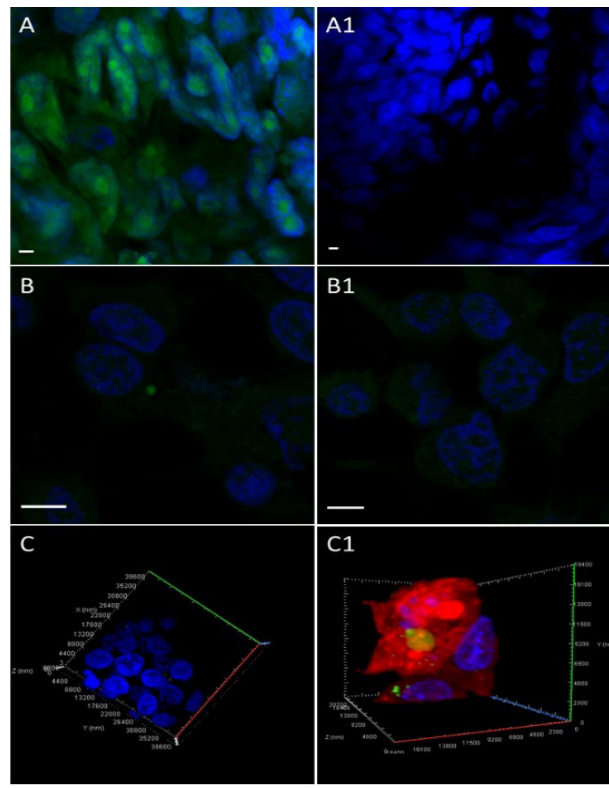


Figure 7. Visualizing the 5-EU labelled transcripts in using Alexa 488
(A) the green florescence of Alexa flour 488 indicates the labelled RNA in trophoblast spheroids. (A1) The fluorescent signal is not observed in the negative control. (B) Endometrial cells co-incubated with labelled trophoblast spheroids showed the presence of transferred transcripts. (B1) The fluorescent signal was not observed in endometrial cells co-incubated with unlabeled spheroids. (C, C1) 3D confocal image of endometrial cells showing that uptaken labelled transcripts (green) are accumulated in the cytoplasm (red). Scale bar 4 μm.

5.4. Identification of potentially transferred transcripts from trophoblast spheroids to endometrial cells

To identify any transferred transcripts between the trophoblast and the endometrium, trophoblast spheroids labelled with 5-Eu were co-incubated with endometrial cells in a non-contact system (experimental group). The controls were prepared by using unlabelled trophoblast spheroids. After incubation for 24h, the RNA from endometrial cells were collected and 5-EU labelled RNA were affinity precipitated. Precipitated RNA was sequenced and differential expression was calculated compared to the control group. The efficacy of the labelling procedure at a whole was calculated using the quantity of labelled RNA in 5-EU labelled spheroids and the quantity of whole RNA. The efficiency of RNA labelling was 12.66% (± 1.01%).

The efficiency of the affinity precipitation was calculated using the quantity of labelled RNA enriched from endometrial cells in the experimental group with labelled spheroids (2.85% ± 0.45%) vs. the quantity of RNA enriched by affinity precipitation in the negative control with unlabelled spheroids (1.13% ± 0.2%). The data show that the efficiency of labelled RNA recovery is low (~3%) and a significant portion (35%) of the recovered RNA were not labelled but were captured due to non-specific binding to the magnetic beads.

On average, the RNAseq procedure resulted in 13.5×10^6 reads per sample and on average 81% of the base pairs exceeded the Phred quality score of 20 (base call confidence > 99%). Reads were aligned to hg19 human reference genome and the average alignment percentages were 51% in experimental groups and 55% in control groups.

Eighteen transcripts of varying origins were identified as significantly enriched in the experimental samples compared to the controls and assumed "putatively transferred transcripts" (Table 1, Fig 8 B). However, given the inherent unspecific nature of the 5-EU labelling and low efficiency of the RNA capture system, the alignments were highly unspecific. Therefore, alignments of these elements were visually inspected using the IGV to confirm that the appearance of alignments as non-random. A transcript was considered specifically enriched when a transcript was

i. Detected in at least three out of four of the biological replicates in the experimental group
ii. Not detected in any of the negative control samples.

Three candidates fulfilled these stringent criteria (Fig 8 C, D). The selected candidates were ZNF81, an exonic region and an intronic region of the long non-coding RNA LINC00478. The transfer of 5-EU labelled transcripts was confirmed with qPCR (Fig 8 E, F, G) and the products of the PCR reactions were sequenced using sanger method to confirm their origin as the identified transferred transcripts.

Table 1. Putatively transferred transcripts.
List of putatively transferred transcripts between trophoblast spheroid and endometrial epithelium. logFC denotes the log expression change of the gene between experimental (endometrial cells co-incubated with labelled trophoblast spheroids) and negative control (endometrial cells co-incubated with unlabelled trophoblast spheroids). logCPM denotes the normalized abundance of each gene in log counts per million reads. FDR denotes the significance of data.

Gene ID	logFC	logCPM	FDR
MUC4	4,962	1,84E+00	1,64E-04
MUC3A	4,09E+00	3,69E+00	1,64E-04
MUC16	3,59E+00	3,57E+00	1,12E-03
MUC12	3,40E+00	2,98E+00	3,41E-03
ZNF81	4,43E+00	-2,96E-01	6,97E-03
RRAGB	4,22E+00	-7,88E-02	8,32E-03
MT-TW	2,84E+00	3,89E+00	2,13E-02
Z95704,5	3,72E+00	1,20E-01	2,48E-02
MT-TS1	2,67E+00	5,02E+00	3,29E-02
ITGAE	3,54E+00	9,15E-02	3,48E-02
RP11-357C3,3	2,98E+00	1,85E+00	3,48E-02
TMEM154	3,45E+00	4,12E-01	3,48E-02
CASP14	3,35E+00	4,68E-01	4,33E-02
ZNF765	3,31E+00	5,09E-01	4,45E-02
LINC00478	3,38E+00	-1,14E-01	4,69E-02
MT-TQ	2,56E+00	7,00E+00	4,85E-02
ANKRD44	3,22E+00	7,80E-01	4,85E-02
ZBED3-AS1	3,29E+00	-1,13E-01	4,85E-02

5.5. The intronic region of LINC00478 was observed in co-culture conditioned media

Conditioned media from the non-contact co-culture system with 5-Eu labelled spheroids and (experimental group) and unlabelled (negative control) were collected and divided into two parts. First portion was subjected to sequential centrifugation to remove cell debris and apoptotic bodies and then EVs were extracted using SEC technology. Collected EVs from each portion and the remaining conditioned media samples were then subjected to RNA extraction and affinity precipitation of labelled RNA followed by qPCR for candidate transcripts. The transcript intronic-LINC00478 was significantly enriched in the experimental conditioned media and to a much-reduced extent in control conditioned media and EV sample prepared by experimental conditioned media (Fig 8 H). ZNF81 and the exonic region of LINC00478 were not detected in any of

the samples. The absence of the two transcripts (ZNF81 and the exonic LINC00478) could be due to and undetectably low copy number.

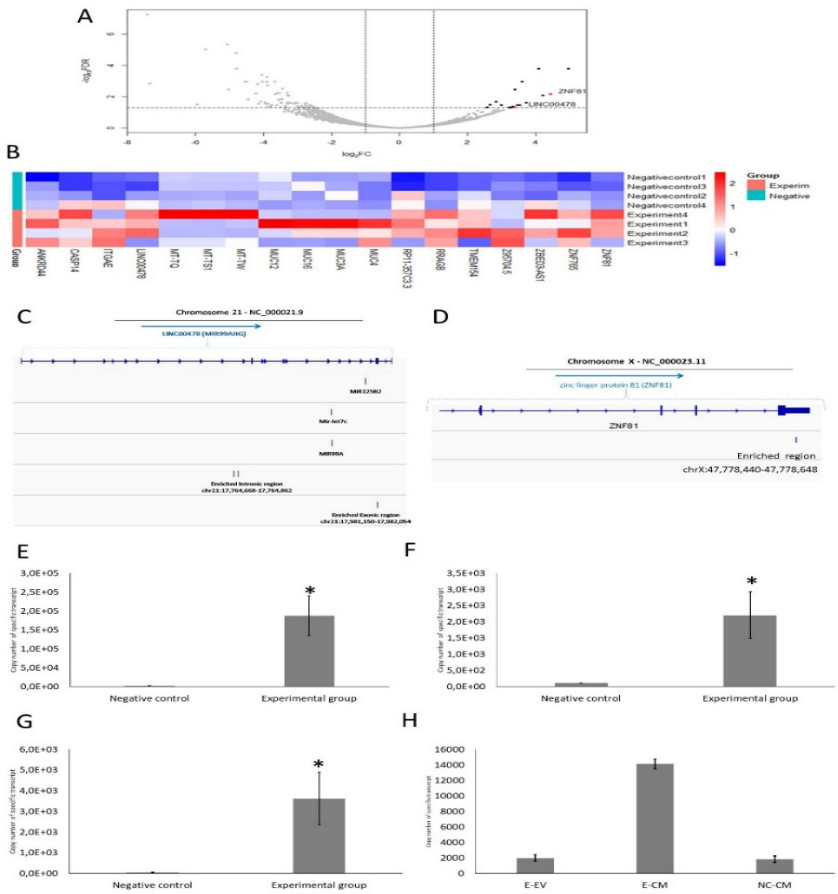


Figure 8. Quantifying the transfer of 5-EU labelled RNA from trophoblast spheroid to endometrial cells

(A) Volcano plot depicting the differentially expressed genes contrasting the affinity precipitated RNA from endometrial cells co-incubated with 5-EU labelled trophoblast spheroids (Experimental group, n=4) *vs.* endometrial cells co-incubated with unlabelled trophoblast spheroids (Negative control, n=4). **(B)** The relative abundance of putatively transferred transcripts. Heatmap presents the z-scores calculated based on normalized read counts. Alongside the heatmap, unsupervised hierarchical clustering of samples based on Euclidean distance calculated from presented z-scores are presented. *(***C)** Position of enriched intronic- LINC00478 and exonic- LINC00478 in relation to chromosome 21. **(D)** Position of enriched ZNF81 in relation to chromosome X. **(E, F, G)** Absolute copy numbers of EU labelled Intronic-LINC00478, Exonic-LINC00478 and the exonic region of the ZNF81 measured in experimental group compared to the

negative control. Data is presented as mean ± SEM. (*) $p < 0.05$ vs negative control. **(H)** Intronic-LINC00478 presence in conditioned media from the experimental co-culture (E=CM), conditioned media from the negative control co-culture (N-CM) and EVS extracted from the E-CM samples (E-EV). Exonic-LINC00478 and ZNF81 were not detected in either group. Data is presented as mean ± SEM.

5.6. Transferred transcripts were significantly downregulated in endometrium

Trophoblast spheroid-endometrial cell non-contact co-cultures were prepared using translucent cell culture inserts separating the trophoblast spheroids and the endometrial monolayer (Fig 5 A, B, C) along with similarly prepared HEK293-endometrial cell non-contact co cultures as a negative control (500 spheroids were co incubated with 4×10^5 cells). No bioorthogonal labelling was used in any of the cultures. Along with co-cultures, endometrial cells were also supplemented with HEK293 and JAr spheroid conditioned media and their derived EVs (particle: cell ratio 50:1) in separate samples. All the samples were incubated for 24h and endometrial RNA was subjected to qPCR to detect the expressions of candidate transcripts.

Interestingly, the candidate transcripts (ZNF81, exonic LINC00478 and intronic LINC00478) were significantly downregulated in trophoblast spheroid co-culture (Fig 9 A, B, C), conditioned medium of trophoblast spheroids and EVs extracted from trophoblast spheroid conditioned medium while any of the HEK293 groups did not exhibit any significant downregulation. Control genes (Beta-2-microglobulin and Beta-actin) did not show any significant differential expression between groups (Fig 9 D, E). Since the candidate genes are re-cognized as transferred from the embryo to the endometrium, an upregulation was expected. However, the copy number of transferred transcripts could be too low for detection with qPCR and the observed downregulation could be indica-tive of a more systematic gene regulation induced by the endometrial EVs.

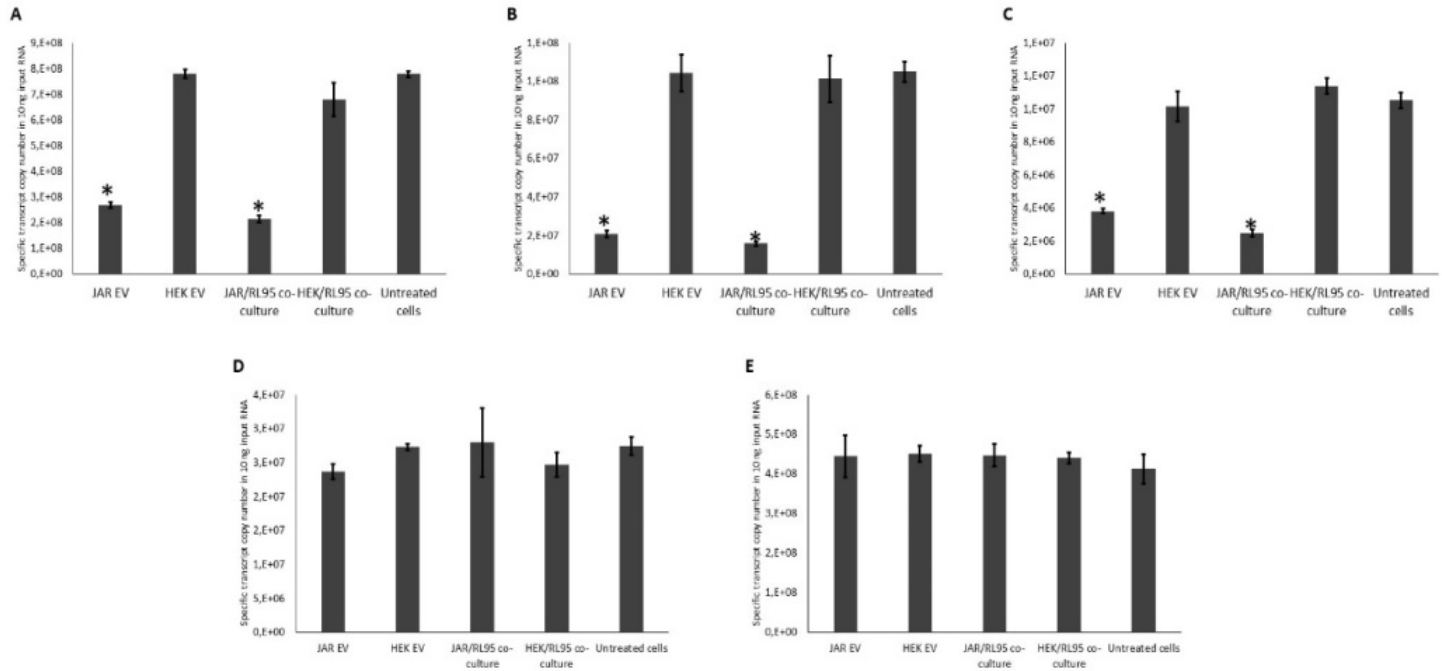


Figure 9. Expression of transferred transcripts in endometrial cells
(A, B, C, D, E) Expressions of Intronic-region of LINC00478, Exonic region of LINC00478, ZNF81, beta actin and beta-2-microglobulin in endometrial cells in 24h of co-incubation time (JAr spheroid or HEK293 spheroid) or 24h EV treatment (JAr EV or HEK293 EV). Data is presented as mean ± SEM. (*) $p < 0.05$ vs untreated control.

5.7. Key findings of Study-1

1. RNA produced by the embryo analogue (JAr spheroid) is uptaken by the endometrial analogue (RL95-2) cells without there being a physical contact between two cells. However, there is no evidence showing that uptaken RNA is released to the cytoplasm of the target cells or if they are biologically active.
2. An intronic-region of LINC00478, an exonic region of LINC00478, and the mRNA ZNF81 were identified as "putatively transferred transcripts" using RNA sequencing data.
3. Interestingly, the putatively transferred transcripts were downregulated in target cells compared to RL95-2 cells not exposed to JAr spheroids. The mechanism of these downregulations is not clear with the data available.

5.8. Experimental plan for Study-2

5.8.1. Determining the effects of JAr and HEK293 cell derived EVs on RL95-2 cellular transcriptome

Endometrial analogue (RL95-2) cells were cultured in 12 well plates until 80% confluency using the culture methods and conditions described above. At the desired confluency, growth medium was removed and 1×10^8 paricles derived from JAr and HEK293 cells were supplemented to the RL95-2 cell monolayer separately in an EV-depleted supplementation medium. Controls were prepared using untreated RL95-2 cells cultured in EV-depleted media. Cells were incubated for 24h. After incubation, the medium was removed and cellular RNA was collected for mRNA sequencing. The experiment was performed on three different days to prepare the three biological replicates.

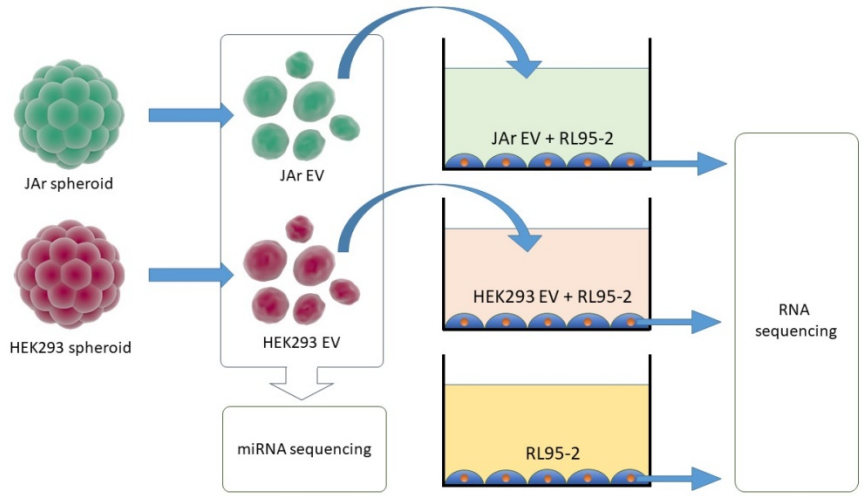


Figure 10. Experimental plan for Study 2 and study 3
Study-2: Endometrial cells (RL95-2) were treated with either JAr spheroid derived EVs (JAr EV) or HEK293 cell derived EVs (HEK293 EV). Resulting transcriptome changes were quantified using mRNA seq. **Study-3**: JAr EV and HEK293 EV RNA was extracted and sequenced for miRNA and mRNA.

5.9. JAr cell derived EVs uniquely induced a significant alteration of RL95-2 transcriptome

JAr spheroid derived EVs and HEK293 spheroid-derived EVs were supplemented to RL95-2 cells as separate samples along with a negative control of untreated RL95-2 cells. After 24h of incubation, the RL95-2 transcriptome was sequenced and differential expression (DE) was calculated with reference to the untreated RL95-2 cells. JAr-derived EVs induced significant alterations in the endometrial transcriptome (1166 upregulated and 588 downregulated genes) compared to the untreated RL95-2 cells. Interestingly, the HEK293 cell-derived EVs could not induce any significant alteration as evidenced by the heatmap of the dataset (Fig 11).

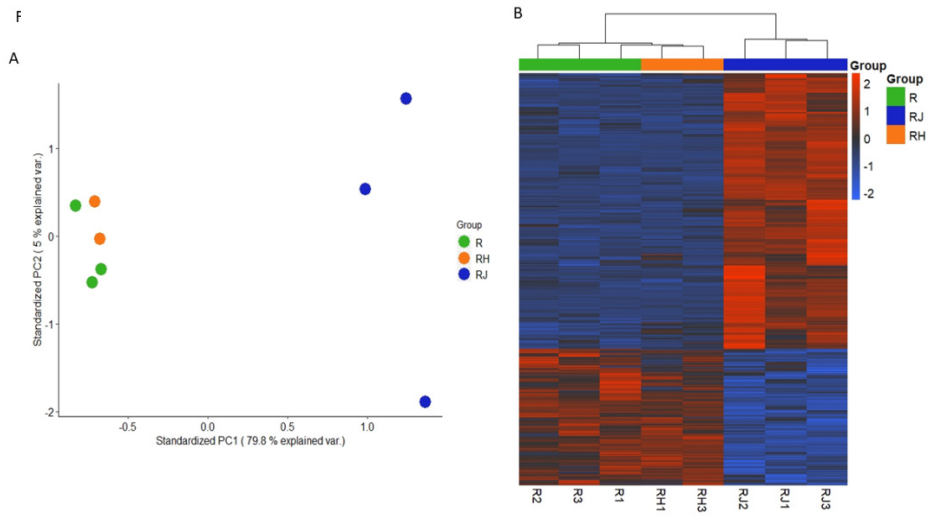


Figure 11. Transcriptomes of RL95-2 cells treated with JAr EVs, HEK293 EVs and untreated control.
(A) Considering all genes above threshold expression, the principal component analysis (PCA) shows that transcriptome of RL95-2 cells supplemented with JAr EVs (RJ) is clearly differentiated from the transcriptomes of RL95-2 cells supplemented with HEK293 EVs (RH) and un-supplemented control RL95-2 cells (R). The first two principal components (PC) are presented. (B) Heatmap and unsupervised hierarchical clustering (Euclidean distance) of the 1,754 differentially expressed genes. (DEGs) in the RJ vs R comparison.

5.10. Gene set enrichment analysis of JAr EV-targeted genes in RL95-2

Two major Reactome pathways were significantly enriched in the JAr EV supplemented RL95-2 cell geneset. They were "extracellular matrix (ECM) organization" (Reactome code – R-HSA-1474244) and "signalling by G-protein coupled receptors (GPCR)" (Reactome code – R-HSA-372790). Reactome pathways are built around a core "reaction" and contain experimentally validated lists of "entities" such as transcripts, genes and proteins that participate in the reaction. Each major pathway has several branch pathways that describes more specific functions. The ECM organization pathway, for example, describes all the "reactions" known to be a part of generating, remodelling and destruction of the ECM. Some branch pathways of both ECM organization pathway and GPCR signalling pathway were also significantly enriched (Fig 12 A, B).

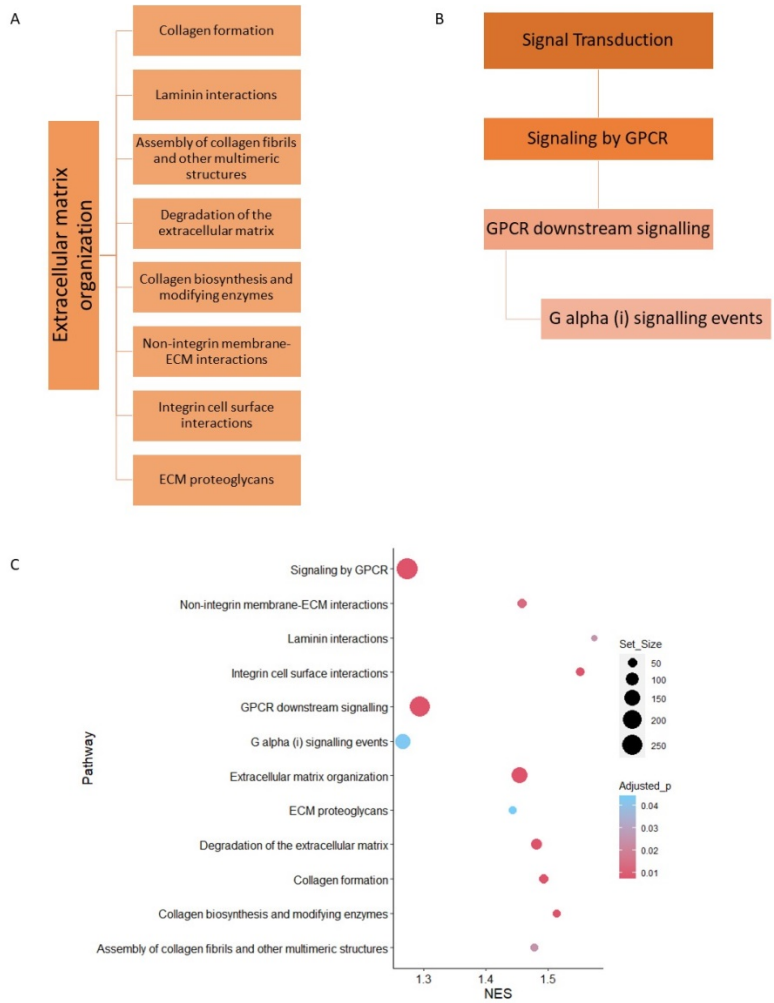


Figure 12. Pathways significantly affected by the transcriptomic changes
Relationships between pathways enriched by the transcriptomic changes (A, B) Pathways are presented against the normalized enrichment score (NES). The colour indicates the degree of significance and the size of the points indicate the size of the gene set enriched (C).

5.11. Key findings of Study-2

1. JAr spheroid derived EVs can induce significant alterations to the RL95-2 cellular transcriptome while HEK293 spheroid derived EVs failed to induce any significant effects.
2. The altered transcriptomes of the RL95-2 cells are significantly enriched in several biochemical pathways important in embryo implantation such as extracellular matrix organization and GPCR based signalling.

5.12. Experimental plan for study 3

5.12.1. Investigating the RNA cargo of EVs

Trophoblast analogue spheroids and HEK293 control spheroids were prepared and incubated for 24h in EV-depleted medium placed on a gyratory shaker to stop the loss of structural cohesion. After incubation, the conditioned media were separated and EVs were extracted using SEC. Concentration of each EV sample, JAr (n=3) and HEK293 (n=3) were measured using NTA. Part of each sample (1×10^8 particles) were used to extract RNA and subjected to mRNA and miRNA sequencing. mRNAseq data were analysed for differential expression and enriched mRNA between JAr and HEK293 and miRNAseq data were analysed for miRNA uniquely expressed in each type of EV sample and their putative targets.

5.13. mRNA cargo of JAr derived EVs is distinct from HEK293 derived EVs

EVs from both JAr and HEK293 were extracted using SEC and included RNA was enriched and sequenced. Read counts of JAR EV sample-derived RNA was compared to read counts of HEK293 EV sample RNA. The population of RNA fragments aligning to known genes was substantially different between the JAr EV RNA and HEK293 EV RNA. 400 genes were found to be significantly enriched among the EV RNA fragments while 501 mRNA were significantly depleted compared to HEK293 EV. Based on these data, the mRNA cargo appears to be significantly dependent on the type of cells producing the EVs (Fig 13).

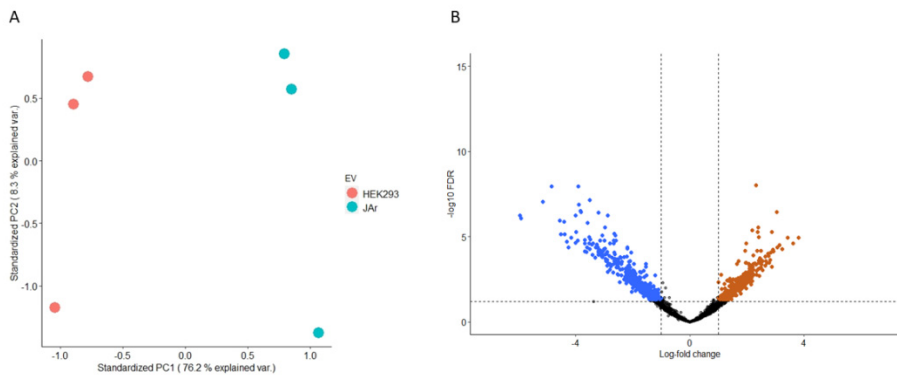


Figure 13. The contrast between RNA cargo of JAr EVs and HEK293 EVs.
(A) Clear separation between RNA cargo of JAr EVs and HEK EVs can be observed in the PCA plot along the PC1. **(B)** Differential enrichment of mRNA contrasted JAr EVs *vs.* HEK293 EVs. mRNA enriched in JAr is coloured orange (log2FC > 1) and mRNA enriched in HEK293 EVs are coloured blue (log2FC < -1).

5.14. miRNA of JAr derived EV samples is distinct from miRNA of HEK293 derived EV samples

The cargo of miRNA carried in JAr EVs were also different from HEK293 EVs. The filtering criteria influenced the unique miRNA found in each type of EV. Eleven miRNAs were found to be unique to JAr EVs when read count threshold was set at 5 and the miRNA was required to be present in 2/3 libraries with only 2 miRNAs were uniquely detected in HEk293 EVs. Since HEK293 EVs did not induce any significant transcriptome alteration in RL95-2 cells, only the uniquely JAr miRNA were used for further analysis (Fig 14).

Endeavouring to explain the JAr EV induced downregulation of genes in RL95-2 cells, uniquely JAr miRNAs were analysed for potential targets from miRDB database with a target score of more than 90. There were 1188 high-confidence targets for the 11 identified miRNA and 744 of which were present in the JAr EV/RL95-2 transcriptome dataset. Most of the putative targets were non-differentially expressed (623 a 6.4% of all non-differentially expressed genes) and only 53 downregulated (9% of total downregulation) and 68 upregulated (5.8% of total upregulation). Furthermore, six out of the eleven miRNAs had a greater number of targets which were downregulated than upregulated, while only four had a greater number of upregulated than downregulated targets. hsa-miR-524-5p had the largest number of putative targets represented in the expression dataset, the 26 downregulated targets of which constituted 4.4% of the total downregulated genes (Fig 15).

The mean log2FC of downregulated target genes displayed a moderate negative correlation with the abundance of a given miRNA in JAr EVs (weighted Pearson's correlation, $r = -0.65$, $p = 0.041$; Figure 5B). The most abundant JAr-specific miRNA was has-miR-1323, the downregulated targets of which had the lowest log2FC of all miRNAs except for hsa-miR-526b-5p, which had only one downregulated target. The negative correlation was found to be influenced both by the target score cut-off and FDR cut-off used to detect downregulated genes.

Downregulated genes were examined to find out whether they constituted high-confidence predicted targets of multiple miRNAs. In this regard, only two downregulated genes were identified and they were the putative targets of at least three JAr-specific miRNAs: *ATF2* (predicted target of hsa-miR-524-5p, hsa-miR-520a-5p, and hsa-miR-525-5p) and *SPTSSA* (predicted target of hsa-miR-524-5p, hsa-miR-526b-5p, and hsa-miR-1323), respectively.

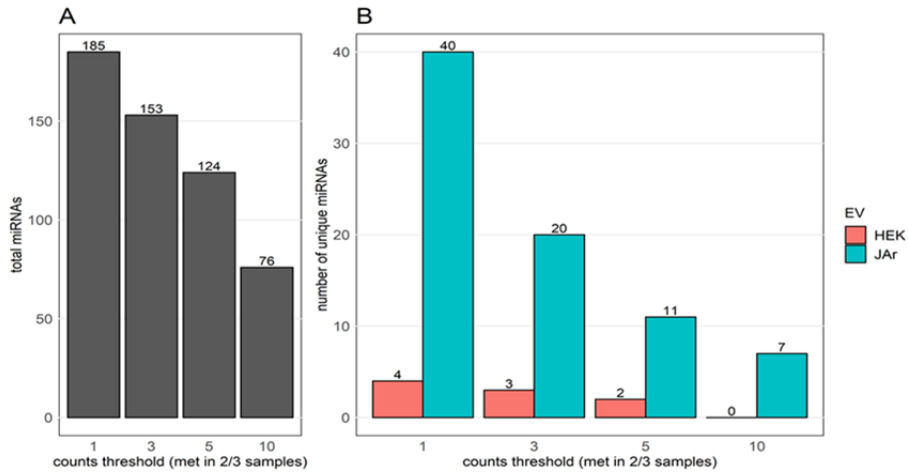


Figure 14. miRNA content of JAr and HEK293 EVs
(A) Number of miRNAs detected at 4 different threshold count values. Each miRNA has to detected at least the number of times indicated in 2/3 of all libraries to be counted. Threshold 5 was selected as a compromise between strictness and representability. **(B)** Numbers of unique miRNA counted in each threshold level. miRNAs were considered unique After passing the required counts criteria for one EV type but absent in the other type of EVs.

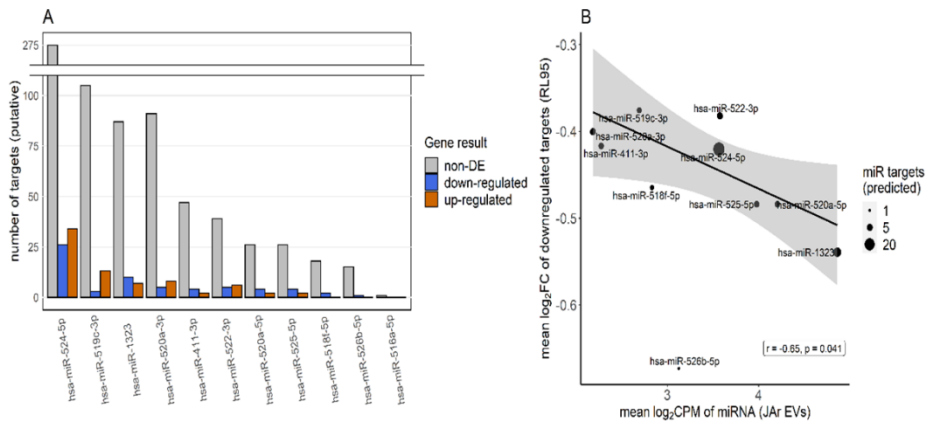


Figure 15. The potential of EV miRNA to change the transcriptome of endometrial cells
(A) JAr EV specific miRNA and their potential high confidence (target score >90) targets in the endometrium with corresponding differential expression data. Non-differentially expressed targets (grey), downregulated targets (blue) and upregulated targets (orange). (B) Relationship between the mean expression of significantly downregulated (FDR ≤0.05) high confidence target fold changes (log2FC) and the abundance of JAr specific miRNA in EVs (log2CPM). Size of the points corresponds to the number of putative targets.

5.15. Key findings of Study-3

1. Both mRNA and miRNA extracted from JAr spheroid derived EVs are significantly different from mRNA and miRNA extracted from HEK293 spheroid derived EVs.
2. There is a negative correlation between the abundance of unique miRNA in the JAr EV samples and the expression of their targets in RL95-2 cells.
3. It can be hypothesized that miRNA transported in EVs have regulated the transcriptome of the target cells. However, the extent of the EV-miRNA based transcript regulation represents a minor percentage of the full effect on the target transcriptome.

5.16. Experimental plan for Study 4

5.16.1. The effect of embryo conditioned media derived EVs on endometrial cells

Conditioned media were collected from individually grown human embryos on day 3 and day 5 post IVF. The samples were “good quality – day 3” (n=4) and “degenerating-day3” (n=4) “good quality – day 5” (n=4) and “degenerating-day 5” (n=4). Conditioned media from each group were pooled and EVs were extracted. Prepared EVs were then supplemented to endometrial cells (Cell: EV was 1:50). Negative controls were prepared using native endometrial cells. After 24 hours of incubation total RNA was extracted from RL95-2 cells, cDNA was prepared and qPCR was performed for candidate transcripts. Beta actin and beta-2-microglobulin were used as control genes.

5.16.2. Supplementation of embryo-derived EVs to the BOEC monolayer culture

Each BOEC monolayer culture was supplemented with EVs extracted from 10 individually cultured bovine embryos or control media samples. The supplemented numbers of particles were 7.99×10^8, 6.48×10^8, and 6.81×10^8 per well for good quality embryo-derived EVs, bad quality embryo-derived EVs and NPs extracted from day 5 control media respectively. After 24h of incubation, RNA from the BOECs were collected and sequenced for mRNA expression.

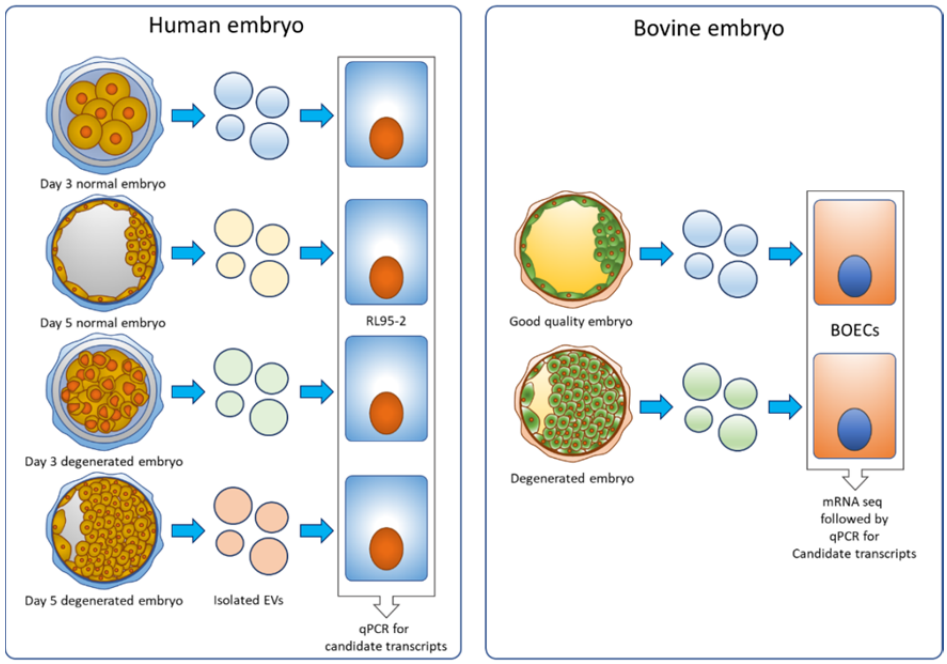


Figure 16. Experimental plan for study-4
Day 3 and day 5 human embryo (both normal and degenerated in quality) were used as the source of EVs. Collected EVs were then supplemented to endometrial analogue (RL95-2) cells for 24h. after incubation, the RNA was collected from the cells and quantified for the expression of candidate transcripts identified in study 1 in an attempt to develop a non-invasive embryo grading methodology. Similarly, good quality and degenerated bovine embryos were also used as the source of EVs. Prepared EVs were supplemented to BOECs and co incubated for 24h. At the end of incubation, RNA was collected and sequenced for mRNA profile. Differentially expressed genes of biological importance were then quantified using qPCR.

5.17. Human embryo derived EVs selectively alter the expression of ZNF81 in endometrial cells

EVs derived from good-quality and degenerating embryos from both days 3 and 5 were supplemented to endometrial cells. After incubation, the expressions of candidate transcripts were measured using qPCR. Both good-quality embryos (day 3 and day 5) derived EVs induced a significant downregulation of ZNF81 transcript compared to both degenerating embryo derived EVs and untreated endometrial cells (Fig 17A). Control genes did not show any significant altered expression as responses to embryo derived EVs (Fig 17 B, C).

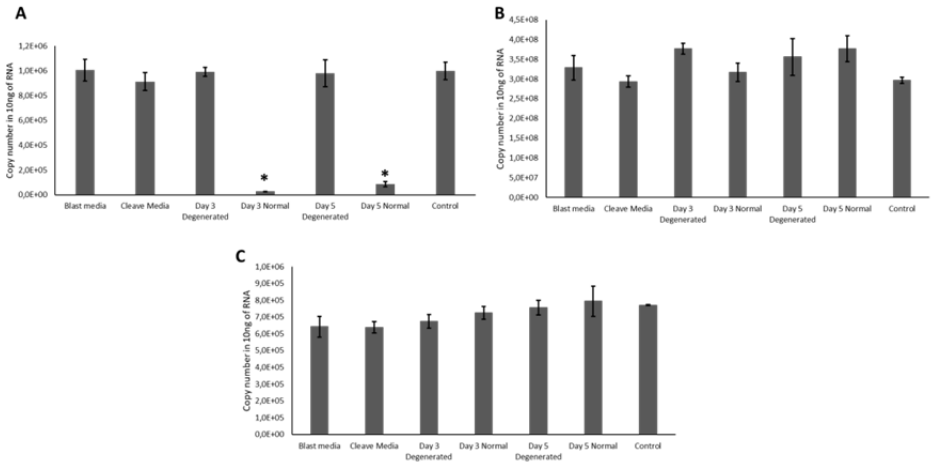


Figure 17. Embryo derived EVs selectively alter the endometrial expression of ZNF81 **(A**) Expression of ZNF81 in endometrial cells when treated with embryo culture media, day3 degenerated embryo derived EV, Day 3 normal embryo derived EV, Day 5 degenerated embryo derived EV, Day 5 normal embryo derived EV and in unsupplemented control. In groups with normal embryos (both day 3 and day 5) significant down-regulation of znf81 was observed. **(B, C)** Gene expressions of **(B)** Beta-2-microglobulin and **(C)** Beta actin in similar situations with no significant changes. Data is presented as mean ± SEM. (*) $p < 0.05$ vs untreated control.

5.18. Bovine embryo derived EVs selectively alter the BOEC transcriptome

RNA sequencing yielded an average of 6.0×10^6 reads per sample, 99.0% ± 0.1% of which were retained for analysis after quality control and aligned to *B. taurus* genome assembly. The mapping rate was 95.4% ± 1.1% and after removing the read counts below threshold in all experimental groups, 10412 uniquely aligned genes were retained for differential expression analysis. A considerable amount of inter-group and intra-group variations were observed in the gene expression profiles (Fig 18).

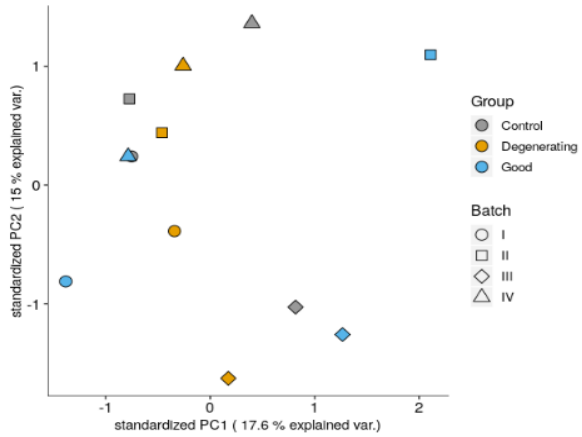


Figure 18. transcriptomic profiles of BOECs treated with bovine embryo derive EVs
Gene expression profile of BOECs supplemented with good quality embryo EVs (blue), degenerating embryo EVs (Orange) and unsupplemented control (Grey) with point shapes representing batches. Two leading principal components of standardized (z-score) counts per million (CPM) values of the expressed genes in the BOECs is presented.

5.19. Four interferon induced genes were consistently and significantly upregulated

The good-quality embryo EVs induced a transcriptional alteration of 7 up-regulations and 18 downregulations in BOEC cells compared to the un-supplemented BOEC cells. Four out of the 7 upregulated genes, were interferon-induced genes (ISG-15, MX1, OAS1Y, LOC100139670). One marginal down-regulation was observed in BOECs supplemented with degenerating embryo EVs (ENSBTAG00000051364, log2FC = 0.83, FDR = 0.046). Good-quality embryo EVs induced a different transcriptomic profile on BOECs compared to degenerating embryo EVs with 4 upregulated genes and 11 downregulated genes. Again, the upregulated genes belonged to the interferon-induced genes.

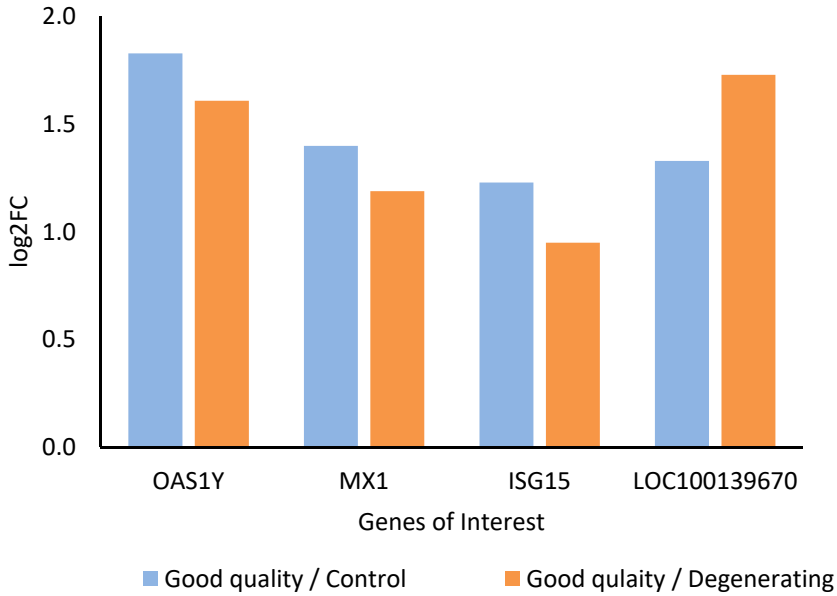


Figure 19. Interferon-induced genes upregulated in BOECs treated with good quality embryo derived EVs.
The interferon induced genes were significantly (FDR < 0.05) upregulated in BOECs treated with good quality embryo derived EVs when contrasted with negative control (grey) and BOECS supplemented with degenerating embryo derived EVs (Orange). The upregulation is virtually similar in each instance indicating similar transcriptomic profile in negative control and the BOECs supplemented with degenerating embryo.

Genes that were considered most relevant to the context of the study (OAS1Y, MX1, and LOC100139670) were subjected to confirmation by qPCR (Fig 20), Compared un-supplemented BOECs (control group) the BOECs supplemented with EVS from good-quality embryos showed a significant upregulation in two of the genes (MX1 and OAS1Y, FDR ≤ 0,05, Mann-Whitney U test, Benjamini-Hochberg Procedure correction).

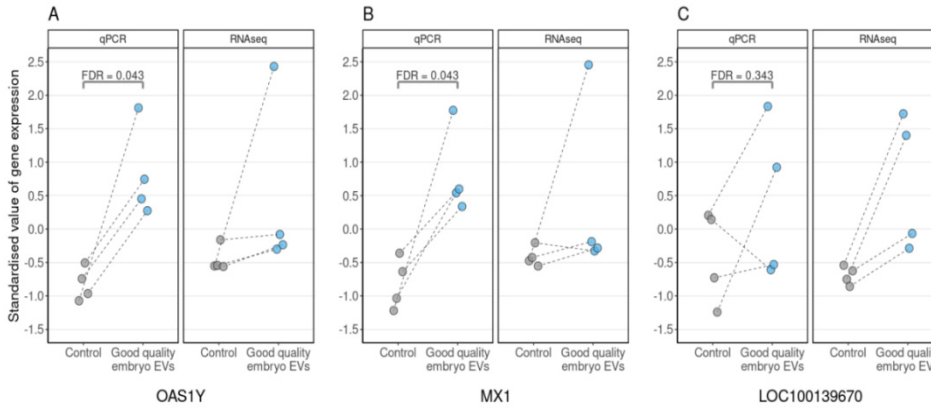


Figure 20. Real time qPCR validation of the transcript changes (A, B, C) Comparison between the gene expressions of OAS1Y, MX1, and LOC100139670 in RNAseq and RT-qPCR. Dashed lines represent the experimental batches. Mann-Whitney U test was used to compare the groups with Benjamini-Hochberg Procedure to correct for multiple testing.

5.20. Key findings of Study-4

1. EVs derived from good-quality human embryo conditioned media (collected on day 3 and day 5 post fertilization) were able to downregulate the putatively transferred transcripts (identified in study-1) in RL95-2 cells. Such downregulation was not observed in RL95-2 cells exposed to EVs collected from degenerating embryo conditioned media.
2. EVs derived from good-quality bovine embryo conditioned media were able to induce significantly different transcriptomic changes in BOECs compared to EV derived from bad-quality bovine embryo conditioned media derived EVs.
3. Genes known to be stimulated by the bovine embryo implantation marker IFN-τ were significantly upregulated in BOECs treated with good-quality embryo derived EVs.

6. DISCUSSION

In broad terms, the aims of the research encompassed within this thesis was to investigate EV mediated RNA transfer between the embryo and the maternal system, to evaluate the overall effect the EVs have on the endometrial transcriptome, to investigate the RNA cargo of EVs produced by trophoblast spheroids in order to elucidate possible mechanisms of EV mediated intercellular communication and finally to apply the knowledge gathered by EV mediated embryo maternal communication to the real world problem of non-invasive embryo quality assessment in the field of assisted reproduction.

Through extensive experimentation with a well-established in vitro system, it was observed that embryonic RNA is indeed transferred to the endometrium without physical contact between the cells and the transferred RNA is readily uptaken into the endometrial cells. EVs derived from the trophoblast cells were capable of transferring trophoblast RNA to the endometrial cells in the absence of trophoblast cells. Transcriptomes of endometrial cells treated with trophoblast derived EVs were significantly altered compared to negative controls. Some of the biochemical pathways enriched by the altered transcriptome were of importance in the process of embryo implantation. The transcriptome alterations were not observed when endometrial cells were treated with EVs derived from a non-trophoblast source implying the effect is specific to the embryonic EVs. The transcriptomic changes were partially correlated to the ncRNA cargo of trophoblast derived EVs. However, since there is no evidence that EV RNA is exposed to cytosol post uptake, this correlation might be indicative of a coincidence and not a biologically significant phenomenon. Interestingly, when endometrial cells were supplemented with EVs collected from good-quality and degenerating embryos, only good quality embryos were able to induce significant effects on endometrial transcriptome. A similar effect was observed in BOECs treated with good-quality and bad-quality bovine embryos implying a general mechanism of embryo derived EVs affecting the maternal tissue transcriptome. It can be proposed that these findings lay the foundation for a non-invasive embryo grading system that can be used in assisted reproduction.

6.1. Transfer of genetic material between cells

Transfer of genetic material between cells has been put forward as a new mechanism of intercellular communication, particularly in the context of RNA based communications. These transfers of information is known to be used as a tool in all forms of life from plants (Mclean, Hempel and Zambryski, 1997; Wu, Weigel and Wigge, 2002; Van Norman, Breakfield and Benfey, 2011; de la Canal and Pinedo, 2018) to animals (Mittelbrunn and Sánchez-Madrid, 2012). RNA transfer between cells is not limited to cells of same species or even cells of same kingdom. miRNA from parasites are known to target the host transcriptome thus altering the gene expression in the host to a state more amenable

to the parasite (Shahid *et al.*, 2018), vice versa, plants are reported to use the transfer of ncRNA as a means to inhibiting fungal growth (Samuel *et al.*, 2015; Regente *et al.*, 2017; Cai *et al.*, 2018). Metastasing cancer cells are known to exchange genetic martials with the target tissues endeavouring to remodel the architecture of the tissues to better support metastasis[39,40]. ncRNA are reported to be utilized in these communications also(Li *et al.*, 2016; J.-P. Wang *et al.*, 2018; Parsons *et al.*, 2018; Probert *et al.*, 2019).

In each case, the objective of RNA transfer seems to be the alteration of the target cell or tissue to better accommodate the requirements of the cells that originates the communications. In the context of reproduction and especially implantation, the aim of any embryonic RNA transfer should be to alter the endometrial transcriptome to a state where the inherently invasive process of implantation is facilitated. There are definite reports of embryo-to-endometrium transfer of RNA(Cuman *et al.*, 2015) and endometrium-to-embryo transfer of RNA(F. Vilella *et al.*, 2015).

6.2. Methods of bio-orthogonal RNA labelling

Bioorthogonal click chemistry was used to capture the labelled RNA in the first study. Using the 5-EU labelled RNA has significant advantages over using other methods of nucleotide labelling such as 5-bromouridine (BrU), 5-iodouridine (IU), or 5-fluorouridine (FU). While these uridine derivatives are equally capable of labelling nascent RNA, the process of detecting them in cells is more complicated and imprecise compared to the click IT reaction that can be performed with 5-EU (Dvořáčková and Fajkus, 2018). The efficiency of tagging is approximately 1 out of 35 nucleotides in 5-UE system which is not significantly different from other available systems (Jao and Salic, 2008). Even with elevated precision of click iT reaction, the percentage of non-specific enrichment of RNA is 35%. Which although high, is an inherent weakness of bioorthogonal RNA tagging and recovery. A possible limitation of the methodology employed in the tracking of labelled RNA is the possibility of capturing RNA that haven't been uptaken by the endometrial cells. Efforts has been taken to ensure that all unbound EVs are washed away from the endometrial cells. However, no agent has been employed to remove labelled RNA attached to the plasma membrane surface of the recipient cells. It was assumed that any labelled RNA permanently attached to the apical surface of the recipient cells were a feature of the non-contact RNA transfer between embryo and the endometrium. The confocal microscopy images did not show any labelled RNA attached to the apical surface of the endometrial cells. Therefore, it was assumed that the captured RNA represented the uptaken RNA.

6.3. Identified transferred transcripts

Three transcripts (ZNF81, an exonic region and an intronic region of LINC00478) were identified as transferred from the embryo to the endometrium because there was a significant presence of these transcripts in endometrial cells co-incubated with trophoblast spheroids in a non-contact manner. As per the route of RNA transfer, it was hypothesized that EVs could make up a major portion of avenues that are available. Only the labelled intronic region of the LINC00478 was identified in the co-culture EVs. This could be due to the low abundance of the other two transcripts and the low efficiency of the 5-EU labelling and capture system. LINC00478, also known as the MIR99AHG associated with miR-99a/let-7c/miR-125b2 cluster, is implicated in tumour suppression (Han *et al.*, 2021) by autophagy regulated via ANXA2 induced ATG16L+ vesicle biogenesis. In cases of 21 chromosome long arm deletion (chromosome 21q), MIR99AHG is downregulated and the tumour suppression capacity is reduced significantly. The zinc-finger proteins as a family have the ability to bind with transposable elements and form an epigenetic regulatory network (Berg, 1993; Trono, 2016; Imbeault Michaël, Helleboid Pierre-Yves and Trono Didier, 2017). ZNF81 protein is capable of binding to long interspersed nuclear elements, a known expression regulator (Imbeault Michaël, Helleboid Pierre-Yves and Trono Didier, 2017).

6.4. Expression of transferred transcripts in the recipient cells

Since new RNA were transferred to the endometrial cells, the expectation was that the post-transfer expression of the transferred transcripts would be upregulated. However, all the transferred transcripts showed a significant downregulated expression compared to untreated cells. Neither any previous reports of transcripts downregulating the expression of similar transcripts when introduced to target cells nor any known mechanism of such action has been found after extensive surveys of literature. Therefore, it can be proposed that there is no connection between the observation that these transcripts were transferred from embryo to the endometrium and the observation that similar transcripts are downregulated in the endometrial cells. It can be further speculated that the candidate transcripts were downregulated due to some gene regulation/ gene silencing effect induced by the EV or EV cargo. The phenomenon of gene expression downregulation as a result of intercellular RNA transfer has been previously observed (Syed and Hecht, 1997; Lloret-Llinares *et al.*, 2018). The mechanism of these downregulations could be attributed to RNA mediated gene regulation among any other methods of gene regulation. RNA-mediated downregulation could be achieved using one of the several pathways, such as post-transcriptional gene silencing, co-suppression, quelling, and RNA interference (RNAi) (Travella and Keller, 2009). Negative feedback mechanisms have been

postulated with reference to lncRNA mediated gene expression regulation (Jiang *et al.*, 2018; Tian *et al.*, 2018; Yan *et al.*, 2018). The phenomenon of downregulation of the transferred transcripts can be explained by RNA mediated gene regulation even though there were no definite evidence to the fact in the dataset analysed in the first study. It should be noted that the same downregulation occurs when the endometrial cells were treated with trophoblast spheroid derived EVs, implying a strong connection between the EVs and the RNA transfer between embryo and the endometrium. Interestingly, only trophoblast spheroid derived EVs were able to induce the effect of transferred transcript downregulation in the endometrial cells compared to HEK293 cell derived EVs. Seemingly, the EV based communication was specific to trophoblast cell derived EVs. These results lead to further experimentation on possible specificity of EV-mediated RNA transfer/ intercellular communication between the embryo and the maternal tract.

Following the study 1 that conclusively showed that embryonic RNA is transferred to endometrium without contact, an attempt was made to produce a more complete image of the intercellular communication that was taking place. The obvious limitations of low efficiency and minuscule amounts of EV derived RNA from the first study restricted the observation of the full effect that embryo mediated EVs were having on the cells of the maternal tract. Using next generation sequencing methods, a more complete model of EV induced transcriptome alterations in embryo-maternal communication context was developed.

6.5. EV-mediated embryo-maternal communication induced transcriptomic alterations

The knowledge base of EV-mediated intercellular communication is increasing in popularity and substance (Yiran *et al.*, 2017). However, the underlying mechanisms of EVs target specificity and the mechanisms of EV-mediated communication is not yet well understood. In the study 2, the same embryo-maternal communication in vitro model was used to detect the mechanisms behind to some of the interesting observations from the first study.

JAr spheroid derived EVs (JAr EVs) representing the trophoblast in the context of these experiments, induced a significant alteration to the endometrial (represented by the RL95-2 cells) transcriptome while the HEK293 derived EVs (HEK293 EVs) failed to do so. Transcriptional alterations induced by the JAr EVs are functionally critical to embryo implantation process. The main pathway affected by the DEGs was the extracellular matrix organization pathway (R-HSA-1474244). Most of the genes associated with the pathway were up-regulated. ECM remodelling is a critical change that the endometrial cells undergo in the process of decidualization. The receptors for the adhesion molecules are overexpressed on the apical surface and the pinopodes to facilitate trophoblast adhesion (Brown and Papaioannou, 1992; Johnson *et al.*, 2003; Kaloglu and Onarlioglu, 2010). Major subunits of the ECM pathway such as

laminin interactions (R-HSA-3000157) (Miner *et al.*, 2004), integrin cell surface interactions (R-HSA-216083) (Reddy and Mangale, 2003; Aplin, 2005; Al-Dossary *et al.*, 2015), and non-integrin membrane-ECM interactions (R-HSA-3000171) (Kirn-Safran, D'Souza and Carson, 2008) also significantly enriched, are all known to be implicated in endometrial modifications in the WOI.

Signalling by GPCRs (R-HSA-372790) was significantly enriched in DEGs as well. G-protein coupled receptors (GPCRs) are coded by nearly 4% of the human genome. They are the most numerous family of receptors (Bjarnadóttir *et al.*, 2006). GPCR ligands are extremely diverse and regulate a wide range of physiological functions (Sharma, Akhade and Qadri, 2013; Saroz *et al.*, 2019). Progesterone and estrogen (membrane receptor) (Thomas and Pang, 2012; Thomas, Pang and Dong, 2014; Wetendorf and DeMayo, 2014), hCG (Casarini *et al.*, 2012), prostaglandin E2 (Salleh, 2014; Niringiyumukiza, Cai and Xiang, 2018), cytokines (Castro-Rendón *et al.*, 2006; Chaouat, Dubanchet and Ledée, 2007; Guzeloglu-Kayisli, Kayisli and Taylor, 2009) are among the ligands that binds to GPCRs. The secondary messengers that regulate endometrial morphology such as the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) (Gentilini *et al.*, 2007; Nakano *et al.*, 2017; Wydooghe *et al.*, 2017; Makker *et al.*, 2018) are initiated by the downstream signalling of GPCR pathway (R-HSA-388396), which was also enriched by DEGs. Results gathered from the gene set analysis of DEGs in JAr/RL95-2 dataset suggest that not only embryonic EVs are modifying the morphology of the endometrium to facilitate implantation, but also priming the receptors of the endometrial epithelium to receive signals from the other regulators of the implantation process such as progesterone and cytokines.

6.6. Functional specificity of EV-mediated embryo-maternal communication

The results demonstrated a clear specificity of function in JAr EVs compared to HEK293 EVs. The specificity of EVs is a yet unresolved topic of scientific inquiry (Mathieu *et al.*, 2019b). Some reports (Fitzner *et al.*, 2011; Chivet *et al.*, 2014) suggests that EVs are specific to the target cells and would not affect any other adjacent cells. However, other reports (Svensson *et al.*, 2013; Costa Verdera *et al.*, 2017; Horibe *et al.*, 2018) posit that EV uptake by target cells is a general phenomenon. The functional specificity of EVs was observed in the study 2 is similar to the observations of some investigators in cancer metastasis (Wu *et al.*, 2017; Bae, Brumbaugh and Bonavida, 2018). The specific nature of the EV function can either be attributed to either uptake specificity or the effect of each type of EVs on the target cells probably linked to the cargo/ surface molecules of the EVs. Many reports suggesting a non-specific EV uptake in many types of target-EV combinations (Mathieu *et al.*, 2019b; Bonsergent *et al.*, 2021) confirms the understanding that latter might be the case in this regard.

6.7. RNA cargo of EVs

Following the deduction that the specificity of function observed between the JAr and HEK293 EVs on RL95-2 cells could be due to the differences in the cargo of the EVs, the RNA cargo of the two EV types was sequenced. Significant differences were observed between the mRNA of HEK293 EVS and JAr EVs. Any connection between the mRNA cargo of EVs and the EV induced transcriptomic alterations remains elusive.

The miRNA cargo of the EVs from each cell type were also distinct. The physiological function of miRNA is to regulate gene expression by destabilizing transcripts (Bartel, 2009; Fabian, Sonenberg and Filipowicz, 2010; Morozova *et al.*, 2012). There are over 2000 miRNAs identified which target about 60% of the human genes (Fromm *et al.*, 2015; Alles *et al.*, 2019; Kozomara, Birgaoanu and Griffiths-Jones, 2019). They play important roles in intercellular communication (Chen *et al.*, 2012; Gross, Kropp and Khatib, 2017; Z. Z. Wang *et al.*, 2018). The involvement of EVs as transport mechanisms for miRNA is also a known fact. Eleven miRNAs were identified as unique to the JAr EVs with more than 5 counts in 2/3 of the libraries. When the selection criteria were relaxed, the number of uniquely JAr EVs found were substantially high. However, the number of uniquely HEK293 EVs remained the same suggesting most of the HEK293 miRNA were also present in JAr EVs. Multiple reports have observed contrasting populations of miRNA in EVs extracted from different cell types (Yáñez-Mó *et al.*, 2015; Liu *et al.*, 2019; Temoche-Diaz *et al.*, 2019).

The action of miRNA is always linked to gene regulation by silencing or inhibition, resulting in a downregulation of the target gene/s. In the DEGs of the RL95-2 cells induced by JAr EVs, the specific targets of the uniquely JAr miRNA were in the both upregulated and downregulated groups. This observation suggests that miRNA induced silencing might not be the primary mode by which JAR EVs induce transcriptomic alterations. However, if miRNA induced silencing was not taking place at all, the high confidence targets of uniquely JAr miRNA should be upregulated or downregulated in similar proportion to the genes that were not targeted by miRNA. The majority of JAr-specific miRNAs targets (confidence > 90) were downregulated. The fold change (log2FC) of downregulated putative targets negatively correlated with the abundance of the uniquely JAr miRNA. Suggesting some measure of miRNA induced gene silencing.

It will be illogical to attribute the substantial effect JAr EVs had on the endometrial transcriptome to the actions of transferred miRNA or mRNA, especially considering the minute amount of RNA contained within EVs (Chevillet *et al.*, 2014; Albanese *et al.*, 2020). Whether the uptaken EVs release their miRNA and mRNA cargo to the cytosol of the endometrial cells is another unresolved aspect of the observed transcriptomic effect. With earlier results (Fig 7) the transfer of embryonic RNA to the endometrium is confirmed. However, there is no evidence that these transcripts are actually released to the cytosol.

Since the methods that attempt to confirm the cytosolic release is still rudimentary and require highly advanced and specialized expertise and equipment, it was assumed that miRNA and mRNA delivered by the EVs are at least partially exposed to the cytosol. Obviously, this assumption requires experimental confirmation and remains a major limitation in the studies comprising this thesis. Another limitation introduced during the experimental procedure is the lack of any treatment intended to remove any RNA not contained within the EVs that were supplemented to the RL95-2 cells. The approach of introducing RNAse to digest extraluminal RNA in EV samples was attempted. Due to the problems of inhibiting the activity of the RNAses used and the detrimental effects induced by the residual RNAse in the EV samples on the target cells, this approach was abandoned. It was assumed that with protein separation technique such as SEC, the amount of extraluminal nucleic acids would be negligible, again introducing another limitation to be considered while interpreting the observations.

However, based on the results of study 3, the role played by EVs, and specifically JAr-derived EVs as the mediator of the effect is clear, even though the molecular mechanisms underlying the majority of the transcriptomic changes remain elusive. Other types of regulatory mechanisms such as other biomolecules carried as cargo to the endometrium such as transcription regulation enzymes or cytokines, molecules bound to EV surface, other non-coding RNA could all play a role in the observed effects between the trophoblast and the endometrium.

Even though the mechanisms of specific effects on endometrial cells are not completely explainable by the collected data, the presence of specificity is undeniable. The data clearly shows that one type of EVs can induce a significantly different effect on recipient cells compared to other types of EVs. In other words, the induced effect can be used as a method of differentiating between the two types of cells. Since EVs can be harvested without damaging or sampling the cells that produce them, this observation has the potential of being utilized as a non-invasive method of categorizing cells that produce EVs given that harvested EVs induce quantifiable effects on the recipient cells. The non-invasive nature of this method increases its usefulness in evaluating precious cellular material such as embryos developed for ART.

6.8. Possible applications in the ART

In ART, it has been the usual practise for the longest time to transfer more than one embryo to the uterus to increase the chances of a pregnancy. However, this practise lead to a significantly high number of multiple births. Single embryo transfer has been gaining popularity to avoid these complications (Bromer and Seli, 2008). In single transfer, it is critically important to choose the best embryo for transfer. The best embryo is usually selected based on morphological criteria or morphokinetic calculations. A novel method of embryo

selection can be imagined using the observations of studies 1 and 3. Combining the identified transferred transcripts from study 1 and the observation of functional specificity seen in study 2, it can be hypothesized that "good" embryos would alter the expression of transferred transcripts in endometrial cells significantly differently compared to "bad" embryos. The expression of transferred transcripts would essentially be a biomarker for the functional competency of the embryo that produced the EVs.

When treated with EVs collected from morphologically good quality embryos (day 3 and day 5) endometrial cells showed a significant downregulation of the transferred transcript ZNF81 compared to both untreated cells and cells treated with morphologically bad quality embryos (both day 3 and day 5). This observation confirms the earlier hypothesis of utilizing the embryo-maternal communication induced transcript alterations as a method of embryo quality evaluation. Even though the study was performed on a limited number of embryos these preliminary results are a promising sign for future development of this methodology.

6.9. Similar observations in bovine reproduction

The field of ART has grown to encompass more than just human reproduction. Bovine ART is a field gaining rapid popularity especially in the cattle breeding industry (Moore and Hasler, 2017). Bovine embryos are also cultured using the same technologies used in human embryo development. Even though bovine embryo development and implantation are drastically different from human reproduction events, the embryo-maternal communication component is reported to be present in cattle reproduction (Valadão, Silva and Silva, 2018). Embryo-maternal communications begins with regulation of gamete production and continues through fertilization and embryo development(Hasan *et al.*, 2020; Reshi *et al.*, 2020). The cross talk that occurs during the period the embryo develops in the oviduct is one of the major aspects of embryo-maternal communications in bovine reproduction. Embryo is able to induce alterations to the oviductal epithelium both in vivo and in vitro (Schmaltz-Panneau *et al.*, 2014; Maillo *et al.*, 2015; Hamdi *et al.*, 2019) in cattle.

In vitro fertilized bovine embryos and BOEC cells were used to evaluate the effects induced by embryo derived EVs on bovine epithelial transcriptome and to examine whether the specificity observed in human embryo-maternal communication is also applicable to bovine reproduction. Good quality embryo derived EVs were able to induce significant transcriptional changes in the BOECs compared to bad quality embryo derived EVs. Perhaps of more importance are the genes upregulated by good quality embryo derived EVs. Collectively termed interferon-stimulated genes (ISGs), ISG-15, MX1, OAS1Y and LOC100139670 are particularly known to be involved with the interferon tau (IFN-τ) pathway. IFN-τ, much like hCG in human reproduction is widely known as a pregnancy recognition signal. It functions by inhibiting the

expression of oxytocin receptors and the synthesis of PGF2α. Similar to hCG, IFN-τ also prevents the breakdown of the corpus luteum (Demmers, Derecka and Flint, 2001). When co-cultured with BOECs, IFN-τ could be detected in the 15 cell stage (day 4 post fertilization) embryos (Talukder *et al.*, 2018) .

The results suggest that the good quality embryos used in the study 3 (15 cell stage morulae) were possibly capable of producing and secreting IFN-τ in sufficient concentration to induce a detectable effect in BOECs while bad quality embryos lacked the capability. Two separate studies specifically report an upregulation of these very genes in BOECs when co-cultured with embryos both in vitro (Schmaltz-Panneau *et al.*, 2014) and in vivo (Smits *et al.*, 2016). In both cases, embryos were physically present in adjacent to the oviductal cells. Since only EVs from embryos were used as supplementation in study 3, it could be hypothesized that the upregulation of ISGs are caused as a result of EV-mediated communication. The fact that NPs extracted from culture medium could not induce this effect further substantiates the claim of embryo derived EV-mediated communication between the embryo and the oviduct.

Most of the genes downregulated as a result of good quality embryo EVs such as UNC13D and ARHGEF2, were involved with the innate immune system (Birkenfeld *et al.*, 2008; Pivot-Pajot *et al.*, 2008; Zhao *et al.*, 2012). Embryos Being essentially foreign entities, immune regulation should be a vital system during the pre-implantation period to avoid the rejection by the maternal system.

Degenerating embryo derived EVs did not induce any significant alterations in the BOECs, exhibiting somewhat similar profile to media derived NPs. There is a possibility that good quality embryos produce a specific type of EVs recognizable by the BOECs due to the very different cellular morphology and biochemistry between the healthy and degenerating embryos (Betts and King, 2001; Betts and Madan, 2008; Graf *et al.*, 2014).

Much like human embryo-maternal communication, the bovine embryo-maternal communication seems to be specific to the functional competency of the embryo initiating the communication. There is a clear possibility of developing a non-invasive method of embryo grading using the effects induced by bovine embryo derived EVs on oviductal epithelial cells.

6.10. Future prospects

Taken together the results of these studies clearly describe the foundations of an embryo-maternal communication based on EV exchange between the maternal system and the pre-implantation embryos. The implications of good quality embryo communicating differently from bad quality embryo with the endometrium and (in bovines) the oviductal epithelium and the evidence of functional specificity between different types of EVs are especially intriguing. The "quality" of an embryo is almost synonymous with "morphological" quality of an embryo in the current usage. Almost all the available systems of embryo

sorting for ART is based on morphological quality of embryos. However, there seems to be an upper limit to the efficacy of morphology-based embryo selection. Since the maternal tract is clearly able to sense the quality of embryos by the effect that embryonic EVs have on the epithelial cells, it could potentially be utilized as a non-invasive method of embryo sorting completely based on embryo functionality as opposed to the morphological characteristics of the embryo. If implemented correctly with further confirmatory steps endeavouring to understand the underlying mechanisms of this unique phenomenon, these observations could be the first step in the path to a completely unique non-invasive system of embryo sorting.

The results show that the effects induced by trophoblast spheroids are focused on altering the endometrial transcriptome to create a more favourable environment for embryo adhesion and the subsequent invasion. The results also show that good quality embryos can induce a more drastic change in the endometrium compared to the bad quality embryos. Based on these observations, it can be hypothesized that this ability to alter the endometrial physiology is what makes a “good” embryo “good” in a purely functional sense of the word. Of course, there needs to be much more evidence to substantiate this hypothesis such as quantifying the effects induced by embryos that were successful in producing a pregnancy to embryos that failed to do so. The mechanisms of such physiological alterations should also be elucidated. However, if sufficiently substantiated, there is a possibility that the endometrium can be tricked into accepting a “bad” embryo as a “good” embryo by pre-treating the maternal tissue with “good” embryo derived EVs that would alter the endometrium sufficiently to allow the “bad” embryo to implant. Even though it appears far-fetched at the present level of knowledge, there has been attempts of utilizing EVs as therapeutic agents that can deliver bioactive compounds to the target tissue without affecting the other tissues in the vicinity. There is a high potential of using EVs as a therapeutic agent against subfertility.

The process of reproduction does not end with a successful implantation. Even though an embryo is implanted, there are many hurdles yet to overcome before a successful birth. Recent attempts to use embryo conditioned media derived EVs as a source for embryonic genome material might prove to be a powerful non-invasive diagnostic tool in detecting genetic abnormalities in the embryo prior to transfer.

The process of ART is commercially, physically and emotionally taxing to the patients and their families. With improved diagnostics, embryo development, embryo selection and therapeutic agents, the time to a successful pregnancy can be reduced.

7. CONCLUSIONS

Study 1: Trophoblast spheroid RNA is transferred from the trophoblast to the endometrial cellular cytoplasm without physical contact between the two cell types. One of the modes of RNA transport is EVs. Following the transfer, the transferred transcripts are downregulated in the endometrial cells.

Study 2: Trophoblast spheroid derived EVs induce significant alterations to the endometrial transcriptome. Overall effect of the transcriptomic change is focused on pathways vital to embryo implantation. The trophoblast spheroid derived EVs appear to prepare the endometrial cells for the imminent adherence by the trophoblast spheroid confirming the functional intentionality of embryo-maternal cross talk.

Non-trophoblast spheroid derived EVs do not induce any significant effects on endometrial transcriptome. This observation suggests that the EV-mediated embryo-maternal cross talk is functionally specific to the trophoblast EVs.

Study 3: Both trophoblast spheroid derived EVs and non-trophoblast derived EVs contain RNA (both mRNA and miRNA). The cargo of RNA in trophoblast spheroid derived EVs is different from the RNA cargo of non-trophoblast derived EVs. miRNA delivered by trophoblast spheroid derived EVs are partially responsible for the transcriptomic changes observed in the endometrial cells.

Study 4: Morphologically good quality human embryo EVs induce a significantly different effect on the identified endometrial transcripts compared to morphologically bad quality embryo EVs. This differential effect further confirms the functional specificity observed in the earlier studies. There is a high potential of developing these findings as a non-invasive embryo grading system to be used in human assisted reproduction.

Similarly, good quality bovine embryo EVs can induce a significantly differential effect on bovine oviductal epithelial cells compared to bad quality embryo EVs, suggesting that a non-invasive embryo grading system based on EV induced maternal transcript alterations has the potential to be applied universally in mammalian assisted reproduction.

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SUMMARY IN ESTONIAN

Rakuväliste vesiikulite vahendatud embrüo-emaka suhtlus – vahend hindamaks implantatsiooni-eelse embrüo talituslikku pädevust

Implantatsioon on imetajate reproduktsioonis oluline etapp, kus kõigepealt kinnitub embrüo endomeetriumi epiteelile ja edasi tungib epiteelialusesse stroomasse. Embrüo nidatsiooni peetakse inimese kunstliku viljastamise kitsaskohaks. Umbes 2/3 nurjunud kunstliku viljastamise katset on põhjustatud implantatsiooni ebaõnnestumisest (Jarvis, 2016). Hoolimata abistava reproduktsioonitehnoloogia (ART) edusammudest jääb embrüo siirdamisest tekkinud rasedus alla 50%, mille peamine põhjus on edutu implantatsioon (Apter et al., 2017). Mitmed teadusuuringud on üritanud mõista ebaõnnestunud nidatsiooni põhjuseid kuna embrüo morfoloogiline kvaliteet ei seostu eduka implantatsiooni ja sünnitusega ka juhtudel, millal emaka keskkond on optimaalne pesastumiseks (Wilcox, Baird and Weinberg, 1999).

Endomeetriumis toimuvad mitmed füsioloogilised ja morfoloogilised muutused, et jõuda embrüo vastuvõtlikkuse staadiumi, mida nimetatakse implantatsiooniaknaks (*window of implantation* – WOI) (Ashary, Tiwari and Modi, 2018). Need muutused põhjustavad emaka immuunkeskkonna täieliku ümberkorralduse, mis on oluline embrüo kinnitumiseks endomeetriumi. Embrüo saab pool genoomi ja osad antigeenid isalt, mis emaka normaalse immuunsüsteemi talitusel tuvastatakse ning mille tagajärjel embrüo eemaldatakse endomeetriumist. Sellest hoolimata on täheldatud, et endomeetriumi immuunsüsteem suudab samal ajal eirata embrüot ja kaitsta implatatsiooni asukohta. Seda protsessi nimetatakse indutseeritud immuunignorantsuseks, mille täpseid esilekutsumise mehhanisme ei teata (Warning, McCracken and Morris, 2011).

Pikka aega on arvatud, et endomeetrium ja embrüo on reproduktsioonis passiivsed või nii nimetatud „vaikivad“ osapooled. Mitmed uuringud on leidnud, et nidatsiooni ja embrüo invasiooni endomeetriumi põhjustavad välised tegurid nagu hormoonid ja tsütokiinid. Viimastel aastakümnetel on aga tuvastatud lisaks uus komponent embrüo implantatsiooni regulatoorsetes mehhanismides – embrüo-emaka suhtlus (Modi ja Bhartiya, 2015).

Uus hüpotees püstitab teooria, kus embrüo ja endomeetrium ei järgi passiivselt endokriinsüsteemi juhiseid, vaid võtavad aktiivselt osa üksteisega suhtlemises ühe või mitme signaalmeetodiga (Bhagwat et al., 2013). Sellest hoolimata teatakse embrüo-emaka suhtlusest vähe. Varasemalt on tõestatud, et lähestikku olevad rakud on võimelised suhtlema erinevate signaalmolekulitega nagu tsütokiinid, hormoonid, eritatud proteiinid ja teised biomolekuid (Nakamura et al., 2019). Mitmed endomeetriumi eritatud ained emaka keskkonnas reguleerivad implantatsiooni mõjutades otseselt blastotsüsti arengut või kaudselt adhesioonimolekulide avaldumist (Ashary, Tiwari and Modi, 2018). Tuvastatud on ka

embrüo päritolu molekule, mis mõjutavad emaka kude (endomeetriumi ja munajuha) põhjustades füsioloogilisi muutuseid ja kutsudes esile WOI.

Eelnevalt nimetatud biokeemilised sõnumitoojad peavad sihtkohta jõudmiseks läbima emaka valendikus oleva rakkudevahelise ruumi. Rakkudevaheline ruum ei ole aga sobilik keskkond osadele biomolekulidele nagu RNA. Mitmed uuringud on pakkunud, et rakuvälised ehk ekstratsellulaarsed vesiikulid (EV) on rakusuhtluseks oluline transportmehhanism, mis kaitseb biomolekule ebasobilikes keskkondades ning tõstab sihtrakku jõudmise spetsiifilisust (Nakamura, Kusama, Suda, et al., 2020; O'Neil et al., 2020).

Kogudes tõendeid EV-de vahendatud embrüo-emaka suhtlusest oleks tulevikus võimalik kasutada emaka rakkudes toimuvaid muutuseid bioloogilise sensorina, mis lubaks kvantifitseerida embrüo-emaka suhtlust. Kunstlikus viljastamises kasutatakse mitmeid meetodeid, et hinnata embrüo kvaliteeti. Embrüo liigitamine kvaliteedikategooriatesse on vajalik kunstlikus viljastamises parima embrüo valimiseks, mis vähendab ohte mitmikute sündimisel. Enamik kasutatavad meetodid põhinevad embrüo morfoloogilistel ja morfokineetilistel hindamistel, mille rasestumise edukus on 35%. Embrüo-emaka suhtlus võimaldab hinnata embrüo funktsionaalseid omadusi, mis pakub teoreetilise võimaluse kasutada embrüo-emaka suhtlust embrüo kvaliteedi hindamiseks lisaks morfoloogilistele metoodikatele.

Doktoritöö eesmärgid

Doktoritöö üldine eesmärk oli uurida EV päritolu mittekodeeriva RNA-l (ncRNA) põhinevat embrüo-emaka suhtlust ja embrüo põhjustatud endomeetriumi füsioloogilisi muutuseid.

Täpsed eesmärgid:

1. Tuvastada inimese trofoblasti analoogist (JAr-rakud) edasiantav RNA inimese kesk-sekretoorse endomeetriumi epiteelrakkude analoogile (RL95-2 rakud) kasutades bioortogonaalset märgistust ja trofoblasti sferoide (uuring 1).
2. Kasutades mRNA sekveneerimist hinnata trofoblasti sferoidi EV mõju endomeetriumi analoogi transkriptoomile. Indutseeritud efekti spetsiifilisust uuriti mittetrofoblasti sferoidide (HEK293 sferoid) EV-dega (uuring 2).
3. Uurida trofoblasti sferoidide EV-des olevat RNA-d ning seostada bioloogiliselt aktiivse ncRNA-d teises uuringus tuvastatud muutustega, et selgitada EV-de põhjustatud muutuste mehhanisme endomeetriumi analoogis (uuring 3).
4. Võrrelda morfoloogiliselt hea ja halva kvaliteediga embrüo EV-de mõju emakakoele kasutades nii inimese kui ka veise (*Bos Taurus*) *in vitro* kasvatatud embrüoid eesmärgiga leida mitteinvasiivne embrüo hindamise meetod (uuring 4).

Metoodika

Inimese embrüote kasutamist katseteks piiravad eetilised ja juriidilised aspektid. Selle tõttu kasutasime *in vitro* embrüo-emaka suhtluseks rakukultuuri mudelit, kus JAr (inimese kooriokartsinoomi rakud) sferoidid asendasid trofoblaste ja RL95-2 (inimese adenokartsinoomi rakud) rakud vastuvõtlikku endomeetriumi. Katsed algasid trofoblastide sferoidide RNA märgistamisega 5-etünüüluridiiniga (uratsiili analoog). Märgistatud sferoide inkubeeriti kultuurisüsteemis endomeetriumi rakkudega, et uurida RNA transporti rakkude vahel. Märgistatud transkriptid koguti retsipentrakkudest (endomeetriumi rakud) klikikeemia meetodil ja sekveneeriti ioontorrentsüsteemiga. Seejärel eraldati sferoidide kasvusöötmest EV suuruskromatograafia meetodil, et tuvastada RNA ülekande viis rakkude vahel. Isoleeritud EV lisati endomeetriumi rakkudele 24h ja rakkudes toimunud transkriptoomi muutused kvantifitseeriti Illumina mRNA sekveneerimisega. Kuna kõige tõenäolisemalt põhjustab EV-põhise embrüo-emaka suhtluse miRNA, siis tuvastati NGS meetodil trofoblasti EV-de miRNA ja mRNA. Negatiivseks kontrolliks kasutati HEK293 EV-sid.

Selleks, et hinnata seost retsipentrakkude muutuste ja embrüokvaliteedi vahel lisati endomeetriumi rakkudele hea ja halva kvaliteediga embrüo EV-sid. Sarnased katsed viidi läbi veise embrüotega, et uurida kas EV-de põhjustatud endomeetriumi rakkude transkriptoomi muutused on sarnased imetajate liikidel.

Tulemused

Uuringus tuvastasime kolm transkripti (mRNA ZNF81, üks ekspresseeruv ja interfereeruv regioon LINC00478), mis kandusid trofoblastidelt endomeetriumi rakkudesse. Huvitaval kombel ülekantud transkriptid reguleerisid alla antud geenide avaldumise endomeetriumi rakkudes. Samasuguseid muutusi täheldati ka trofoblasti EV-de lisamisel endomeetriumi rakkudele, mis näitab et EV-d osalevad rakkudevahelises suhtluses.

Endomeetriumi rakkude transkriptoomi muutis märgatavalt trofoblastide EV. HEK293 rakukultuuris, kuhu oli lisatud EV-sid, avaldusid erinevalt 1754 geeni võrreldes HEK293 rakukultuuriga, kuhu EV-sid ei lisatud. Geeni rikastamise analüüsil tuvastati, et transkriptoomi muutused olid implantatsiooniks olulistel molekulradadel nagu rakuvälise maatriksi ümberkorraldus. Trofoblasti ja HEK293 EV-des olev mRNA ja miRNA olid erinevad, kuid tuvastatud miRNA-d põhjustasid vähem kui 10% transkriptoomi muutuseid.

Hea kvaliteediga embrüote EV suutsid reguleerida alla endomeetriumi rakkudesse ülekantud transkripte. Sama ei teinud halva kvaliteediga embrüote EV. Antud tulemus viitab, et embrüo kvaliteet ja rakkudevaheline suhtlus on omavahel seotud. Sarnaseid embrüo kvaliteedil põhinevaid erinevusi täheldati ka veise embrüo ja munajuha rakkude vahelises suhtluses.

Arutelu

Imetajate sigimine on keeruline protsess. Oluliseks aspektiks reproduktsioonis peetakse embrüo-emaka suhtlust. Doktoritöös kirjeldatud uuringutes tõestati, et trofoblasti rakud suhtlevad endomeetriumi rakkudega implantatsiooni-eelsel perioodil, kus ncRNA transportimiseks rakkude vahel kasutatakse EV. Rakkudevahelises suhtluses muutub endomeetriumi transkriptoom füsioloogiliselt olulistes mustrites, mida osaliselt põhjustab EV-des olev miRNA. Suhtluse aktiivsus (mõõdetud transkriptide muutuste hulgana) on seotud embrüokvaliteediga nii inimese kui ka veise reproduktsioonis. Antud tulemus näitab võimalust kasutada embrüo-emaka suhtlust embrüo talitusliku pädevuse hindamiseks. Küll on vaja antud meetodit edasi arendada ja läbi viia kliinilisi uuringuid, et hinnata selle mitteinvasiivse embrüo hindamise meetodi praktilise kasutamise võimalust kunstlikus viljastamises.

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