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Embryo- and endometrium-derived exosomes and their potential role in assisted reproductive treatments – liquid biopsies for endometrial receptivity

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Abstract

Multiple pregnancies resulting from the transfer of more than one embryo pose a significant threat to offspring born through Assisted Reproductive Treatments (ART). Transferring one embryo at a time would eliminate this risk. However, current approaches of identifying the highest quality embryo to transfer are either unreliable (*e.g.* morphology assessment) or highly invasive and potentially detrimental to embryos (*e.g.* PGD). Approaches for non-invasive embryo selection would be a major advancement that would increase efficiency and reduce both the costs and the risks associated with ART. Exosomes are a particular subtype of extracellular vesicles (EVs) that are secreted from a wide range of cells, including placental and endometrium cells. Exosomes are very stable vesicles that contain a broad spectrum of molecules, including proteins, mRNAs and miRNAs. Very little is known about this form of cell-to-cell communication in the context of ovarian follicular biology and implantation, but emerging data suggest that exosomes secreted by the blastocyst could influence gene expression and receptivity of endometrial cells thereby controlling its own implantation. Here we review emerging findings regarding exosomal signalling in reproductive biology and the prospects for mapping blastocyst-derived exosomal profiles as a means for supporting single embryo transfer policies.

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Introduction

Implantation involves intricate communication between embryos and the maternal endometrium. Increasing interest is centred on extracellular vesicles (EVs) and their contained cargo, particularly microRNAs (miRs), as important mediators of this dialogue [1, 2]. Recently, a role for EVs in cell-to-cell communication has been established [3]

EVs are classified according to their size and origin into exosomes and microvesicles (MVs). Microvesicles are released from budding of plasmatic membrane (PM) while exosomes originate in the endosomal compartment (early and late endosomes) (Figure 1). Exosomes circulate inside the cells as intraluminal vesicles (ILV), which are incorporated in the early to late endosome and multivesicular bodies (MVB) and then travel to the PM and are released by fusion of the MVB and PM to the extracellular environment as exosomes [4]. Exosomes and MVs contain a wide array of molecules, including proteins, nucleic acids, and lipids. Protein constituents of EVs have been widely studied due to their roles in signalling cascades. As such, the delivery of proteins via exosomes may shape the bioactivity of target cells and tissues. For example, EVs contain matrix metalloproteinases, commonly secreted in the EVs of tumours in particular leading to potentially oncogenic effects. It has also been found that exosomes may contain a subset of proteins that is dependent on the cell type of origin. On the other hand, exosomes also contain proteins such as membrane-derived proteins and endosomal proteins that are ubiquitously found in most exosomes. Contrastingly, proteins derived from other organelles such as the Golgi apparatus and the endoplasmic reticulum are not included in most exosomes [4]. Some proteins highly abundance in EVs, particularly exosomes, include ALIX, TSG101, CD63, and CD81. MHC II molecules are also found abundantly in MVs. Nucleic acids are also a notable component within EVs. Valadi *et al*. uncovered the presence of mRNA and miRNA in exosomes, thus further broadening the understanding of exosomal content [5]. mRNA has also been reportedly found in MVs. Thus,

this content suggests EVs, particularly MVs and exosomes, may be a pathway for the transfer of genetic information and the alteration of gene expression in recipient cells. Notably, mRNA inside EVs is resistant to digestion by RNAse treatment [6]. Lipid composition of EVs has not been as extensively studied. However, it has been found that EVs tend to be enriched in sphingomyelin, saturated fatty acids, and cholesterol. Exosomes are particularly thought to be enriched with ganglioside GM3 and ceramide derivatives. Variations in lipid content may be indicative of different origins from the plasma membrane, including lipid rafts. It is also noteworthy that the composition of EVs changes based on the extracellular environment, as illustrated by various studies. For example, RNA and protein content of EVs may be altered due to vascular injury or acidic environments as occurs in cancer.

The mechanisms involved in the incorporation of specific molecules such as proteins and RNAs in EVs remain to be established, however, there exists a paucity of data showing that changes in the microenvironment milieu regulates the secretion and composition of this vesicles in a wide range of cells including placental cells [7-10]. Moreover, placental exosomal signalling has been characterised across gestation [11-13], and placental exosomes are involved in the maternal immunological response during pregnancy [8, 14].

Here we review recent findings pertaining to EVs secreted by embryos and endometrial epithelium with a particular focus on instances in which mechanistic pathways have been elucidated. Because miRs are a prominent cargo of EVs mediating many of their effects [4], reference will also be made to new findings involving miRs. For more extensive accounts of miRs during implantation the reader is referred to recent reviews on this topic [15, 16].

Overview of implantation and extracellular vesicles

Successful pregnancy depends on proper implantation involving three recognised stages, apposition, attachment (or adherence) and invasion. Completion of these stages in turn depends on a receptive endometrium, a viable embryo at the appropriate developmental stage and a well-orchestrated dialogue between the two. Inadequate implantation can result in spontaneous miscarriage whilst defects in trophoblast invasion required for proper placental formation predispose to complications of later pregnancy such as pre-eclampsia and intrauterine growth restriction. Surprisingly little is known about the molecular details underpinning a productive embryo-endometrium rapport.

In recent years, there has been increasing interest in the role of EVs, particularly microvesicles (MVs) and exosomes, in mediating the embryo-endometrial cross-talk [1]. EVs contain diverse cargo including cell surface receptors, lipids, messenger RNAs (mRNAs), miRs, proteins and even DNA, and are identified by their size and the presence of cell surface markers such as the tetraspanins, CD9, CD81 and CD63 [4]. EVs are increasingly recognised as an important mode of cell-to-cell communication as they can transfer their contents to other cells thereby altering the recipient's behaviour [17].

Embryo-derived pathways involving EVs and their cargo

Surprisingly little is known about EVs secreted by preimplantated mammalian embryos. Indeed, in a review in 2014, it was noted that embryo-derived EVs had not yet been reported [2]. Since then, two papers have investigated embryo-secreted EVs whilst the majority have studied embryo-secreted miRs, a well-known EV cargo. miRs are small (18-23 nucleotide) non-coding RNA sequences that are largely regarded as post-transcriptional silencers of gene expression through engaging the 3' untranslated region of target mRNAs via complementary base-pairing leading to their degradation or repression [18].

Embryo-derived EVs exert extra- and intra-embryonic effects

Two papers have shown that EVs produced by embryos contain cargo that can influence the behaviour of neighbouring cells [19, 20]. In one case the cargo is mRNA with the potential to impact the viability of other embryos [20] whilst in the other case EVs are proposed to shuttle protein from embryonic stem cells (ES cells) of the inner cell mass (ICM) to trophectoderm cells [19].

Exosomes and MVs have been isolated from conditioned media following culture of parthenogenetic porcine embryos. These EVs were found to contain mRNAs for the pluripotency genes, Oct4, Sox2, Klf4, c-Myc and Nanog [20]. The authors found that the *in vitro* blastocyst development rates of cloned embryos (produced by somatic nuclear transfer into enucleated oocytes) were more than doubled by co-culturing them with parthenogenetic embryos. Notably, co-cultured cloned embryos exhibited increased expression of Oct4, Klf4 and Nanog. Since these were the cargo of EVs isolated from conditioned media, this raised the possibility that the transfer of pluripotency factors via EVs could have a role to play in improved development. As proof-of-concept that embryos could assimilate EVs from the surrounding environment, purified exosomes/MVs derived from parthenogenetic embryo conditioned media were labelled with the green fluorescent dye, PKH67 (which stably incorporates into lipid regions of cell membranes). Culturing cloned embryos with PKH67-labelled EVs for 22 hours led to the appearance of green fluorescence signals in cloned blastomeres consistent with uptake of EVs from the media. Interestingly, although it appeared that transfer of EVs during co-culture improved cloned porcine embryo development, bolus addition of parthenogenetic embryo conditioned media was not beneficial [20]. The authors speculated that this could be because of the need for continuous mRNA transfer due to the

active destruction and reduced translational potential of foreign mRNA. Taken together, these data show that mammalian embryos secrete EVs into their surrounding environment and support the notion that such EVs can be taken up by neighbouring cells to influence their development.

A very recent paper found that MVs were shed by mouse ES cells, which are derived from the ICM of the blastocyst [19]. These MVs were shown to express the extracellular matrix proteins, fibronectin and laminin α5. Moreover, MV fibronectin and laminin interacted with integrin receptors on trophoblast cells in turn leading to the activation of focal adhesion kinase (FAK) and c-Jun N-terminal kinase (JNK), which are often implicated in promoting cell migration. Indeed, using an *in vitro* migration assay, the authors could show that either ES cell spent media or EVs purified from spent media promoted trophoblast migration that was abolished when FAK and JNK activation were inhibited. To determine whether ES cell-derived MVs could promote trophoblast migration and invasion of intact murine blastocysts, an elegant approach was used involving ES cells expressing a plasma membrane-targeted green fluorescent protein (PM-GFP), which produced fluorescent MVs. Fluorescence that was restricted to the trophectoderm following microinjection of blastocysts with purified fluorescent MVs from PM-GFP-expressing ES cells supported that trophectoderm could indeed internalise MVs produced by cells in the ICM. Strikingly, following transfer to the uteri of surrogate females, blastocysts injected with MVs from ES cells exhibited significantly increased implantation rates entirely in keeping with *in vitro* data showing that ES cell MVs augmented trophoblast migration [19]. Unlike the majority of studies that have focused on communication between maternal tissue and embryonic trophoblast during implantation, here the authors reveal a novel pathway of signalling between the two major compartments of the preimplantation embryo that ultimately promotes implantation.

Evidence for miRNA secretion from human embryos

It has been established that exosomes provide a protective and enriched source of miRs [6]. The foregoing studies demonstrated that EVs are secreted by mouse and porcine embryos. Human embryos are routinely cultured as part of *in vitro* fertilisation (IVF) treatment and therefore present an opportunity to investigate their secretory products, especially since analysis of spent media does not incur any risk to the embryo or compromise the patient's treatment success. Significantly however, EVs have not yet been shown to be secreted by human embryos. In addition, initial attempts to isolate miRs from spent human blastocyst media were unsuccessful. However, in recent years, miRs have been identified in spent media from human blastocysts derived from IVF treatment [21-25].

Kropp et al. probed for the presence of 5 miRs and identified miRs in spent media following culture of human and bovine embryos [23]. Around the same time, Rosenbluth and co-workers profiled 754 miRs and detected 10, of which only two (miR-372 and miR-191) were specific to human embryo conditioned media [25]. Both miRs were increased in spent media derived from embryos that were fertilised using intracytoplasmic sperm injection (ICSI) compared to those fertilised by standard insemination. Three miRs (miR-372, miR-191 and miR-645) were differentially expressed in conditioned media from non-ICSI cycles depending on whether or not the blastocyst led to a successful pregnancy whilst miR-191 alone was enriched in spent media from aneuploid embryos versus euploid ones [25].

Capalbo et al. found that 59 out of 377 miRs could be detected in 3 out of 5 replicates of spent media from human blastocysts [21]. Furthermore, 57 of these 59 miRs (96.6%) could also be detected in biopsied trophectorderm cells suggesting their release from blastocysts into media. They next sought to determine whether miR profiles in spent media might correlate with implantation potential. To rule out confounding effects of embryonic

aneuploidy, analyses were restricted to embryos that were proven to be euploid by comprehensive chromosome screening of trophectorderm biopsies. Spent media from 53 euploid blastocysts were prospectively analysed, 25 of which resulted in an on-going pregnancy. Two miRs (miR-20a and miR-30c) were found to be increased in media from implanting blastocysts and 5miRs (miR-220, miR-146b-3p, miR512-3p, miR-34c and miR-375) were preferentially detected with implanted blastocysts. Interestingly, based on in silico prediction and experimentally validated miR targets, miR-20a and miR-30c miRNAs would be predicted to influence endometrial cell growth and proliferation [21].

The same group subsequently compared the profiles of 377 miR sequences in spent media from "twin" human blastocysts created by artificial embryo splitting at the cleavage stage with that of control blastocysts that produced healthy pregnancies [24]. They found 59 miRs secreted by control blastocysts and 48 miRs by twin embryos of which, only 22 were shared. Interestingly, in twin embryo spent media, they found significantly lower levels of miR-30c, which they previously identified as a putative biomarker of implantation potential.

Embryo-mediated regulation of the uterine epithelium

Studies showing that embryos secrete EVs and/or miRs, which target genes predicted to mediate cellular activities such as adhesion and migration, suggest that embryos could potentially modify implantation events. The next question pertains to whether secreted miRs/EVs can be internalised by uterine cells and can alter endometrial function and implantation.

Cuman et al. showed that human blastocysts secrete miRs into media and went one step further by interrogating the mechanisms by which the secreted miRs might influence implantation [22]. Using a 784 miR array panel, they found that the miR profile of spent

media from human blastocysts failing to implant differed markedly from implanting media and identified miR-661 as the most highly differentially expressed miR. *In vitro* studies showed that miR-661 was readily internalised by primary human endometrial epithelial cells (HEEC) cultured in non-implanting spent media supporting that human endometrial cells could take up blastocyst-secreted miR-661. Following ultracentrifugation of spent media, miR-661 did not co-segregate with the pellet containing MVs but was enriched in the supernatant where it co-immunoprecipitated with the RNA binding complex (RBC) protein, Argonaute 1, indicating that miR transport involved RBC-binding rather than encapsulation within MVs. In silico analyses revealed that miR-661 targets included genes involved in adhesion/invasion. Significantly, the protein levels of two of these genes, *PVRL1* and *MTA2*, which were shown to be expressed in human endometrial glandular and luminal epithelial sections, were down-regulated in HEEC exposed to miR-661-containing conditioned media. Collectively, these data indicated that miR-661 could be secreted from blastocysts and taken up by endometrial cells to reduce the expression of key pro-implantation factors. To further test this, the authors employed an *in vitro* adhesion assay and showed that adhesion of spheroids to HEEC was severely hampered following miR-661 treatment and importantly, that protecting *PVRL1* from being targeted by miR-661 could rescue adhesion [22]. Although MV-dependent pathways are the focus of this review and miR-661 in this paper partnered with RBC rather than MVs, it nevertheless provides compelling proof-of-concept that embryos actively modulate endometrial receptivity via miR-mediated pathways and offer a paradigm by which EV cargo might also influence implantation through their miR cargo. It is possible that miR cargo might act at the epigenetic level to bring about changes in endometrial gene expression. In line with this, transient over-expression of miR-30d in HEEC led to changes in the levels of regulatory factors involved in DNA methylation such as DNA methyltransferase 1 (DNMT1)[26].

Whilst miRs and/or EVs might be taken up *in vitro* by cultured cell-lines as shown in the above study, does this also apply in the *in vivo* context of a 3-dimensional uterine cavity? Recent data from the ovine model provide evidence in this regard [27]. In sheep, attachment to the uterine epithelium begins by Day 16 post-mating at the filamentous stage, an elongated stage that arises 8 days after blastocyst hatching. Conditioned media obtained following *in vitro* culture of elongated Day 14 conceptuses for 24 h was found to contain EVs with an average diameter of 150 nm and a size range consistent with both exosomes and MVs. Mass spectrometry analysis of EV content identified 231 proteins while RNA sequencing detected 512 mRNAs. To investigate which cells were targeted by embryo-secreted EVs, an *in vivo* model was used in which EVs isolated from spent media were first labelled *in vitro* with the PKH67 green fluorescent dye and then infused into the uterine horn using a catheter and osmotic pump from Days 8-14 postestrus prior to necropsy [27]. Distinct green fluorescence signals were observed in cross-sections of luminal epithelium and superficial glandular epithelium of the uterine horn but not in the uterine stroma or myometrium or more distant sites such as ovary, parametrial lymph nodes or lung. Thus, these findings provide evidence that uterine epithelia can take up EVs secreted by embryos *in vivo*.

Extracellular vesicles secreted by the endometrium

The success of human pregnancy is dependent of the interaction between blastocyst and endometrium. The human endometrium is a complex tissue in which the implantation takes place [28]. The endometrium exhibits several morphological changes that allow the interaction with the blastocyst. Interestingly, it has been proposed that exosomes secreted from the endometrium influence the blastocyst to attach and invade the endometrial epithelium [29, 30]. Synchronous crosstalk between the endometrium and blastocyst in the

placental developmental and pre-implantation phase is essential for initiating pregnancy. It has previously been suggested that the endometrial luminal epithelium may become more or less receptive to extracellular signals through molecular exchange by exosomes and other EVs. Ng et al., suggested that exosomes could be released from the endometrial epithelium, thereby transferring molecular cargo to the blastocyst or the endometrium. Exosomes as well as MVs were found to be present in preparations of uterine fluid/mucus and endometrial epithelial cells. miRNA were found to be sorted into exosomes/MVs, with 13 of the 227 isolated miRNAs being exclusively found to the EVs. Has-miR-200c, has-miR-17 and has-miR-106a were found at the highest levels within the EVs. Bioinformatic analysis revealed that these particular miRNAs may have roles in biological processes associated with implantation [30]. The endometrium is marked by cyclical changes, including the transitions between the proliferative (nonreceptive) and secretory (receptive) phases throughout menstruation. During the non-receptive phase, these transitions are modulated by estrogen. On the other hand, progesterone is the key modulatory factor during the receptive phase. Recently, Greening *et al.*, have establish that the exosomal cargo is regulated by both hormones, as well as the phase of the menstrual cycle during which the exosome is packaged and secreted [29]. Interestingly, uptake of exosomes and release of exosomal content has been associated with changes in the properties of trophoblasts. For example, exosomal uptake has been linked to increased trophoblast adhesive capacity at the time of implantation.

Proteomic analysis in exosomes isolated from endometrial cells suggest that glycoproteins (fibulin-1, in particular) and integrins in exosomes are associated with cell adhesion factors, cell migration, and remodeling of the ECM. Furthermore, Fibulin-1, was expressed ~9-fold higher in estrogen/progesterone exosomes, compared to estrogen exosomes [29]. This suggests selective packaging of integrins into endometrium-derived exosomes. Taken together, these results suggest that endometrium exosomes may play an important role in cell-

to-cell communication crosstalk between the endometrium and blastocyst during implantation. Therefore, exosomes of endometrial origin may be a platform for potentiating implantation of the embryo and enhancing fertility and gestational outcomes.

Potential clinical applications

Recent IVF data for Australia show that of 71,516 initiated IVF cycles in 2013, only 23.8% resulted in a clinical pregnancy and even fewer, 18.2%, in a live delivery. To increase success rates, the temptation is to transfer more than one embryo but at the risk of increasing perinatal morbidity well-known to be associated with multiple pregnancies. A major reason for poor success rates is failed implantation. Surprisingly however, very little is known regarding the molecular embryo-endometrium "cross-talk" required for successful implantation. Exosomes mediate communication between different cell-types via their content of signalling molecules including miRNAs and mRNAs. An intriguing possibility is that the miRNA profile of exosomes from high-quality blastocysts is pivotal for their higher implantation potential and that defining such a profile could improve embryo selection capability and greatly refine Assisted Reproductive Treatment (Figure 2). The capacity to identify a predictive biomarker from spent media – and therefore at no risk to the embryo – would be a powerful non-invasive innovation. This can be contrasted with current selection approaches involving tedious scoring systems for embryologic morphology, which have notoriously poor predictive value, and invasive embryo biopsy for chromosomal analyses, which are traumatic to embryos. It should be noted however, that EVs have been reported to be present in IVF culture media alone [2]. Furthermore, some findings indicate that media components such as protein supplements could be miRNA carriers [23, 25]. Use of spent media would therefore need to take into account possible contaminants from the media itself, which could vary from

one lab to the next if different media formulations are used, making it important to incorporate steps for ensuring that only embryo-derived products are being analysed. Given that exosomes secreted by the endometrium might promote implantation, another potential clinical application could be the delivery of specific cargo via exosomes into the uterine cavity for the purposes of enhancing embryonic implantation and placentation.

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Figure 1. Biogenesis and content of exosomes and microvesicles. Exosomes are nanosized vesicles (30-150 nm) of endocytic origin that are released from cells into the extracellular space by exocytosis following the fusion of multivesicular bodies with the cell membrane. MVs are larger (100 nm – 1.5 µm) and are produced by direct budding of the plasma membrane. Exosomes and microvesciles contain proteins, lipids and nucleic acids, mediating intercellular communication to modify the different biological function of target cells.

Figure 2. Single embryo selection via exosomes profile. Current approaches for identifying the highest quality embryo to transfer are either unreliable (e.g. morphology assessment) or highly invasive and damaging to embryos (PGD). Approaches for non-invasive embryo selection would be a major advance that would increase efficiency and reduce both cost and risks associated with ART. We suggest that embryo-derived exosomes profile may be used to single embryo selection to in vitro fertilisation implantation potential as a means for deriving a novel non-invasive biomarker which will greatly advance embryo selection and single embryo transfer capability.

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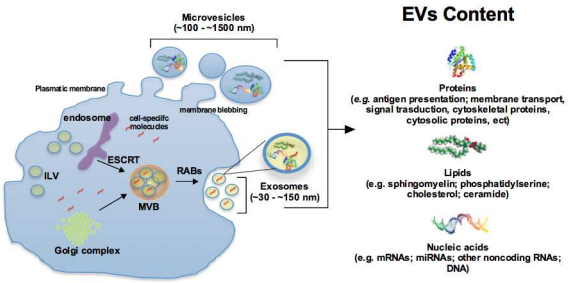

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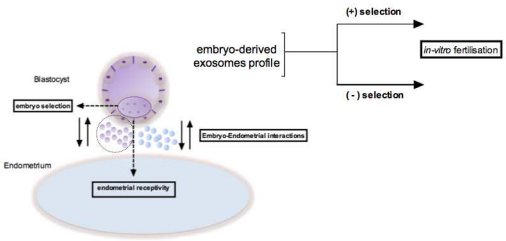

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