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Extracellular vesicles: roles in gamete maturation, fertilization and embryo implantation

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BACKGROUND: Extracellular vesicles (EVs) are membrane-bound vesicles, found in biofluids, that carry and transfer regulatory molecules, such as microRNAs (miRNAs) and proteins, and may mediate intercellular communication between cells and tissues. EVs have been isolated from a wide variety of biofluids, including plasma, urine, and, relevant to this review, seminal, follicular and uterine luminal fluid. We conducted a systematic search of the literature to review and present the currently available evidence on the possible roles of EVs in follicular growth, resumption of oocyte development and maturation (meiosis), sperm maturation, fertilization and embryo implantation.

METHODS: MEDLINE, Embase and Web of Science databases were searched using keywords pertaining to EVs, including 'extracellular vesicles', 'microvesicles', 'microparticles' and 'exosomes', combined with a range of terms associated with the period of development between fertilization and implantation, including 'oocyte', 'sperm', 'semen', 'fertilization', 'implantation', 'embryo', 'follicular fluid', 'epididymal fluid' and 'seminal fluid'. Relevant research articles published in English (both animal and human studies) were reviewed with no restrictions on publication date (i.e. from earliest database dates to July 2015). References from these articles were used to obtain additional articles.

RESULTS: A total of 1556 records were retrieved from the three databases. After removing duplicates and irrelevant titles, we reviewed the abstracts of 201 articles, which included 92 relevant articles. Both animal and human studies unequivocally identified various types of EVs in seminal, follicular and ULFs. Several studies provided evidence for the roles of EVs in these biofluids. In men, EVs in seminal fluid were linked with post-testicular sperm maturation, including sperm motility acquisition and reduction of oxidative stress. In women, EVs in follicular fluid were shown to contain miRNAs with potential roles in follicular growth, resumption of oocyte meiosis, steroidogenesis and prevention of polyspermy after fertilization. EVs were also detected in the media of cultured embryos, suggesting that EVs released from embryos and the uterus may mediate embryo-endometrium cross-talk during implantation. It is important to note that many of the biologically plausible functions of EVs in reproduction discussed in the current literature have not yet been substantiated by conclusive experimental evidence.

CONCLUSIONS: A detailed understanding of the contributions of EVs in the series of events from gametogenesis to fertilization and then on to implantation, in both normal and pathological cases, may enable the development of valuable tools to advance reproductive health. Because of the early stage of the field, it is unsurprising that the current literature includes not only growing experimental evidence, but also as-yet unproven hypotheses pertaining to the roles of EVs in key reproductive processes. In this review, we present a comprehensive survey of the rapidly expanding literature on this subject, highlighting both relevant findings and gaps in knowledge.

Key words: extracellular vesicles / oocyte / sperm / fertilization / exosomes / ovarian follicle / embryo / implantation

Introduction

Interest in intercellular communication has risen in recent years, with an increasing awareness of the complexity of its contributions to diverse physiological processes, including regulation of cell proliferation, differentiation, gametogenesis, embryogenesis and development. In particular, the identification of extracellular vesicles (EVs) as novel mediators of intercellular communication has re-focused research efforts in the field. Traditionally, intercellular communication has included three mechanisms, i.e. contact-dependent signaling via membrane-bound signaling molecules (receptors) or gap junctions, short-range paracrine signaling via secreted soluble molecules, such as cytokines and chemokines, and long-range endocrine signaling via secreted hormones. Recent studies have uncovered the existence of EVs, which are released by cells into the extracellular environment and can serve as vehicles for the transfer of proteins, lipids and RNAs between cells both locally (autocrine and paracrine) and remotely (Zhang *et al.*, 2009; Raposo and Stoorvogel, 2013). EVs are released by a wide range of cell types under both normal and pathological conditions. EVs and their cargoes may play

key roles in numerous aspects of biology, including reproduction, as candidate biomarkers of health and disease, and as potential targets for therapeutic interventions (Gould and Raposo, 2013).

Gametogenesis, fertilization, implantation and early embryo development are complex processes that are highly dependent on communication between cells and organs. Oogenesis is a multistep process that occurs over an extended timeframe (decades in the human), and involves interactions between the developing oocyte and the cumulus and granulosa cells that surround it in the follicle. Sperm undergo maturation, capacitation and the acrosome reaction, which facilitate their binding and fusion with the released oocyte, thus enabling fertilization to occur. After the developing embryo migrates into the uterus, apposition and subsequent adherence of the blastocyst to the endometrial luminal epithelium, followed by endometrial invasion, must occur to result in successful implantation (Cuman *et al.*, 2014). Detection of EVs in the reproductive biofluids points to possible roles for them in the intercellular communication necessary for and after conception (Table I). The aim of this review is to present the currently available evidence on the roles of EVs in conception and implantation and demonstrate key gaps in our present knowledge.

Table I Key studies implying a role for extracellular vesicles in reproduction.

Process	Main findings	Species	Reference
Sperm maturation	EVs from epididymal fluid (epididymosomes) contain proteins associated with sperm maturation, such as macrophage migration inhibitory factor (MIF) and aldose reductase (AKRBI)	Bovine	Frenete <i>et al.</i> (2003)
Prevention of premature acrosome reaction and premature capacitation	The sperm membrane becomes enriched with cholesterol, sphingomyelin, and saturated glycopospholipids after fusion with EVs (prostasomes) in the seminal fluid. The fluidity of the sperm membrane decreases, preventing premature acrosomal reaction	Human	Arienti <i>et al.</i> (1998a, b) Carlini <i>et al.</i> (1997)
	EVs (prostasomes) in seminal fluid inhibit early capacitation and spontaneous acrosome reaction	Human	Pons-Rejraji <i>et al.</i> (2011)
	EVs (epididymosomes) contain GPX5, which protects the sperm against premature acrosome reaction	Bovine	Rejraji <i>et al.</i> (2002)
Capacitation, acrosome reaction, and fertilization	CD9-labeled EVs from the plasma membranes of oocytes are able to transfer proteins to the fertilizing sperm in the perivitelline space (PVS) before fertilization. Transfer of these EVs is crucial for the reorganization of sperm membrane and fusion with the oocyte	Mice	Barraud-Lange <i>et al.</i> (2007)
	A significant increase in the acrosome reaction occurs in sperm incubated with EVs isolated from seminal plasma compared with control sperm	Porcine	Siciliano <i>et al.</i> (2008)
Prevention of polyspermy	After fertilization, Juno is shed from the oolemma and is redistributed in EVs. These EVs can bind and neutralize acrosome-reacted sperm and prevent polyspermy	Mice	Bianchi and Wright, (2014)
Communication between embryos	Co-culture of porcine embryos significantly improves the <i>in vitro</i> development of cloned embryos. Labeled EVs from porcine embryos are internalized by the NT embryos	Porcine	Saadeldin <i>et al.</i> (2014)
Endometrial embryo cross-talking	EVs are present in the uterine fluid. EVs isolated from the uterine fluid of pregnant sheep can transfer RNAs including endogenous beta-retroviruses RNAs, which play a role in the regulation of conceptus trophoctoderm development, to other cells	Sheep	Burns <i>et al.</i> (2014)

EVs: extracellular vesicles; NT: nuclear transfer.

Methods

For this review, we performed a systematic online literature search of MEDLINE, Embase and Web of Science databases. We searched all articles published since database inception through July 2015. We used the following query: ('extracellular vesicles' OR 'microvesicles' OR 'microparticles' OR 'exosomes' OR 'epididymosomes' OR 'prostatosomes' OR 'oviductosomes' OR 'uterosomes') AND ('oocyte' OR 'sperm' OR 'semen' OR 'capacitation' OR 'nidation' OR 'fertilization' OR 'fertilisation' OR 'implantation' OR 'embryo' OR 'follicular fluid' OR 'epididymal fluid' OR 'seminal fluid'). MeSH and Emtree terms were also used where applicable. The terms 'apoptotic bodies' and 'apoptotic blebs' were excluded as these types of EVs might have functions independent of reproductive processes (Caselles et al., 2014). Both animal and human studies were considered eligible for this review. We applied database filters for 'English language' and 'full length articles' and eliminated any duplicate articles. After application of the search limits defined above, we reviewed the titles and abstracts of each publication. Studies found to be irrelevant after screening their titles and/or abstracts were excluded. The remaining full-length articles were retrieved. Only studies whose scope included EVs of all types plus conception/reproduction were included. References from these articles were also used to obtain additional articles. A total of 1556 records were retrieved from the three databases. After removing duplicates and irrelevant titles, the abstracts of 201 articles were reviewed and 92 were considered relevant and included in this review.

Extracellular vesicles

EVs are membrane-bound vesicles released by every prokaryotic (Kim et al., 2015) and eukaryotic (Regente et al., 2009; Oliveira et al., 2010; Mantel and Marti, 2014; Cocucci and Meldolesi, 2015) cell type that has been studied to date. Different terms have been used to classify subtypes of EVs, which have been often intentionally or unintentionally employed to designate overlapping categories of EVs (Gould and Raposo, 2013). Here we report a commonly used nomenclature, recognizing that it is not universally adopted or enforced (Gould and Raposo, 2013), largely because it is usually not possible to determine the specific biogenesis mechanism of any given population of EVs. Exosomes, which are typically 40–100 nm in diameter (Crescitelli et al., 2013), are formed within cells by inward budding of late endosomes, called multi-vesicular bodies, and are then released into the extracellular environment by fusion of the multi-vesicular body with the plasma membrane (Denzer et al., 2000; Laulagnier et al., 2004). Microvesicles bud directly from the plasma membrane, and are usually 50–1000 nm in diameter. Apoptotic bodies have a particularly wide range of size, having been described to range from 800 to 5000 nm (Thery et al., 2009; Crescitelli et al., 2013; EL Andaloussi et al., 2013; Traver et al., 2014). Given the overlapping size ranges, it is well appreciated that EV preparations are generally quite heterogeneous and it is difficult to distinguish between the different subtypes of EVs. Historically, the term 'exosomes' has been commonly used to describe any type of vesicle found in an extracellular biofluid, but we prefer the term EV (Lötvall et al., 2014). EVs have also been labeled according to the tissue/biofluid in which they are detected. Based on this nomenclature, prostatosomes or prostasomes, epididymosomes, oviductosomes and uterosomes have been used to indicate vesicles isolated from seminal fluid, epididymal, oviduct and uterine fluids, respectively (Ronquist and Brody, 1985; Saez et al., 2003; Griffiths et al., 2008; Al-Dossary et al., 2013). EVs have been shown to contain proteins, lipids (specifically high levels of sphingomyelins), DNA, and a variety of RNA species,

including microRNAs (miRNAs) and mRNA fragments (Thery et al., 2009). Proteins commonly found on EV membranes include tetraspanins, specifically CD63, CD9, CD81, heat-shock proteins (HSP70) and glycosphosphatidylinositol-anchored proteins. Tissue-specific molecular mediators characteristic of the parent cell can be found both on EV membranes and within EVs (Wubbolts et al., 2003; Laulagnier et al., 2004; Subra et al., 2007; Valadi et al., 2007; Luo et al., 2009; Simpson et al., 2009; Record et al., 2011; Sullivan and Saez, 2013; van der Grein and Nolte-t Hoen, 2014). Molecules on the surface of EVs promote interaction with other cells through adhesion of the vesicles to lipids and ligands on the surface of the recipient cell, internalization of the whole vesicle into recipient cells, or fusion of the EV membrane with the plasma membrane of the recipient cell (Thery et al., 2009; Record et al., 2011).

Extracellular vesicles and sperm maturation

The ability of sperm to fertilize an oocyte is gradually acquired during transit through the epididymis, interaction with the seminal fluid during ejaculation, passage in the vagina, contact with the epithelium of the oviduct and fusion with the oocyte (Caballero et al., 2010). The epididymis has several functions, including sperm transport, sperm maturation and storage of gametes (Cooper, 1996; Jones, 2004). The epididymis is divided into three main segments: the caput, the corpus and the cauda. Each segment forms its own microenvironment with different protein secretions and gene expression, optimized for each stage of sperm maturation (Dacheux et al., 2012; Belleannée et al., 2013). The caput and the corpus are responsible for sperm maturation, while the cauda acts as a sperm reservoir (Sullivan et al., 2005; Sullivan and Saez, 2013). During their transit through the epididymis, sperm lose their cytoplasmic droplets and their plasma membrane surface proteins undergo remodeling (i.e. changes in phospholipid composition and in the cholesterol/phospholipid ratio, an increase in total negative charges, and modification of surface proteins). Upon ejaculation, sperm is mixed with seminal fluid, which consists of secretions from the prostate, seminal vesicles and bulbo-urethral glands (Aalberts et al., 2013). From these secretions, the sperm acquire the ability to survive and move in the hostile acidic vagina, bind the zona pellucida and fuse with the oocyte plasma membrane. The changes in sperm morphology and function all result from interactions between the sperm and the intraluminal fluid along the epididymis, and depend on the secretion and uptake of proteins and lipids from the surrounding microenvironment (Caballero et al., 2010).

Prevailing data demonstrate that proteins and miRNAs in the epididymal fluid associated with post-testicular sperm maturation are transferred to the sperm by EVs. EVs were first identified in the human seminal fluid as organelles expelled with prostatic secretions, and named 'prostosomes' (Ronquist and Brody, 1985; Ronquist et al., 1978a, b; Ronquist et al., 2009), which measure 50–500 nm and contain proteins, lipids and nucleic acids. Human prostosomes transport at least 140 proteins, including prostate-specific proteins (e.g. PSA and PAP), prostate stem cell antigen, structural proteins, signal transduction proteins, Guanosine triphosphates proteins and Adenosine triphosphates (Utleget al., 2003; Ronquist et al., 2012) (Fig. 1a). These proteins have antimicrobial, antioxidant and immune-regulatory effects (Rooney et al., 1993; Saez et al., 1998, 2000; Carlsson et al., 2000; Pons-Rejraji et al., 2011; Li et al., 2013; Madison et al., 2014). Prostosomes have an

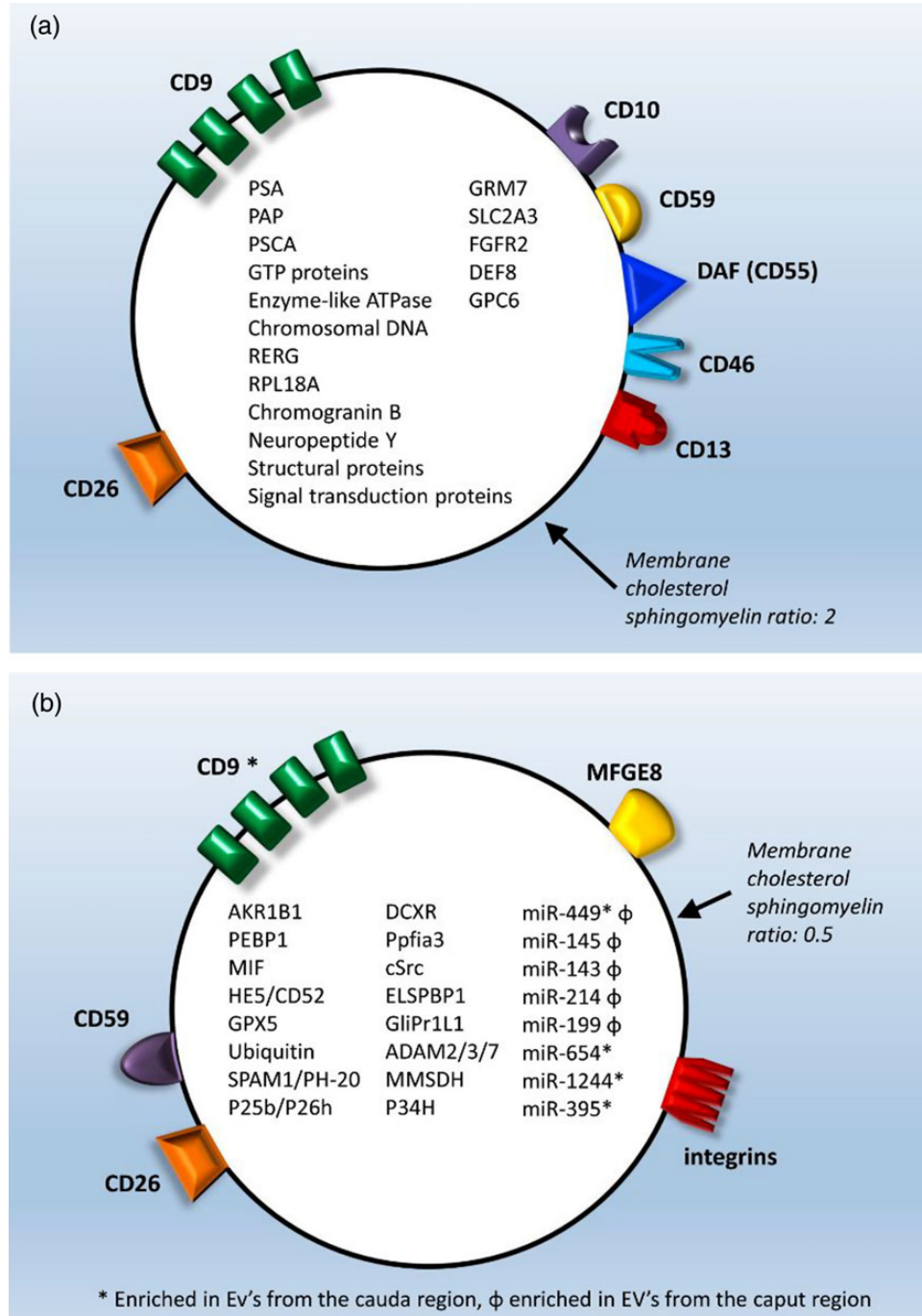


Figure 1 (a) Prostasome structure and content. (b) Epididymosome structure and content.

active role in controlling the timing of capacitation and acrosome reaction. In particular, prostasomes participate in avoiding premature sperm capacitation and premature acrosome reaction. In fact, the prostasome membrane is enriched in cholesterol and sphingomyelin, with a high cholesterol/phospholipid ratio, which contributes to its stability in the acidic vaginal environment (Arvidson *et al.*, 1989; Fabiani and Ronquist, 1995; Arienti *et al.*, 1998a, b, 1999; Kravets *et al.*, 2000; Carlsson *et al.*, 2003; Pons-Rejraji *et al.*, 2011). Furthermore, *in vitro* experiments have shown that prostasomes also contribute to capacitation and

acrosome reaction. *In vitro* incubation at a slightly acidic pH results in fusion of human sperm with prostasomes (Carlini *et al.*, 1997), which results in decreased sperm membrane fluidity, making the sperm receptive to subsequent fertilization signals (Carlini *et al.*, 1997; Ikawa *et al.*, 2010).

Epididymosomes are EVs that are released from epididymal epithelial cells via apocrine secretion (Yanagimachi *et al.*, 1985; Sullivan *et al.*, 2005; Belleannee *et al.*, 2013; Sullivan and Saez, 2013). Epididymosomes have been isolated from hamster, rat, ram, mouse and bovine species, as well

as from humans (Yanagimachi et al., 1985; Fornes et al., 1995; Frenette and Sullivan, 2001; Rejraji et al., 2002; Gatti et al., 2005; Sullivan and Saez, 2013). Epididymosomes contain adhesion molecules, such as tetraspanins, integrins and milk fat globule-epidermal growth factor 8 protein (MFGE8) (Thimon et al., 2008; Thery et al., 2009; Girouard et al., 2011) (Fig. 1b). Bovine epididymosomes contain several proteins that participate in the acquisition of sperm motility, fertilization ability and protection against reactive oxygen species (Frenette et al., 2006a, b; Frenette et al., 2004; Vernet et al., 2004). These include aldo-keto reductase family I, member B1 (aldose reductase) (AKR1B1), phosphatidylethanolamine binding protein 1 (PEBP1), macrophage migration inhibitory factor (MIF), enzymes of the polyol pathway, HES/CD52, type 5 glutathione peroxidase (GPX5), ubiquitin, sperm adhesion molecule 1 (SPAM1)/PH-20 and P25b/P26h (known in humans as dicarbonyl/L-xylulose reductase (DCXR) or P34H) (Fig. 1b). These proteins are involved in sperm maturation and fertilization: MIF and enzymes from the polyol pathway promote sperm motility (Frenette et al., 2003, 2004, 2006a, b); GPX5 prevents premature acrosome reaction (Rejraji et al., 2002) and, together with ubiquitin, protects sperm against oxidative stress. AKR1B1 and PEBP1, found in caudal epididymosomes, help to maintain epididymal sperm in a quiescent state during transit (Frenette et al., 2010). SPAM1/PH20 is a hyaluronidase that increases sperm penetration through the cumulus cell layer around the oocyte and is involved in sperm-zona pellucida adhesion (Zhang and Martin-DeLeon, 2003; Chen et al., 2006; Kimura et al., 2009). P25b/P26 h is important for the binding of sperm to the zona pellucida (Berube and Sullivan, 1994; Frenette and Sullivan, 2001; Sullivan et al., 2007).

In the bovine system, epididymosomes from different epididymis regions carry different cargoes (Frenette et al., 2010; Belleannee et al., 2013). Some miRNAs, for instance, such as miR-449, are enriched in epididymosomes from both the caput and cauda regions, while others are

differentially expressed between these regions, with miR-145, miR-143, miR-214 and miR-199 being more abundant in epididymosomes from the caput, while miR-654, miR-1224 and miR-395 are expressed mostly in epididymosomes from the cauda region (Belleannee et al., 2013).

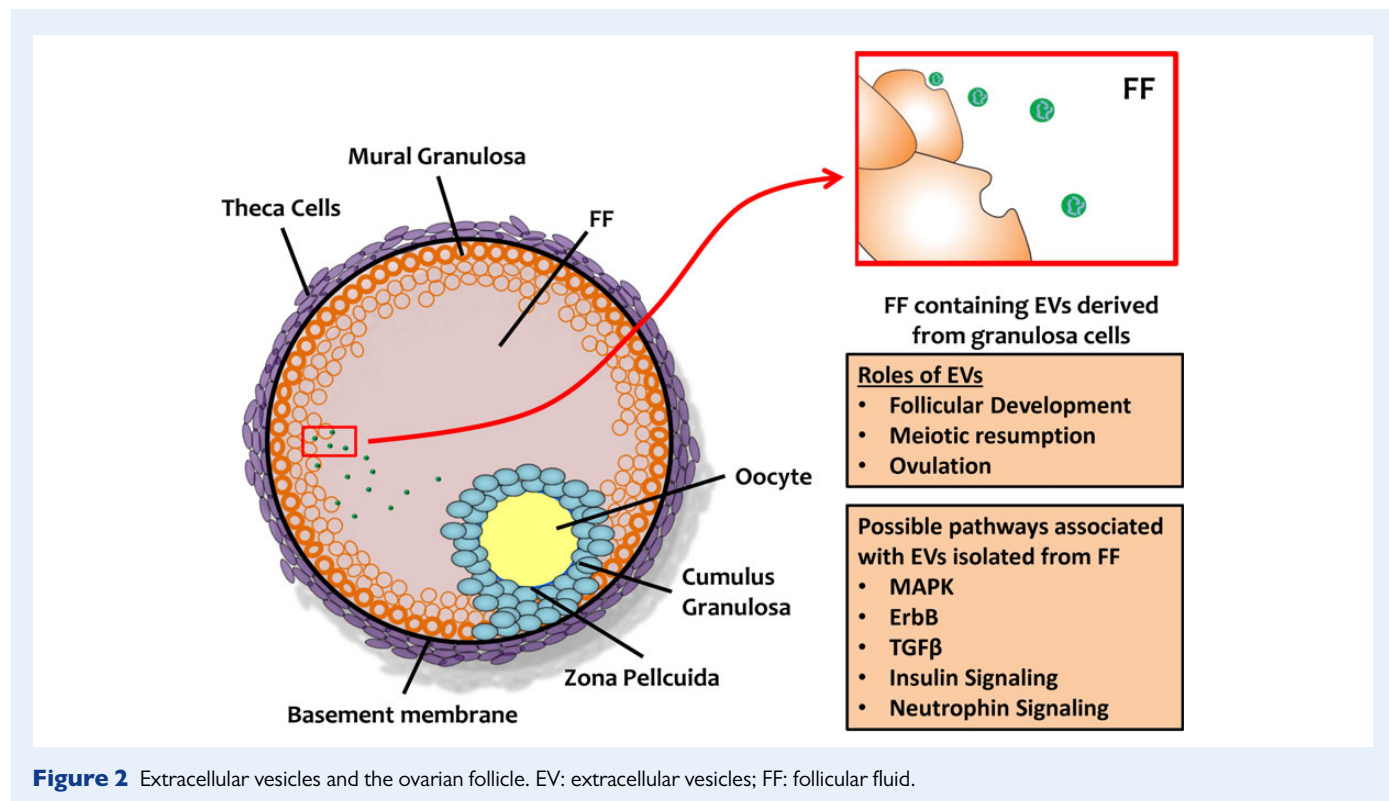
EVs in the seminal fluid are less well studied, although they originate from several cell types in the male genital tract (Renneberg et al., 1997; Vojtech et al., 2014) and fuse with the sperm membrane to transfer molecules that assist with sperm survival in the acidic environment of the vagina (Arienti et al., 1997, 1999).

Interactions between sperm and EVs are pH-dependent (Arienti et al., 1997; Carlini et al., 1997; Frenette et al., 2002). This fits well with the known alkaline pH of prostatic secretions, which prevents early EV/sperm fusion, and the acidic pH of the vagina, which acts as a trigger for fusion.

These animal and human studies provide solid evidence for the contribution of EVs to sperm maturation. However, there is as-yet limited evidence for the roles of specific types of EVs.

Extracellular vesicles, communication in the ovarian follicle and oocyte maturation

The mature ovarian follicle is composed of the oocyte, the somatic cells (cumulus granulosa, mural granulosa and theca cells) and follicular fluid (FF) (Fig. 2). FF derives from constituents of circulating plasma that cross the blood-follicular barrier via theca capillaries, as well as from granulosa and theca cell products, including hormones, proteins, amino acids and anti-apoptotic factors (Fortune, 1994; Revelli et al., 2009). Bidirectional communication between gametes and granulosa cells in the follicle occurs either directly by a network of gap junctions



or through paracrine, autocrine and endocrine signaling factors in the FF (Eppig *et al.*, 1997; Matzuk *et al.*, 2002). These interactions are critical for normal follicular growth, proliferation and differentiation of granulosa cells, oocyte maturation, modulation of transcriptional activity, fertilization and preimplantation embryonic development (Brower and Schultz, 1982; Buccione *et al.*, 1990; Adashi, 1994; Chesnel *et al.*, 1994; Eppig, 2001; Eppig *et al.*, 2002; Matzuk *et al.*, 2002; Senbon *et al.*, 2003; Hamel *et al.*, 2008). EVs add an additional layer of transmission and control, as they carry miRNAs that are predicted to target key elements in pathways related to follicular growth and oocyte maturation in mammals, such as wingless signaling pathway (WNT), transforming growth factor beta (TGF β), mitogen-activated protein kinase (MAPK), neurotrophin, epidermal growth factor receptor (ErbB) pathways and ubiquitin-mediated pathways (da Silveira *et al.*, 2012; Sohel *et al.*, 2013; Santonocito *et al.*, 2014). WNT molecules are glycoproteins involved in follicular growth, luteogenesis and steroidogenesis. Members of the TGF β superfamily, including inhibin, activin, bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9), are expressed in the oocyte from very early stages and are responsible for follicular growth and oocyte maturation (Knight and Glister, 2003, 2006; Boyer *et al.*, 2010; da Silveira *et al.*, 2012). The MAPK pathway stimulates granulosa cell proliferation and cumulus expansion. Furthermore, together with the ERbB pathway, the MAPK pathway promotes the resumption of meiosis in the oocyte (Zhang *et al.*, 2009; Conti *et al.*, 2012). The ubiquitin-mediated pathway modulates oocyte meiotic maturation (Huo *et al.*, 2004) and early mitotic division in embryos (Suzumori *et al.*, 2003), while the neurotrophin-signaling pathway regulates oogenesis and follicle formation (Dissen *et al.*, 2009).

Equine, bovine and human studies have all demonstrated the presence of EVs in FF (da Silveira *et al.*, 2012; Sohel *et al.*, 2013; Santonocito *et al.*, 2014). Within the ovarian follicle, EVs transport RNAs, miRNAs and proteins to recipient cells (da Silveira *et al.*, 2012; Sohel *et al.*, 2013; Santonocito *et al.*, 2014). Some miRNAs are found only in FF EVs, while others can be detected also in the non-vesicular fraction of FF (da Silveira *et al.*, 2012; Sohel *et al.*, 2013). Interestingly, both equine and bovine EVs found in the FF contain miRNAs predicted to target transcripts of proteins associated with focal adhesion and regulation of the actin skeleton

(da Silveira *et al.*, 2012; Sohel *et al.*, 2013). In the bovine system, relevant pathways associated with exosomal miRNAs include the ubiquitin-mediated pathway, MAPK signaling, insulin signaling and neurotrophin signaling (Sohel *et al.*, 2013). In the equine, miRNAs related to the MAPK pathway are found in larger EVs, i.e. microvesicles rather than exosomes (da Silveira *et al.*, 2012). In humans, most of the EV miRNAs are predicted to act on targets that regulate the WNT, ErbB, MAPK and TGF β signaling pathways, all of which operate across the different compartments in the ovarian follicle and can contribute to follicular development, meiotic resumption and ovulation. In general, granulosa and/or cumulus cells can take up miRNAs from the FF EVs both *in vivo* and *in vitro* (Sohel *et al.*, 2013). Table II lists the miRNAs detected in FF EVs to date.

Data linking EVs with follicular maturation and oocyte competence are currently associative and descriptive. A recent bovine study contrasted the profiles of EV-encapsulated miRNAs from follicles containing mature oocytes against those of follicles with immature oocytes. EVs from FF surrounding immature oocytes contained higher numbers of up-regulated miRNAs, suggesting the presence of a higher transcriptional activity during oocyte growth (Sohel *et al.*, 2013). It is unclear whether the higher numbers of miRNA detected results from either an increase in the release of exosomes-contained RNA during oocyte growth or from an increase in miRNA concentrations. The predicted targets for these up-regulated miRNAs include the ubiquitin, neurotrophin, MAPK and insulin signaling pathways, which are linked with ovarian follicular growth, oocyte meiotic maturation and mitotic division of early embryos (Sohel *et al.*, 2013). In equine FF, miRNAs from exosomes can regulate gene expression of the TGF β superfamily in granulosa cells during oocyte maturation (da Silveira *et al.*, 2014). In several species, FF exosomes contain miRNAs, such as miR-30b, let-7, miR-181A, miR-375, miR-503 and miR-513a-3p, that may contribute to follicular growth and oocyte maturation (Murchison *et al.*, 2007; Nagaraja *et al.*, 2008; Lei *et al.*, 2010; Xu *et al.*, 2011; da Silveira *et al.*, 2012). A recent human study compared the exosomal profile of plasma and pooled FF from 15 women younger than 35 years who underwent *in vitro* fertilization (IVF) due to male factor infertility (Santonocito *et al.*, 2014). The authors identified 37 miRNAs up-regulated in FF compared with plasma, 32 of them carried by exosomes. Some

Table II Expression of miRNAs in exosomes and microvesicles in the follicular fluid and their target pathways.

Species	miRNAs Enriched in Exosomes	Pathways	Reference
Human	miR-10b, miR-21-5p, miR-31, miR-95, miR-99b-3p, miR-134, miR-135b, miR-140-3p, miR-190b, miR-203, miR-218, miR-337-5p, miR-339-3p, miR-370, miR-449a, miR-455-5p, miR-483-5p, miR-489, miR-493, miR-503, miR-542-5p, miR-654-3p, miR-874, miR-886-5p, miR-887	WNT MAPK ErbB TGF β	Santonocito <i>et al.</i> (2014) Diez-Fraile <i>et al.</i> (2014)
Bovine	miR-21, miR-26b, miR-30b, miR-33a, miR-132, miR-155, miR-191, miR-199a-5p, miR-221, miR-324-3p, miR-363, miR-373, miR-449b, miR-450b-3p, miR-451, miR-491-5p, miR-526b, miR-573, miR-582-5p, miR-640, miR-654-5p, miR-873, miR-1272, has-let-7c	Ubiquitin-mediated Neurotrophin MAPK Insulin signaling	Sohel <i>et al.</i> (2013)
Equine	miR-20a, miR-20b, miR-22, miR-23a, miR-23b, miR-30b, miR-93, miR-101, miR-152, miR-215, miR-223, miR-378, miR-433, miR-483-3p, miR-499-3p, miR-513a-3p miRNAs enriched both in exosomes and microvesicles miR-17, miR-19b, miR-21, miR-24, miR-25, miR-30a, miR-92a, miR-92b, miR-132, miR-135a, miR-135b, miR-181a, miR-181c, miR-191, miR-192, miR-195, miR-197, miR-320a, miR-323-5p, miR-363-5p, miR-375, miR-421, miR-485-3p, miR-486-5p, miR-523	WNT MAPK Focal adhesion TGF β	da Silveira <i>et al.</i> (2012)

TGF β : transforming growth factor beta; MAPK: mitogen-activated protein kinase; ErbB: epidermal growth factor receptor.

of the miRNA isolated from FF exosomes are also found in cumulus and granulosa cells. Pathway analysis revealed that these miRNAs targeted factors, such as WNT, MAPK, ERbB and TGF β , that contribute to follicular development and meiotic resumption. This study, however, analyzed pooled FF, which is expected to contain follicles from different maturation stages, and therefore could not provide information on the role of FF EVs across the different steps of oocyte maturation. Describing the dynamic transitions of FF EVs miRNAs throughout follicle maturation requires further studies investigating EVs from follicles at each stage of oocyte maturation. These studies require invasive sampling procedures and cannot be easily performed in the general population. Valuable information could be obtained from animal experiments or, in humans, by recruiting patients undergoing IVF and obtaining FF and paired data on oocyte maturation from oocyte retrievals.

The catalog of the macromolecules contained in FF and FF EVs is continuously expanding, yet direct evidence for EV communication within the ovarian follicle is still lacking. Current studies have several limitations. Because of the relatively high costs of FF harvesting and analysis, to contain laboratory costs, *in vivo* studies of FF EVs typically have very small sample sizes and/or have pooled together FF samples from multiple follicles. These studies therefore often lack statistical power to draw conclusive results. Further, the *in vivo* studies conducted to date are mostly observational in nature, have generated descriptive information, and do not provide experimental evidence on the functions of EVs within the FF. *In vitro* functional studies will be fundamental to elucidate FF EV functions. The EV community has used *in vitro* studies to investigate the transfer of EVs from source into recipient cells. Similar studies are urgently needed for FF EVs. For instance, future investigations could harvest EVs from granulosa cells cultured *in vitro* and transfer them to an immature oocyte to determine whether EVs can induce maturation and fertilization. Findings from *in vivo* descriptive studies can directly inform experimental investigations. Several groups have reported or are actively identifying molecular mediators encapsulated in FF EVs *in vivo*. The growing capabilities for engineering EVs with predetermined contents could be leveraged to increase fertilization rates and improve embryo quality with the ultimate goal of increasing IVF success rates.

Other studies have examined the miRNA content of FF EVs in relation to female age. In equines, exosomal miRNA expression profiles vary with animal age and are associated with differences in fertility. Two studies reported high levels of miR-181A, miR-375 and miR-513a-3p in exosomes from older versus younger mares. These three miRNAs all target TGF β , can suppress the TGF β pathway, and lead to impaired oocyte maturation in older females (Knight and Glister, 2006; da Silveira et al., 2012). A human study also observed different miRNA profiles of EVs isolated from FF of three younger (<31 years old) compared with three older women (>38 years old) (Diez-Fraile et al., 2014). Specifically, four miRNAs were differentially expressed in FF of young versus older women: miR-21-5p was expressed only in the FF from young women, miR-190b and miR-99b-3p were exclusively in EVs from older patients, and miR-134 was significantly enriched in EVs from older patients. The observed correlations between miRNAs in FF EVs and female age open new fundamental questions: do miRNAs transported across the FF contribute, or even just track along, oocyte aging? If FF EVs were found to influence oocyte viability, could we use FF EVs miRNAs that are detected in younger women to develop new therapeutic interventions and attenuate the effects of age on fertility? These are forward-looking questions, yet easily testable *in vitro*. If the

miRNAs that differ between younger and older patients also had a role in fertilization and embryo quality, the addition of EVs from younger women to the FF of older women, or preferably the addition of the relevant miRNAs or other biomolecules that may be involved in maintaining oocyte viability in younger women, could potentially improve fertilization as well as the rates of top quality embryos in older patients.

Extracellular vesicles in fertilization

Fertilization consists of sequential events, including cumulus cells expansion facilitating sperm passage through them, followed by sperm binding to the zona pellucida (Jin et al., 2011). The interaction of capacitated sperm with the zona pellucida induces the acrosome reaction, resulting in biochemical changes to the sperm head membrane and the release of proteases and hyaluronidase from the acrosome, enabling the sperm to recognize the zona pellucida and to enter the perivitelline space (PVS) (Chang, 1951; Austin, 1952; Kirchoff et al., 1997; Aitken and Nixon, 2013). Recently, PMCA4a, a protein that helps maintain sperm Ca²⁺ homeostasis, was isolated from CD9-positive EVs in the oviduct (oviductosomes) and uterus (uterosomes). *In vitro*, sperm acquired PMCA4a after incubation with isolated exosomes from the luminal fluid. These findings suggest that EVs may prevent premature sperm capacitation (Al-Dossary et al., 2013).

After the acrosome reaction, the inner acrosomal membrane adheres to the areas of the oocyte that are covered with microvilli, and the sperm fuses with the oocyte membrane (Kaji et al., 2000). These microvilli display CD9 (Runge et al., 2007). Fusion of the sperm and oocyte leads to depolarization of the oolemma and triggers the release of cortical granules that are found under the oocyte surface, blocking the penetration of other sperm. The molecular basis underlying fusion remains obscure. In an *in vitro* porcine model, EVs are capable of fusing with sperm and inducing an acrosome reaction (Siciliano et al., 2008). Barraud-Lange et al. (2007) have shown in a mouse model that EVs are transferred from the oocyte to the sperm in the PVS before direct interaction between the oocyte and the sperm. The presence of EVs in the PVS was confirmed also by using electron microscopy (Barraud-Lange et al., 2012).

CD9-positive EVs are found on the plasma membrane of the oocyte, especially on the oocyte microvilli at the sperm attachment site. CD9 also localizes on the surface of the fertilizing sperm at the time of membrane binding and fusion and is required for sperm-oocyte fusion. CD9 null oocytes have altered microvilli and are unable to fuse with sperm (Runge et al., 2007). In a mouse study, fusion between the sperm and CD9 null oocytes was rescued when they were co-incubated wild-type oocytes. The authors concluded that fusion between the sperm and the oocyte is mediated by exosome-like vesicles containing CD9, which are released from the oocyte into the PVS and then transferred to the sperm (Miyado et al., 2008). However, two other studies in hamster and mice did not find any increase in the ability of CD9 null oocytes to fuse with sperm when they were inseminated in the presence of wild-type oocytes or medium that contained CD9-associated EVs (Gupta et al., 2009; Barraud-Lange et al., 2012).

Another tetraspanin, CD81, primarily produced by cumulus cells and localized mainly in the inner region of the zona pellucida, may also participate in fertilization, mainly in fusion-related events prior to membrane fusion and specifically in acrosome reaction (Tanigawa et al., 2008). Both

CD81 and CD9 appear to be transferred to the sperm via EVs when the sperm penetrates the PVS (Miyado *et al.*, 2008; Ohnami *et al.*, 2012). CD81 may help transfer CD9 from the oocyte into the sperm membrane before sperm-oocyte fusion (Ohnami *et al.*, 2012). In 2005, a new protein called Izumo was identified on the surface of sperm that had undergone an acrosome reaction (Inoue *et al.*, 2005). Sperm lacking this protein are unable to fuse with oocytes. During oocyte and sperm fusion, Izumo I binds to a folate receptor on the oocyte called Juno (Bianchi and Wright, 2014). Surprisingly, after fertilization, Juno, which is highly expressed on oocytes before fertilization, is shed from the oocyte membrane and redistributed in the PVS as EVs that are likely derived from the microvillus-rich oolemma. EVs may bind and neutralize subsequent acrosome-reacted sperm, providing a possible mechanism to prevent polyspermy (Bianchi and Wright, 2014; Bianchi *et al.*, 2014b). *In vitro* studies have shown that Juno-deficient oocytes are unable to be fertilized (Bianchi and Wright, 2014). These findings provide evidence that the Izumo I and Juno interaction facilitates the adhesion between the sperm and oocyte required for fertilization (Bianchi and Wright, 2014).

Extracellular vesicles and paracrine communication among embryos

Although controversial, it has been reported that *in vitro* culture of embryos is more successful when the embryos are kept in large groups during the whole culture period (Ferry *et al.*, 1994; Hoelker *et al.*, 2009). Embryos may generate their own microenvironment by secreting growth factors, which constitute a 'secretome' with both

autocrine and paracrine effects (Katz-Jaffe *et al.*, 2006; Ratajczak *et al.*, 2006; Saadeldin *et al.*, 2014). Cloned embryos cultured with porcine parthenogenetic embryos show a significant increase in their developmental competency (i.e. increased number of blastomeres and better blastocyst formation) compared with cloned embryos cultured alone. Analysis of culture media from porcine embryos cultured individually detected 30–120 nm vesicles differing in size according to the embryo's age (<40 nm in cultures from two-cell embryos and <120 nm in cultures from blastocysts). These vesicles express the exosomal marker CD9 and contain mRNAs, including *OCT4*, *SOX2* and *KLF4*, which vary according to the stages of embryo development. Further experiments have shown that these exosomes/microvesicles can pass through the zona pellucida and are internalized by blastomeres (Saadeldin *et al.*, 2014). The potential functional roles of EVs in embryo development have yet to be demonstrated. In humans, replicating the results from porcine studies have been challenging since the culture media used for *in vitro* fertilization contains significant quantities of EVs derived from the synthetic serum substitutes that are used to supplement the developing embryo, making it difficult to isolate EVs that are secreted by the embryo (Tannetta *et al.*, 2014).

Extracellular vesicles and endometrial-embryo cross-talk during implantation

Successful implantation is dependent on coordination between the embryo and the endometrium, and EVs may participate in this required cross-talk (Fig. 3). It has been suggested that the endometrial epithelium release EVs that are involved in the transfer of signaling miRNAs and

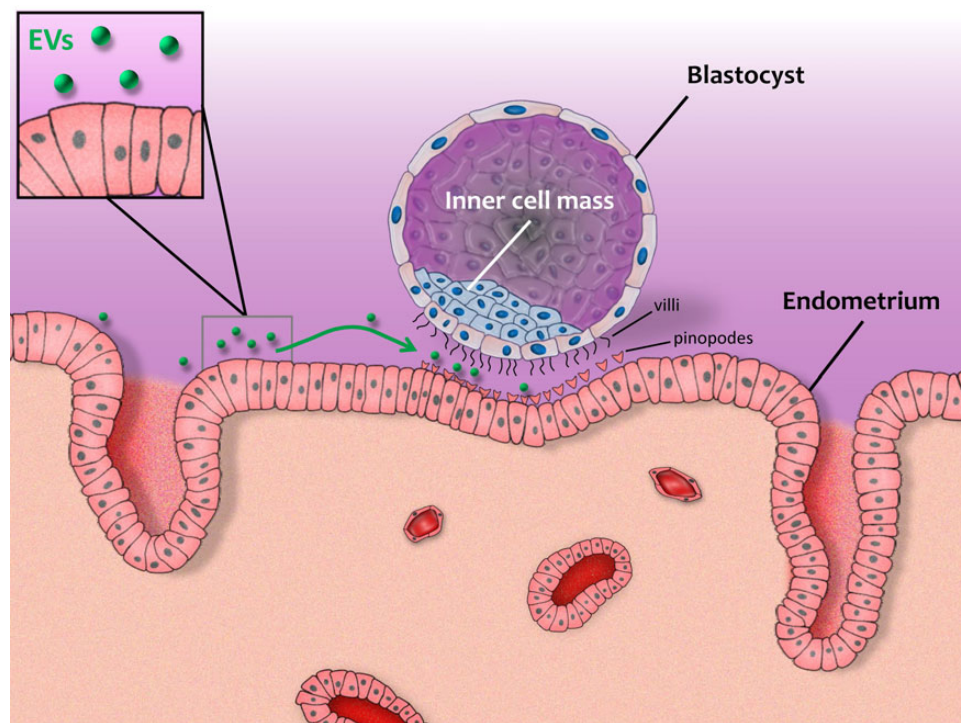


Figure 3 Extracellular vesicles and the cross-talk between the blastocyst and endometrium during implantation.

adhesion molecules either to the blastocyst or to the adjacent endometrium into the uterine cavity, which in turn can affect endometrial receptivity and implantation. EVs positive for the exosomal markers CD63 and HSP70 have been isolated from the uterine luminal fluid (ULF) of cyclic and pregnant sheep (Burns et al., 2014). Analysis of these vesicles revealed miRNAs and proteins expressed both by the conceptus trophoblast and by the endometrial epithelium, such as cathepsin L1 and prostaglandin synthase 2. In the sheep, endogenous beta-retroviruses (enJSRVs) play a critical role in regulating conceptus trophoblast development and placental growth (Varela et al., 2009; Burns et al., 2014). *In vitro* studies have shown that EVs isolated from the ULF of pregnant sheep can transfer RNAs (including enJSRV RNA) to other cells. These findings indicate that EVs present in the ULF are likely to have a biological role in the interaction between the embryo and the endometrium. In human studies, EVs have been isolated from uterine fluid from women in different phases of the menstrual cycle. Both the luminal and glandular apical surfaces of endometrial epithelial cells express CD9 and CD63 exosomal markers, thereby showing that the endometrial epithelium are possible source of the exosomes found in the uterine cavity (Ng et al., 2013). The identification of EVs in the uterine fluid suggests a possible role of EVs in transferring information from the endometrium during implantation, although this hypothesis still needs to be proven.

Possible future clinical implications

The study of EVs in reproduction has the potential for expanding our current understanding of the normal physiology of reproduction, i.e. identifying high-quality sperm and oocytes or pathological conditions such as implantation failure. Specifically, EVs have potential for identifying non-invasive biomarkers and for developing novel therapies to increase reproductive success.

In the last decade, multiple efforts have been performed to develop non-invasive methods to assess oocyte quality, top quality embryos and blastocyst formation and to identify the right embryo(s) to transfer in order result in a successful pregnancy. New techniques such as morpho-kinetic parameters, and attempts to establish proteins or secreted metabolomes in the culture medium have been added either clinically or experimentally to the classic morphological parameters for embryo selection, however, birth rates after assisted reproduction technologies have remained almost unchanged (Barkalina et al., 2014; Rodgaard et al., 2015). Recently several studies have focused on miRNAs in FF or culture media as possible biomarkers for increasing IVF success rates (Rosenbluth et al., 2014). EV-encapsulated miRNAs, in particular, are shielded from degradation and are remarkably stable in biological fluids. This property may greatly facilitate the translation of the growing understanding of miRNA biology into clinical applications. Investigating EV-encapsulated miRNAs also has key biological advantages over analysis of total miRNAs. While total miRNAs in human biofluids or supernatants from cell cultures may be released from apoptotic cells or cell debris, EV miRNAs are actively released by viable cells and are expected to represent an active means of communication between cells and tissues locally or systemically. Specifically miRNAs that are encapsulated by EVs might have a different role compared with miRNAs in biofluids as they transfer biological information to recipient cells.

A question of particular interest is about whether EVs carry and transport DNA from the developing embryo and the fetus to the maternal circulation. In the past few years, non-invasive prenatal testing using cell-free DNA (cfDNA) in maternal plasma has modified the clinical paradigm of prenatal screening for the common fetal aneuploidies and Y-chromosome (Bianchi et al., 2015). If cfDNA were to be carried by EVs, and embryonic and/or fetal DNA can be identified either in the culture media or maternal circulation, EVs could be used as biomarkers for Y-chromosome specific DNA. Further, EVs could be used for non-invasive prenatal genetic diagnostic techniques to discover aneuploidy before embryo transfer or in the very early stages of pregnancy, instead of embryo biopsy as in today's practice (Wright and Burton, 2009; Kahlert et al., 2014; Saadeldin et al., 2015).

EVs are being actively investigated for their potential clinical applications, particularly for targeted drug delivery as an alternative to nanoparticle-mediated delivery. In reproduction, nanoparticles have been used experimentally to load sperm with exogenous genetic material that is transferred to the oocyte during fertilization (Kim et al., 2010; Campos et al., 2011). However, the long-term consequences of using exogenous nanoparticles during conception are unknown. In this respect, EVs, as endogenous carriers of biomolecules, may be particularly advantageous. EVs are naturally present in human biofluids, but may also be engineered for tissue-specific transfers, such as the transfer of selected compounds into gametes and embryos to increase reproductive success (Barkalina et al., 2015).

Conclusions

Research in the field of intercellular communication in the reproductive tract has increased exponentially in the last decade. EVs and their contents, including miRNAs and proteins, have been characterized in several relevant body fluids. While in many cases, their functional roles are not entirely understood, there is growing evidence in the literature that intercellular communication mediated by EVs may contribute to oocyte and sperm maturation, fertilization, prevention of polyspermy and embryo implantation. Most studies of EVs in reproduction have used animal models, although relevant human research is emerging. As discussed throughout this review, much of the current published work is correlative. There is a critical need for further functional and mechanistic studies to provide conclusive experimental evidence for EVs and their cargoes as mediators of intercellular communication. In human reproduction, a major challenge is presented by limitations in relevant biological materials, which limits our ability to perform studies that utilize perturbations of pathways to confirm cause and affect relationships.

Authors' roles

R.M. designed the review, performed the literature research and wrote the manuscript. L.L. designed the review, performed revisions and critically discussed the complete manuscript. A.A.B. designed the manuscript, supervised, and critically reviewed the complete manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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