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Role of exosomes in the reproductive tract Oviductosomes mediate interactions of oviductal secretion with gametes/early embryo

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1. ABSTRACT

The oviductal epithelial membrane releases into the luminal environment extracellular vesicles (EVs) which are pleomorphic in nature and fall into two categories: exosomes and microvesicles. Both of these membrane vesicles are referred to as Oviductosomes (OVS), and to date have been identified in the murine and bovine species. Bovine EVs derived *in vivo* and from *in vitro* culture show differences in their protein cargo which includes CD9 and HSC70 biochemical markers and fertility-modulating proteins such as oviduct-specific glycoprotein (OVGP) and Plasma Membrane Ca^{2+} ATPase 4 (PMCA4). PMCA4, an essential multifunctional sperm protein, is hormonally-regulated with elevated levels seen in proestrus/estrus. OVS deliver PMCA4 to sperm via a fusogenic mechanism involving the interaction between CD9 and integrins which are present on their surfaces. Studies of OVS are needed to determine the components of their cargoes and their interaction with oocytes and the very early embryo. Based on our present knowledge of their interaction with sperm, they are expected to play pivotal roles in regulating fertility and promise to inform the current IVF practice.

2. INTRODUCTION

It has been well-established that mammalian sperm have molecular interactions with the female tract, as recently reviewed (1). While in the storage reservoir in the isthmus, the distal region of the oviduct, sperm bind to the oviductal membrane (2), an interaction that helps to maintain their fertilizing ability as revealed by *in vitro* incubation of bull sperm with oviductal epithelium (3,4). In addition to direct interaction, indirect molecular interactions occur via secretions from the oviductal secretory epithelial cells (1). As the reproductive secretory fluids in the uterus (5,6) and the male tract (7,8) contain membranous extracellular vesicles (EVs) that interact with sperm, it was expected that the same would be the case for the oviductal luminal fluid. However, it was not until 5 years after the 2008 discovery of uterine EVs (Uterosomes) by Griffiths *et al*. (5) that Al-Dossary *et al*. showed the existence of these particles in the oviductal luminal fluid and dubbed them 'Oviductosomes' (6). Although EVs have been described for more than 3 decades (9, 10), it has only been in the past few years that interest in them has intensified. This interest is due to the finding that they mediate cell-cell communication by transferring proteins and genetic factors (RNA and microRNAs) to recipient cells (11). This review deals with oviductal EVs in cycling mammalian females, the components and impact of their cargo identified to date, and the mechanism of cargo delivery to the sperm surface.

3. CLASSIFICATION AND BIOGENESIS OF OVIDUCTOSOMES

Secreted from epithelial linings, EVs have lipid bilayers and are pleomorphic in nature, with sizes varying from <100 nm (exosomes) to 100 nm-1 μm (microvesicles) in diameter (12). They are characterized by: a) the presence of biochemical markers e.g. CD9 tetraspanin, which is found on the surface, and HSC70 which has a sub-surface location; and b) membrane orientation with the cytoplasmic-side inward (12-14). The secretion of these vesicles occurs via two distinct processes: 1) a pathway involving multivesicular bodies (MVBs) whose outer membrane fuses with the apical

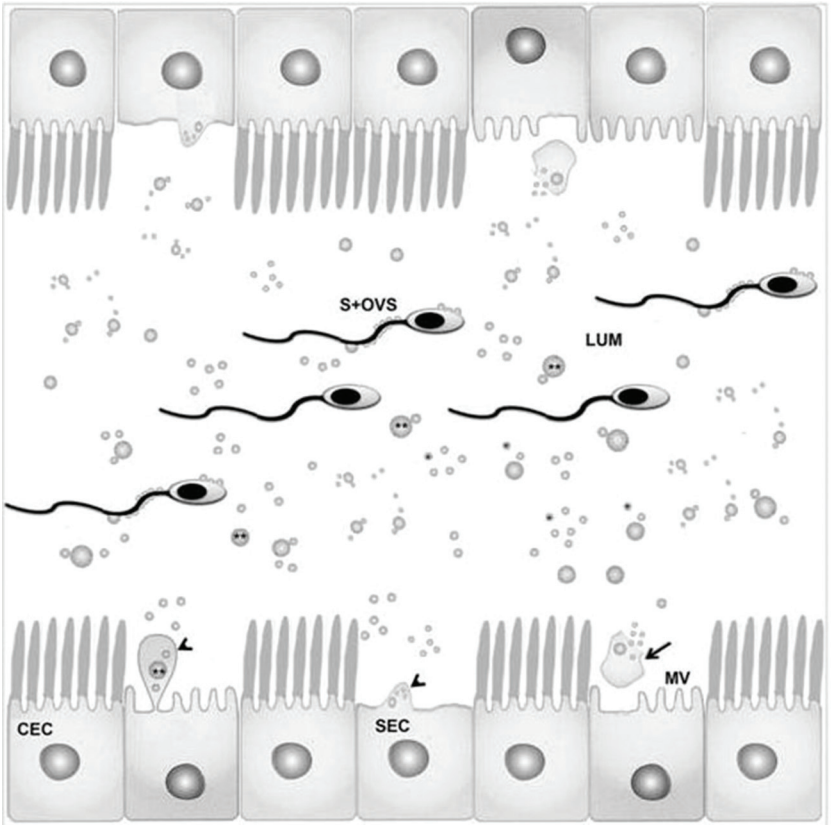

Figure 1. Diagram shows how oviductal epithelial cells communicate with sperm via extracellular vesicles produced by apocrine secretion. At their apical surface, secretory epithelial cells form blebs containing both microvesicles and exosomes. After the blebs are dislodged, they release the EVs which bind to, and fuse with, sperm. CEC = ciliated epithelial cells, SEC = secretory epithelial cells, arrow heads show attached blebs, arrow shows detached blebs releasing EVs. MV = microvilli, LUM = lumen, S+OVS = sperm with bound OVS.

membrane to release their contents (13-16), and b) an apocrine pathway which involves the formation of vesicle-containing blebs protruding from the apical side of epithelial membranes into the lumen. These blebs become dislodged from the epithelium and ultimately release the vesicles into the lumen (17, 18); The apocrine pathway, in which both exosomes and microvesicles are produced, is involved in the production of epididymosomes (which are found in the epididymal luminal fluid) (17,18), while prostasomes (found in the prostatic secretion) appear to arise with approximately equal frequency from both pathways (19). Similar to these mammalian reproductive EVs, oviductosomes appear to arise from the apocrine pathway (Figure 1) (unpublished data). However, it should be noted that in *Drosophila* male reproductive glands, secondary cells of the accessory glands secrete exosomes that are generated inside endosomal MVBs (20).

4. ISOLATION AND CHARACTERIZATION OF OVIDUCTOSOMES (OVS)

The term oviductosomes (OVS) is used to refer to both exosomes and microvesicles detected in the oviductal luminal fluid (6). Using perfusion fixation, murine OVS have been detected *in situ* in oviductal sections analyzed by TEM (unpublished data). They were identified for the first time in murine oviductal luminal fluid recovered from cycling females by flushing or mincing the oviduct in PBS, and fractionating the purified fluid by ultracentrifugation (6). Using the established characterization criteria for EVs; namely, negative staining, shape, size, membrane orientation (cytoplasmic-side inward), and the CD9 exosome biomarker (12-14), TEM revealed OVS ranging in sizes from 25-100 nm (exosomes) and 0.1-1μm (microvesicles) in diameter (6, Figure 2). Western analysis revealed the presence of the CD9 biomarker and immunogold labeling detected its localization on the exterior of the membrane (6), verifying the exosomal nature of OVS which can be distinguished from endosomes which have the cytoplasmic side of the molecule in the exterior orientation.

In addition to murine OVS, there have been recent reports of the purification of bovine oviductal EVs from *in vivo* and *in vitro* origin (21,22). Preliminary results have shown that exosomes collected via serial

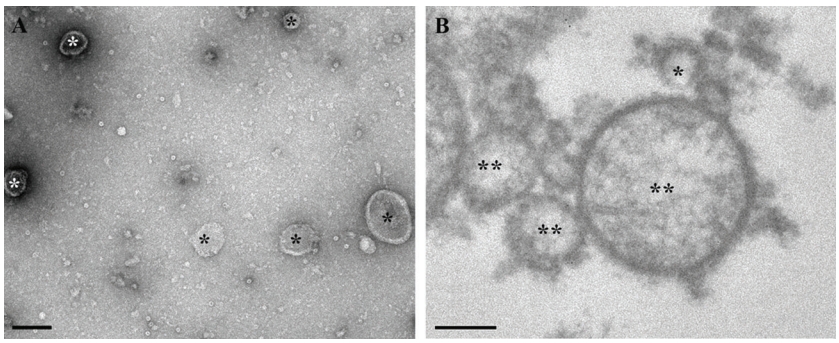

Figure 2. Electron micrographs of mouse oviductosomes isolated from oviductal luminal fluids. Oviductosomes such as exosomes and microvesicles were isolated using differential ultracentrifugation and imaged via transmission electron microscopy (Zeiss LIBRA 120, operated at 120 kV), using uranyl acetate as a phospholipid stain. (A) Exosomes (*) size < 100 nm in diameter. (B) Microvesicles (**) ranging in size from 0.1. μm to 1 μm in diameter. Bar = 100 nm and 200 nm, respectively. Adapted with permission from (30).

ultracentrifugation from the bovine oviductal fluid and from conditioned media of primary cultures of oviductal epithelial cells varied in sizes. The size distributions parallel those for exosomes and microvesicles in the *in vivo* preparation, but were mostly those of microvesicle populations in the *in vitro* preparation (22). Interestingly, SDS-PAGE revealed quantitative and qualitative differences among bovine EVs with respect to their cells of origin and the milieu (conditioned media or flushing), although both those of *in vivo* and *in vitro* origin were positive for the HSC70 biochemical marker (22).

5. PROTEIN COMPONENTS OF OVIDUCTOSOMAL CARGOES

Sperm Adhesion Molecule 1 (SPAM1) was found to be secreted during murine estrus in both the oviduct and the uterus (23). In the latter where it was shown to be associated with EVs (5), it was demonstrated to have the ability to bind to sperm and to enhance hyaluronic acid-binding and cumulus dispersal abilities (24). These findings for the uterus suggested that SPAM1 is also present on oviductal EVs. Recent preliminary results are in agreement with this, as bovine exosomes were shown to carry SPAM1 in their cargo (21). Other preliminary proteomic findings in bovine exosomes showed a difference in the cargo of those secreted *in vitro* and *in vivo*. While the latter showed the presence of oviductal-specific glycoprotein (OVGP) and heat shock protein A8 (HSPA8) which have known functions in fertilization and in the early embryo, only HSPA8 was detectable in *in vitro*-derived exosomes (22). It should be noted that it has been well-established that OVGP is a zona pellucida-associated protein with a role in pre-fertilization zona pellucida hardening that reduces polyspermy (25). In general, proteomic studies of OVS are in their infancy, with mostly preliminary studies, and although EVs have been shown to carry in their cargoes several types of components, including, RNAs, microRNAs, and lipids (11) only proteins have been investigated to date.

In mice, Al-Dossary *et al*. (6) have shown that in addition to CD9 tetraspanin (an adhesion molecule) and αV integrin (a cell surface receptor), OVS carry in their cargo Plasma membrane Ca^{2+}-ATPase 4 (PMCA4) (6) which is the major Ca^{2+} efflux pump in murine sperm (26). There is absolute requirement for this sperm protein for fertility, since deletion of its encoding gene, *Pmca4,* leads to male sterility resulting from loss of progressive and hyperactivated motility (27,28). A 10-pass transmembrane protein, PMCA4 was immunolocalized to OVS after gentle permeabilization of the vesicles in order for the anti-PMCA4 antibody to gain access to, and recognize an epitope on, the cytoplasmic-side of the molecule (6). This localization also served to confirm that OVS are exosomes and not endosomes, as also shown above by the localization of CD9. PMCA4 in the combined female luminal fluid was shown to be very abundant during proestrus/estrus and was only marginally present in metestrus/diestrus (6). Importantly, in proestrus/estrus the levels were 9- and 4-fold higher in the oviductal fluid than in the uterine and vaginal fluids (6). These remarkable differences in the abundance of PMCA4 in different locations in the female tract suggest that PMCA4 plays an important role in the oviduct. This conclusion is bolstered by the finding that oviductosomal PMCA4 could be delivered to caudal sperm *in vitro* following their co-incubation with OVS (6), suggesting that it is delivered to sperm *in vivo* in the oviduct at a time when the intracellular Ca^{2+} levels are elevated in sperm.

6. PMCA4 IN EVS IN THE MALE AND FEMALE TRACT: A CRUCIAL ROLE IN THE OVIDUCT

Our Lab has shown that in mice PMCA4 transcripts are produced in spermatogonia, spermatocytes and spermatids (29), and that during spermiogenesis it is distributed to the inner acrosomal membrane as well as the plasma membrane, as revealed by Super-resolution Structured illumination Microscopy

(SR-SIM) (30). When sperm leave the testis, the amount of PMCA4 on the sperm plasma membrane is augmented during epididymal maturation, as significant amounts are acquired from epididymosomes (29). It should be noted that sperm acquisition of additional amounts of PMCA4 and of motility in the cauda epididymis are parallel events that are consistent with the association of the presence of PMCA4 and sperm motility (29). This association can be explained by the fact that in its Ca^{2+} efflux role PMCA4 exchanges 1 Ca^{2+} ion for 1 H^{+} and thereby regulates both intracellular Ca^{2+} and pH (31), both of which are involved in sperm motility (32). PMCA4 is also secreted in the male accessory organs, as it is present in the cargo of human prostasomes which are able to deliver it to sperm when they are co-incubated with seminal plasma (33). This finding suggests that upon ejaculation further additional PMCA4 is added to the sperm surface from the seminal plasma (34) and is complemented by that acquired from uterosomes from the uterine fluid (6). Thus progressive motility of sperm is ensured, allowing them to arrive at the fertilization site in the oviduct.

In the female tract the series of changes that mammalian sperm undergo to gain fertilizing competence, capacitation, and the subsequent acrosome reaction are both dependent on elevated levels of intracellular Ca^{2+}concentration ($(Ca^{2+})_i$) (35,36). To prevent premature capacitation during epididymal transit and at ejaculation, sperm acquire decapacitation factors (DF) (37, 38). One of these in murine sperm stimulates Ca^{2+}-ATPase activity to lower $(Ca^{2+})_i$ (39), a task performed primarily by PMCA4 (26). During capacitation, DF are lost and the $(Ca^{2+})_i$ is elevated (37,38). Thus acquisition of additional PMCA4 in the oviduct via OVS would be advantageous to ensure adequate Ca^{2+} efflux to promote sperm viability: 1) during their storage in the sperm reservoir where they adhere to the oviductal epithelium prior to ovulation to avoid premature capacitation (2); and 2) after Ca^{2+} influx required for hyperactivated motility, that releases sperm from the oviductal epithelium (36, 40), and the acrosome reaction (41). Thus the massive secretion of oviductal luminal fluid upon ovulation not only facilitates sperm release and transport to the ampullary-isthmus junction (the fertilization site (2)), but provides OVS with increased amounts of PMCA4 (1) to ensure a return to resting $(Ca^{2+})_i$ levels (50-100 nM (42)) after the required Ca^{2+} spikes (41).

It should be noted that for both equine and human sperm, direct contact with the oviductal epithelium improved sperm viability *in vitro* and in the latter it was shown to be due to the maintenance of low levels of $(Ca^{2+})_i$ (43,44) which delays capacitation during storage in the reservoir (2). The precise mechanism of how this occurs is unknown. However, based on the identification of PMCA4 in the cargo of the newly discovered OVS which are secreted from the apical surface of the oviductal secretory cells where PMCA4 is abundant (6), it can be postulated that this effective Ca^{2+} efflux mechanism is involved in this direct contact. Thus, the oviductal epithelium creates a unique surface and luminal fluid microenvironment with PMCA4-bearing OVS that delay sperm capacitation and enhance their motility (43, 44). Further, after the demand for the high $(Ca^{2+})_i$, required for hyperactivated motility (2) and for the acrosome reaction (35), the acquisition of this efflux pump via OVS serves to restore Ca^{2+} homeostasis and to ensure viability. In addition to regulating $Ca^{2+}$and intracellular pH, PMCA4 is known to negatively regulate Ca^{2+}/calmodulin-dependent enzymes such as endothelial nitric oxide synthase and neuronal nitric oxide synthase (45-47), both of which are present in sperm. Thus the negative regulation of these enzymes which have the potential to produce toxic levels of nitric oxide, is likely to contribute to the maintenance of sperm viability resulting from the acquisition of PMCA4 via OVS (33). It can therefore be concluded that in carrying PMCA4, OVS contain in their cargo a multifunctional essential fertility-modulating protein produced in elevated levels when sperm are likely to be present in the oviduct. This underscores a crucial role played by OVS at the fertilization site.

7. SPERM-OVIDUCTOSOME INTERACTION IN CARGO DELIVERY

For some time it was not known how individual EVs interact with recipient cells to deliver their cargoes, although it was thought that different mechanisms are involved depending on cell type (48). For example, while endocytosis has been proposed as a mechanism in somatic cells, it would not be feasible for sperm where this process does not occur (49). Martin-DeLeon and co-workers, in studying cargo delivery to sperm via uterosomes and epididymosomes, reported that these EVs dock on the sperm membrane during the delivery of SPAM1, a glycosyl phosphatidylinositol (GPI)-linked protein (5). This docking was postulated to facilitate hydrophobic interactions between the GPI anchor and the outer leaflet of the lipid bilayer of the sperm membrane (5). While docking may be a prerequisite for fusion, it cannot explain the delivery of transmembrane proteins such as PMCA4. However, Schwarz *et al*. proposed a fusogenic mechanism for the delivery of PMCA4 from epididymosomes to bovine sperm (50). A recent investigation of the fusogenic mechanism for cargo delivery from OVS to sperm, using PMCA4 as a model, provided strong evidence for its existence (30). Al Dossary *et al*. (30), using nanoscale super-resolution imaging in the plasma membrane, lipophilic staining of OVS, and high magnification TEM, provided conclusive evidence that the presence of fusion-competent sites in the form of CD9 and integrins on the surface of OVS and sperm mediates fusion in cargo delivery (30). TEM provided evidence for an intermediate step in the fusion process, formation of fusion stalk (30), and fusion could be blocked by exogenous ligands for $\alpha5\beta1$ and $\alpha v\beta3$, namely

fibronectin and vitronectin, their RGD recognition motif, and anti-αv antibodies (30). Thus, using co-incubation assays, both physical and molecular interactions were revealed in the dynamic process of EV-sperm interaction. While the model we presented was shown to facilitate fusion during capacitation and the acrosome reaction, it would also be applicable to cargo delivery to sperm from reproductive EVs in the male tract and the uterus.

8. SUMMARY AND PERSPECTIVES

An understanding of OVS and their role in the communication between the oviductal epithelial membrane and gametes/embryos is only just emerging. While we have a clear understanding of the mechanism of their interaction with sperm in delivering their cargo, nothing is known of their interaction with oocytes or the early embryo. Additionally, our current knowledge of the contents of their cargoes is limited to only proteins, although it is suspected that, like other reproductive EVs, OVS also contain genetic factors. Based on the finding that they carry essential fertility-modulating proteins, which can be delivered to sperm, they appear to be critically important for sperm maturation and function in the oviduct. It is highly likely that they may be the primary mediator of molecular interactions occurring between the oviductal membrane and the gametes and early embryo, to regulate fertility. Further studies on OVS are highly warranted to extend our knowledge of their role in fertilization *in vivo* and early embryonic development, a knowledge which can be directly extrapolated to *in vitro* fertilization to improve its efficiency.

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