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Uterosomes: Exosomal cargo during the estrus cycle and interaction with sperm

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1. ABSTRACT

The term “uterosomes” was first used to classify extracellular membrane vesicles released into the uterine luminal fluid. These extracellular vesicles (EVs), varying in sizes, fit the classification of exosomes and microvesicles on the basis of size, the presence of the CD9 biochemical marker, and lateral orientation of the membrane. Uterosomes appear to be formed by the apocrine pathway, similar to other reproductive EVs. In the murine system, the protein cargo carried by uterosomes includes glycosyl phosphatidylinositol (GPI)-linked and transmembrane proteins and these are hormonally regulated, appearing at high levels during proestrus/estrus and only marginally present at diestrus/metestrus. Uterosomes have been shown to deliver proteins in their cargo to sperm, with a functional impact, and are thought to participate in promoting sperm capacitation. Further studies are warranted, particularly those aimed at identifying the contents of their cargo during the estrus and menstrual cycle and the role they play n sperm maturation.

2. INTRODUCTION

In the mammalian female, sperm must traverse a long distance in the uterus before they reach the oviduct where fertilization occurs. During their transit in the female genital tract, they are in intimate association with the luminal fluids which are responsible for a sequential series of biochemical modifications on their plasma membrane. Collectively, the processes leading to these modifications which result in the remodeling of the sperm surface are referred to as capacitation (1). The series of membrane and metabolic transformations in the physiological activity of capacitation play a crucial role in fertility, as it contributes to the functional maturity of sperm and endows them with the ability to effect fertilization. Some molecular components secreted in the female luminal fluids are also synthesized in the testis as membrane proteins (2) while others are specific for the female (3-5). Many of these proteins are transferred to the sperm surface and are therefore pivotal in the final maturation of sperm (3,5), rendering them fertilization competent. The 2003 discovery of the iterative nature of the expression of Sperm adhesion molecule 1 (SPAM1) in the uterine fluid during estrus (2), subsequent to its expression in the epididymis during epididymal maturation (6-9), efferent duct, vas deferens, the prostate and seminal vesicles (10), and the testis (6), led to investigations which revealed the existence of SPAM1-bearing membrane vesicles (uterosomes) that mediate transfer of macromolecules to sperm (11). While the extracellular organelles for transferring macromolecules from the male reproductive fluids have been well-characterized with respect to their structure, contents, and their crucial role in sperm maturation and fertility (12,13), information on uterosomes is only just emerging. This review deals with these extracellular organelles in the uterine fluid prior to the establishment of a pregnancy, and the role they may play in sperm maturation and the regulation of fertilization.

3. CLASSIFICATION AND BIOGENESIS OF UTEROSOMES

It now appears that almost all biofluids contain EVs secreted from both the glandular and luminal epithelial lining. Consisting of a lipid bilayer, these EVs vary in sizes, with the larger ones (diameter of 100 nm -1 μm) referred to as microvesicles, while smaller ones (<100 nm) are termed exosomes (14). The latter are also characterized by: a) the presence of

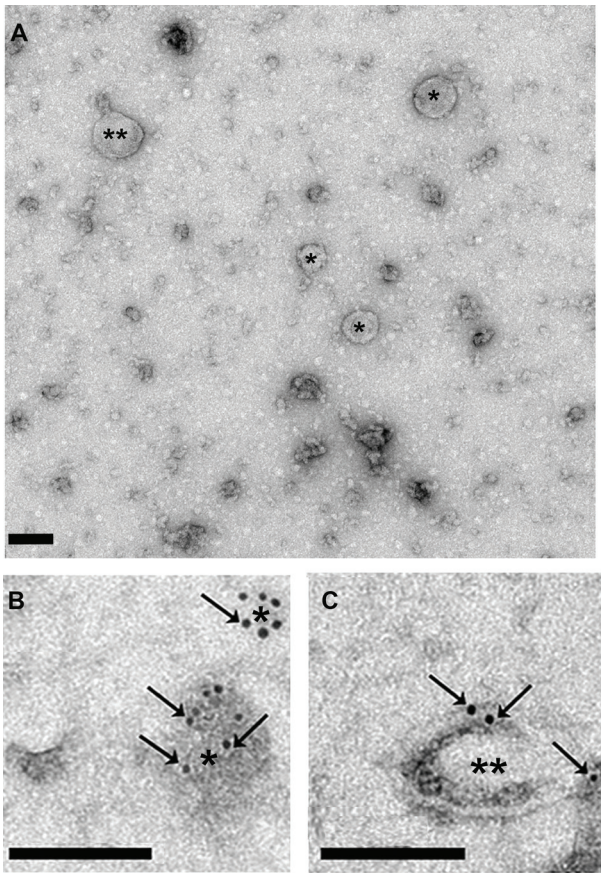

Figure 1. Electron micrographs of murine uterosomes. Uterosomes, exosomes* and microvesicles**, were isolated using differential ultracentrifugation. (A) An overview image of negatively stained samples with uranyl acetate was acquired by transmission electron microscopy. Larger vesicles with sizes ranging from 0.1. μm to 1 μm in diameter are termed as microvesicles and the smaller exosomes are < 100 nm. (B and C) Immunogold labeling of PMCA4a (arrows) show ultra-small gold particles on both exosomes and microvesicles. Samples were stained with uranyl acetate prior to imaging with transmission electron microscopy. IgG control (not shown) had no immunogold particles. Images were acquired with a Zeiss LIBRA 120 TEM at 120kV. Bars= 100 nm.

CD9 tetraspanin, a biochemical marker and adhesion molecule, on their surface and b) membrane orientation with the cytoplasmic-side inward (15-17). EVs from the non-pregnant uterus have been studied in mice where the fluids were analyzed at stages during the estrus cycle (11,18). Based on the above classification, in the two published reports on murine uterine EVs (11,18), both microvesicles and exosomes were detected in the fluids (Figure 1A) and both types are referred to as uterosomes, a term coined in 2008 by Griffiths *et al.* when they were first identified and shown to transfer SPAM1 protein to the sperm surface (11,19). This term was chosen to align with the terms used for exosomes/microvesicles identified in the male reproductive tract; namely, epididymosomes and prostasomes which are both involved in post-testicular sperm maturation (12,13). It should be noted that although in their 1999 paper Habiba *et al.* detected in women a large protein that was secreted from the glandular and luminal epithelia of the endometrium and could later be detected in the lumen, the protein was never seen in EVs (it was seen in intracellular vesicles) (20). In a more recent study in women, the isolation and characterization of exosomes and microvesicles from the uterine luminal fluids was demonstrated (21). Ng *et al.* reported that in women exosomes were shed in the uterine fluid and associated mucus and could be recovered from uterine flushings as well as from a cultured human uterine epithelial cell line (21).

3.1. Possible formation and methodology of isolation of uterosomes

While the specific pathway for the formation of uterosomes has not yet been studied, it seems likely that of the two distinct pathways for generating EVs; namely, one involving multivesicular bodies (15,17,22,23) and the other an apocrine pathway (24,25), the latter is likely to be relevant (18). The apocrine pathway involves the formation of vesicle-containing blebs protruding from the apical side of the membrane into the lumen, with the blebs ultimately releasing the vesicles into the lumen (24,25). The identification of exosomal biochemical markers as CD9 and CD68 in the apical surface of human endometrial epithelium (21) is consistent with the involvement of the apocrine pathway in the genesis of uterosomes, which have been shown to be CD9- and CD63-tetraspanin positive (18,21). There is evidence that the formation of epididymosomes which are generated by the apocrine pathway is hormonally–controlled (26). Similarly, evidence suggests that the expression of macromolecules in uterosomal cargo may be estrogen-regulated (2,18), as mentioned below.

Uterosomes have been isolated from the uterine fluids via ultracentrifugation (11,18,21), and analyzed by transmission electron microscopy (Figure 1A) following negative staining (11,18), or by FACS and immunofluorescence staining after exosome binding to beads (21). Their structural integrity has been confirmed by their resistance to hypo-osmotic and freeze-thaw stresses (11) which are likely not to be tolerated by resealed plasma membrane.

4. UTEROSOMES IN THE ESTRUS CYCLE: EVIDENCE FOR PROTEOMIC DIFFERENCES AND IMPACT OF DELIVERY TO SPERM

The identification of the presence of proteins in the uterosomal cargo first came from hypothesis-driven studies. Based on knowledge that murine epididymosomes and/or prostasomes carry glycosyl phosphatidylinositol (GPI)-linked SPAM1 (8-10) as well as Plasma membrane calcium ATPase 4a ((PMCA4a), a ten-pass transmembrane protein that is essential for

fertility (27-29)), studies were performed to investigate their presence in uterosomes. Western blot analysis and immunoelectron microscopy revealed that uterosomes carry both SPAM1 (11) and PMCA4a (18, Figure 1B,C). In the case of PMCA4a, as expected, 100% of the protein resides in the uterosomal fraction of the ULF (18); while SPAM1 is divided between the uterosomal and soluble ULF fractions (11). When Western blot studies were performed on the combined female luminal fluids (which included that from the oviduct and the vagina, with the majority consisting of ULF) from naturally cycling females, PMCA4a was shown to be highly expressed in uterosomes at pro-estrus/estrus and only marginally present at metestrus/diestrus (18). A similar pattern of estrogen-regulated expression of uterine SPAM1 was also detected (2). Thus the expression of these proteins detected in uterosomes, appear to be hormonally-regulated. In addition to being present in uterosomes, these proteins were also identified in EVs in the oviduct (18), as well as in the oviductal and vaginal epithelia and/or fluid (2,18,30) where they are likely to appear in EVs. Interestingly, the relative levels of these proteins in the three regions of the female genital tract were reported to be markedly different: PMCA4a was shown to be significantly lower in uterosomes than in oviductal EVs, increasing from the vagina to the oviduct (18) while SPAM1 increased from the oviduct to the vagina (2). This distribution of these proteins may be relevant to their physiological roles in sperm interacting with the uterosomes during estrus.

Uterosomes were shown to transfer both SPAM1 and PMCA4a *in vitro* to mature caudal epididymal sperm under neutral pH conditions (11, 18). This suggests that *in vivo* transfer may occur during sperm transit in the female tract and that this transfer may be a component of the capacitation process. Although both proteins are expressed in spermatids (6,27) and in epididymosomes which were also shown to deliver them to the sperm surface *in vitro*, consistent with *in vivo* acquisition during epididymal maturation (9,11,27), the finding that caudal sperm are able to acquire them from uterosomes *in vitro* indicates that the proteins exist on caudal sperm in amounts below the levels of saturation. Therefore it is feasible to envisage that sperm acquire additional amounts from the uterine secretion during their journey in the female genital tract.

In a recent review of EVs in the prostate (prostasomes) by Aalberts *et al.*, it was hypothesized that in the ejaculate prostasomes bind to capacitated sperm in the female tract under neutral or slightly alkaline pH conditions and later fuse with the sperm surface when they encounter more acidic pH, possibly in the cumulus matrix in the ampulla of the oviduct (31). This hypothesis does not consider the finding that the female tract contains endogenous EVs or uterosomes which have been shown to bind to the sperm surface under neutral pH conditions (11,18). It should be noted that this hypothesis inadvertently does not attribute the prostasome-sperm interaction in the female tract to the induction of capacitation, but rather to a post-capacitation activity since it states that the prostasomes bind to capacitated sperm (31). However, the acquisition of SPAM1, a hyaluronidase, by caudal sperm from uterosomes appears to be involved in the maturational process of capacitation since it enhances hyaluronic-acid-binding ability and cumulus penetration efficiency (19) which are essential steps in the fertilization process. Additionally, it has been proposed that selective sperm uptake of GPI-linked proteins such as SPAM1 from uterosomes may assist in the stabilization of the sperm membrane during cholesterol efflux (32). The latter is well–known to be associated with remodeling of the sperm membrane during capacitation (see 32). Thus the impact of the delivery of SPAM1, and likely other hyaluronidases (30), to the sperm surface by uterosomes provides evidence that the latter play a role in the sperm surface re-modeling that characterizes capacitation (33-34).

To date, studies of the impact of transfer of PMCA4a to the sperm surface by uterosomes has not yet been reported. However, based on its role as the major Ca^{2+} efflux pump in murine sperm (35), it can be predicted that its acquisition from uterosomes would facilitate efficient Ca^{2+} handling that is required to meet the high demand for Ca^{2+} during capacitation (36-38). With respect to the hypothesis proposed above by Aalberts *et al.* (31), it seems likely that a mixture of uterosomes and prostasomes in the uterine fluid deliver sperm proteins that contribute to triggering the metabolic processes and membrane transformations associated with capacitation. In support of this, is the finding that PMCA4 which regulates hyperactivated sperm motility (28,29) is present on human prostasomes (unpublished observation) as well as epididymosomes in mice (27) and bulls (39). Further, both uterosomes and prostasomes deliver their cargo to sperm under the same pH condition. It thus seems pertinent to ask the question: What is the significance or physiological relevance of the iterative expression and delivery of these fertility modulating proteins to the sperm via EVs in the male fluid and subsequently the uterine fluid?

Griffiths *et al.* argued that since SPAM1 is implicated in the acrosome reaction, it is advantageous to acquire maximal levels on the surface of sperm only after arrival in the ampulla, as fertilization occurs at the ampullary-isthmic junction. Thus the incremental acquisition of SPAM1 on sperm prior to arriving in the ampulla serves to prevent premature acrosome reaction before arriving at the fertilization site (19). In

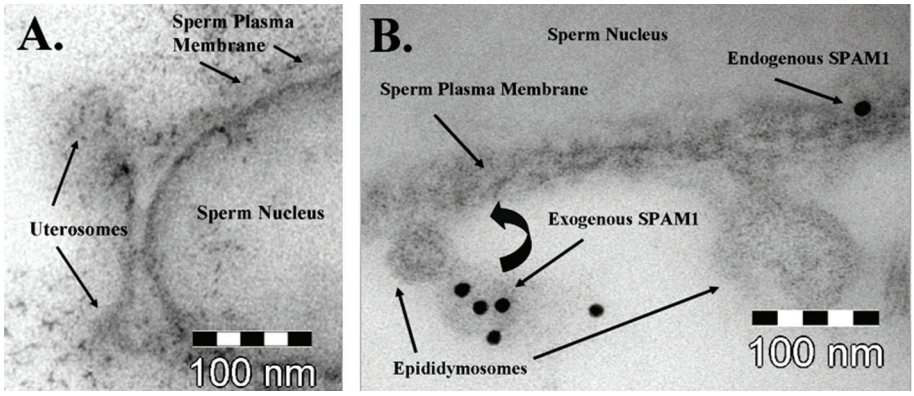

Figure 2. A) Transmission electron microscopy reveals two uterosomes docking on the plasma membrane over the sperm head following co-incubation of murine sperm and non-immunolabeled uterosomes. In B), epididymosomes immunolabeled for SPAM1are seen interacting with the murine sperm membrane. While the smaller vesicle is labeled, the larger whose membrane appears to be fusing with the sperm membrane has no immunogold particles. Reproduced with permission from ref # 11.

the case of PMCA4, its acquisition from uterosomes may also be adaptive, with respect to the loss of decapacitation factors during capacitation. To prevent premature capacitation during epididymal transit and at ejaculation, sperm acquire decapacitation factors (DF) (40,41). One of these in murine sperm stimulates Ca^{2+}-ATPase activity to lower cytosolic Ca^{2+} concentration ($(Ca^{2+})_c$) (41), a task performed primarily by PMCA4 (35). During capacitation, DF are lost and the $(Ca^{2+})_c$ is elevated (40,41). Thus acquisition of additional PMCA4 via uterosomes would be advantageous to ensure adequate Ca^{2+} efflux that is required to promote sperm viability.

Another pertinent question that might be asked is: What are other fertility modulating proteins that are carried by uterosomes and can be delivered to the sperm during their passage in the uterus? Proteomic studies using mass spectrometry on proteins from cycling and pregnant ewes have shown a variety of proteins in EVs (42). Interestingly, many of the proteins identified are those that do not have a signal peptide for classical secretion or are considered intracellular proteins. Thus, EVs are thought to provide a unique mechanism for transmission of proteins (42). However, apart from the study of Burns *et al.* (42) there have been no reports of proteomic studies to identify proteins that may be carried on uterosomes and transferred to sperm. These studies are important and are highly warranted.

In addition to proteins, microRNA and mRNAs have been found in uter somal cargoes (21, 42). MicroRNA (miRNA) analysis of human cultured endometrial epithelial cells, ECC1 cell line, and their EVs revealed that the latter have specific miRNAs that could be transferred to neighboring cells (21). A study of EVs from the ovine uterine fluid by Burns *et al.* (42) confirmed the findings for miRNA and extended them to RNAs. Importantly, significant differences were detected between the cargoes of pregnant and non-pregnant sheep (42). This suggests that uterosomes play physiologically important roles and could contribute significantly to the final maturation that sperm undergo before they are competent to effect fertilization. However, more transcriptomic as well as proteomic work needs to be done to analyze uterosomal cargoes, to determine their impact on the fertilizing ability of transiting sperm or on other regions of the reproductive tract. Such studies on the impact of the fertilizing ability of sperm hold promise for informing the current practice of *in vitro* fertilization which has a success rate of only 32% (43).

5. MECHANISM(S) OF UTEROSOMAL CARGO DELIVERY

Although there are significant gaps in our understanding of the mechanism of exosomal cargo delivery to sperm (44), it has been shown that uterosomes containing GPI-linked SPAM1 dock on the sperm plasma membrane (Figure 2A). This indicates that SPAM1 transfer from the uterine fluid is partly vesicle–mediated (11), in addition to being delivered to the sperm surface via a soluble membrane-free mechanism involving lipid carriers such as ApoA and ApoJ (32). It was proposed that the docking could allow GPI-anchored proteins on the vesicles to be shuttled onto the sperm plasma membrane where they are inserted via hydrophobic interactions (11). However, in Figure 2B there is evidence for vesicle-plasma membrane fusion with the involvement of an epididymosome. Thus it is highly likely that uterosomes fuse with the sperm membrane to deliver their cargo, as now appears to be the case for human prostasomes (45,46), bovine epididymosomes (39), and oviductosomes (47). It

should be noted that recent studies of the proteomes of bovine and human epididymosomes have identified a number of transmembrane proteins (48,49), including CD9 tetraspanin which mediates plasma membrane fusion (14,15,50). Since it is unlikely that transmembrane proteins such as PMCA4 could be shuttled on to the sperm PM and be inserted via hydrophobic interactions, vesicle fusion is the likely mechanism for cargo delivery to sperm via uterosomes. Indeed Al-Dossary *et al.*, with the use of 3D super-resolution structured illumination microscopy and dye-labeled EVs, recently showed sperm-EV fusion simultaneously with immunolabeled PMCA4a delivery to the murine sperm surface (47, 51).

While there have been reports that EVs can deliver their cargo via endocytosis (52, 53), such a mechanism would not occur in sperm which are devoid of cytoplasm and do not undergo this process (54). A fusogenic mechanism which is now supported by studies in prostasomes, oviductosomes, and epididymosomes (39,45-47,51) is able to deliver cargo containing GPI-linked, transmembrane, and membrane-associated proteins which are the predominant ones found in sperm in the absence of the vast majority of the cytoplasm. Further, it appears to deliver proteins in their functional complexes as seen from co-immunoprecipitation studies of PMCA4 and CASK (Ca^{2+}/CaM serine kinase) in murine epididymosomes (27) and PMCA4 and nNOS (neuronal nitric oxide synthase) in human prostasomes (unpublished data, Martin-DeLeon Lab). Delivering proteins in a complex from EVs to sperm is not only efficient, but ensures that stoichiometric amounts of interacting proteins are available for functional activity.

6. SUMMARY AND FUTURE WORK

Although it is now established that uterosomes are secreted into the uterine luminal fluid and are capable of transferring fertility modulating proteins to transiting sperm, the studies are limited. More work needs to be done on characterizing uterosomal cargo with respect to the types of macromolecules that they carry, as well as how the content varies during the estrus or menstrual cycle. The genesis of uterosomes and their mechanism of cargo delivery appear to be similar to that of the well-studied reproductive EVs, prostasomes and epididymosomes. Further studies of these vehicles of cell-to-cell communication hold promise for identifying proteins of physiological relevance to the capacitation process of sperm and thus might have a translational impact in the IVF clinic.

7. ACKNOWLEDGEMENTS

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