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# Epididymosomes: Role of extracellular microvesicles in sperm maturation

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## 1. ABSTRACT

The spermatozoa of vertebrate species that practice internal fertilization have to transit along the epididymis after leaving the testis. This epididymis is a single, long convoluted tubule that links the testis to the vas deferens (1). During this transit, the male gametes acquire their fertilizing ability and their forward motility properties. Collectively, these modifications known as sperm maturation depend on a series of well-orchestrated biochemical modifications imposed upon the transiting male gamete (2). These modifications are in part regulated by extracellular microvesicles called epididymosomes that are found in the intraluminal epididymal compartment (3, 4). In this review, the biochemical composition of epididymosomes, their mode of secretion, the mechanisms underlying their interactions with the male gamete, and how they are involved in sperm maturation will be described.

## 2. INTRODUCTION

Two main functions are associated with the male gonad: production of hormones assuring the male phenotype and delivery of differentiated male gametes. In vertebrate species practicing internal fertilization, spermatozoa exiting the testis harbor all of the cytological features of functional gametes, but are incapable of fertilization. In order to acquire fertilizing ability, they must transit a single, long convoluted tubule linking the efferent ducts to the vas deferens (5). The epididymal length varies from one mammalian species to another, as does the sperm transit time, which can reach 90 m and 15 days in bulls, respectively. Anatomically, the epididymis is divided into three segments: the proximal caput, corpus and distal cauda epididymidis. In rodents, a proximal «initial segment» with histological characteristics distinct from the caput is thought to play an essential role in sperm physiology (6). The epididymis is part of the excurrent duct originating from the Wolffian (mesonephric)(7-11) duct. The excurrent duct in virilised males is formed by the efferent ducts, the epididymis, the vas deferens, the ejaculatory duct, and seminal vesicles. As a result of the pioneering work of Orgebin-Crist and Bedford in the ’60s the epididymis is known to be essential for the acquisition of fertilizing capacity and forward motility by the male gamete; changes collectively known as sperm maturation. Functions of the epididymis include water reabsorption from fluid originating from seminiferous tubules with subsequent sperm concentration, sperm protection against the immune response, and sperm storage in the optimal milieu of the distal segment (12). The epididymis is a feature of male reproductive tracts of mammalian phyla practicing internal fertilization. Coupled with the knowledge that copulation is not always synchronized with ovulation, it is hypothesized that the epididymis generates a heterogeneous population of male gametes with optimal maturity occurring at different time points post-copulation in order to increase the fertility window of a given ovulatory event. Thus, the epididymis is a complex organ playing key functions in sperm physiology (12).

## 3. THE EPIDIDYMIS

The epididymis is a single, long convoluted tubule and is formed by a pseudostratified epithelium of epithelial cells named principal cells. The epididymal

epithelium is highly active in protein secretion; the principal cells harbor pseudociliary projections at the apical pole in order to optimize this secretory activity. Sophisticated tight junctions between these cells assure the blood–epididymal barrier and allow formation of a unique intraluminal milieu. Principal cell height decreases along the epididymis, whereas the lumen diameter of the epididymal tubule increases. Although principal cells are the major cell type forming the epididymis, other cells types are also found within the epithelium such as: clear cells, which express V-ATPases and are involved in acidification of the intraluminal milieu; basal cells, which harbor cytoplasmic extensions that sense the composition of the epididymal fluid; and halo cells, which are more abundant in the proximal segments and are thought to represent a mixture of immune cells (12).

The epididymal intraluminal compartment is distinctive in comparison with other biological fluids. For example, the epididymal fluid pH is around 6.5.; the osmotic pressure can reach 400 mOsmol/kg and even higher in some species; the electrolyte composition is unique in comparison with other biological fluids, and the high Zn concentration is characteristic of the epididymis (11). Very tight gap junctions forming the blood–epididymal barrier assure the maintenance of the unique «epididymal sperm nursery» and assure the unique intraluminal environment necessary for sperm maturation (13).

The epididymis response to androgens stimulation involves a marked increase in translational and protein secretion activities. The epididymal fluid protein composition has been of interest since the ’80s because interactions between epididymal intraluminal macromolecules and the sperm surface modulate sperm fertilizing ability and forward motility. A particular feature of the epididymis is the variability of the fluid proteome along the organ (14-16). A subset of soluble epididymal proteins is thought to be added to the maturing spermatozoa, or to be involved in modification to, or depletion of sperm proteins during epididymal transit (11). Furthermore, with regard to the proteome, each epididymal segment displays its own transcriptomic signature (8,17,18). Thus, sperm maturation involves sequential modifications to the transiting male gamete with a changing epididymal intraluminal milieu. The nature of the epididymal proteins involved in sperm maturation has been the subject of intense research by many laboratories (19). The mechanisms governing the targeting of specific sperm membrane subdomains by individual epididymal proteins remain puzzling (11). It rapidly became obvious that epididymal proteins secreted into the lumen of the epididymal tubule interact with spermatozoa in a manner that is incompatible with the classical merocrine secretion pathway. For example, certain epididymal proteins behave as integral membrane proteins once transferred to the maturing spermatozoa, others are anchored by glycosylphosphatidylinositol (GPI) (20) to the sperm surface, and yet others are integrated into intracellular compartments of the maturing male gamete (21).

## 4. EXTRACELLULAR MICROVESICLES NAMED EPIDIDYMOSOMES

In 1985, Yanagimachi *et al.* first described small membrane microvesicles in close contact with spermatozoa in the hamster epididymis (22). As they were enriched in cholesterol, it was hypothesized that these microvesicles could be involved in sperm membrane cholesterol/phospholipid changes occurring during maturation. Since this pioneering work, such vesicles have been described as constituents of the epididymal fluid in hamster (23) mice (24,25), rats (26), bulls (27,28), rams (29, 30), and humans (31). These vesicles, known as epididymosomes, are spherical in appearance and heterogeneous in size with diameters ranging from 50–250 nm.

Their diameter and appearance at the electron microscopic level vary along the epididymis, at least in the bull (32). They have recently been visualized by high-resolution helium ion microscopy; these micrographs suggest that they «fuse» with the epididymal spermatozoa in laboratory rodent species (33). There is also electron micrographic support for this concept. Other experimental evidence at the light microscopic level proposes that epididymosomes interact preferentially with the acrosomal cap and the midpiece of epididymal spermatozoa in the bull (28, 34). Although the interaction and/or fusion of epididymosomes with epididymal spermatozoa is poorly documented at the microscopic level, there is substantial experimental evidence to support the concept that proteins, miRNAs and possibly other macromolecules such as phospholipids are transferred from epididymosomes to maturing spermatozoa (3).

A complex mixture of proteins is associated with epididymosomes. The protein composition of epididymosomes differs from the protein composition of the fluid fraction collected in the same epididymal segments (10,28,35). To date, the epididymosome proteome has been published using ram (35) and bull (10) tract tissues. A proteomic analysis has been performed on human microvesicles prepared from fluid aspirated from the proximal scrotal portion of the vas deferens, with the assumption that it is enriched in microvesicles originating from the epididymal fluid. A more exhaustive study compares the proteomes of caput and cauda epididymosomes from a bovine model. Using ES–MS/MS technology, 555 and 438 proteins were identified in caput and cauda bovine epididymosomes, respectively; 231 were common to both epididymosome populations (10). Biological networks of these proteomes show that epididymosome compositions and underlying functions vary along the epididymis. This study reveals

that some of the identified proteins are involved in sperm maturation as they are implicated in sperm–egg interactions and sperm motility. Of interest is that enzymes involved in glycosylation and in the acquisition of the GPI-anchor are also associated with epididymosomes. Rab and SNARE adhesion molecules are also found in the epididymosome proteomes and could be involved in mediating microvesicle adhesion to epididymal spermatozoa (10).

## 5. SECRETION OF EPIDIDYMOSOMES

Merocrine secretion is the most common pathway used by cells to secrete proteins. During this process, a signal peptide targets proteins to be secreted to the endoplasmic reticulum (ER) where they are synthesized. Full-length proteins transit the Golgi apparatus where they undergo post-translational modifications. They are then packaged in secretory vesicles and released into the extracellular milieu after subsequent fusion with the apical plasma membrane of secretory cells.

Apocrine secretion is an alternative secretory mechanism of the merocrine pathway. It consists of the formation of cytoplasmic protrusions, which are referred to as apical blebs. These apical blebs detach from the secretory cells whereupon they breakdown to release their content; proteins synthesized on cytoplasmic free ribosomes and membrane-bound microvesicles. This secretion pathway was described as early as 1922 by Schiefferdecker cited by Anmuller *et al* (36), but was subsequently disregarded as apical blebs were considered by many to be fixation artifacts (36,37). It is now generally accepted that the rat coagulating gland, the prostate, the seminal vesicles, the vas deferens and the epididymis use the apocrine pathway to secrete proteins within the male reproductive tract (36). Formation of the underlying apical blebs at the apical pole of these epithelial cells is hormone-dependent. It is widely acknowledged that epididymosomes undergo apocrine secretion from epididymal principal cells (38). Rejraiji *et al.*, provide clear electron micrographic illustrations of the existence of cytoplasmic blebs containing microvesicles at the apical pole of epididymal principal cells (25).

Other sources of microvesicles present in the epididymal intraluminal compartment cannot be excluded. This is particularly true when considering the heterogeneity of epididymosome diameter and their appearance at the electron microscopic level. Testicular origin of a subpopulation of epididymosomes has not yet been evaluated and cannot be excluded. On the other hand, different types of extracellular microvesicles present in different biological fluids have been described and classified according to their size, mode of secretion and presence of specific markers (39). One of the most studied types of microvesicle is the exosome and we cannot overlook the possibility that the epididymal epithelium secretes this category of extracellular vesicle. Secretion by multivesicular bodies is one criterion used to discriminate exosomes from other microvesicles along with the presence of the tetraspanin CD9 surface protein. Whereas CD9 is characteristic of a subpopulation of epididymosomes in bovine epididymis, the presence of multivesicular bodies (40,41), as reported in a limited number of studies, does not appear to be the hallmark of the epididymal principal cells. Further investigation is necessary to understand the secretory pathway of epididymosomes.

## 6. SPERM–EPIDIDYMOSOME INTERACTIONS

Protein transfer from epididymosomes to spermatozoa has been studied *in vitro* using biotinylation of epididymosome surface proteins. A complex pattern of proteins accessible to biotinylation in bovine cauda epididymosomes is revealed by SDS-PAGE. Only a subset of these proteins is transferred to caput spermatozoa after co-incubation during *in vitro* experiments (28). This transfer shows a particular specificity as it is saturable and unlabeled epididymosomes compete with their biotinylated counterparts when added to the co-incubation medium. The transfer is temperature- and pH-dependent. Of interest is that the protein transfer efficiency reaches a maximum at pH 6.0.–6.5., which is the physiological pH of the intraluminal epididymal fluid. Whereas addition of divalent cations such as Ca, Mg, and Mn has no effect on protein transfer from cauda epididymosomes to caput spermatozoa, Zn potentiates the quantity of proteins transferred in a dose-dependent manner (28,42). Thus, with the knowledge that Zn is highly concentrated in epididymal tissues, this observation has physiological significance (43).

Studies using different animal models have highlighted epididymosome-associated proteins that are transferred to the maturing spermatozoa (3) e.g. P26h/P25b (23,27), Sperm adhesion molecule 1 (SPAM1) (24, 44), glioma pathogenesis-related protein 1(GPR1L1) (45), and A desintegrin metalloproteases (ADAM2, ADAM3, ADAM7) (46), all of which are involved in fertilization processes. Glutathione peroxidase 5 (GPX5) (47), ubiquitin (UBC) (48), epididymal sperm binding protein 5 (CD52) (49), and epididymal sperm binding protein 1 (ELSPBP1) (50, 51) are epididymosome-associated proteins that play a role in sperm protection or elimination. Additional proteins acquired by the maturing spermatozoa via their interaction with epididymosomes include: Plasma membrane Ca ATPase 4 (PMCA4) involved in sperm intracellular homeostasis; c-Src kinase, a player in the capacitation signaling cascade (52); macrophage migration inhibitory factor (MIF) (28,53-55), a dense fiber-associated protein involved in sperm motility control; and liprin alpha 3 (Ppfia3), which plays a role in the acrosome reaction. Methylmalonate-semialdehyde

dehydrogenase (56) and cathepsin D (CAT-D) (57), are also epididymosomal proteins transferred to spermatozoa during epididymal transit; however, their functions in sperm physiology remained to be determined. Thus, there is increasing evidence that epididymal microvesicles interact with maturing spermatozoa by modulating their protein composition.

The male gamete also undergoes changes in lipid composition during epididymal transit. Experimental evidence suggests that epididymosomes could be, at least in part, involved in this sperm membrane remodelling. The lipid composition of epididymosomes per se shows some peculiarities: in mice and bulls the ratio of cholesterol: phospholipids in epididymosomes increases by 1.5. along the epididymis (10). Reijraji *et al.* show that murine epididymosomes are enriched in polyunsaturated fatty acid and sphingomyelin; the sphingomyelin concentration accounts for half of the phospholipid content of microvesicles collected in the cauda epididymidis (25). Cholesterol and sphingomyelin, which are abundant in bovine epididymosomes, are concentrated in epididymosome lipid rafts. These membrane domains are critical for protein transfer from epididymosomes to specific sub-compartments of spermatozoa (58). Fusion of epididymosomes with spermatozoa was studied *in vitro* using epididymosomes labeled with octadecyl rhodamine-B; a membrane lipid probe. Following *in vitro* fusion of immature bovine spermatozoa with epididymosomes, the decrease in the sperm plasma membrane cholesterol/phospholipids ratio is similar to that observed during epididymal transit of the bovine male gamete (34). This decrease in sperm membrane cholesterol/phospholipids ratio following co-incubation with epididymosomes described by Schwarz *et al* (34) is puzzling when one considers the high cholesterol content of epididymosomes. Thus, there is compelling evidence that microvesicles secreted in the intraluminal compartment play a major role in protein and lipid transfer to spermatozoa and that these macromolecules are involved in sperm maturation.

The mammalian sperm proteome is complex; in a given species more than 3, 000 different plasma membrane proteins can be identified by modern proteomic technologies. These proteins are highly segregated as they are associated with highly defined plasma membrane subdomains (11,59). This is also true for epididymal secreted proteins acquired by the transiting spermatozoa. These proteins can be loosely bound to the sperm surface by electrostatic interactions, whereas others behave as integral membrane proteins or as GPI-anchored molecular entities (11,23). Even more challenging is that some secreted proteins found in the intraluminal epididymal compartment become intracellular constituents of the maturing spermatozoa. This holds true for MIF, which is added to the sperm tail dense fibers where it regulates formation of disulfide bonds within these intracellular structures (55,60). We performed a series of experiments using a bovine model to show that some epididymosome-associated proteins are exposed externally, whereas others are contained within the vesicles. Moreover, a subset of externally exposed epididymosome membrane-associated proteins is a constituent of raft membrane domains, whereas others are excluded from these membrane subdomains. The different localizations of epididymosome-associated proteins dictate the sperm domains to which they will be transferred; the raft-associated proteins will be transferred to sperm raft membrane domains and internal proteins will become constituents of intercellular compartments (58). Therefore, it appears that the manner in which proteins are segregated in the epididymal microvesicles or epididymosomes, determines the sperm sub-compartment targets to which they will be transferred in order to mediate the functions known to be acquired during epididymal sperm maturation. For example, the sperm P26h/P25b proteins involved in binding to the egg's zona pellucida, are transferred from epididymosome raft domains to rafts of sperm plasma membrane covering the acrosome, whereas MIF, contained within bovine epididymosomes, is transferred to the internal dense fibers of the sperm flagellum (21).

## 7. EPIDIDYMOSOME HETEROGENEITY

As mentioned above, epididymosomes comprise a heterogeneous population of vesicles with diameters ranging between 50–250 nm at the ultrastructural level. Whereas microvesicles called prostasomes, contained in human seminal plasma, can be fractionated into different subpopulations, a limited number of attempts have been made to isolate potential subpopulations of epididymosomes (61). Forbes *et al.* (1995) subjected rat epididymal fluid to sucrose gradient centrifugation to separate two populations of membranous vesicles that differed in size and enzymatic composition. With diameters in the micrometer range these vesicles likely differ from epididymosomes, which are characterized by diameters of less than 250 nm as described by a number of laboratories using different animal models (62,63). The existence of extracellular microvesicles has raised great interest during the last decade as a result of their potential roles in cell–cell communication and their possible implication in multiple pathophysiological situations. These vesicles have been tentatively classified according to their origin i.e. their mode of secretion, their size and their macromolecular composition. The best-characterized extracellular microvesicles are exosomes that harbor tetraspanin CD9, which is used as a marker of this type of microvesicle (39). Using an exosome purification protocol, we have been able to distinguish at least two distinct populations of microvesicles in an epididymosome preparation. These two populations of vesicles differ in size with the smaller one being characterized by CD9. This is analogous to exosomes

studied in other biological fluids. The tetraspanin domains of these small epididymosomes (exosomes) are involved in membrane fusion with spermatozoa since antibodies against CD9 and CD26, a CD9 partner, inhibit protein transfer when epididymosomes and epididymal spermatozoa co-incubated *in vitro*. The remaining population of larger CD9-negative epididymosomes contains a high level of epididymal sperm binding protein 1 (ELSPBP1) (32). The latter is highly expressed in the proximal bovine epididymis and is found in the intraluminal compartment in association with epididymosomes. Epididymal sperm binding protein 1 is specifically transferred to dead spermatozoa in the epididymis and remains associated with them following ejaculation. It was first described in humans as HE12, and orthologs exist in the dog, horse, pig and bull. Whereas the structure of this protein, characterized by type 2 fibronectin domains, is known, its function remains elusive. Of interest is that Zn, a cation that is highly concentrated in the epididymis, potentiates the association of ELSPBP1 with its partners and interaction with spermatozoa. The identification of ELSPBP1 partners suggests that binding of ELSPBP1 to epididymal sperm may be involved in the protection of live spermatozoa against detrimental molecules generated by dying spermatozoa within the epididymis (50,51). Thus, it appears that epididymosomes contain at least two distinct populations of microvesicles; an exosome-like CD9-positive population that transfers proteins involved in sperm maturation by fusion e.g. P25b and GliPr1L1 involved in zona pellucida recognition, MIF which modulates flagellum beating, and AKR1B1 (32). The remaining population harbors ELSPBP1, which binds to dying spermatozoa within the epididymis in order to protect the surviving male gametes. Further analyses of epididymosome subpopulations are expected to reveal additional functions associated with extracellular microvesicles present in the intraluminal compartment of the epididymis.

## 8. miRNAS IN EPIDIDYMOSOMES

Recent work from Belleannée *et al.* shows that miRNAs are another constituent of epididymosomes. Comparison of epididymosomes collected from the caput and cauda segments of the bovine epididymis reveals that the epididymosome population in each segment has its own miRNA signature. In a given epididymal segment, the population of epididymosomal miRNAs differs from the miRNA signatures of epididymal tissues from which the epididymosomes were collected. This suggests the existence of a selection mechanism for epididymosome-associated miRNAs (64). A number of *in vitro* experiments support the concept that epididymosomes can transfer their miRNA content to epididymal epithelial cells and thereby negatively modulate the presence of specific mRNAs. Therefore, it appears that epididymosomes secreted in the proximal region of the epididymis may modulate gene expression of epididymal principal cells in more distal segments of the excurrent duct (65, 66). It remains to be determined whether epididymosomes transfer miRNAs to spermatozoa during their journey along the male tract.

## 9. CONCLUSION

Epididymosomes form a complex mixture of extracellular microvesicles that are involved in sperm maturation and also in gene expression regulatory mechanisms along the epididymis. Further work is needed to appreciate the complex physiological processes that occur in the male reproductive tract under regulation by extracellular microvesicles.

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