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·*Review*·

Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit

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Abstract

During epididymal transit, spermatozoa acquire new proteins. Some of these newly acquired proteins behave as integral membrane proteins, including glycosylphosphatidylinositol (GPI)-anchored proteins. This suggests that the secreted epididymal proteins are transferred to spermatozoa by an unusual mechanism. Within the epididymal lumen, spermatozoa interact with small membranous vesicles named epididymosomes. Many proteins are associated with epididymosomes and the protein composition of these vesicles varies along the excurrent duct and differs from soluble intraluminal proteins. Some epididymosome-associated proteins have been identified and their functions in sperm maturation hypothesized. These include P25b, a zona pellucida binding protein, macrophage migration inhibitory factor, enzymes of the polyol pathway, HE5/CD52, type 5 glutathione peroxidase, and SPAM1 or PH-20. The electrophoretic patterns of proteins associated to epididymosomes are complex and some of these proteins are transferred to defined surface domains of epididymal spermatozoa. Epididymosomes collected from different epididymal segments interact differently with spermatozoa. This protein transfer from epididymosomes to spermatozoa is time-dependent, temperature-dependent and pH-dependent, and is more efficient in the presence of zinc. Some proteins are segregated to lipid raft domains of epididymosomes and are selectively transferred to raft domains of the sperm plasma membrane. Some evidence is presented showing that epididymosomes are secreted in an apocrine manner by the epididymal epithelial cells. In conclusion, epididymosomes are small membranous vesicles secreted in an apocrine manner in the intraluminal compartment of the epididymis and play a major role in the acquisition of new proteins by the maturing spermatozoa. *(Asian J Androl 2007 July; 9: 483–491)*

Keywords: apocrine secretion; epididymis; epididymosomes; spermatozoa; sperm maturation

1 Introduction

The epididymis is part of the anatomy of all vertebrate species in which reproduction involves internal fertilization [1]. This organ is a single convoluted tubule located between the vasa efferentia and the vas deferens. Spermatozoa leaving the testicles have to transit along this 3–12-m-long tubule (depending on the species) before reaching the vas deferens. Classically, the epididymis is divided in three segments: the head (caput), an elongated region (corpus) and a terminal bulbous region (cauda). In some species, a differentiated initial segment incorporated in the caput is present [2].

The epididymis is responsible for sperm transport, concentration, storage and maturation. Sperm maturation involves the acquisition of forward motility and fertilizing ability [3]. During this process, the male gamete will undergo many biochemical modifications that are modulated by the epididymal intraluminal composition, in particular secreted epididymal proteins [4]. In fact, it has been known for a few decades that the epididymal epithelium bordering the intraluminal compartment

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secretes, under androgenic stimulation [5, 6], proteins that interact with the maturing spermatozoa to generate fully functional gametes [7–9]. The pattern of gene expression varies along the epididymis, encoding proteins secreted in the intraluminal compartment [10–12]. The composition of the intraluminal epididymal milieu encountered by spermatozoa varies from one segment to the other [8, 13]. The sperm maturation process involves a sequence of well-orchestrated biochemical events that will sequentially modify spermatozoa during their journey along the epididymis [14, 15].

The major modifications undergone by maturing spermatozoa include: changes in membrane phospholipid composition and in cholesterol/phospholipid ratio, increases in disulfide bonds and in net surface negative charge, relocalization of surface antigens, and modification, elimination and addition of surface proteins. Functions of epididymal proteins added to spermatozoa during the maturation process have raised considerable interest [3, 7, 15]. These proteins are usually referred to as coating proteins, which are proteins binding to the sperm membrane through electrostatic interactions. By definition, these proteins can be washed from the sperm surface with a high ionic strength solution [16, 17]. Interestingly, many epididymal proteins behave like integral membrane proteins when spermatozoa are subjected to different biochemical treatments [15, 18]. Many of these proteins associated to the sperm surface have glycosylphosphatidylinositol (GPI) anchors, for example HE5 (CD52) in humans [19], SPAM 1 and hyaluronidase in mice [20–22], and the orthologs P26h [16] and P25b [23] in hamsters and bulls, respectively. According to one cell biology dogma, a protein GPI-anchored to a cell plasma membrane has to transit through the endoplasmic reticulum-Golgi apparatus-secretory vesicle pathway, or merocrine secretion, to be GPI-anchored. According to the classical secretion pathway, proteins from the extracellular compartment cannot be GPI-anchored to a cell surface. Therefore, proteins of epididymal origin and GPI-anchored to the sperm plasma membrane have to be secreted in an unusual fashion [18, 24, 25]. Furthermore, recent evidence shows that secreted epididymal proteins can be incorporated into intracellular subcompartments of the sperm cell [26–28]. Taken together, these observations suggest that secreted epididymal proteins are transferred to spermatozoa by unusual mechanisms [15, 29].

2 Epididymosomes

Epididymal proteins secreted in a merocrine fashion by the epithelial cells are expected to be soluble in the intraluminal compartment. These proteins contain in their sequence a signal peptide trafficking them to the endoplasmic reticulum [30]. Some proteins present in the intraluminal compartment of the epididymis can be pelletted if submitted to ultracentrifugation. In fact, these proteins are associated with small membranous vesicles of 50–500 nm in diameter [16]. Yanagimachi *et al.* [31] were the first to describe at the electron microscopic level membranous vesicles in the intraluminal epididymal fluid that interact with the hamster sperm surface (Figure 1). Knowing that spermatozoa undergo many membrane modifications during epididymal transit, these authors hypothesized that these vesicles might be involved in the transport of sperm plasma membrane cholesterol. These vesicles, which have been recently named "epididymosomes", have been described in many mammalian species, including the hamster [16, 31], the bull [23, 32], the mouse [33], and the rat [26, 34]. Epididymosomes are characterized by a very high cholesterol/phospholipid ratio and sphingomyelin is the major phospholipid constituent [33]. Similar vesicles with a comparable lipid composition have been described as constituents of semen. These vesicles have been named "prostasomes" because they were first described as a secretory product of the prostate [29, 35–37]. Many different proteins are associated with both prostasomes [38] and epididymosomes [39].

The protocol used for epididymosome purification resembles that used to prepare microsomal fractions from homogenized tissues [32]. Epididymosomes can be prepared from epididymides of small laboratory animals by mincing tissues from each epididymal segments. Epididymosomes prepared this way will inevitably be contaminated by microsomes. To study epididymosomes we used epididymides dissected from bull testicles freshly collected from the slaughterhouse. Uncontaminated epi-

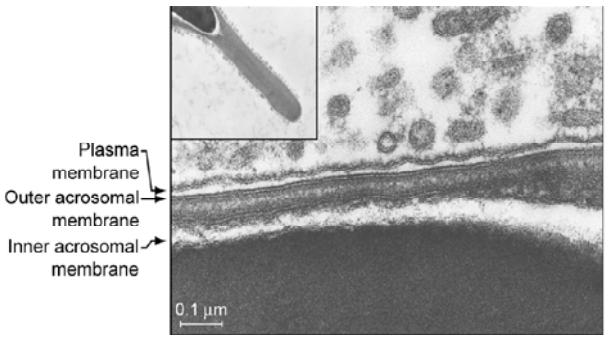

Figure 1. Electron photomicrographs showing Chinese hamster epididymosomes surrounding the plasma membrane of a spermatozoon. The inset shows the general appearance of the acrosome surrounded by theses vesicles. Original photos were kindly provided by Dr Yanagimachi (University of Hawaii). Scale bar = 0.1 μm. Reproduced with permission from Saez *et al.* [29].

didymal fluids can be collected by retrograde luminal flushing to allow preparation of pure population of epididymosomes. Two-dimensional electrophoresis revealed that epididymosome protein composition was different from the electrophoretic patterns of soluble intraluminal proteins collected from the same epididymal segment [32]. Patterns of proteins associated with epididymosomes collected from different segments of the epididymis also show great differences. The protein composition also shows great differences between epididymosomes prepared from the cauda epididymidis and similar vesicles purified from ejaculated semen [39]. This suggests that epididymosomes do not contribute significantly to the populations of vesicles present in the ejaculate. Treatment of epididymosomes with 0.1% Triton is inefficient for dissociating these proteins, showing that the association of proteins to epididymosomes is very strong. This is probably because of the unusual composition of these membranous vesicles, especially the high cholesterol/phospholipid ratio. Some of these proteins have been identified and their functions in sperm maturation hypothesized [39, 40].

3 Proteins associated with epididymosomes and their hypothesized functions

Very few proteins associated with epididymosomes have been identified. This is the reason why functions of epididymosomes remain to be determined [41, 42]. P25b is the bovine ortholog of P26h, a sperm protein showed by our laboratory to be involved in sperm–zona pellucida interaction in the hamster [43, 44]. Like P26h [16], P25b is GPI-anchored to epididymosomes and transferred to spermatozoa during epididymal transit [23]. The accumulation of P25b or P26h, respectively, on the acrosomal cap of maturing bovine and hamster spermatozoa is correlated with the ability of the male germ cell to bind the zona pellucida: a key step leading to fertilization [9, 45, 46].

Macrophage migration inhibitory factor (MIF) was first defined as a T cell cytokine. MIF is now known to have a wide tissue distribution [47]. Depending on the differentiation status and the type of cell expressing this protein, it will play different functions [48, 49]. Enzymatic activities, such as tautomerase [50] and thiol-protein oxido-reductase [51], are part of MIF functions. When secreted by Leydig cells, MIF modulates inhibin production by Sertoli cells [52, 53]. The MIF sequence has three cysteines present as free thiols [54] and the N-terminal lacks a signal peptide [55]. MIF is expressed by epididymal epithelial cells in rats [26, 27], humans [28] and bulls [40, 56]. It is secreted as a protein associated with epididymosomes that interact with maturing spermatozoa [26, 40]. In the intraluminal epididymal compartment, these epididymosomes are in close contact with the sperm plasma membrane and then MIF is transferred to spermatozoa as a new component of the flagellar outer dense fibers [26]. How MIF is translocated from epididymosomes to an intracellular component of the sperm flagellum remains to be determined. It has been hypothesized that during epididymal transit, MIF free thiol groups chelate zinc associated with the outer dense fibers, allowing formation of disulfide bounds between structural proteins of this flagellar structure (Figure 2) [27]. This mechanism could be involved in the modulation of sperm motility that occurs during the epididymal transit [28].

The enzymes involved in the polyol pathway, an aldose reductase and sorbitol dehydrogenase, are two other protein components of the epididymosomes [57–59]. This sugar pathway can be another mechanism related to epididymosomes that modulates sperm motility during epididymal transit. The first step of the polyol pathway involves an aldose reductase that uses NADPH as an electron donor to reduce glucose to sorbitol. In the second step, sorbitol dehydrogenase uses NAD^+ as an electron acceptor to generate fructose [60]. In the bull, both enzymes of the polyol pathway are associated with epididymal spermatozoa and epididymosomes. Aldose

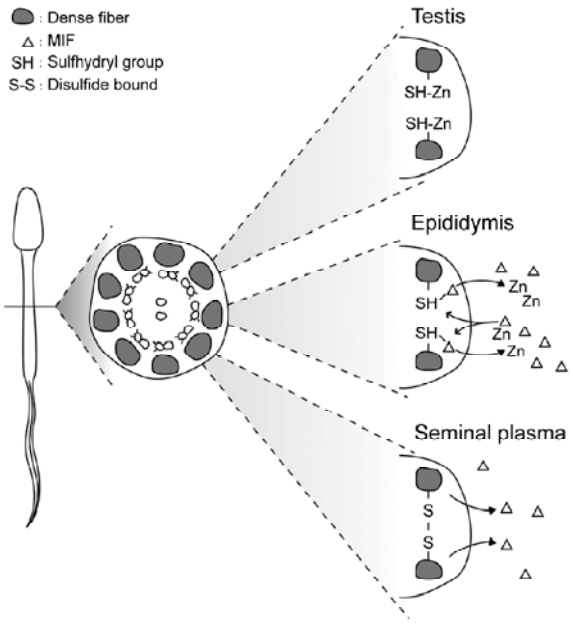

Figure 2. Schematic representation of macrophage migration inhibitory factor (MIF) interaction with spermatozoa along the male reproductive tract. Drawn based on our results and Eickhoff *et al.* [27]. Reproduced with permission from Frenette *et al.* [28].

reductase activity is high along the epididymis except in the distal cauda and the vas deferens. The optimum pH of epididymal aldose reductase activity for reduction of glucose to sorbitol is 6.0–6.5, the pH of the epididymal fluid [61]. The higher sorbitol dehydrogenase activity is in the distal cauda epididymidis and in the vas deferens. Therefore, sorbitol production is favored along the epididymis, except in the distal portion and in the vas deferens where the high sorbitol dehydrogenase activity oxidizes sorbitol to fructose. We hypothesize that sorbitol is enriched in the intraluminal milieu almost all along the epididymis (Figure 3). Sorbitol, unlike glucose and fructose, is a linear alcohol. For this reason, the sperm plasma membrane is poorly permeable to sorbitol. The high aldose reductase activity deprives the sperm intracellular compartment of an energy source. In the distal portion, sorbitol dehydrogenase generates fructose that can be metabolized by the spermatozoa ready to be ejaculated [57]. It has also been hypothesized that sorbitol in the epididymal lumen acts as an osmolyte required for volume regulation of the sperm cell [62]. Therefore, epididymosomes through MIF and enzymes of the polyol pathway that are associated with them, can modulate motility of spermatozoa while they are transiting along the epididymis [42].

HE5 was identified as a product of a gene preferentially expressed in the human epididymis [63]. Sequencing revealed that this protein is CD52, a surface protein of human lymphocytes [64]. This protein is GPI-anchored to the sperm surface during epididymal maturation [18]. It has been hypothesized that this protein secreted is associated with epididymosomes and that these vesicles are involved in HE5/CD52 transfer to sperm plasma membrane. HE5/CD52 is thought to be associated to immunological infertility [19, 65, 66].

Type 5 gluthatione peroxidase (GPX5) is another protein secreted by the caput epididymidal epithelial cells in association with epididymosomes [67, 68]. GPX5 is an atypical gluthatione peroxidase because it lacks selenocysteine, an uncommon amino acid that is a signature of other GPXs. GPX5 might be involved in protecting the transiting epididymal spermatozoa against oxidative stress. However, this function remains to be demonstrated [69]. Glutathione-S-transferase is another protein secreted by epididymal principal cells that is associated with insoluble material (epididymosomes) in the intraluminal compartment. This enzyme can also be involved, as GPX5, in protecting spermatozoa from free radical injury [70]. As for HE5/CD52, it is thought that epididymosomes are responsible for the transfer of secreted GPX5 to sperm surface covering the acrosome. Interestingly, GPX5 and HE5 lack a signal peptide in the N-terminal of their respective deduced amino acid sequence.

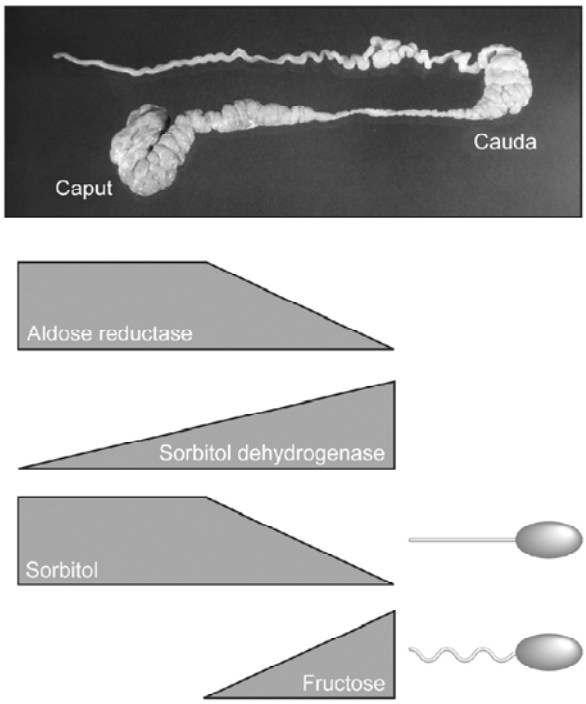

Figure 3. Schematic representation of aldose reductase and sorbitol dehydrogenase enzymatic activities along the bovine epididymis as well as the relative concentrations of sorbitol and fructose, two products of the polyol pathway. Sorbitol is hypothesized to deprive spermatozoa from energy sources to minimize sperm motility during the epididymal transit. Sorbitol dehydrogenase activity generates fructose in the distal part of the epididymis contributing to sperm motility restoration in the distal excurrent duct. Upper panel: a photograph of the bovine epididymis (Dr C. Reyes-Moreno, Université du Québec à Trois-Rivières, QC, Canada). Schematic representation of enzymatic activities is drawn from previously published results. Reproduced with permission from Frenette *et al.* [57].

"Murine sperm adhesion molecule 1", SPAM1 or PH-20, is synthesized by epididymal principal cells [71]. This protein is a constituent of intraluminal fluid and behaves as an insoluble protein. It is another protein using epididymosomes for its transfer to spermatozoa [22]. SPAM1 is GPI-anchored to epididymosomes and once transferred to spermatozoa is thought to play a dual role in sperm–egg cumulus complex interaction [72]. Ubiquitin is another example of a protein associated with bovine epididymosomese [73, 74]. As for the other proteins, ubiquitin is transferred to spermatozoa during epididymal transit and can be involved in the elimination of defective spermatozoa.

Epididymosomes are involved in the transfer of secreted epididymal proteins to different sub-compartments of the transiting spermatozoa. These proteins are in-

volved in the acquisition of zona pellucida binding ability (P26h/P25b) and cumulus-oocyte complex interactions (SPAM1), in the modulation of sperm motility within the epididymis (polyol pathway enzymes and MIF), in modulation of immunological fertility (HE5/CD52), in the protection against oxidative stress (GPX5), and the elimination of defective spermatozoa (ubiquitin). Many other proteins associated with epididymosomes and transferred to spermatozoa remain to be identified to understand fully the complex functions of these membranous vesicles in sperm maturation.

The protein composition of epididymosomes shows differences along the epididymis in the bull. Partial proteomic analysis of membranous vesicles collected at different levels of the epididymis reveals that some proteins are unique to epididymosomes collected in the caput epididymidis, and that those proteins associated with cauda epididymosomes are also present in vesicles collected in the caput epididymidis. These proteins include calcium binding proteins, proteins involved in calcium signalling, and many chaperone proteins [39]. This suggests that epididymosomes play numerous functions in sperm maturation and that these functions vary along the epididymis.

4 Interactions between spermatozoa and epididymosomes

Yanagimachi *et al.* [31] first described epididymosomes at the electron microscopic level and hypothesized that they could be involved in sperm membrane cholesterol efflux. Only indirect evidence supports the idea that lipid exchange occurs between epididymosomes and maturing spermatozoa [33]. By contrast, protein transfer between these vesicles and the epididymal spermatozoa has been well documented [23, 29, 32, 39–42, 75]. When epididymosomes collected from the cauda portion of the bovine epididymis are co-incubated *in vitro* with caput epididymidal spermatozoa, only selected proteins associated with epididymosomes are transferred to spermatozoa [32, 40]. These proteins become associated with specific surface domains of spermatozoa, mainly the membrane covering the acrosome and the midpiece. At least one protein, MIF, has been shown to be translocated in the sperm intracellular compartment and to become associated to outer dense fibers [26–28].

Surface proteins associated with epididymosomes can be biotinylated to document their transfer to spermatozoa. These transferred proteins can be visualized by using avidin-peroxidase to probe western blots of proteins of spermatozoa previously co-incubated with labeled epididymosomes [32]. When co-incubated with caput spermatozoa, epididymosomes prepared from the caput or cauda epididymal fluids transfer different protein patterns. This transfer of proteins from epididymosomes to spermatozoa shows certain specificity. In fact, transfer of biotinylated proteins from cauda epididymosomes to caput spermatozoa decreases in a dose-dependent manner when biotinylated epididymosomes are diluted with unbiotinylated membranous vesicles. Caput epididymosomes added in excess in the co-incubation medium do not affect the transfer of biotinylated proteins from cauda epididymosomes to caput spermatozoa. Furthermore, addition of unbiotinylated cauda epididymosomes does not displace already transferred biotinylated proteins. Therefore, epididymosomes collected from different segments of the epididymis interact differently with spermatozoa and the protein transfer between these two epididymal fluid components shows some specificity in their interactions [39].

The amount of proteins transferred from epididymosomes to spermatozoa *in vitro* increases in time to reach a plateau after 120–150 min of co-incubation. This transfer is temperature-sensitive, being more efficient at 37ºC than at 22ºC. The transfer is also affected by the pH; being 2.5-fold more effective at pH 6.0–6.5 compared with pH 7.5. This is physiologically relevant if we consider that bovine epididymal fluid has a pH of 6.5 [61]. The presence of zinc at a concentration of 0.1–1.5 mmol/L in the co-incubation medium favors the transfer of proteins from epididymosomes to epididymal spermatozoa. Other divalent cations such as calcium and magnesium have no effect. It should be noted that zinc is found at high concentration in the epididymis [76].

Epididymosomes are particularly rich in sphingomyelin. In the mouse, sphingomyelin associated with these membranous vesicles increases along the epididymis, representing 50% of phospholipids of epididymosomes in the cauda [33]. These epididymal vesicles are also rich in cholesterol, resulting in a cholesterol/phospholipid ratio near 2.0 (Sullivan *et al*., unpublished data). These two characteristics are signatures of lipid raft microdomains. Lipid rafts are specialized domains of all somatic cells plasma membrane. They are characterized by high cholesterol and ordered phospholipids, such as sphingolipids. Acylated and lipid-modified proteins, GPI-anchored and lipidified signalling molecules are segregated in these microdomains [77–79]. In fact, epididymosomes are characterized by lipid raft microdomains. Some proteins are specifically associated with raft domains of epididymosomes, such as the bovine P25b protein, whereas others (aldose reductase and MIF) are localized in the Triton-soluble fraction of these vesicles. Interestingly, proteins associated with rafts of epididymosomes will be transferred to raft domains of the matur-

ing spermatozoa. This segregation is also true for aldose reductase and MIF, which are transferred to the non-raft domains of epididymal spermatozoa (Sullivan *et al*., unpublished data). Therefore, the raft microdomains can be used to segregate some epididymally originating proteins to specific subdomains of the sperm plasma membrane (Figure 4).

5 Apocrine secretion of epididymosomes by the epididymal epithelium

As already mentioned, many proteins associated with epididymosomes and eventually with spermatozoa lack an N-terminal signal peptide in their deduced amino acid sequence. These proteins cannot be translocated to the endoplasmic reticulum and will be synthesized in the cytoplasm on free ribosomes. They will end up in the intraluminal compartment of the epididymis via apocrine secretion. This pathway of secretion, first described in mammary and sweat glands, was first thought to result from a fixation artefact. It is now recognized as an alternative mode of secretion and has received considerable research attention in the male reproductive tract [24, 25, 80, 81]. Apocrine secretion has been particularly well-studied in the vas deferens [82, 83] and the epididymis [24].

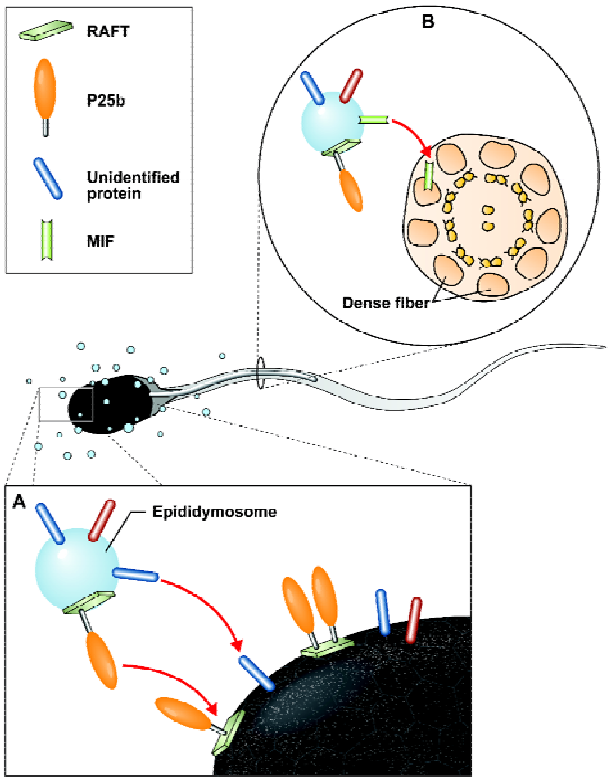

Figure 4. Schematic representation of protein transfer from epididymosomes to caput epididymal spermatozoa. In Panel A, the epididymal bovine protein P25b is mainly associated with lipid raft microdomains of epididymosomes and is transferred to the lipid raft microdomains at the sperm surface. Others unidentified biotinylated proteins are completely excluded from the lipid raft and are transferred at the sperm surface. These unidentified proteins are not associated with lipid rafts of spermatozoa. In Panel B, other proteins associated to epididymosomes, such as macrophage migration inhibitory factor (MIF), are internalized and linked to dense fibers of the sperm flagellum. RAFT, cholesterol and sphingolipid-enriched lipid microdomain.

Apocrine secretion involves formation of protrusions of the apical cytoplasm of principal cells (Figure 5). These protrusions form blebs at the apex of the principal cells between microvilli. These apical blebs segregate cytoplasmic organelles and contain only few endoplasmic reticulum cisternae, free ribosomes and small membranous vesicles: epididymosomes. The presence of free ribosomes suggests that newly synthesized proteins without an N-terminal signal peptide use these apical blebs for secretion. Electron micrographs of the murine epididymis and vas deferens suggest that the apical blebs eventually detach from the apex of principal cells and than breakdown to liberate their content into the intraluminal epididymal compartment; including epididymosomes that will than interact with sperm surface [24, 33]. How epididymosomes or similar vesicles secreted by other types of tissues are assembled before the apical blebs are detached in the intraluminal compartment remains to be determined.

6 Conclusion

Small membranous vesicles with unusual lipid composition are a product of apocrine secretion activity of the epididymal principal cells. Complex patterns of proteins are associated with these vesicles named “epididymosomes”. The protein composition of epididymosomes varies from one epididymal segment to the other and selected proteins from epididymosomes are transferred to spermatozoa during the epididymal maturation. Therefore, the interaction between epididymosomes and spermatozoa is an important aspect of epididymal sperm maturation. Studies of these interactions will contribute to the understanding of how new proteins are added to the spermatozoa during its maturation in the excurrent duct.

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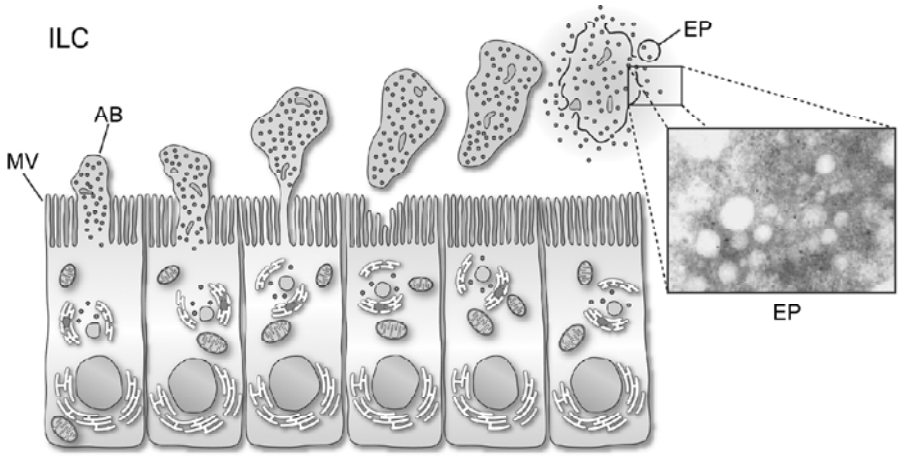

Figure 5. Schematic representation of apocrine secretion in principal cells of the epididymis. The inset shows electron micrograph of epididymosomes. AB, apical bleb; EP, epididymosomes; ILC, intraluminal compartment; MV, microvilli.

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