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The sperm journey in the excurrent duct: functions of microvesicles on sperm maturation and gene expression along the epididymis

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Abstract

Mammalian spermatozoa are differentiated, but unable to fertilize as they leave the testis. In order to acquire fertilizing ability and forward motility properties, the male gamete has to transit the epididymis. This process is collectively known as sperm maturation. The epididymis is a single convoluted tubule located between the vas efferens and the vas deferens; sperm epididymal transit takes 3 to 15 days depending on the species. Protein synthesis in the epididymis is highly active in response to androgen stimulation, and the underlying gene expression pattern shows great variability along this organ. For decades it has been recognized that interactions between the transiting spermatozoa and the intraluminal compartment of the epididymis govern complex modifications of sperm macromolecular composition that are necessary for sperm maturation. There is increasing interest in extracellular microvesicles that modulate cell-cell interactions in many physiological systems: such vesicles are found in the intraluminal compartment of the epididymis are called epididymosomes. We have shown that the epididymosome protein composition varies along the epididymis, and that a subset of these proteins is selectively transferred to the male gamete. These proteins are targeted to different sperm sub-compartments and are proposed to be involved in both the acquisition of fertilizing ability and forward motility properties. More recently, we used a bovine model to show that different subpopulations of epididymosomes are present in the fluid of a given epididymal segment. One of these subpopulations is proposed to be involved in the transfer of specific proteins by a membrane fusion process mediated by tetraspanin complexes, whereas another population of epididymosomes is involved in a mechanism that protects epididymal sperm against degenerating ones. Micro RNAs (miRNAs) are constituents of epididymosomes and microarray analyses have shown that the miRNA population characterizing epididymosomes varies with the site of collection along the epididymis. With the knowledge that miRNAs modulate transcriptional activity and that the pattern of gene expression shows great variation along the epididymis, we provide experimental evidence to support that miRNAs associated with epididymosomes modulate gene expression in the distal portion of their region of secretion along the male tract. Taken together, our work shows that microvesicles are one of the major players in the mechanisms underlying sperm maturation and modulation of gene expression along the epididymis. Clinical applications of these results will also be presented. The work described in this abstract was supported by grants from the Natural Sciences and Engineering Research Council of Canada.

Keywords: epididymis, epididymosomes, male reproductive tract, microvesicles, sperm maturation.

Epididymis

In vertebrate species practicing internal fertilization, the male gamete has to transit the epididymis in order to acquire its fertilizing ability. The epididymis is a convoluted tubule localized between the efferent ducts and the vas deferens. Collectively, these post-testicular structures are called the excurrent duct. Anatomically, the epididymis is divided into three segments: the proximal caput, the corpus, and terminal cauda epididymidis. In some rodent species, a more proximal segment called the "initial segment" is histologically distinct from the caput epididymidis and is thought to play a significant role in sperm physiology. Spermatozoa transiting the excurrent duct are fully differentiated, but unable to fertilize. During the epididymal transit, they acquire their forward motility properties and their ability to efficiently penetrate the oocyte. Collectively, these physiological modifications are called sperm maturation (Cooper, 1996).

The epididymal tubule is formed by a pseudostratified epithelium; the epithelial cells bordering the lumen are linked by tight junctions that allow formation of an intraluminal compartment that differs significantly from other body fluids. Other cell types form part of the epididymal epithelium and confer different properties assuring diverse functions such as immunological protection and acidification of the epididymal fluid (Belleannee *et al*., 2012b). In response to stimulation by sexual steroids, the epididymal epithelial cells, called principal cells, play active roles in protein synthesis and secretion. Thus, during epididymal transit, the maturing spermatozoa interact with an intraluminal compartment that is rich in secreted proteins, other macromolecules, and electrolytes

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(Dacheux and Dacheux, 2002; Dacheux et *al*., 2003).

Pioneering work (Yanagimachi *et al*., 1985), in addition to more recent microarray analyses have shown that the transcriptome is highly regulated along the epididymis (Johnston *et al*., 2005; Dube *et al*., 2007; Thimon *et al*., 2007). In fact, the pattern of gene expression varies along the excurrent duct assuring an intraluminal protein composition that will interact sequentially with the male gamete during post-testicular transit. The epididymal spermatozoa undergo modifications to their biochemical properties essential for their maturation (Cooper, 1998; Jones, 1998; Caballero *et al*., 2010). The identification of secreted epididymal proteins involved in sperm maturation, and the mechanisms by which they interact with the male gamete have been studied by many laboratories since the '80s. It rapidly became obvious that some epididymal proteins added to spermatozoa during the maturation process are secreted by epididymal principal cells in a non-classical manner and interact with the male gamete by an unusual mechanism (Hermo and Jacks, 2002). Early works propose that particulate material or microvesicles are found in the intraluminal compartment of the excurrent ducts. The presence of such vesicles in seminal plasma has been exhaustively documented and, at least in humans, the prostate seems to be the principal source of what has been called prostasomes (Sullivan and Saez, 2013). It appears that the epididymis also contributes to the population of microvesicles present in human semen. In fact, microvesicles named epididymosomes appear to be a constituent of the epididymal fluid in many of the mammalian species investigated so far such as hamsters, rats, mice, horses, rams, and bulls; epididymosomes from the latter species have been a particular focus of our group (Sullivan and Saez, 2013).

Epididymosomes

Epididymosomes are heterogeneous membranous microvesicles with a diameter of 20-100 nm. *In situ* micrographs at the EM level suggest that these vesicles interact with epididymal spermatozoa (Yanagimachi *et al*., 1985). Electron microscopic observations reveal that epididymal principal cells use an apocrine secretion pathway to liberate epididymosomes in the intraluminal compartment. Apocrine secretion consists of the formation of a bleb at the apical pole of principal cells. These blebs contain microvesicles that are released in the extracellular milieu following detachment of the blebs from cells and subsequent disintegration (Rejraji *et al*., 2006). This mode of secretion appears to be common within the male reproductive tract as it has been described in the prostate, seminal vesicles, epididymis, and the vas deferens (Aumuller *et al*., 1999). In the epididymis, epididymosomes represent a heterogeneous population of microvesicles. Thus, we cannot exclude the possibility that they are secreted by different pathways and that they originate from different cell types. A better understanding of the nature of extracellular microvesicles, which is currently a focus of intense research, will be necessary to understand the physiology of epididymosomes in the male reproductive tract.

As epididymosomes are rich in cholesterol, it has been hypothesized that they are involved in a membrane stabilization process by transferring sterol to the sperm plasma membrane. This hypothesis remains to be supported by experimental data. In fact, the lipid composition of epididymosomes varies along the epididymis (Rejraji *et al*., 2006). In particular, the ratio of cholesterol: phospholipids in epididymosomes increases by 1.5 along the excurrent duct. A complex mixture of proteins is associated with epididymosomes, and the composition of this mixture changes along the epididymis (Frenette *et al*., 2006; Girouard *et al*., 2009). The epididymosome protein composition differs from the soluble fraction of the epididymal fluid from which it is purified (Frenette *et al*., 2002; Gatti *et al*., 2005). Proteomic analyses of bovine epididymosomes permitted the identification of 555 and 438 different proteins associated with caput and cauda epididymosomes, respectively; 231 of these proteins were common to both types of epididymosome. Of interest is that the epididymosome proteome includes proteins previously suggested to be involved in sperm-egg interactions and in sperm motility, which supports the hypothesized role of epididymal microvesicles in sperm maturation processes. The presence of Rab and SNARE is another important feature of epididymosomes, at least in bovines. As these proteins are involved in vesicle trafficking and fusion, their association with epididymosomes suggests that a mechanism of transfer between epididymal microvesicles and transiting spermatozoa is involved in the acquisition of fertilizing potential by the male gamete (Girouard *et al*., 2011). Furthermore, *in vitro* co-incubation of isolated epididymosomes with epididymal spermatozoa clearly demonstrates that protein transfer occurs. Of particular interest is that only a subset of proteins associated with epididymosomes is transferred to spermatozoa. This transfer is saturable and temperature-dependent, and is optimal at pH 6.5 in presence of Zn; however, no other divalent cations potentiate sperm uptake of proteins associated with epididymosomes. These optimal *in vitro* conditions for protein transfer reflect the intraluminal composition of the epididymis (Frenette *et al*., 2002). In particular, glycosylphosphatidylinositol (GPI)-anchored proteins are acquired by epididymal spermatozoa via epididymosomes. These include the P25b/P34H orthologous proteins, which are epididymal proteins acquired by spermatozoa and are essential for sperm-zona pellucida interactions (Legare *et al*., 1999; Frenette and Sullivan, 2001). Other proteins are subcompartmentalized in epididymosomes; membranous or surface proteins are associated with raft or non-raft domains, whereas others are not exposed to the epididymosome surface. It is interesting that following

transfer, both raft and non-raft proteins are transferred to similar sperm membrane domains (Girouard *et al*., 2009), where as internal proteins are transferred to specific sperm intracellular compartment e.g. MIF associated to flagellar dense fibers (Frenette *et al*., 2005). Thus, epididymosomes transfer a subset of proteins to specific sperm subdomains during epididymal transit.

Epididymosomes: a heterogeneous population of microvesicles

As mentioned earlier, epididymosomes are heterogeneous in size. On the basis of different biophysical characteristics, we have been able to isolate different subpopulations of epididymosomes collected in bovine epididymal fluid. Interestingly, these microvesicle populations differ in their protein composition. Two populations have been studied: one is enriched in ELSPBP1 (epididymal sperm binding protein 1) and the other contains CD9, P25b, GliPr1L1, and MIF (Caballero *et al*., 2013). When ELSPBPB1-positive epididymosomes are co-incubated with bovine spermatozoa, ELSPBP1 is only transferred to epididymal spermatozoa that are already dead. The transfer of ELSPBP1 is enhanced by the presence of Zn in the incubation medium in a similar manner to the transfer of other epididymosomal proteins to epididymal spermatozoa (D'Amours *et al*., 2012). Therefore, this raises the hypothesis that ELSPBP1 acts as a tag for the recognition of dead spermatozoa during epididymal transit. This raises the question why the epididymis dedicates specific resources to spermatozoa that won't participate to the fertilisation process. An immunoprecipitation study has shown that biliverdin reductase A (BLVRA) is an ELSPBP1 partner involved in the reduction of biliverdin to bilirubin. Bilirubin being a major physiological antioxydant, it can be hypothesized that BLVRA is involved in an ROS scavenging system in the epididymis that protects live spermatozoa from degenerating ones. Co-incubation of spermatozoa with CD9-positive epididymosomes labelled with fluorescent membrane probes results in the transfer of fluorescence to spermatozoa, demonstrating fusion between the male gamete and microvesicles of epididymal origin. Addition of anti-CD9 antibodies to the co-incubation medium inhibits the fusion process as does an anti-CD26 antibody, a known partner of CD9. CD26 and CD9 are known to be associated with tetraspanin webs in the plasma membrane. In other biological systems, tetraspanin-enriched microdomains mediate adhesion of exosomes to their target cells. Thus, it appears that tetraspanin complexes are involved in the fusion of epididymosomes to the maturing spermatozoa. CD9-positive epididymosomes are enriched in P25b, GliPr1L1, and MIF (Caballero *et al*., 2012, 2013; Belleannee *et al*., 2013b. These three proteins play significant roles in sperm physiology: P25b and GliPr1L1 are involved in sperm binding to the egg's zona pellucida, and MIF is involved in control of sperm motility. Hence, it would appear that different subpopulations of epididymosomes play distinct roles in the epididymis: ELSPBP1-containing microvesicles protect live spermatozoa against deleterious molecules generated by dying sperm cells in the excurrent duct, and CD9-positive epididymosomes are involved in sperm maturation by transferring proteins essential for sperm functions.

Epididymosomes and gene expression

Gene expression is highly regulated along the epididymis. There is growing interest in miRNAs, which are involved in gene expression regulatory mechanisms. In particular, miRNAs associated with exosomes have been shown to be involved in cell-cell interactions in different physiological systems. This has led to our search for miRNAs associated with epididymosomes. In bovines, the relative amount of miRNAs associated with epididymosomes is markedly higher in the caput when compared with the cauda epididymidis. Each of these two populations has its own miRNA signature, suggesting that they modulate expression of different mRNA subsets. Furthermore, the miRNA repertoires associated with epididymosomes differ from those of their parent cells. As described in other biological systems, miRNAs released from epithelial cells in the extracellular milieu are selectively sorted in this manner. These observations suggest that distinct miRNA repertoires are secreted in the intraluminal epididymal compartment in a segment-specific manner and could be involved in an intercellular communication mechanism throughout the epididymis via epididymosomes (Belleannee *et al*., 2013b). Distinct miRNA repertoires are also characteristic of epididymal segments in humans. Using immortalized human epididymal cell lines, these miRNAs have been shown to be involved in the regulation of expression of specific genes (Belleannee *et al*., 2012a). A subset of miRNAs containing epididymosomes is present in the seminal plasma in human: these epididymosomes are undetectable in vasectomized men and reappear in the seminal plasma of vasovasostomized men (Belleannee *et al*., 2013a). These miRNA-containing epididymosomes present some characteristics of epididymal functionality and excurrent duct permeability, and as such could be potential markers of certain male fertility conditions.

Conclusions

In conclusion, the epididymal fluid contains microvesicles named epididymosomes. These vesicles are heterogeneous in nature and play multiple functions in epididymal physiology and sperm maturation. They convey miRNAs that modulate gene expression along the excurrent duct; they protect transiting spermatozoa

against deleterious molecules generated by dying spermatozoa and, by fusing with spermatozoa, allow the male gamete to acquire its fertilizing ability and forward motility. Further work is needed to fully appreciate the role of epididymosomes in male reproductive function.

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