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The epididymis and its role on sperm quality and male fertility

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Abstract

The epididymis is a complex organ where spermatozoa acquire motility and ability to fertilize the egg. Epididymal maturation lasts 1 or 2 weeks and exposes the immature spermatozoa to a sequentially modified milieu, promoting intense interactions with secretions by the epididymal epithelium. Sperm surface modifications in response to interactions with epididymal secretions are key steps to achieve fertility ability. However, the precise molecular mechanisms that convert an immotile and infertile gamete into a highly motile cell capable of fusion with an oocyte are still unknown. Recent data on proteomics and transcriptomics of epididymal fluid and epididymosomes brought new ideas of the physiological roles of proteins and miRNAs in epididymal maturation in spermatozoa. This review focuses on the recent discoveries on epididymal fluid composition and its role on sperm maturation and preservation, linking to their survival and fertility potential.

Keywords: epididymis, epididymosomes, spermatozoa, proteomics, sperm maturation.

Introduction

Modern livestock production systems rely on efficient reproductive management and the continuous search for maximum efficiency has demanded new methods for animal selection and semen evaluation. Our understanding of male reproductive physiology is still expanding and the role of the epididymis on sperm maturation is considered today a key factor for male fertility. To effectively identify spermatozoa fertilizing ability, it is indispensable to determine whether the sperm cells have completed their maturation process. This complex event is tightly regulated and operated mainly by proteins secreted by the testis and epididymis, and these proteins are considered potential candidates as molecular markers. Biomarker-based semen analysis can be used not only in single ejaculate evaluation but also in both sire fertility and breeding soundness assessment (Sutovsky, 2015). The goals of this new approach are to optimize and increase the use of single sire in different livestock systems. The present review will discuss aspects of epididymal physiology and bring new information learned through genomic proteomic studies, discussing how the comprehension of sperm epididymal maturation could contribute to the development of new reproductive biotechnologies.

A brief introduction to epididymal physiology

Viable spermatozoa are produced inside several biological tubes lumina, where complex processes of cell differentiation and maturation occur. Spermatogenesis takes place in the walls of the seminiferous tubules and, after spermiation, the immature spermatozoa begin interacting with a sequence of distinct luminal environments during their transition along the excurrent ducts (Hess, 2002). Seminiferous tubules connect to the rete testis, a cuboidal epithelium forming an anastomotic network that connects to the efferent ducts, a series of 4 to 20 tubules depending on the species (Robaire et al., 2006). These tubules converge to empty into the epididymis, a single coiled duct with over 1 m length in the mouse, 3 m in the rat; and 6 m in the human (reviewed by Hinton et al., 2011). It is usually divided into four anatomical regions: the initial segment, head (caput), body (corpus), and tail (cauda). Each region of the epididymis is organized into lobules separated by connective tissue septa that serve not only as internal support for the organ but also as a functional separation which allows for selective expression of genes and proteins within each individual lobule (Robaire et al., 2006; Turner et al., 2003).

Both anatomical and physiological aspects of the epididymis are hurdles to overcome when studying the epididymis. Comparing proteomic or gene expression data between regions is tricky since each region behaves differently and apparently independently. In fact, the Hinton group suggested very recently that despite being formed by one single Wolffian duct, the epididymis seems to be a series of organs placed side by side (Domeniconi et al., 2016). This compartmentalized gene expression results in segment-specific protein secretions into the luminal fluid, creating sperm microenvironments where specific modifications in spermatozoa happen. The composition of the intra luminal milieu is defined by the pseudostratified epithelium composed of six cell types possessing distinct physiological functions: narrow and apical cells found in the proximal region of the epididymis and principal cells, clear cells, machophages, dentritic cells, basal cells and halo cells found throughout the organ (Breton et al., 2016; Da Silva and Barton, 2016; Kempinas and Klinefelter, 2014; Shum et al., 2014). Each cell type contributes to the establishment and regulation of a unique luminal environment for the concentration, maturation, storage, and viability of spermatozoa.

Luminal acidification is achieved by epithelial cells that have specific roles depending on their location along the epididymis (Shum et al., 2011). The importance of luminal acidification was observed in c-ros knockout (KO) male mice, which are infertile and have an abnormally elevated epididymal luminal pH

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(Yeung et al., 2004). Similarly, a KO male mouse model for Foxi1 (a transcription factor specifically expressed in clear cells that controls the expression of proteins involved in acid secretion) presented a higher epididymal luminal pH compared with its wild type counterpart, being also infertile because of an inability of their spermatozoa to reach the oviduct and fertilize the egg (Blomqvist et al., 2006; Vidarsson et al., 2009). A practical observation of inhibition of luminal acidification is the exposure to environmental pollutants, such as tobacco smoke and heavy metals, which induce a reduction in male fertility (Shum et al., 2011).

Clear cells are present in caput, corpus and cauda regions of the epididymis and proximal vas deferens participating in the resorption of luminal content. Also, they contribute with the net proton secretion via the proton-pumping ATPase (V-ATPase), which is highly expressed in their apical plasma membrane (Beaulieu et al., 2005; Da Silva et al., 2007a; Da Silva et al., 2007b; Pastor-Soler et al., 2008; Shum et al., 2008). These cells are significantly more numerous in the cauda epididymis compared with the caput region, suggesting a major acidifying role in the distal region, where spermatozoa are stored (Shum et al., 2011).

Furthermore, clear cells of the human epididymis also express the V-ATPase (Da Silva et al., 2007a; Herak-Kramberger et al., 2001), indicating that the acidifying role of these cells occurs across species. The a4 promoter is under the control of the Foxi1 transcription factor, and several V-ATPase subunits are absent in clear cells from Foxi1 KO mice (Blomqvist et al., 2006; Vidarsson et al., 2009), indicating the importance of the V-ATPase in male fertility. Clear cells might contribute to the reestablishment of an acidic resting luminal pH following principal cell activation (Shum et al., 2011).

At alkaline luminal pH, the V-ATPase is mainly located in well-developed apical microvilli, and at acidic pH, it is actively recycling between sub-apical endosomes and the apical membrane. Intracellular production of bicarbonate (HCO_3^-) is essential for the alkaline pH-induced response and cAMP induces an apical translocation of V-ATPase identical to that induced by alkaline pH. Alkalinization of luminal pH, followed by an increase in intracellular pH in clear cells, leads to an elevation of intracellular bicarbonate concentration (Pastor-Soler et al., 2003). Bicarbonate elevation activates soluble adenylyl cyclase (sAC) and triggers cAMP production, which in turn leads to the accumulation of V-ATPase in apical microvilli. Clear cells also have the ability to respond to an increase in luminal bicarbonate concentration at constant pH, presumably due to entry of bicarbonate across the apical membrane, and subsequent elevation in intracellular bicarbonate concentration followed by sAC activation (Pastor-Soler et al., 2003).

The epididymal epithelium cells are also responsible for water removal from the lumen with three main consequences. First, there is an increase in luminal sperm concentration from 10^8 spermatozoa/mL in the rete testis fluid to 10^9 in the deferent duct. Second, there is a constant modulation in protein concentration, with a 10-fold increase from the rete testis fluid and the initial segment of the epididymis (2 to 4 mg/ml) to the distal caput (50-60 mg/ml) (Belleannee et al., 2011b; Dacheux et al., 2012; Fouchecourt et al., 2000).

The third consequence is related to osmolality, since the hypothesis that immature spermatozoa have a reduced ability to regulate their volume in osmotic challenging environments was considered (Cooper and Barfield, 2006). The increasing osmolality with which spermatozoa are confronted in the epididymis, if acting to reduce cell volume, should lead to an uptake of secreted permeant epididymal osmolytes during the process of regulatory volume increase. Recently, it was demonstrated that both caput and cauda sperm cells regulate volume, but cauda spermatozoa were more effective (Damm and Cooper, 2010). The osmotic stress is of great concern when developing sperm preservation media, like semen extenders routinely used in artificial insemination programs in livestock production. Despite all advances observed in sperm cryopreservation, some species like swine still face hurdles in post thaw sperm survival.

The development of motility by spermatozoa during epididymal transit

The remarkable biochemical changes in the epididymal sperm surroundings induces progressive and controlled modifications both biochemically and functionally. Motility is the most evident change in the epididymal sperm, with irregular and asymmetric flagella beating in the caput epididymis becoming symmetrical with propagation of waves on each side of the flagella, inducing forward motility of the spermatozoa when it reaches the cauda (Bork et al., 1988; Chevrier and Dacheux, 1992; Dacheux and Dacheux, 2014). However, such motility is only observed *in vitro* when epididymal sperm have been washed free of epididymal fluids and extended in a proper artificial medium. *In vivo*, weak beating flagella can be seen in the rete testis and efferent duct fluids, but after the increase in sperm concentration in the epididymal fluid, most of the spermatozoa maintain quiescent motility whatever its location in the epididymis (Dacheux and Dacheux, 2014).

The sperm flagellum machinery at the output of the testis is molecularly functional but inactivated *in vivo*, however the precise mechanism of activation of sperm motility in the epididymis is still unclear. This cell function is dependent on the intracellular cAMP generated by adenylyl cyclase, and on subsequent successive protein phosphorylations including protein kinase A, A-kinase anchor proteins and many other phosphorylated proteins (Esseltine and Scott, 2013; Perino et al., 2012; Turner, 2006).

Throughout the epididymal lumen, the sperm's intracellular cAMP level increases from the corpus to the cauda (Dacheux and Paquignon, 1980; Hoskins et al., 1974; Pariset et al., 1985), simultaneously with metabolic capacity and ATP production (Inskeep and

Hammerstedt, 1982). In addition, [HCO_3^-] and [Ca^{2+}] are key factors of the luminal epididymal fluid, which could directly control intracellular cAMP concentrations in the epididymal spermatozoa and consequently activate protein phosphorylation and motility (Xia et al., 2007). Calcium combined with bicarbonate ions is able to accelerate the flagellar beating of mature sperm *in vitro*, changing to symmetrical flagellar wave propagation, directly associated with the level of internal [Ca^{2+}] (Carlson et al., 2007; Lindemann et al., 1991). Instead, the removal of Ca^{2+} prevents this speeding up of flagellar beating (Carlson et al., 2003). Therefore, the low concentration of luminal and intracellular sperm [Ca^{2+}] found in the terminal part of the epididymis contributes to maintaining mature sperm motility quiescent (Dacheux and Dacheux, 2014). These findings point out that the control of the intracellular cAMP of spermatozoa and, consequently, protein phosphorylation is the key to understand the progressive acquisition of motility during epididymal transit. Determining the precise way sperm acquire motility in the epididymis could result in the development of better semen processing protocols in cases of male infertility.

Regulation of secretory activity in the epididymis

While half of the epididymal proteins are under positive or negative control by androgens, other proteins (43%) are modulated by local factors or are not influenced despite the physiological conditions (Syntin et al., 1999). The influence of lumicrine factors on the epididymis has been investigated after efferent duct ligation (EDL), avoiding testicular factors from entering the epididymis (Robaire et al., 2006). As a consequence, several phenomena were observed like epididymal gene expression changes (Hermo et al., 2000; Hinton et al., 1998; Lan et al., 1998), decreased protein synthesis and secretion (Holland et al., 1992) and apoptosis of epithelial cells of the initial segment (Abe and Takano, 1989; Fan and Robaire, 1998; Nicander et al., 1983). After EDL, secretion in the proximal caput is the most highly affected region with a 50% decrease in the specific proteins found in this region, such as RNase-Train A (Dacheux et al., 2005).

Testicular factors play a role in the control of protein secretion in the first part of the epididymis. Specific proteins of the epididymal caput, such as glutathione peroxidase (GPX) and hexosaminidase (HEX), are positively regulated by testosterone. In the distal caput and corpus, lactoferrin, Niemann–Pick type C protein also called Cholesterol Transfer Protein or HE1 (NCP2/CTP/HE1) and E-RABP are also stimulated by androgens, whereas clusterin is inhibited (Dacheux et al., 2005). Indeed, several genes have been shown to depend on testicular factors with diverse bioactivities such as gamma-glutamyl transpeptidase (Palladino and Hinton, 1994a; Palladino and Hinton, 1994b), GPX (Rigaudiere et al., 1992; Vernet et al., 1997), 5-alpha-reductase (Viger and Robaire, 1996), and ADAM7 (Cornwall and Hsia, 1997).

Also, the epididymal physiology is strongly regulated by steroid hormones. Several papers described the influence of orchiectomy and EDL in the epididymis, with dramatic changes in morphology and function (Brooks, 1976; Ezer and Robaire, 2003; Fawcett and Hoffer, 1979; Turner et al., 2007a; Turner et al., 2007b). Therefore, there is a proven dependence on testicular and circulating factors for proper epididymal function (Belleannee et al., 2011a). Testosterone produced by Leydig cells affects metabolism, ion transport, synthesis and secretion of epididymal proteins and sperm maturation, transport and storage (Bilinska et al., 2006). In rabbits, testosterone leaves the testis through the efferent ducts and is incorporated into principal cells of the epididymis by endocytosis of the complex testosterone–androgen binding protein (ABP) (Danzo et al., 1977). There, it can be quickly metabolized to dihydrotestosterone (DHT) by 5-alpha reductase, and to estradiol by aromatase P450 (Robaire and Hamzeh, 2011; Robaire et al., 2006).

In epididymal cell lines androgenic regulation of gene expression involves the androgen dependent signaling pathways MEK, ERK1/2 and CREB, (Hamzeh and Robaire, 2011). Additionally, formation of an androgen–androgen receptor (AR) complex regulates transcription of androgen response elements (AREs) containing-genes (Heinlein and Chang, 2002a; Heinlein and Chang, 2002b; Janne et al., 2000). Interestingly, like androgens, estrogens are present at a very high concentration in the testis and are generated from the aromatization of testosterone both in Leydig cells (Hess et al., 2001a; Hess et al., 2001b) and in epididymal spermatozoa (Hess et al., 1995; Joseph et al., 2011). Estrogens' biological effects are induced via interaction with ESR1 or ESR2 nuclear receptors and subsequent recognition of estrogen response elements (EREs) located in the promoters of target genes (Joseph et al., 2011).

Role of epididymal proteome in sperm maturation

The epididymal fluid is described as the most sequentially modified milieu of the body. And the most important change in this luminal fluid is induced by water reabsorption (Dacheux and Dacheux, 2014). Together with secretory activities of the tubular epithelium, these changes modulates metabolic pathways, such as second messenger and intracellular protein phosphorylation levels, energy metabolism, plasma membrane transport (Dacheux et al., 1989; Ecroyd et al., 2004; Jones et al., 2007; Saez et al., 2011; Voglmayr et al., 1985) (for review, refer to (Cornwall, 2009; Robaire et al., 2006)). This "passive" maturation is necessary because spermatozoa have no or very low capacity to synthesize new components, owing to their condensed DNA (Belleannee et al., 2012b).

The outstanding contributions of the Dacheux group to the characterization of seminal plasma and epididymal fluids of different species - including monotremes (Nixon et al., 2011), opened new roads for the study of male reproductive physiology. Hundreds of epididymal proteins have already been identified from the epididymal fluid of different species. For all these

epididymal fluids characterized, so far no more than twenty proteins are present in high concentrations, representing 80 to 90% of the total luminal proteins (Dacheux and Dacheux, 2014). Several of these proteins are common to different species such as lactoferrin, lipocalin 5, clusterin, glutathione peroxidase 5, prostaglandin D2 synthase, transferrin, Niemann-Pick disease, type C2, phosphoethanolamine binding protein 4, beta-N-acetyl-hexosaminidase, glutathione S transferase, gelsolin, actin, beta galactosidase (Dacheux and Dacheux, 2014). Specifically in man, 77% of the total luminal proteins are represented by albumin (43.8%), clusterin (7.6%), NCP2/CTP/HE1 (6%), lactoferrin (5.9%), extracellular matrix protein (3.2%), α1-antitrypsin (2.7%), prostaglandin D2 synthase (2.2%), transferrin (1.3%), and actin binding protein (1.2%) (Dacheux et al., 2006).

Significant variations among species both in concentration and in regionalization of epididymal proteins were described. For example, lactoferrin, mannosidase, prostaglandin D2 synthase, and albumin are present in high concentrations in the stallion, boar, ram, and human, respectively, but glutathione peroxidase and prostaglandin D2 synthase are virtually absent from the human and boar, respectively (Guyonnet et al., 2011).

Souza and coworkers detected 113 ± 7 spots per gel of cauda epididymal fluid from rams by means of 2D SDS-PAGE analysis (Souza et al., 2012). The most abundant proteins in these gels appeared as albumin and transferrin. These proteins corresponded to 27.3% of all spot intensities and were present as trains of multiple isoforms. Such molecular heterogeneity is linked to post translational modifications (mostly different degrees of glycosylation), which occur in the secretory cells and that induce the secretion of series of isoforms of the same protein (Dacheux et al., 2005). Other proteins identified include clusterin, alpha-1-anti-trypsin, prostaglandin D synthase, alpha-2-HS glycoprotein and actin.

In bulls, Moura et al. identified 114 ± 3 spots in the cauda epididymal fluid protein maps and the most abundant proteins were albumin (21.1%), epididymal secretory protein E1 (10.5%), prostaglandin D-synthase (7.6%) and gelsolin (6%) (Moura et al., 2010). Many proteins appeared in the CEF map as groups of isoforms, such as clusterin (11 spots), transferrin (6 spots), N-acetyl-β-glucosaminidase (6 spots), cauxin (7 spots), prostaglandin D-synthase (4 spots), gelsolin (3 spots) and glutathione peroxidase (2 spots). Other proteins identified were acidic seminal fluid protein (aSFP), aldehyde reductase, α-L-fucosidase, α-1-β-glycoprotein, apolipoprotein A-1, β actin, calmodulin, cathepsin D, cystatin E/M, α-1-antitrypsin, enolase, galectin 3- binding protein, leucine amino-peptidase and a 49-kDa nucleobindin.

In boars, the proteomic profile of the epididymal fluid also showed a very high polymorphism, both in molecular mass and p*I*, as principal characteristic of the proteins secreted in the lumen. A total of 125 epididymal proteins were identified by mass spectrometry covering 187 spots (Syntin et al., 1996). The number of isoforms secreted for a given protein varied according to the epididymal region. Additionally, post-secretory modifications such as partial proteolysis and deglycosylation may occur, thus, influencing the number of isoforms present in the lumen for a given protein as it transits along the epididymis. The secretory activity of the caput region is 6– 8 times greater than that of the caudal region (Dacheux et al., 2005). Protein secretion in the caput, corpus and cauda, thus, represents 83, 16 and 1% of the overall secretion of the epididymis, respectively. The maximum secretory activity corresponds to the maximum concentration of sperm in the fluid and a minimum volume surrounding them. The most abundant proteins are RNase-Train A, GPX, NCP2/CTP/HE1, retinoic acid binding protein (E-RABP), mannosidase, hexoaminidase, clusterin, cathepsin L, lactoferrin. Only one protein is specific for the distal corpus, train H (36–40 kDa), while two minor proteins appear in the cauda (Dacheux et al., 2005; Syntin et al., 1996).

In the stallion, the protein concentration of 35 mg/ml in the proximal caput is around 5 times higher than in rete testis fluid (Fouchécourt et al., 2000). The concentration increases to a maximum value of 60–80 mg/ml in the distal caput, and then decreases progressively toward the corpus and cauda to reach 20–30 mg/ml. A total of 324 different spots distributed throughout the epididymis were detected, corresponding to 201 isolated spots or trains of different proteins. The 5 major polymorphic proteins secreted by testicular tissue were an isoform of clusterin, an isoform of prostaglandin D-synthase (30 kDa, pI 5.5–6), train T1 (67–80 kDa, pI 4–6.2), train T2 (94 kDa, pI 6.5), and train T3 (43 kDa, pI 6.5–7.8). Trains T1, T2, and T3 were specific to the testis. The caput was the most active region, accounting for 73% of the total epididymal secretion. The corpus was the next most active, secreting 20.5%, followed by the cauda, 6.5%. Lactoferrin and clusterin were the most abundant proteins, making up 41.2% and 24.8% of the total epididymal secretion, respectively. Procathepsin D (5.2%), CTP/HE1 (3%), hexosaminidase (2.9%), prostaglandin D-synthase (2.3%), and GPX (1.4%) were the other major compounds secreted (Fouchécourt et al., 2000).

After secretion, these proteins display different fates, such as accumulation of or changes in their modifications that give rise to many different isoforms. Not all of these major secreted proteins remain in the fluid: in the boar, RNAse 10 is present in the anterior caput fluid but is reabsorbed in the next zones, and almost no protein can be found distal to the caput region (Castella et al., 2004). The same pattern was observed for chaperones belonging to the protein disulfide isomerase (PDI) family in sperm and epididymal fluid from boars (Schorr-Lenz et al., 2016).

This resorptive activity is mostly visible in the vas deferens and the proximal caput, where the fluid is mainly composed of testicular fluid and a few epididymal secreted proteins (Guyonnet et al., 2011). Most of the proteins originating from the testis, such as albumin, transferrin, and testicular clusterin, are rapidly

reabsorbed in the efferent ducts, and generally almost all testicular proteins are removed from the fluid before the posterior part of the epididymis (Clulow et al., 1994; Dacheux et al., 2009; Dacheux et al., 2003; Syntin et al., 1996; Veeramachaneni and Amann, 1991). However, some variations among species occur, such as in humans, for which albumin and transferrin remain in large quantities throughout the duct (Dacheux et al., 2006).

Today, proteomic methods became more powerful using shotgun mass spectrometer approaches, with higher sensitivity and throughput results (Amaral et al., 2013; Amaral et al., 2014). Using advanced mass spectrometry and a proteomics platform, Wang et al. (Wang et al., 2013) identified 4675 proteins from human sperm, which is about 4-fold greater than the previously estimated number, demonstrating the unexpected complexity of the human sperm protein composition. With such a large-scale sperm proteome, it is possible to analyze functional pathways in sperm, not only helping the study of sperm function, but also in the development of contraceptive drugs. Annotation of drug targets showed that 500 human sperm proteins are known targets (Wang et al., 2013). The list of sperm proteins can be a rich resource for the development contraceptive drugs. For example, the identified Angiotensin-converting enzyme (ACE) has been shown to be important for male fertility in mice (Krege et al., 1995). Thus, its inhibitors, such as enalapril and quinapril and other 13 different drugs, could be tested as part of new human contraceptive approaches (Wang et al., 2013).

Proteomic research of both sperm and seminal plasma opened doors for a better understanding of the role of specific proteins in semen quality and fertility. Despite the association of several proteins with semen traits (for example the characterization of bovine osteopontin as a seminal plasma fertility-associated protein (Cancel et al., 1997)), suggested molecular markers for sperm quality did not become part of routine ejaculate examination and semen processing. It is accepted that the current methods used in sperm quality assessment are unsatisfactory to correctly predict sperm fertility potential. Also, they do not provide enough information for diagnosing and overcoming possible clinical infertility situations (Payan-Carreira et al., 2013). Therefore, there is a significant window of opportunity for the biotechnological use of semen protein markers to select for or to improve semen quality and fertility in both in human and animal reproduction.

The crosstalk between epithelial cells and spermatozoa during epididymal transit

The acquisition of intraluminal epididymal proteins by transiting spermatozoa occurs by means of hydrophobic interactions (Cooper, 1998). Some sperm proteins acquired during maturation do not behave as coating proteins when sperm cells are experimentally submitted to different biophysical treatments (Thimon et al., 2008b). Some of them behave as integral membrane proteins, including GPI (glycosylphosphatidylinositol) anchored-proteins (Kirchhoff et al., 1996; Legare et al., 1999), while others are integrated inside sperm cell compartments during the maturational process, probably involved in sperm motility (Eickhoff et al., 2004; Eickhoff et al., 2006; Eickhoff et al., 2001).

The incorporation of epididymal proteins by spermatozoa cannot be explained by the classical secretory pathway (Cooper, 1998; Sullivan et al., 2007; Thimon et al., 2008b). Many sperm proteins acquired during the epididymal transit behave as integral membrane proteins when cells are treated with different extraction procedures. Apocrine secretion and exosomes are the main mechanism of transfer of epididymal-secreted proteins to the sperm plasma membrane (Sullivan et al., 2005). The epididymosomes are blebs released from the apical pole of principal cells, and their contents appear to be segregated; only free ribosomes, endoplasmic reticulum cisternae, and small membrane vesicles are visualized (Hermo and Jacks, 2002; Rejraji et al., 2006). The presence of these 20 nm diameter vesicles in the apical cytoplasm of cells forming blebs, as well as in the intraluminal compartment, suggests that they are liberated in the epididymal lumen once blebs detach from principal cells (Sullivan and Saez, 2013). The cholesterol:phospholipids ratio of these vesicles can be as high as 2, and sphingomyelin is the major phospholipid constituent (Sullivan et al., 2005). Epididymosomes in the epididymal fluid have been reported in rat (Eickhoff et al., 2001; Fornes et al., 1995), bovine (Fraile et al., 1996; Frenette et al., 2003; Sutovsky et al., 2001), mouse (Rejraji et al., 2002), horse (Sostaric et al., 2008) and man (Thimon et al., 2008b).

The protein composition of epididymosomes is complex and varied along the epididymis. In rams (Gatti et al., 2004; Gatti et al., 2005) and bulls (Frenette et al., 2003; Frenette et al., 2002), epididymosome protein concentration differs from fluids or spermatozoa collected in the same segment of the epididymis. The proteome of epididymosomes collected in the caput and cauda epididymis in bovine is composed of 555 and 438 proteins respectively, being that 231 proteins are common to both types of epididymosomes (Girouard et al., 2011). Proteins proposed to be involved in sperm–egg interaction and motility, as well as proteins involved in remodeling of sperm components or potentially involved in sperm protection and elimination, were shown to be associated with epididymosomes (Sullivan and Saez, 2013). The proteome of cauda epididymosomes collected from the vas deferens during surgical vasectomy reversal in men showed that epididymosomes transit along the epididymis in humans and that vesicles collected distally represent a mixed population of vesicles secreted all along the epididymis (Thimon et al., 2008a; Thimon et al., 2008b).

The fusogenic properties of bovine epididymosomes with spermatozoa were investigated by Schwarz et al. who observed that spermatozoa isolated from the epididymal caput showed a higher fusion rate

(both pH and time dependent) than those taken from the cauda (Schwarz et al., 2013). Also, the lipid and protein content in spermatozoa changed during epididymal transit and after *in vitro* fusion with epididymosomes. The authors also demonstrated the transfer of epididymis-derived PMCA4 to spermatozoa via epididymosomes.

In a recent paper from the Sullivan group, the study of epididymal sperm binding protein 1 (ELSPBP1) in dead spermatozoa shed some light in how epididymosomes transfer proteins to sperm. To date, ELSPBP1 function remains unclear but its sequence similarity with binder of sperm proteins (BSPs) suggests a role on modulation of phospholipids and cholesterol in sperm membrane (Ekhlasi-Hundrieser et al., 2007; Manjunath et al., 2007; Manjunath and Thérien, 2002). With an elegant approach, D'Amours and coworkers showed that ELSPBP1 is secreted by principal cells in association with epididymosomes, which specifically transfer ELSPBP1 only to spermatozoa already dead before incubation (D'Amours et al., 2012). This receptivity was enhanced by the presence of zinc in the incubation medium. As a result, a subpopulation is formed suggesting an underlying mechanism involved, in which ELSPBP1 could be a tag for the recognition of dead spermatozoa during epididymal transit.

In the last decade, small non-coding RNAs such as microRNAs (miRNAs) have gained attention as critical regulators of gene expression in several biological processes, including differentiation of the epididymal epithelium and acquisition of male fertility (Bjorkgren et al., 2012; Hawkins et al., 2011; Papaioannou and Nef, 2010). Consisting of about 22 nucleotides, miRNAs regulate posttranscriptional gene expression by targeting mRNAs for cleavage or translational repression (Bartel, 2009). Distinct miRNA signatures in the caput, corpus and cauda of human epididymis were found to correlate with epididymal gene expression pattern (Belleannee et al., 2012a).

The Sullivan group was the first to report the ability of epididymosomes to transport miRNAs, extending the current knowledge with regards to the role of epididymosomes in the transfer of proteins to maturing spermatozoa. In a bovine model, small RNAs were found to be threefold more abundant in epididymosomes from the proximal region compared to those in the distal region (Belleannee et al., 2013). Overall, 1645 miRNA sequences from 74 different species and 82 bovine miRNA sequences were detected with strong signal intensity in epididymosomes. Among these, 178 miRNAs were differentially detected between caput and cauda epididymosomes, including 92 miRNAs enriched in caput epididymosomes and 86 enriched in cauda epididymosomes. miRNA populations from epididymosomes did not mirror the miRNA profiles of their surrounding epithelium, suggesting that instead of being passively released by epididymal epithelial cells, subpopulations of epididymal miRNAs may employ selective and distinct biogenesis and secretion pathways that are regulated in a region-specific manner along the epididymis. Similarly, other studies proposed that the miRNA content of extracellular vesicles does not simply imitate the miRNA repertoire of the cells of origin (Guduric-Fuchs et al., 2012; Mittelbrunn et al., 2011).

Concluding remarks

During the first one or two weeks of post-testicular life, spermatozoa is incarcerated in a long and highly coiled biological tube, nevertheless experiencing different environments until ejaculation. These subtle sequential changes in the luminal milieu are a result of regionalized specialization of the epididymal epithelium. Coordinated secretions and endocytosis lead to an intense modification of the epididymal fluid protein profile, exposing the gametes to factors that will modulate sperm physiology. Moreover, apocrine secretions of principal cells produce epididymosomes that deliver target proteins, miRNAs and lipids to spermatozoa, a fundamental event for gamete recognition and fusion. Research on the molecular processes driving the vesicle-dependent maturation of spermatozoa, as well as several other luminal components, will increase the understanding of sperm maturation in the epididymis, opening doors for the discovery of new markers for semen quality and fertility.

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