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Abstract

In order to acquire progressive motility, complete maturation and compaction of chromatin, regulate their volume, and acquire molecules necessary for fertilization, spermatozoa released from the testis must transit through the epididymis, a long convoluted tubule that connects the efferent ducts to the vas deferens, where they undergo several molecular modifications. Sperm modifications occurring during transit in the three segments that compose the epididymis (caput, corpus, and cauda) are accomplished by epididymal epithelium secretions, including epididymosomes (extracellular microvesicles enriched in cholesterol and proteins), miRNA, and other macromolecules. Epididymal pH and electrolytes composition of the luminal fluid are also important for a correct sperm maturation. Epididymal secretions are regulated by a variety of factors, mostly androgens and estrogens, to create a different luminal environment in each epididymal segment supporting progressive sperm maturation and allowing maintenance of sperm viability and motility during storage in the cauda. Finally, epididymal contraction allows sperm emission at ejaculation. Overall, the role of epididymis on the development of sperm functions is essential for male reproduction, and alterations in any of its functions may lead to subfertility or infertility. Due to its importance for a successful male reproductive function, the epididymis appears to be a promising target for post-testicular male contraception.

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Introduction

Although fully differentiated into a highly specialized cell within the testis, the male gamete needs to undergo, following spermiation, several maturation events in order to become fully functional and able to fertilize the oocyte. Such events occur during transit both in the male and female genital tracts. In the former, spermatozoa complete chromatin maturation, acquire the ability to swim progressively, and come into contact with several proteins which are needed for sperm-oocyte interaction and other functions. In the female genital tract, by interacting with uterine and tubal epithelial cells and fluids, spermatozoa undergo the complex process of capacitation (De Jonge 2005), which allows development of a special type of motility (known as hyperactivation and required to penetrate oocyte vestments) and renders the spermatozoa able to respond to stimuli inducing acrosomal exocytosis and to fuse with the oocyte. In this complex series of events necessary for the reproductive function, the epididymis has a central role, accomplishing most of the post-testicular maturation events and representing the structure where spermatozoa are stored before ejaculation. In such a situation, it appears clear that any problem causing epididymal dysfunction or abnormal development of the organ may generate disturbances of male fertility. In addition, understanding the molecular basis of epididymal function is critical to the generation of novel approaches to alleviate male infertility or for the development of new methods for male contraception. It is important to underline that most of the knowledge on the functions of this complex organ derives from animal studies because of the difficulty of obtaining human epididymis and studying epididymal functions in human beings. In view of the differences in the molecular events involved in the reproductive functions in mammalian species, it cannot be excluded that part of the information collected so far is specific for a given specie and not necessarily true for humans.

Anatomical Background

The epididymis is located along the posterolateral surface of the testis and connects efferent ducts to vas deferens. In humans, epididymis has a total length of 6 m (Robaire and Chan 2010) and can be grossly divided into three regions: caput (head), consisting mainly of efferent ducts, corpus, (body) and cauda (tail) which evolves in the vas deferens (Fig. 1). Besides showing histological differences, each epididymal region carries out distinctive functions: the caput and corpus accomplishing, respectively, early and late sperm maturational events, and the cauda region primarily serving as a storage site for functionally mature spermatozoa. The epididymis derives from the Wolffian duct and consists, at birth, mainly of mesenchymal tissue. After birth, the epididymis undergoes considerable remodeling leading to duct elongation and convolution until reaching, at puberty, its fully differentiated state consisting of a highly tortuous tubule lined by epithelial cells (Rodriguez et al. 2002). The development of a fully differentiated epithelium is dependent not only on androgens but requires also the influence of luminal (lumicrine) factors secreted by the testis or other epididymal segments (Rodriguez et al. 2002).

The epididymal epithelium consists of several cell types, namely, principal, basal, clear, narrow, apical, and halo, which are pseudostratified and delimitate the intraluminal compartment (Fig. 2). The most represented cell type throughout the

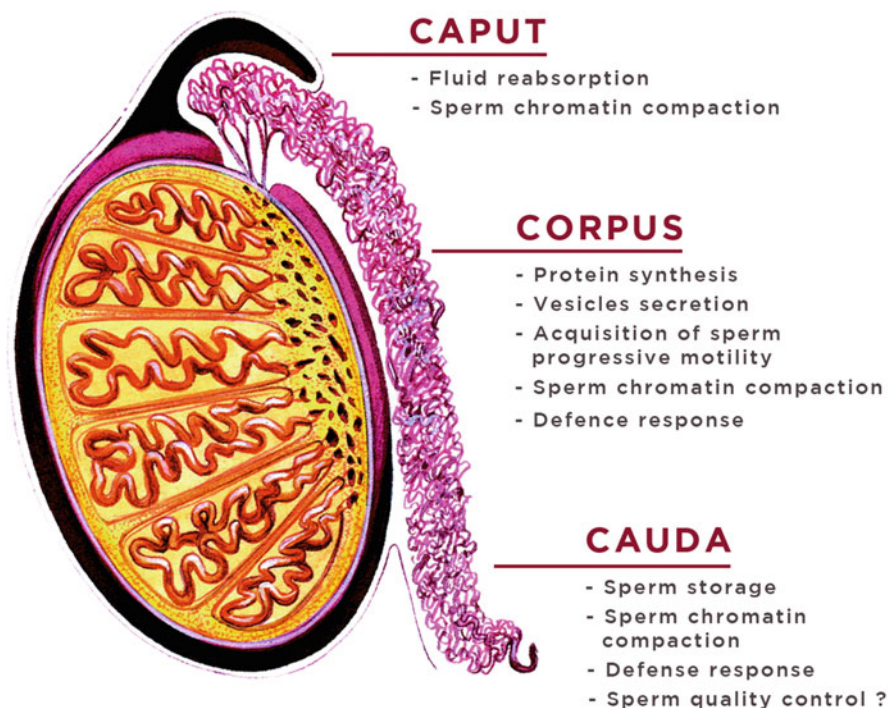


Fig. 1 Regions of the human epididymis: caput (including the initial segment), corpus, and cauda. The distinctive functions of each region is indicated

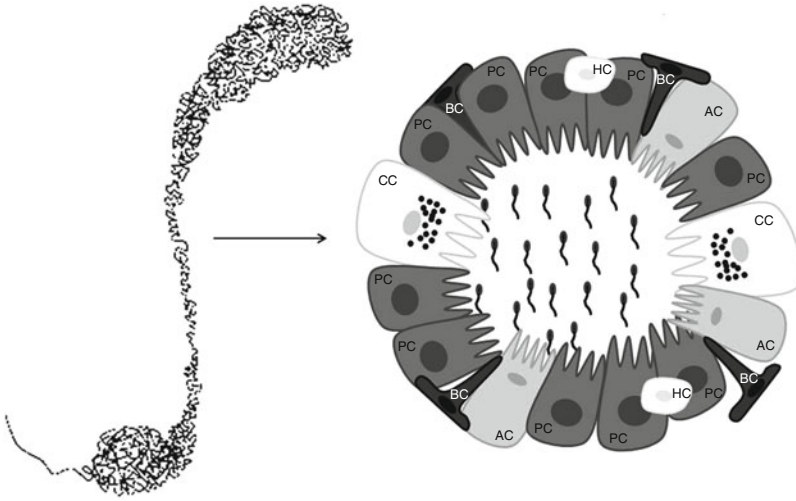


Fig. 2 Representation of the different cell types present in the epithelium of the corpus epididymis. *PC* principal cell, *AC* apical cell, *CC* clear cell, *BC* basal cell, *HC* halo cell

tubule is the principal one which constitutes 80% of the epithelium and is, by far, the most studied, since it is responsible for the secretion of the bulk of proteins, ions, and organic molecules that are present in the lumen. These cells are distributed along the entire duct but show structural differences in each region, in particular, the luminal diameter increases and the cell height decreases from caput to cauda. Narrow, apical, and clear cells contain the vacuolar type H^+ -ATPase (V-ATPase) which secretes protons into the lumen participating in acidification of the epididymal fluid (Pietrement et al. 2006; Kujala et al. 2007); in particular, clear cells are large and show endocytotic properties being likely responsible for clearance of proteins from the epididymal lumen. Basal cells possess thin processes that extend along the basement membrane and do not have direct access to the lumen of the duct. They express a number of antioxidant proteins and are thought to play a role in protecting from oxidative stress and other possible environmental factors that may affect sperm integrity. Finally, halo cells consist of T helper lymphocytes, cytotoxic T lymphocytes, and monocytes playing a role in immune protection of spermatozoa during epididymal transit (Serre and Robaire 1999). The epididymal epithelium is characterized by a unique set of tight junctions that forms the blood–epididymis barrier allowing an intraluminal milieu with a composition of electrolytes and macromolecules different from that of the circulating body fluids and creating an immunoprotective environment within the epididymal lumen.

The Epididymis as a Secretory Organ

The epididymal epithelium is very active in protein synthesis and secretion. Importantly, each epididymal region presents specific patterns of gene expression and protein secretions leading to different composition of the luminal fluid in caput,

corpus, and cauda to accomplish the different maturation steps of spermatozoa. Besides androgens and other steroid hormones (see below for regulation of epididymal secretion), region-dependent gene and protein expression are under control of many paracrine and lumicrine secretions of the testis. Several epididymal luminal proteins have been identified and characterized. Many of these proteins are implicated in sperm maturation processes and are acquired by mammalian spermatozoa during epididymal transit (Cornwall 2009). In some cases, such proteins become integral membrane proteins of spermatozoa, being glycosylphosphatidylinositol (GPI) anchored (Cooper 1998).

As mentioned, most of protein secretions derive from the principal cells of the epididymal epithelium. These cells have been shown to release epididymosomes as part of a process known as apocrine secretion. Apocrine secretion consists of blebbing of the apical part of secretory cells forming vesicles that are then released in the lumen. In the male genital tract, apocrine secretion has been described also for the prostate, which secretes prostasomes. Epididymosomes are highly heterogeneous in protein content and various in sizes (from 50 to 250 nm; Sullivan 2015). They transit along the epididymis, and those reaching the caudal segment represent a mixed population of vesicles secreted in the three segments. In some species, epididymosomes have been shown on sperm surface, bound to the acrosome, where they might be involved in cholesterol exchange with the sperm membrane (Rejraji et al. 2006). Indeed, epididymosome membranes are rich in cholesterol (Sullivan and Saez 2013), and its amount increases during epididymal transit (Rejraji et al. 2006) in contrast to spermatozoa where cholesterol is reduced (see below). Analysis of the proteins associated with epididymosomes reveals protein profiles quite different from that of proteins present in the lumen. In addition, protein and lipid composition of epididymosomes is species specific and varies in the different epididymal compartments. Among proteins found in epididymosomes, some are involved in sperm–egg interaction (such as SPAM1; Kimura et al. 2009), development and maintenance of motility (such as aldose reductase; Frenette et al. 2004), and protection from oxidative stress (such as glutathione peroxidases; Drevet 2006).

Sullivan (2015) separated, by serial centrifugation steps, two distinct populations of epididymosomes characterized by different size and different protein content. One population contains the tetraspanin CD9 and other members of the tetraspanin family and preferentially binds to live spermatozoa, likely exerting a protective role against oxidative stress and other insults that may occur during transit in epididymis. The other population contains epididymal sperm binding protein 1 (ELSPBP1) which is transferred to unviable or dying spermatozoa and remains associated with them following ejaculation, likely to prevent the release of molecules with detrimental effects on viable spermatozoa. Epididymosomes contain also tRNA fragments (Sharma et al. 2016) and miRNA (Belleannée et al. 2013) although it is not clear whether these molecules are transferred to spermatozoa. Overall, epididymosomes play an essential role to produce male gametes with optimal fertilizing ability (Sullivan et al. 2005).

A summary of the most characterized proteins (luminal and within epididymosomes) having a role in sperm maturation and acquisition of fertilizing ability is provided in Table 1.

Table 1 Proteins secreted by epididymis and associated with spermatozoa with known or proposed functions in the fertilization process

Protein name	Protein functions	References
ADAM 2, 3, 7 (a disintegrin and metalloproteinase 2, 3, 7)	Sperm–oocyte interaction	Oh et al. (2005)
Clusterin	Sperm motility and sperm aggregation	Han et al. (2012)
CRISP1 (cysteine-rich secretory protein 1)	Participates in the binding of spermatozoa to the zona pellucida	Da Ros et al. (2015)
Kinases cSrc	Sperm capacitation	Krapf et al. (2012)
GPX4, GPX5 (glutathione peroxidase 4, 5)	Protection against oxidative stress	Drevet (2006)
HE2 (human epididymis protein 2)	Antimicrobial protein	Yenugu et al. (2004)
HE5/CD52 (human epididymis protein 2/ cluster of differentiation 52)	Protection against immune attack	Kirchhoff and Hale (1996)
Lactoferrin	Immune system defense protein	Guyonnet et al. (2011)
Liprin α 3	Acrosome reaction	Joshi et al. (2012)
MIF (macrophage migration inhibitory factor)	Inhibition of sperm motility in the epididymis	Frenette et al. (2005)
NPC2 (Niemann–Pick disease type C2)	Cholesterol transport during sperm maturation	Kirchhoff et al. (1998)
PGDS (prostaglandin D2 synthase)	Lipophilic ligand-binding protein	Moura et al. (2006)
EPPIN	Antimicrobial protection, binding seminogelin involved in sperm motility	O'rand et al. (2004)
HE6 (human epididymal protein 6)	Regulation of fluid reabsorption	Davies et al. (2004)
SED1	Binding to zona pellucida	Raymond et al. (2010)
SPAM1 (sperm adhesion molecule 1)	Sperm–oocyte interaction	Kimura et al. (2009)
Rnase10	sperm-oocyte interaction	Krutsikh et al. (2012)

Regulation of Epididymis Secretion

Changes in fluid composition in each segment of epididymis are the result of different expression of a wide spectrum of genes (Turner et al. 2003; Cornwall 2009). In a recent paper, Browne et al. (2016), by evaluating gene expression profiles on tissue and cultured epithelial cells derived from each segment of the human epididymis, demonstrated a different expression of genes in caput with respect to

corpus and cauda, whereas only few genes were differentially expressed in the latter segments. In particular, genes related to ion transport and those involved in the response to hormone and urogenital tract development are predominant in the caput, whereas genes related to responses to environmental insults are more represented in the corpus and cauda. A higher expression of ion transport proteins in the caput epididymis is likely involved in maintaining a low bicarbonate and acidic environment in the caput lumen favoring development of progressive motility and maturation but assuring quiescence of spermatozoa (see below) (Browne et al. 2016).

Many genes expressed in the epididymis are regulated by sex hormones (estrogens and androgens). In the epididymis, testosterone derived from the testis is converted into 5 α -dihydrotestosterone (DHT) (Turner 1991) by 5 α -reductase or into estradiol by the aromatase P450 enzyme. Androgen receptor (AR) and estrogen receptor- β (ER β) are expressed at similar levels along the entire epididymis, whereas ER α is primarily expressed in the efferent ducts and the caput of the epididymis (Zhou et al. 2002). In the absence of androgens (such as following androgen deprivation or orchidectomy), spermatozoa become immotile, lose the ability to fertilize, and die (Dyson and Orgebin-Crist 1973) due to alterations of the secretory function of principal cells and, consequently, of the epididymal fluid composition. In addition, an apoptotic process is triggered throughout the epididymis (Fan and Robaire 1998). Re-administration of DHT results in up- and downregulation of many gene families (involved in solute transport, cell communication, cell proliferation and apoptosis, signal transduction, proteolysis, peptidolysis, and development) restoring most of the histological features of the organ (Robaire et al. 2007). Clearly, any androgen-deprived condition results also in lack of estrogens, and thus these studies could not discriminate between estrogen and androgen effects on epididymis. Generation of ER α KO mice (Lubahn et al. 1993) allowed to understand that several proteins that are important in fluid/ion equilibrium in the epididymis, such as solute carrier family 9 member 3 (SLC9A3), carbonic anhydrase 2 (CAR2), and two aquaporin water channels, AQP1 and AQP9 (Zhou et al. 2001; Ruz et al. 2006), are estrogen regulated. The absence of ER α results in lack of reabsorption of the large volume of fluid secreted by the testis leading to infertility due to lower motility and inability to fertilize of spermatozoa. On the contrary, the ER β KO mouse is fully fertile (Krege et al. 1998). More recent studies have shown that estrogens regulate also epididymal contractility by upregulating the calcium-sensitive Ras homolog gene family, member A (RhoA)/Rho-associated protein kinase (ROCK) pathway in epididymal smooth muscle cells, increasing responsiveness of oxytocin and endothelin-1 receptors (Fibbi et al. 2009) (see also below).

Other possible mechanisms could regulate the activation of gene transcription beside androgens and estrogens. Among these, miRNAs (small noncoding RNAs that control gene expression posttranscriptionally) were shown to be implicated in the epithelium differentiation and in the regulation of sex steroid signaling in epididymis (Bjorkgren et al. 2012). In particular, miR-200a (Wu et al. 2012), miR-200c (Wang and Ruan 2010a), miR-335 (Wang and Ruan 2010b), and miR-29a (Ma et al. 2012) were demonstrated to regulate epididymal development. miRNAs display a different expression among the epididymal regions in many

species including the human (Belleannee et al. 2012; Ma et al. 2012; Nixon et al. 2015). As an example, in rats, miR-200 family members are more expressed in the caput with respect to the cauda epididymis, contributing to the distinct physiological function in sperm maturation/storage of the two segments (Chu et al. 2015). Gene transcription in the epididymis may also be regulated by DNA methylation. It has been shown that ion transportation-related, sexual reproduction-related, and spermatogenesis-related genes resulted to be methylated throughout the epididymis (Chu et al. 2015), but, intriguingly, such methylation is not related, in the mature epididymis, to a repression of gene transcription. However, it is possible that DNA methylation of such genes plays a role in the early epididymal differentiation (Chu et al. 2015).

Functions of Epididymis

As mentioned, each epididymis region has specific properties and different functions in order to guarantee sperm maturation during epididymal transit and safe storage before ejaculation (Fig. 1). A spontaneous and rhythmic contraction of smooth muscle cells surrounding the epididymal ducts guarantees sperm movement through caput and corpus up to the cauda. The passage through human epididymis and consequently all the biochemical modifications underlying sperm maturation last 10–15 days (Johnson and Varner 1988). The essential processes for sperm maturation and acquisition of motility take place mostly in caput and corpus, whereas the cauda represents mainly a storage structure of mature spermatozoa. There is also evidence for a role of epididymis in sperm protection and in elimination of defective sperm cells (Fraile et al. 1996; Sutovsky et al. 2001), although the existence of an epididymal “sperm quality control” is highly debated (Cooper et al. 2002).

Sperm Chromatin Compaction

During spermatogenesis a complex process of chromatin remodeling occurs in order to obtain a rigid sperm nucleus required for protection of sperm chromatin integrity, successful transport in the female reproductive tract, and penetration of the oocyte (Huszar et al. 1999). In particular, histones associated with sperm DNA are replaced, during spermiogenesis, first by transition proteins and later by protamines. The process of chromatin compaction is completed during epididymal transit, when redox-mediated intra- and intermolecular disulfide bridges within protamines are established resulting in a tightly compacted nucleus. This process occurs thanks to the pro-oxidative environment present in the epididymis. The efficiency of the formation of disulfide bridges depends also on the correct binding of protamines to DNA, which, in turn, is dependent on phosphorylation-dephosphorylation of protamines occurring in the testis (Marushige and Marushige 1978; Balhorn et al. 1984). The stability of the chromatin is determined by the number of –S–S– cross-links formed between thiol groups of adjacent protamine chains. Evaluation of the

number of disulfide bonds in protamines from spermatozoa coming from the different epididymal segments has shown that the number of bridges increases passing from caput to cauda (Auger and Dadoune 1993) which coincides with a greater stabilization of chromatin during epididymal transit. The entire process of chromatin compaction is of vital importance for spermatozoa, as a less compacted nucleus may be more vulnerable and can suffer from DNA damage. Since the main mission of spermatozoa is to deliver a fully intact and functioning paternal genome to the oocyte, occurrence of DNA damage can compromise the reproductive outcome both in natural and assisted reproduction (Tamburrino et al. 2012). Maintenance of equilibrium between beneficial and detrimental sperm oxidation in the epididymis relies on the many antioxidant substances present, from small metabolites to enzymes. Among them, the glutathione peroxidase (Gpx) family plays a key role, as these enzymes act as reactive oxidative species (ROS) scavengers to protect spermatozoa (Drevet 2006). For instance, although not presenting evident signs of infertility, Gpx5^{-/-} mice are characterized by an excess in free radicals that could compromise sperm DNA integrity. Indeed Gpx5^{-/-} mice display higher incidence of miscarriages and embryo developmental defects when they were mated with wild-type females. The targeted epididymis GPx5 knockout model brought some clear evidence that GPx5 is a true ROS scavenger protecting epididymis-transiting spermatozoa from oxidative damage (Chabory et al. 2009). Similarly, the mouse mGPx4^{-/-} model (KO for mitochondria-associated isoform of GPx4) shows impaired sperm integrity, including structural malformations of the midpiece, a significant reduction of forward motility, and of the mitochondrial membrane potential, resulting in infertility (Noblanc et al. 2011). A reduced expression of GPx4 was found in apolipoprotein E receptor-2 (ApoER2) knockout mice, which are also infertile. ApoER2 is highly expressed in the initial segment of the epididymis and has a crucial role in sperm maturation, in particular in the acquisition and development of sperm motility, by regulating the expression of sperm GPx4 (Andersen et al. 2003).

It appears clear that if sperm nuclear compaction is not optimal (e.g., defective protamination in the testis), or if dysfunctions in epididymal secretions occur (e.g., low levels of GPx enzymes), the long periods of epididymal transit and storage may represent challenging moments when spermatozoa could be at risk of oxidative damage.

Volumetric Regulation of Spermatozoa

Progressive fluid reabsorption, driven by aquaporins (see also above), allows an increase of sperm and luminal protein concentrations that facilitates interactions of the sperm surface with the secretory products of the epididymis and influences the time of sperm storage. Besides AQP9 and AQP1 (Badran and Hermo 2002; Da Silva et al. 2006), sodium transporters at the apical pole (including the sodium–hydrogen exchanger 3, sodium–glucose transporter, and sodium–nucleotide transporters (Leung et al. 2001; Zhou et al. 2001)) are involved in such a process. Another

consequence of water removal from the lumen is the increase in osmolality of the lumen fluid that reaches the highest levels in the cauda (Cooper and Yeung 2003), and that is essential to regulate sperm volume. High osmolality prevents osmotic sperm dehydration and, likely, provides a reserve of osmolytes which is useful to maintain the volume when spermatozoa enter in contact with the hypotonic seminal plasma. A spermatozoon which fails volume regulation changes its flagellar shape by coiling or angulation in order to avoid excessive stretching of the plasma membrane. Inability to maintain straight flagella and consequent infertility has been demonstrated in *c-ros* (gene encoding proto-oncogene tyrosine-protein kinase) KO mice, where a failure in pubertal differentiation of the epididymal initial segment leads to changes in expression of some proteins, including epithelial transporters. However, caudal spermatozoa from *c-ros* KO mice are able to fertilize eggs *in vitro* indicating that they maintain their ability to interact with eggs (Cooper et al. 2003).

Acquisition of Motility and Membrane Modifications

In the caput of epididymis, spermatozoa show immature tail movements characterized by thrashing beats in wide arcs that result in little forward progression. Within the corpus the frequency of beat increases and the amplitude decreases resulting in a more progressive motility. Within the cauda most spermatozoa present a mature motility pattern. The key factors involved in this process are calcium ions (Ca^{2+}), bicarbonate (HCO_3^-), and cyclic adenosine monophosphate (cAMP). The calcium concentration in the epididymal fluid decreases from 0.8 mM in caput to 0.5 mM in the cauda (Jenkins et al. 1980), and also intracellular sperm calcium decreases during epididymal transit (Vijayaraghavan et al. 1989). Low intracellular calcium is required to maintain a quiescent status and avoid premature hyperactivation (Dacheux and Dacheux 2013). Although bicarbonate concentration of the luminal fluid increases from 2 to 5–7 mM from caput to cauda epididymis, it is maintained at critical concentrations to keep a low luminal pH (in the cauda pH is about 6.8 vs 6.5 in the caput) and to avoid premature activation of hyperactivated motility or capacitation, which are supposed to occur in the female genital tract, where bicarbonate concentration and pH rise up to 90 mM and 7.4, respectively (Pastor-Soler et al. 2003). In the caput, lumen acidification is maintained, thanks to bicarbonate reabsorption by principal cells and proton secretion by clear cells through the V-ATPase pump. Conversely, principal cells in the cauda secrete bicarbonate through cystic fibrosis transmembrane conductance regulator (CFTR), which induces a mild alkalization of the epididymal fluid in this region and is believed to “prime” spermatozoa before ejaculation. Bicarbonate activates an intra-sperm soluble adenylate cyclase (sAC) (Chang and Oude-Elferink 2014) involved in development and maintenance of sperm motility. cAMP generated following sAC stimulation activates a cAMP-dependent protein kinase A (PKA) that phosphorylates Ser and Thr residues on proteins that are involved in various sperm functions including motility. Several studies have demonstrated that Ca^{2+} and HCO_3^- act synergistically on sAC to stimulate motility (Wennemuth et al. 2003; Liu et al. 2012). Perturbations

in these processes result in reduced male reproductive health and consequent subfertility or infertility. Mutations of the CFTR gene are associated with a reduced sperm capacitation due to a disruption of HCO_3^- -dependent events, including increase in intracellular pH, cAMP production, and membrane hyperpolarization (Xu et al. 2007). Moreover, 60–70% of men with mutations of CFTR display congenital bilateral absence of vas deferens (CBAVD) resulting in obstructive azoospermia. Although subjects with CBAVD can father a child after percutaneous epididymal sperm aspiration, recent evidence suggests that pregnancies initiated by these men have an increased risk of miscarriages and stillbirth (Lu et al. 2014), suggesting alterations of sperm integrity likely due to low bicarbonate concentrations in the epididymis.

During epididymal sperm maturation, several membrane modifications in lipid and protein composition occur (Robaire et al. 2000; Cornwall 2009). Spermatozoa collected from cauda epididymis of mouse, rat, hamster, and ram display a 50% reduction in cholesterol levels compared with those retrieved from the caput (Rejraji et al. 2006; Hall et al. 1991; Awano et al. 1993; Parks and Hammerstedt 1985). Furthermore, most mammals, including the human, show a change in the sperm fatty acid composition (from saturated to polyunsaturated forms) during epididymal transit (Rejraji et al. 2006; Hall et al. 1991; Awano et al. 1993; Parks and Hammerstedt 1985; Nikolopoulou et al. 1985; Haidl and Opper 1997; Pyttel et al. 2014). The addition of polyunsaturated fatty acids (PUFAs), in combination with decreased levels of cholesterol during the epididymal sperm maturation, causes an increase in membrane fluidity, which is required for proper sperm motility and fertility (Hall et al. 1991; Haidl and Opper 1997, Evans and Setchell 1979; Aveldaño et al. 1992). If epididymal PUFA synthesis is perturbed, spermatozoa may lose their fertilizing ability as it occurs in mice with conditioned KO of *Dicer1* (a gene encoding an endoribonuclease involved in miRNA formation), characterized by a decrease in the synthesis of PUFAs together with an increased expression of factors involved in cholesterol synthesis in the epididymal epithelium leading to an increase of cholesterol/PUFA ratio in the sperm membrane and, consequently, to membrane instability (Björkgren et al. 2015).

Modification of sperm membrane proteins during epididymal transit is a complex event implying contact with luminal secretions and epididymosomes. First, spermatozoa lose some surface proteins through the action of different proteolytic activities of the epididymal fluid. The second important modification is the appearance of new proteins at the sperm surface. Using 2D electrophoresis gels (Belleannée et al. 2011) and mass spectrometry, many proteins have been identified as being added/removed to sperm surface during epididymal transit (see Table 1 for those having an established role in sperm functions and fertilizing ability). In particular, it has been found that during epididymal transit, about 732 proteins were acquired and 1,034 proteins lost by spermatozoa. Interestingly, in terms of the number of proteins, sperm proteome complexity rose from caput (1,536 proteins) to a maximum in the corpus (1,720) and then decreased in the cauda (1,234). Most of these proteins are common between species although their concentration is variable (Gatti et al. 2004; Dacheux et al. 2005). Only clusterin has always been found in high amounts throughout the

epididymal tract in all studied species (see review by Dacheux and Dacheux 2013). The interaction between a protein secreted by the epididymis and sperm membrane can be characterized by loose binding (potentially involved in maintaining a quiescent state in the epididymis), tight binding (potentially needed for functions in the female tract or for fertilization), or by insertion/modification of integral membrane proteins (involved in masking/unmasking of membrane proteins and sperm decapacitation). Indeed, some integral membrane proteins become detectable only in spermatozoa retrieved from the cauda epididymis or in the ejaculate. A glycosylation process is likely involved in unmasking surface proteins in mature spermatozoa. Moreover, some of the proteins which are transferred to spermatozoa are anchored by GPI to the sperm plasma membrane (Ilio and Hess 1994; Kirchhoff and Hale 1996; Ecroyd et al. 2005). The transfer of proteins to specific membrane domains of spermatozoa depends on temperature and pH (Sullivan et al. 2005). The presence of zinc in the medium, but not of calcium or magnesium, has been shown to increase the efficiency of protein transfer (Frenette et al. 2002).

As mentioned, among the proteins acquired by spermatozoa during epididymal transit, some are essential to guarantee the transit in the female genital tract and oocyte fertilization, as demonstrated by knockout (KO) studies (Table 1). Sperm adhesion molecule 1 (SPAM1), a protein with hyaluronidase activity, is likely involved in sperm penetration through the cumulus matrix. SPAM1^{-/-} mice are fertile, but the lack of SPAM1 results in a remarkably increased accumulation of spermatozoa on the surface or outer edge of the cumulus (Kimura et al. 2009). SED1, a protein involved in cell–cell interaction, is secreted by the initial segment of the epididymis, and its KO in mice leads to a failure to regulate the epididymal fluid and an inability of sperm to bind and fertilize eggs (Raymond et al. 2010). ADAM7, a disintegrin and metalloprotease associated with epididymosomes (Oh et al. 2009), is involved in sperm motility, and KO animals show reduced fertility due to anomalies in epididymal caput structure and reduced sperm motility associated with abnormalities in tail morphology and tyrosine phosphorylation of proteins. Cysteine-rich secretory proteins (CRISP) are present on sperm surface and involved in acquisition of sperm fertilizing ability. CRISP family members can be of testicular (such as Crisp 2) or epididymal (such as Crisp1, Crisp3, and Crisp4) origin. CRISP1-deficient spermatozoa show defects in their ability to increase protein tyrosine phosphorylation during capacitation and impaired interaction with the oocyte *in vitro*. Despite this, they are fertile probably due to a compensatory mechanism by other members of the family (Da Ros et al. 2015).

Sperm Storage

The principal role of cauda epididymis is sperm storage. The storage period varies from days to weeks depending on the sexual and mating behavior of the specie (Jones et al. 2007). As mentioned above, during the passage from testis to efferent ducts and initial region of epididymis, about 90% of water is reabsorbed (Wong and Yeung 1978; Turner 1984). Water reabsorption is essential to achieve good storage

conditions and to preserve viability of spermatozoa. Other factors involved in maintaining sperm motility and viability are androgens and scrotal temperature. A low scrotal temperature with respect to body and testis (Brooks 1973) preserves viability and motility. Conversely, an increase in the scrotal temperature of only 2 °C for 4 days decreases sperm motility and embryo viability after ART (Mieusset et al. 1991). Higher temperatures modify water, Na⁺, K⁺, and Cl⁻ channels in the cauda epididymis (Wong and Yeung 1978) and determine the disappearance of several proteins typically present in its secretions (Bedford 1991; Regalado et al. 1993). Moreover high temperatures reduce the diameter and length of the duct in the cauda region (Foldesy and Bedford 1982) and so its storage capacity. Among mammalian species, human is the one that presents the lowest storage capacity. Sperm survival in the cauda epididymidis is influenced also by androgens. Removal or decline of androgens triggers mechanisms that lead to a malfunctioning epithelium and activate death pathways that ultimately dissolve spermatozoa (Jones 2004). Other important factors involved in sperm survival are a low oxygen content (Free et al. 1976) and absence of glucose (Annisson et al. 1963). The protective role of epididymis on spermatozoa is demonstrated by studies showing that spermatozoa from epididymis stored at 4 °C for several days after the death of the animal (Abella et al. 2015) or cryoconserved in the epididymides for up to 48 h (Takeo et al. 2014) maintained high fertilization potential.

Sperm Emission

During the emission phase a strong contractility of the epididymis and vas deferens occurs provoking a rapid transport of spermatozoa toward more distal regions. Caput and corpus transfer spermatozoa by spontaneous, peristaltic-like contractions, whereas the cauda is characterized by a rich adrenergic innervation which coordinates the muscular contractile activity necessary for emission phase of the ejaculatory process (El-Badawi and Schenk 1967). The group of Maggi (Vignozzi et al. 2008) demonstrated that epididymal contractile activity is mediated by neuronal and nonneuronal factors and hormones. Among the latter, oxytocin and endothelin-1 are two well-characterized factors and are involved in creating an autocrine/paracrine loop which supports the autonomous peristaltic movements of the epididymis and favors sperm progression throughout the duct. Another essential factor to maintain the epididymal sensitivity to these locally produced contractile factors is estradiol, which increases RhoA/ROCK signaling (Fibbi et al. 2009), involved in regulating the myogenic tone of smooth muscle cells.

Impact of Epididymal Alterations on Male Fertility

The essential role played by the epididymis in sperm maturation and development of key sperm functions suggests that alterations of epididymal functions may lead to sub- or infertility. In particular, infections and inflammations may cause tissue

damage impairing epididymal secretory function (Cooper et al. 1990). In addition, recruitment of phagocytic cells to the site of the infection leads to the release of cytokines and other inflammatory mediators and an increased generation of ROS (Azenabor et al. 2015; Lotti and Maggi 2015) affecting functions and integrity of both epididymis and transiting spermatozoa. It is estimated that infections and inflammations of the genital tract constitute about 15% of all cases of male factor infertility and epididymitis together with combined epididymo-orchitis is the major contributor. Infection/inflammation of epididymis may result from microbial invasion, genital trauma, and sterile reflux, but bacterial invasion appears to be the most prevalent (Schagdarsurengin et al. 2016). The occurrence of epididymitis leads not only to a reduction of sperm number (due to induction of apoptosis after exposure to microbes and leukocytes for long time) but also premature acrosome reaction within the epididymal lumen (due to the presence of α -hemolysin and other toxins which damage the acrosome) (Schagdarsurengin et al. 2016). In addition, toxins may increase generation of ROS that alter membrane integrity, reduce motility, and produce oxidative DNA damage and DNA fragmentation in spermatozoa transiting the epididymis. Epididymal alterations may be also induced by environmental factors, in particular, endocrine disruptors can directly affect hormonal regulation of luminal secretions or induce epigenetic modifications of gene expression and, consequently, alterations in the composition of the luminal fluid.

Common abnormalities of the epididymis are cysts and spermatoceles, benign formations mainly located in the head of the organ. They are reported in one out of four men undergoing ultrasound examination. Cysts appear as anechoic avascular spherical formations and spermatoceles as slightly hypoechoic inhomogeneous abnormalities (Lotti and Maggi 2015). Their exact etiology is not clear, but they might be due to a blockage in one of the tubes that transports spermatozoa. The association between cysts or spermatocele and male infertility is doubtful, as their involvement in complete epididymal obstruction and obstructive azoospermia has never been proven. Spermatoceles are generally painless and are filled with milky or clear fluid that usually contain spermatozoa, representing, when large enough, a reservoir of viable and motile spermatozoa that can be used in assisted reproduction (Hirsh et al. 1996; Müller-Tyl et al. 1990).

Epididymis as the Target of Male Contraception

Apart from condoms and vasectomy, contraceptive methods for men are still unavailable. An ideal method of contraception should be rapid, fully and quickly reversible as well as without side effects. Recently, researchers begun to focus their attention on a nonhormonal approach that should display high selectivity. In particular, two principal technical approaches are available to interfere with essential functions in male fertility: immunocontraception and a drug-based contraception. The identification of target proteins specifically expressed in the male reproductive tract and showing a fundamental importance for sperm functions may allow the development of highly selectively acting drugs with excellent safety profiles. The

fact that sperm maturation changes, occurring during epididymal transit, are prerequisites for a successful reproductive function highlights the epididymis and epididymal secretions as important targets for male contraception. Interfering pharmacologically or immunologically with epididymal sperm maturation process could be indeed a good prospective for developing a post-testicular contraception drug. In particular, an epididymal antigen, as candidate, would be optimal for immunocontraception since it allows inhibiting only post-testicular sperm maturation events, without affecting testicular function and so avoiding a possible effect on germ cells. Recently, some studies focused on possible epididymal targets of immunocontraception. O'rand et al. (2004), immunizing seven monkeys against recombinant eppin (an epididymal protease inhibitor involved in modulating prostate-specific antigen activity, providing antimicrobial protection and binding semenogelin, thereby inhibiting sperm motility), obtained a complete, but reversible, contraception in five animals, highlighting the role of the protein in male fertility. Immunization of rat males and females with either native or recombinant CRISP1 (see above and Table 1) produced specific antibodies in over 90% of the animals, resulting in a reversible inhibition of fertility in both sexes (Da Ros et al. 2015). Other possible epididymal candidates for immunocontraception could be β -defensin proteins, involved in regulation of sperm motility (Dorin and Barratt 2014). It has been demonstrated that deletions or mutations in defensin genes lead to, respectively, male sterility and subfertility (Zhou et al. 2013; Tollner et al. 2011; Björkgren et al. 2016).

It should be mentioned that an immunological approach to contraception raises several concerns such as the possibility of provoking autoimmunity and the reversibility of the infertility status. In addition a variability in both the degree and duration of response among individuals has been demonstrated. An attractive alternative would be the development of pharmacological inhibitors to target epididymal proteins. Applying a set of indication-specific criteria, putative drug targets can be efficiently identified. Such criteria include tissue-selective expression, crucial biological function in fertility, and druggable properties. Proteins have first to fulfil these criteria before a drug discovery process can be initiated. Possible targets using such approach could be epididymis-specific disintegrin and metalloproteases containing proteins (ADAMs), especially ADAM7 (see Table 1) and the G-protein-coupled receptor HE6 (Table 1).

Even though a number of novel potential drug targets are emerging, male contraception based on epididymal function is in its infancy from a clinical point of view, since delivering potential contraceptive drugs to the epididymis appears difficult because of the presence of the blood–epididymis barrier. Development of shuttle molecules able to deliver drugs through the blood–epididymis barrier as well as to target them to specific tissues/cells is under investigation.

Conclusion

The epididymis is exerting an essential role in male reproductive functions, allowing poorly motile spermatozoa released from the testis to achieve the progressive motility necessary to reach the oocyte as well as to complete their chromatin

compaction, regulate their volume, and acquire competence to fertilize. Such roles are accomplished by the variegated secretory activities of the epithelium which are under regulation of sex steroid hormones and other epididymal and testicular factors and are specialized in the different segments. Finally, the epididymis guarantees the safe storage of spermatozoa and supports their emission at ejaculation. In view of such important functions, the epididymis is involved in male infertility and represents an attractive target for development of novel contraceptive strategies.

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