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REVIEW

New insights into epididymal function in relation to sperm maturation

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Abstract

Testicular spermatozoa acquire fertility only after 1 or 2 weeks of transit through the epididymis. At the end of this several meters long epididymal tubule, the male gamete is able to move, capacitate, migrate through the female tract, bind to the egg membrane and fuse to the oocyte to result in a viable embryo. All these sperm properties are acquired after sequential modifications occurring either at the level of the spermatozoon or in the epididymal surroundings. Over the last few decades, significant increases in the understanding of the composition of the male gamete and its surroundings have resulted from the use of new techniques such as genome sequencing, proteomics combined with high-sensitivity mass spectrometry, and gene-knockout approaches. This review reports and discusses the most relevant new results obtained in different species regarding the various cellular processes occurring at the sperm level, in particular, those related to the development of motility and egg binding during epididymal transit.

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Introduction

The formation of fertile spermatozoa is the result of spectacular stages of cell differentiation that begin in the male gonad and finish in the female tract. The initialization and formation of the male gamete take place in the epithelium of the seminiferous tubules and are characterized by a series of genetically time-controlled cellular modifications. The spermatozoon developed at the output of the testis is morphologically complete but immotile and unable to fertilize an oocyte. For all mammals, this fertilization ability of the spermatozoon is the result of discrete post-gonadal differentiation stages that occur during transit along the several meters of the epididymal tubule. These modifications occur in a male gamete that lost its transcriptional and translation abilities during the last stages of spermatid differentiation. It is postulated that such post-gonadal sperm differentiation is controlled by the surrounding environment. Such extracellular control of gamete differentiation, also called 'epididymal sperm maturation', appears to be unique in the body. Several investigations have been undertaken over more than 60 years to understand the mechanisms involved in the development of fertility of the male gamete.

The post-gonadal stages of sperm differentiation are set up by successive modifications that occur when the gamete transits specific parts of the epididymal tubule. At the same time, outside the gametes, the composition of the luminal epididymal environment also changes sequentially throughout the epididymis. In view of these two parallel events, most investigations have involved assessing the relationships between these two events and identifying the epididymal signals able to control spermatozoon fertility.

The most recent research using new techniques such as transcriptomic and proteomic approaches has involved identification of these events occurring on/in the sperm and in their surrounding epididymal environment. The role of certain epididymal and sperm proteins involved in this sperm maturation has also been investigated by gene deletion in mice. Furthermore, as epididymal sperm maturation is a common phenomenon in mammals, comparative studies have been developed to identify mechanisms common between species that could be the fundamental mechanisms of post-testicular differentiation.

The epididymal sperm environment: a specific continuously modified milieu

From their haploid stage, the male germ cells are isolated from the rest of the body by a blood barrier that is present from the testis to the deferent duct. Such restrictive exchange results in the formation of a unique sperm environment controlled successively by Sertoli cells and by epididymal epithelium activity. The epididymal fluid is certainly the most sequentially modified milieu of the body in which the spermatozoa are bathed for 1 or

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2 weeks. The most important change in this luminal fluid is induced by water reabsorption. This water movement occurs principally in the efferent ducts where almost 80–90% of the testicular fluid is reabsorbed and continues at a lower level up to the first part of the epididymis. These water changes through aquaporin channels are driven by the transepithelial movement of Na^+, Cl^- and HCO_3^- and also result in important modifications in the ionic composition of the lumen fluid throughout the epididymal tubule (Da Silva *et al*. 2006).

The first consequence of such water movement is a spectacular increase in the luminal sperm concentration, increasing from 10^8 spermatozoa/ml in the rete testis fluid to 10^9 in the deferent duct, with a maximum in the first part of the epididymis. The second consequence is the modification of the protein concentration from 2 to 4 mg/ml in the rete testis fluid and the initial segment of the epididymis to a maximum of 50–60 mg/ml in the distal caput. It then returns to 20–30 mg/ml in the more distal regions of the organ (Fouchecourt *et al*. 2000, Belleannee *et al*. 2011*b*, Dacheux *et al*. 2012). For all the mammals studied, these changes occur in the anterior part of the epididymis and are always associated with the first sperm modifications such as the migration of the cytoplasmic droplet, the beginning of flagellum beating and binding to the zona pellucida (ZP). As most of these sperm modifications have never been successfully induced *in vitro* for testicular spermatozoa, their triggers may have an epididymal origin. Among the epididymal factors potentially involved, it is becoming clearer that the surrounding proteins may be directly or indirectly involved in the changes in sperm properties during their transit through this organ.

Composition, identification and origin of the luminal epididymal proteins

Defining the protein composition of the epididymal fluid has been the aim of several studies since the 1970s–1980s (Turner *et al*. 1979, Brooks 1981). The first research in this field was carried out mostly to find specific epididymal proteins directly involved in sperm maturation and available as immunological targets for male contraception.

In such research, protein identification was based only on isolated proteins. Our understanding of epididymal proteins has increased spectacularly with genome sequencing and new proteomic technology using large-scale analysis in the last 10 years. Today, several hundred proteins can be identified using mass spectrometer technology in a single study. However, due to the huge range of protein concentrations in the epididymal fluid, spanning around 12 orders of magnitude, only four to six orders can be measured in a whole sample using the current most sensitive mass spectrometer. The complete identification of all luminal epididymal proteins thus remains a technological challenge.

Several hundred epididymal proteins have already been identified from the epididymal fluid of different species. All these epididymal fluids are characterized by the presence of proteins in high concentrations, and no more than 20 proteins represent 80–90% of the total luminal proteins (Fig. 1). Several of these proteins are common to different species such as lactoferrin (LTF), lipocalin 5 (LCN5, E-RAPB), clusterin (CLU), glutathione peroxidase (GPX5), prostaglandin D2 synthase (PTGDS), transferrin (TF), Niemann–Pick disease, type C2 (NPC2), phosphoethanolamine-binding protein 4 (PEBP4), β-*N*-acetyl-hexosaminidase (HEXB), glutathione S-transferase (GST), gelsolin (GSN), actin (1.4%), and β-galactosidase (GLB1). For the human epididymis, 77% of the total luminal proteins are represented by albumin (ALB) (43.8%), CLU (7.6%), NPC2 (6%), LTF (5.9%), extracellular matrix protein (ECM1) (3.2%), α1-antitrypsin (SERPINA1 (A1AT)) (2.7%), PTGDS (2.2%, 1.7%), TF (1.3%), and actin (ACT) (1.2%) (Dacheux *et al*. 2006). Most of these epididymal proteins are highly polymorphic due to glycosylation and other post-translational modifications.

There are wide variations in the concentrations of these major proteins between species. No phylogenic studies have been carried out between mammalian species, but the major proteins seem to be a proteomic signature for closed species, as observed for bovine and ovine species (Dacheux *et al*. 2012). However, there is considerable divergence between extreme mammalian classes such as eutherians and monotremes. For monotremes such as the platypus, the luminal protein composition in the epididymis is unique among mammals, with numerous species-specific proteins such as E-OR20 (a new lipocalin), SPARC, and PXN-FBPL (Dacheux *et al*. 2009).

Most of the luminal proteins are actively secreted by the epididymal epithelium, but their secretion is dependent on the region of the organ. Such sequential secretion along the epididymal tubule results in a continuous change in the epididymal fluid proteome (Fig. 1). Furthermore, the secretion pattern is species specific, but several proteins can be found in similar epididymal regions (e.g. PTGDS, GPX5, and RNASE10 in the proximal epididymal regions and LT, NCP2, GSN, and several glucosidases in the middle and distal parts of the organ). However, for several other proteins, such as LCN5, principally secreted in the epididymal caput in the mouse but in the corpus cauda in the bull, ram, and boar, the secretory epididymal region is different (Dacheux *et al*. 2012).

Of all the major epididymal secreted proteins, the most commonly secreted is CLU. This protein, originally found to be a Sertoli cell secretion (Fritz *et al*. 1983), represents around 30% of the total epididymal secretion for all the species studied to date. Furthermore, this

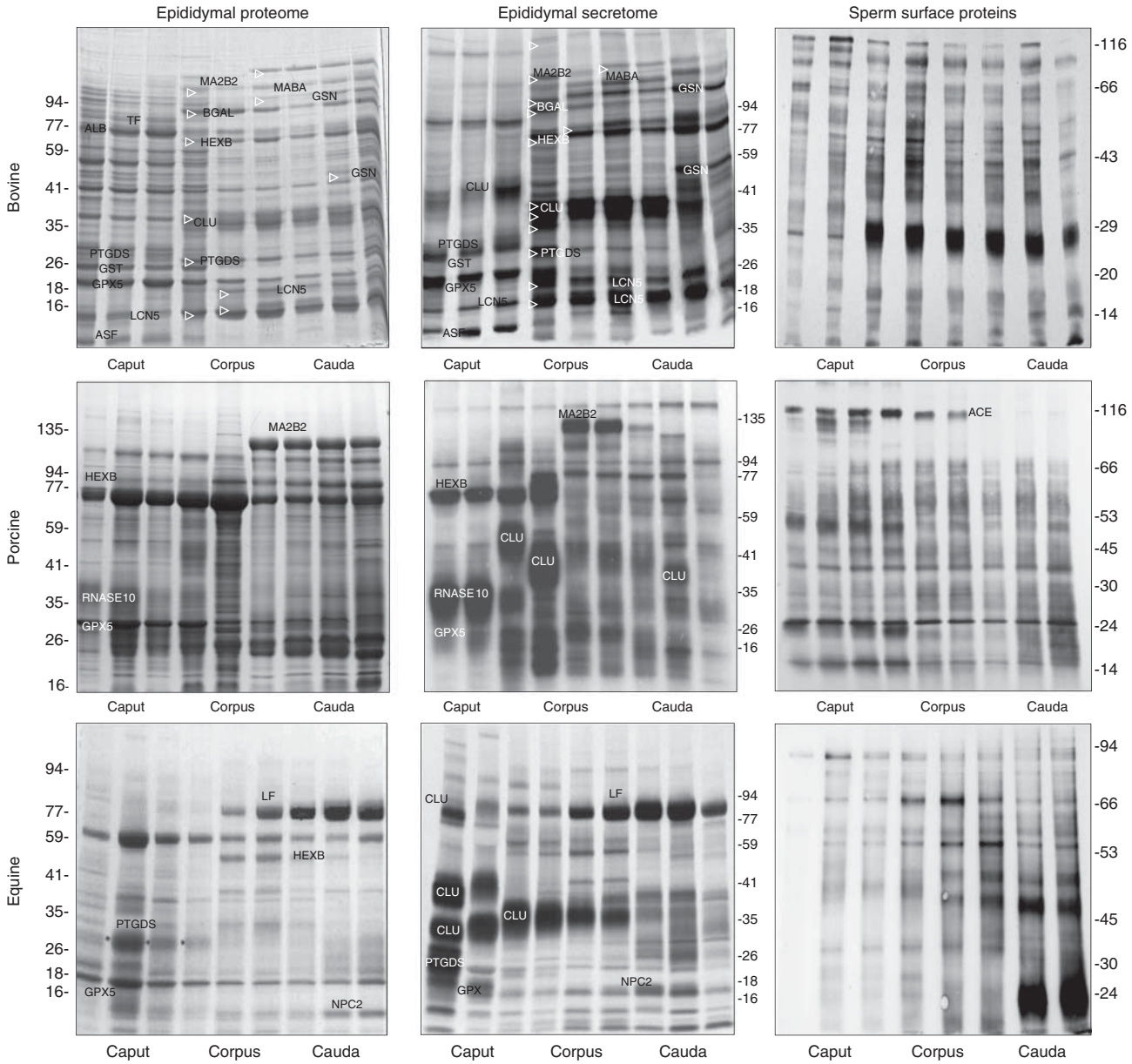

Figure 1 Epididymal proteome and secretome and sperm surface proteins of three species. For the proteome, the same quantity of epididymal proteins from nine zones of the epididymis corresponding to the three principal regions was separated by 1D gel electrophoresis and silver stained. The plates illustrating the secretory activities correspond to the autoradiograms of the same 1D gel separations presented for the proteome of the boar (Syntin *et al.* 1996), stallion (Fouchecourt *et al.* 2000), and bull (Belleannee *et al.* 2011*b*). The sperm surface proteins correspond to the 1D SDS–PAGE, western blot, and streptavidin peroxidase detection of the NP40 extracts of the sulpho-NHS-biotin-labelled surface membrane proteins of spermatozoa collected from eight or nine epididymal zones (C Belleannnée and J L Dacheux, 2006 unpublished data).

protein can be secreted at different levels of the epididymis and as several isoforms (three in the horse and at least two in the bull and the ram).

The local concentration of a protein in the luminal fluid is generally related to the secretory activity of the adjacent epithelium. Thus, a major protein found in the luminal fluid corresponds to a major secretion. However, the concentration of each secreted protein is linked not only to its secretion but also to its reabsorption in specific epithelium regions. Some proteins or isoforms are present only in their secreted region (e.g. RNASE10, porcine, ovine and murine species (Castella *et al.* 2004), and some isoforms of CLU in equine species (Fouchecourt *et al.* 2000)). For other proteins such as GPX5, several glucosidases, and PTGDS (Fouchecourt *et al.* 2000, Belleannee *et al.* 2011*b*), the luminal concentration is modulated by their reabsorption intensity or by their accumulation during epididymal

transit (e.g. lactoferrin in equine species and LCN5 in bovine species) (Dacheux *et al*. 2012).

Most of these major secretory proteins are soluble in the luminal fluid and secreted by conventional and unconventional secretion pathways for the proteins lacking signal peptides. However, several secreted proteins have been found to be highly hydrophobic such as the 17 and 22 kDa ram epididymal proteins (extracted as lipoprotein) (Gatti *et al*. 2000), prion protein (PRP; Gatti *et al*. 2002, Ecroyd *et al*. 2005), and several glycosylphosphatidylinositol (GPI)-anchored proteins, such as CD52 (Kirchhoff 1996). The secretion mechanism of such hydrophobic proteins in the luminal fluid is still unclear. Some of these proteins have been found to be associated with luminal membrane vesicles. These vesicles are liberated from the surface of the principal cells as vesicular blebs (Ilio & Hess 1994, Frenette *et al*. 2002, Hermo & Jacks 2002, Gatti *et al*. 2005) and named 'epididymosomes' (see review by Sullivan & Saez (2013)). However, secreted hydrophobic proteins are not only associated with such luminal membrane vesicles but also found in soluble high-mass protein complexes such as CLU, cauxin, PRNP, 17 kDa hydrophobic protein, and several glycosidases (Ecroyd *et al*. 2005).

Functions of the most abundant luminal epididymal proteins

Most of the major epididymal proteins are also found in other tissues, and thus their general functions can be identified. However, their presence at high concentrations is surprising in the sperm environment. This is the case for the huge amounts of enzymes such as glycosidases (α-D-mannosidase, β-hexosaminidase, GLB1, α-glucosidase, β-glucuronidase, etc.), numerous proteases (kallikrein, cathepsin A-D-H-L-S-L, gACE, ADAM2–7, furin, MMP2, etc.) and several protease inhibitors (serpin, eppin, CRES, serpin2, HE4, etc.). Most of these enzymes have been found to be active *in vitro*, except for certain specific epididymal proteins, such as RNASE10 (Castella *et al*. 2004). Several of these enzymes, such as different matrix metalloproteases (MMPs), are secreted as a pro-form and activated after processing in the fluid (Metayer *et al*. 2002).

However, the presence of such concentrations of active enzymes *in vivo* suggests the presence of enormous quantities of substrates, but to date such substrates remain hypothetical. It is also possible that the function(s) of these enzymes may be different from those displayed in other tissues. This can be illustrated by glutathione-independent PTGDS that is intensively secreted in the epididymal caput and known to catalyse the conversion of prostaglandin H2 (PGH_2) to prostaglandin D2 (PGD_2), but in the epididymis its role is only as a carrier for hydrophobic substrates in the luminal fluid (Fouchecourt *et al*. 2002).

The other intriguing finding is the fact that some enzymes and their specific inhibitors (such as proteases and protease inhibitors) are found together in the same luminal fluid. This suggests that the enzymes' activities could be controlled and functional only in a limited part of the epididymis and blocked by specific inhibitors downstream during transit through the organ. Such sequential enzyme activity is highly probable, but it has not been demonstrated to date.

Thus, in addition to the sequential changes in the protein composition of the epididymal fluid, the activities of these proteins could also be sequentially controlled. Such duality between the presence and activity of an epididymal protein in a specific region makes their understanding in relation to spermatozoa more complex than expected.

Nevertheless, by analogy with the activity of these proteins in other tissues, several general functions of these proteins can be suggested in the epididymis. The most realistic function is protection of the sperm during epididymal transit. Several proteins successively secreted in the epididymal fluid may be involved in the reduction of reactive oxygen species in the luminal fluid such as GPX5 (Chabory *et al*. 2010, Taylor *et al*. 2013), thioredoxin, GSTM1–3, SOD1, and PRDX2–5. This protective function may also be illustrated by the presence of several proteins and peptides such as several β-defensins (Yamaguchi & Ouchi 2012), lipocalins, and CRES proteins against bacterial attack (Wang *et al*. 2012).

The epididymal proteins may also be involved directly in the protection of active sites on the sperm surface, illustrated by the fact that when epididymal sperm are washed free from epididymal proteins, sperm head-to-head agglutination occurs very rapidly. Such protection may be attributed to several proteins with binding properties such as CLU, lactoferrin, TF, apolipoprotein A-1, PEBP, and some other glycoproteins involved in the glycocalyx composition of the gamete (Fabrega *et al*. 2012).

Other important functions of epididymal proteins may be linked to molecular exchange or as carriers of hydrophobic components (e.g. cholesterol, retinoic acid, and androgen). Several epididymal proteins may be involved individually, such as NCP2, LCN5, PTGDS, androgen-binding protein (SHBG (ABP)), vitamin D-binding protein, lactoferrin, TF, and ceruloplasmin (Guyonnet *et al*. 2011), or combined together in a soluble high-molecular mass lipophilic complex (Ecroyd *et al*. 2005).

Several potential roles may thus be attributed to these major luminal proteins, but they are still mostly hypothetical. In the mouse, the knockout (KO) gene for several of these proteins (e.g. lactoferrin, SOD1, GSTM1, CLU, GPX5, PTGDS, LCN5, hexosaminidase, CRISP1, and CRISP4) does not result in a reduction in male fertility *in vivo*, except for the inactivation of the *Rnase10* gene.

The exact functionality and importance of all these major epididymal proteins, therefore, remain to be evaluated in relation to sperm survival, maturation in the epididymis and species diversity.

Epididymal spermatozoa: continuous epididymal sperm surface modifications

As for epididymal fluid composition, the spermatozoa are sequentially modified throughout their transit in this organ. One of most visible morphological changes is the migration of the cytoplasmic droplet, a remnant of the germ cell cytoplasm. This structure slides from the beginning to the end of the intermediate piece of the flagella in the middle part of the epididymal caput. The mechanism involved in this cytoplasmic migration has not been elucidated, but it is worth noting that the transport of this droplet occurs from a very restricted epididymal region that corresponds to the maximum concentration of spermatozoa in the lumen and where the epithelium protein secretions are the most active. The relationship between epithelium functions and molecular mechanisms of such cellular modification remain to be identified. Such migration is an important sign of sperm maturation, since any defect in droplet migration is related to a decrease in sperm fertility (Cooper 2005) but not to the activation of sperm motility in the epididymis.

The other relevant but less visible sperm modifications involve the membrane and sperm surface composition. Over several decades (Scott *et al*. 1967, Dacheux & Voglmayr 1983), many studies have shown that numerous sequential changes occur in the lipid and protein composition of the sperm membrane during epididymal transit.

The characterization of these modifications was first oriented to those directly involved in egg-binding and -fertilization ability of spermatozoa. Based on the inhibition of sperm–oocyte binding/fusion with the use of specific antibodies, several surface proteins such as CRIP1 (protein D/E), acrosin, fertilin, PH20, IZUMO, and SPAM1 were identified (Supplementary Table 1, see section on supplementary data given at the end of this article). Several of these surface proteins are processed during epididymal transit by a cascade of proteolytic cleavages such as the well-documented ADAM family proteins (ADAM1/α-fertilin, ADAM2/β-fertilin, and ADAM3/cyritestin). Most of these cleavages occur as soon as the gamete enters the epididymis or in the first part of this organ. The cleaved peptides of these proteins are either relocated in different plasma membrane domains (e.g. ADAMs) or released into the surroundings, as is the case for ACE, which becomes a major protein in the epididymal fluid for several species (Gatti *et al*. 1999).

Several sperm membrane proteins such as luminal epididymal proteins that bind to the surface of gametes (e.g. CRISP1, cathepsin, ADAM7, EPPIN, MAN2A2, SPAM1, MFGE8, GPX5, CLU, and MIF) have also been identified (Supplementary Table 1). Some of these proteins are adsorbed at the sperm surface by electrostatic interaction or are integrated into the plasma membrane. For hydrophobic and GPI-anchored proteins, the exchange between the epididymal secretion and the sperm membrane has not been completely resolved yet. It may be mediated by soluble intermediates such as the epididymal vesicles (epididymosomes) or soluble hydrophobic protein complexes present throughout the epididymal tubules. The quantity of epididymosomes appears to be relatively low and limited compared with the total number of spermatozoa (no more than 0.5% of the total volume of spermatozoa). Furthermore, the exact mechanism of protein transfer remains to be clarified, since the exchange is protein specific and not all proteins of the vesicles are transferred or retrieved on the sperm surface. Thus, if these vesicles are the potential major route of hydrophobic protein transfer, it may be via very subtle exchange mechanisms that remain to be elucidated.

Using these earlier identification approaches, sperm membrane modifications were studied protein by protein. However, other major protein modifications can be visualized on the sperm surface using more global surface labelling techniques such as surface protein biotinylation and 1D or 2D electrophoresis gel separation (Figs 1 and 2). Such approaches demonstrate that modifications occur continuously at the surface membrane level. It is assumed that most of the surface proteins originating from testicular gametes are modified or disappear and new compounds can be visualized in the terminal regions of the epididymis. Such modifications occur for all the mammalian species, but the protein characteristics and the patterns of the sequential changes involved are different between species (Fig. 1). Such species differences raise the question of whether rodents are the most appropriate model to study epididymal sperm maturation in other species.

The current challenge is the systematic identification of the sperm surface proteins involved in epididymal maturation, whatever the species, and particularly for human spermatozoa. Proteomic analysis is a promising approach to identify epididymal proteins. The first global proteomic studies on the male gamete were based on 2D gel electrophoresis separation. Such an approach has the advantage of separating and quantifying several thousand compounds and isoforms, but mainly the most abundant compounds and less well the high-molecular weight and hydrophobic proteins. Nevertheless, coupled with 2D LC–mass spectrometry, such proteomic approaches provide the opportunity to identify several proteins of the mature spermatozoon (see review by Brewis & Gadella (2010)).

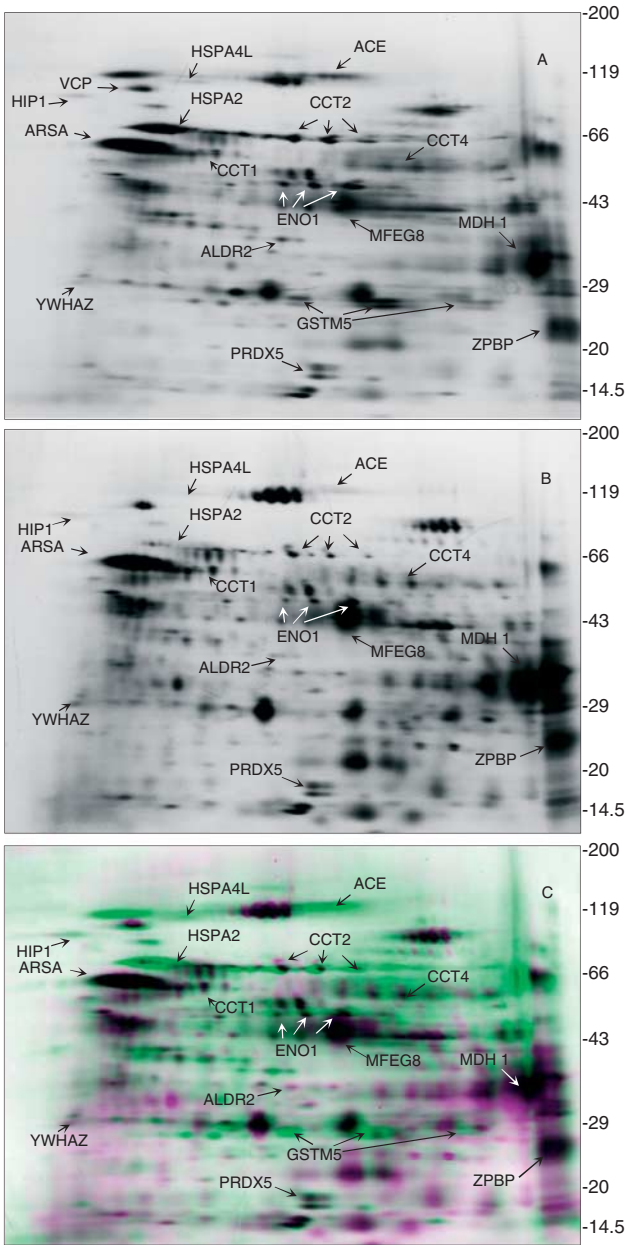

Figure 2 Silver staining of 2D separation of purified surface proteins from immature (A) and mature (B) epididymal boar sperm. (C) Composite 2D gels from immature (green) and mature (red) sperm. Adapted from **Belleannee C, Belghazi M, Labas V, Teixeira-Gomes AP, Gatti JL, Dacheux JL, Dacheux F** 2011*a* Purification and identification of sperm surface proteins and changes during epididymal maturation. *Proteomics* **11** 1952–1964.

The recent improvements in mass spectrometer technology provide the opportunity to identify sperm proteins directly from the whole lysate of mature sperm without previous electrophoresis separation. In these 'deep proteomic analyses' (Oliva *et al*. 2009), several thousand proteins have been collected from mature spermatozoa (epididymal or ejaculated) of different species such as the mouse (Baker *et al*. 2008*b*, Chauvin *et al*. 2012), rat (Baker *et al*. 2008*a*), bull (Peddinti *et al*. 2008), and human (Johnston *et al*. 2005). The maximum number of proteins identified by this technique in human sperm is around 5000 (Wang *et al*. 2013).

Several methods of analysis have been developed to increase the identification of the low-abundance proteins on subcellular parts of the spermatozoa such as the head and flagellum (Cao *et al*. 2006, Amaral *et al*. 2013, Baker *et al*. 2013), acrosomal matrix, (Guyonnet *et al*. 2012), detergent-resistant membrane (Sleight *et al*. 2005, Nixon *et al*. 2009), phosphorylated proteins (Platt *et al*. 2009), and proteins involved in capacitation (Ficarro *et al*. 2003) and motility (Zhao *et al*. 2007, Martinez-Heredia *et al*. 2008, Chan *et al*. 2009) and of sperm–egg interactions (Stein *et al*. 2006, Petit *et al*. 2013). In spite of the lack of a dynamic range of mass spectrometers to identify the low-represented proteomes, these 'shotgun' mass spectrometer approaches provide the opportunity to identify additional proteins involved in new or unexpected metabolic pathways for mature sperm (Amaral *et al*. 2013).

Few systematic proteomic studies have been published about epididymal sperm maturation. Most of them have combined 2D gel electrophoresis and MS on the total extracts of immature and mature epididymal sperm from the mouse (Ijiri *et al*. 2011), rat (Guo *et al*. 2007), or hamster (Kameshwari *et al*. 2010), or on subcellular compartments such as the head and flagella (Suryawanshi *et al*. 2011), acrosomal and membranous proteins (Park *et al*. 2012), and phosphopeptides (Baker *et al*. 2012), or on purified sperm surface proteins (Belleannee *et al*. 2011*a*). About 20 proteins involved in sperm maturation have been identified by these studies, of which only two or three have been found to be common between two studies or species. In view of the numerous changes visualized by specific labelling of the surface proteins during epididymal transit, many more proteins and their isoforms remain to be identified (Fig. 2).

Relationship between epididymal activity and sperm maturation

The principal characteristics of a 'mature' spermatozoon can be summarized as a male gamete that is able to activate its motility (forward and hyperactivated), be capacitated, bind to the pellucida, fuse to the oocyte membrane, and be able to result in a viable embryo. How can epididymal activity be involved in these essential sperm functions during transit of the gamete?

Epididymal surroundings and sperm motility

The most visible and quantifiable change in the epididymal sperm is the development of motility, the activation of which is the result of progressive steps.

It begins with an increase in asymmetry and irregular flagella beating in the anterior part of the epididymis to reach symmetrical propagation of waves on each side of the flagella, inducing the forward motility of the spermatozoa in more distal parts of the organ (Bork *et al*. 1988, Chevrier & Dacheux 1992). However, such motility is only observed *in vitro* when epididymal sperm have been washed free of epididymal fluids and diluted and incubated in an 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 the position in the epididymis.

Thus, for the majority of mammalian species, sperm motility in the epididymis is balanced by two important components: one concerning the final differentiation of the flagella machinery, which can be analysed only *in vitro* after its activation, and the other controlling or repressing this machinery to avoid sperm movement.

In vitro, sperm motility increases progressively from the corpus to the cauda epididymis, with testicular and caput epididymal sperm showing only irregular curvatures of the flagella. The progressive maturation of the complex interactions between tubulin and dynein molecules should occur during epididymal transit. However, it has been known for many years that forward motility can be induced in immature sperm *in vitro*. Such activation can be achieved with intact testicular spermatozoa (Dacheux *et al*. 1979, Okamura *et al*. 1991, Jaiswal & Majumder 1996*b*), intact epididymal sperm (Acott *et al*. 1983, Smith *et al*. 1996, Vijayaraghavan *et al*. 1996), or demembranated immature sperm (White & Voglmayr 1986, Ishijima & Witman 1991, Patil *et al*. 2002). Such observations indicate that the flagellum machinery of the spermatozoon at the output of the testis is molecularly functional but inactivated *in vivo*.

Although these results have been known for several years, the exact mechanism of the activation of sperm motility in the epididymis is still unclear, particularly the influence of the epididymal surroundings. A few studies on the epididymal maturation of sperm motility have been published recently, with most of them being more focused on the potential capacitation of the epididymal sperm than on the motility process.

The motility of mature spermatozoa is dependent on the intracellular cAMP generated by adenylyl cyclase and on subsequent successive protein phosphorylations including protein kinase A (PKA), A-kinase anchor proteins (AKAPs) and many other phosphorylated proteins (see review by Turner (2006)). A cascade of very short (in the order of nanoseconds) phosphorylations and dephosphorylations of dynein arms is generated by serine/tyrosine kinases and serine/tyrosine phosphatases. Such activities induce active bend propagation and regular flagella beating by alternate sliding of microtubules along the length of the flagellum (Morisawa 1994).

During epididymal transit, the sperm's intracellular cAMP level increases from the corpus to the cauda (Hoskins *et al*. 1974, Dacheux & Paquignon 1980, Pariset *et al*. 1985), simultaneously with metabolic capacity and ATP production (Inskeep & Hammerstedt 1982). The production of cAMP is generated by an atypical sperm soluble adenylyl cyclase (sAC or SACY; Okamura *et al*. 1985, Wandernoth *et al*. 2010), which is biochemically distinct from the transmembrane adenylyl cyclases located throughout the sperm midpiece (Hess *et al*. 2005) and regulated by bicarbonate and calcium (Buck *et al*. 1999, Chen *et al*. 2000, Xie *et al*. 2006). The role of cAMP and adenylate cyclase in spermatozoa is now genetically established by KO of the *Adcy10* (*sAc*) gene, which induces male sterility linked to a sperm motility defect (Esposito *et al*. 2004).

The control of the intracellular cAMP levels of the spermatozoa and consequently protein phosphorylation are the keys to understanding the gradual (potential) activation of motility during epididymal transit. The bicarbonate and Ca^{2+} concentrations are two important components of the luminal epididymal fluid that could directly control the intracellular cAMP concentrations in the epididymal spermatozoa and consequently activate protein phosphorylation and motility.

Role of luminal bicarbonate in epididymal sperm motility

The bicarbonate originating from the testis (20 mM) in the lumen of the epididymal tubule is partially reabsorbed between the seminiferous tubules and the caput of the epididymis by the HCO_3^- transporter (Breton 2001, Liu *et al*. 2012) and by carbonic anhydrase activity (Hermo *et al*. 2005). In the rat epididymis, the bicarbonate concentration is around 2–6 mM (Levine & Marsh 1971), with a slight increase in the cauda and vas deferens being linked to bicarbonate secretion by the principal epididymal cells (see review by Shum *et al*. (2011)).

The intracellular concentration of bicarbonate in the epididymal spermatozoa is constitutionally regulated by HCO_3^- transporters (SLC4 and SLC26 families), by HCO_3^--permeable transmembrane proteins (AE2 and CFTR) (Liu *et al*. 2012), and by carbonic anhydrase activity. All these regulatory enzymes are already present and potentially activated in testicular sperm except for carbonic anhydrase IV (CAR4). This enzyme appears on the sperm surface only when the gamete transits through the epididymal corpus (Ekstedt *et al*. 2004, Wandernoth *et al*. 2010) and is retained on the membranes of mature epididymal sperm (Stein *et al*. 2006) and ejaculated sperm (Ficarro *et al*. 2003).

During epididymal transit, the transport of [HCO_3^-] and HCO_3^- in the spermatozoa decreases significantly.

In the caput epididymis, the transport is over three times higher than that in the corpus and cauda epididymis (Okamura *et al*. 1988). For mature spermatozoa, the intracellular [HCO_3^-] is also regulated by the diffusion of CO_2, but the significance of such a regulatory pathway is still unclear (Carlson *et al*. 2007) since *Cah4*-KO male mice remain fertile (Shah *et al*. 2005).

sAC is now well established as a bicarbonate chemosensor for immature and mature spermatozoa. *In vivo* bicarbonate modulates the enzymatic activity of sAC directly to produce cAMP under pH-independent conditions (Chen *et al*. 2000). A high concentration of bicarbonate should thus be associated with high cAMP production and the reverse. However, the relationship between bicarbonate and sAC activity has not been elucidated for immature sperm. Indeed, for testicular sperm and for spermatozoa from the initial part of the epididymis, the luminal HCO_3^- concentration is the highest and therefore the (cAMP) level is predicted to be high also, but in fact all the values available are the opposite. Furthermore, the presence of 25 mM bicarbonate has been shown to stimulate the cAMP content of cauda spermatozoa more than the caput content (Baker *et al*. 2003). Therefore, the luminal bicarbonate concentration affects the activity of the sperm sAC, but a high concentration of bicarbonate is not always associated with high intracellular cAMP content *in vivo* and an increase in bicarbonate content *in vitro* does not initiate the motility of caput spermatozoa.

Low levels of extra- and intracellular bicarbonate in the epididymis in mature sperm reduce sAC activity, and consequently the intracellular cAMP levels, and maintain the sperm in a quiescent state during transit and storage *in vivo* (Jones & Murdoch 1996). An increase in bicarbonate concentration activates sAC in a few minutes in a pH-independent manner for mature sperm (Chen *et al*. 2000) and rapidly reverses the motility quiescence (Okamura *et al*. 1985), accelerating flagella beat frequency (Wennemuth *et al*. 2003*b*) and changing the membrane characteristics (Gadella & Harrison 2002).

Role of luminal calcium in epididymal spermatozoa motility

The concentration of Ca^{2+} is another important factor in the control of epididymal sperm motility through the regulation of sAC (Morton *et al*. 1974, Armstrong *et al*. 1994) and through other calcium pathways independent of cAMP and PKA such as the calmodulin (CaM) pathway (Jaiswal & Conti 2003).

Significant luminal variations in Ca^{2+} concentrations surround spermatozoa during epididymal transit. Between the caput and the distal cauda epididymis, free luminal ionic calcium concentration decreases from 0.8 to 0.25 mM in the rat epididymis (Jenkins *et al*. 1980). The luminal Ca^{2+} ion is mostly absorbed by the epithelium TRPV6 channel, with the Ca^{2+} concentration being increased tenfold in the caudal epididymal fluid when the *Trpv6* gene is deleted (Weissgerber *et al*. 2012).

Cellular calcium levels have been reported to be six times higher in caput spermatozoa than in caudal spermatozoa, and the rate of calcium uptake in caput spermatozoa to be about two to three times higher than that in caudal spermatozoa (Vijayaraghavan *et al*. 1989, White & Aitken 1989). The high calcium concentration in caput spermatozoa could be linked to a higher rate of mitochondrial calcium accumulation in caput sperm than in caudal sperm (Vijayaraghavan *et al*. 1989), to the presence of the cytoplasmic droplet, which possesses relatively high levels of calcium, and also to the immaturity of the calcium regulatory pathways (Okamura *et al*. 1992).

The low retention of intracellular calcium by the sperm is controlled by at least two Ca^{2+}-ATPases (Wennemuth *et al*. 2003*a*). The first, ATP2A (SERCA)1–3, a sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, sequestrates calcium in the acrosome, mitochondria, and probably also in the cytoplasmic droplet. The other, ATP2B1–4, a plasma membrane Ca^{2+}-ATPase, is the most active in Ca^{2+} clearance, exporting Ca^{2+} outside the gamete and mainly located on the membrane of the proximal principal piece of the flagellum. The activity of this Ca^{2+}-ATPase pump increases significantly from the caput sperm to the epididymal cauda sperm (Sanchez-Luengo *et al*. 2004). The transfer of epididymal Ca^{2+}-ATPase isoform 4 (PMCA4a) from the apical membrane of the cauda epididymal epithelium to the sperm membrane has recently been reported to be associated with the low Ca^{2+} concentration in cauda sperm (Brandenburger *et al*. 2011).

Several constitutive channels have been evidenced for cellular Ca^{2+} entry (see review by Darszon *et al*. (2006)) such as voltage-gated Ca^{2+} channels (CaVs; Benoff *et al*. 2007) and sperm-specific cation channels known as CatSper (CatSper1–4 and CatSperβ, CatSperγδ and CatSperγ) (Quill *et al*. 2001, Ren *et al*. 2001, Lobley *et al*. 2003, Wang *et al*. 2009, Chung *et al*. 2011).

The role of Ca^{2+} in the maturation of sperm motility in the epididymis is unclear. *In vitro*, physiological [Ca^{2+}] directly activates sAC and increases (cAMP) production by mature sperm (epididymal or ejaculated) independently of CaM binding to sAC (Jaiswal & Conti 2003). Furthermore, an additive effect of HCO_3^- and Ca^{2+} on sAC activity has also been observed on mature epididymal sperm (Jaiswal & Conti 2003). The influx of Ca^{2+} is required to activate motility in most caudal epididymal sperm samples. This Ca^{2+} entry is not immediately linked to the CatSper channel, since the deletion of any genes of CatSper isoforms does not prevent the activation of quiescent epididymal sperm (Ren *et al*. 2001, Quill *et al*. 2006).

Calcium combined with HCO_3^- ions is able to speed up the flagellar beating of mature sperm *in vitro*

(Carlson *et al*. 2007) and to change the symmetrical flagellar wave propagation, according to the level of internal [Ca^{2+}] (Lindemann *et al*. 1991). On the other hand, the removal of Ca^{2+} prevents this speeding up of flagellar beating (Carlson *et al*. 2003). The low concentration of luminal and intracellular sperm [Ca^{2+}] in the terminal part of the epididymis thus contributes to the maintenance of sperm motility quiescent for mature epididymal sperm.

The action of Ca^{2+} is more complex for immature sperm. As for [HCO_3^-], high [Ca^{2+}] content in the anterior part of the epididymis is expected to activate sAC and consequently cAMP and flagellar activity, but such activation has never been described. However, the calcium concentration gradient in the luminal fluid seems to be important. After the deletion of the TRPV6 calcium channel, which prevents absorption of luminal Ca^{2+}, the motility of mature caudal sperm cannot be normally activated (Weissgerber *et al*. 2012). Recently, several β-defensins (Supplementary Table 1) specifically secreted in epididymal caput have been shown to be associated with the activation of Ca^{2+} channels and initiation of motility of immature spermatozoa *in vitro* (Zhou *et al*. 2004).

For these immature spermatozoa, the progressive decrease in [Ca^{2+}] concentration during epididymal maturation and appropriate regulation of intraluminal Ca^{2+} concentration by the epididymal epithelium are thus essential for the establishment of potential sperm motility, but the exact effect of such changes in Ca^{2+} concentration is still unclear.

Sperm protein phosphorylation during epididymal transit

The flagellar movement is regulated by a network of kinases and phosphatases that induce numerous post-transcriptional protein modifications, mostly phosphorylations, in various compartments of the flagellum and axonema. Two signalling pathways involved in the phosphorylation of these proteins, the cAMP–PKA and calcium signalling pathways, are generally recognized to be essential for the regulation of mammalian sperm motility (Suarez *et al*. 1987, Ho & Suarez 2001).

In the epididymis, the development of a cAMP–PKA-dependent pathway leading to protein tyrosine phosphorylation has often been reported in concert with the sperm's ability to undergo capacitation. The increase in cAMP production induced by the spermatozoa in the epididymis is associated with the progressive development of a cAMP–PKA-dependent signalling pathway, which is parallel to the potential activation of motility (Pariset *et al*. 1985, Jaiswal & Majumder 1996*a*, Baker *et al*. 2003). PKA subunits are associated with the flagella. After activation by cAMP, the PKA RII regulatory subunit transits to a specific location between the coarse fibres (Pariset *et al*. 1989), bound to the sperm-specific AKAP3 and/or AKAP4, the main constituents of fibrous sheath proteins of the sperm flagellum (Miki *et al*. 2002; see review by Luconi *et al*. (2011)). The free catalytic subunits of PKA induce serine/threonine (Ser/Thr) phosphorylation for several proteins including AKAPs and activate several tyrosine kinases downstream, such as ABL1 (ABL), CSK, SRC (cSRC), and TEC, and many others that remain to be identified (Battistone *et al*. 2013).

The rates of tyrosine phosphorylation and phosphorylated proteins appear to be different between immature and mature spermatozoa. In the rat, immature epididymal spermatozoa exhibit more tyrosine phosphorylation than mature sperm (Lewis & Aitken 2001, Baker *et al*. 2003), but most of this phosphorylation is not related to cAMP or bicarbonate concentrations (Baker *et al*. 2003). On the other hand, in the mouse, the cAMP-dependent tyrosine phosphorylation is more active in mature epididymal sperm (Visconti *et al*. 1995, Ecroyd *et al*. 2004, Lin *et al*. 2006). The cAMP signal transduction pathway appears not to be fully functional in immature sperm, whatever the species differences.

The low activity of the cAMP-dependent tyrosine phosphorylation pathway in immature sperm has been attributed in part to the inhibitory effect of high levels of $[Ca^{2+}]_i$ (Ecroyd *et al*. 2004). However, the decreasing $[Ca^{2+}]_i$ content in caput spermatozoa does not activate motility, although the cAMP-mediated signal transduction pathway is effectively activated (Ecroyd *et al*. 2004).

However, the initiation and stimulation of motility for caput epididymal spermatozoa have been shown to be induced, independently of calcium, by the inhibition of Ser/Thr-protein phosphatase I (PPI) activity (Vijayaraghavan *et al*. 1996). In most cells, PPI activity is controlled by the presence of specific inhibitors such as inhibitor-2 (I2), which forms a PPI–I2 complex, and by glycogen synthase kinase 3 (GSK3), which can reactivate PPI by dissociation of this complex. In epididymal sperm, the presence of twofold higher levels of PPI activity, identified as PP1γ2, and sixfold higher levels of GSK3 activity than in mature motile caudal sperm can be linked to the weak activity of the cAMP-mediated signal transduction pathway in immotile caput epididymal sperm (see review by Fardilha *et al*. (2011)).

Recent studies on the mature spermatozoa of mice (Baker *et al*. 2006, Krapf *et al*. 2010) and humans (Battistone *et al*. 2013) have revealed that a cSrc family kinase (SFK) induced the inactivation of Ser/Thr phosphatases and could be involved in the signalling pathways associated with sperm capacitation and also in sperm motility. This tyrosine kinase is detected in the flagellar midpiece of mature mouse sperm, but surprisingly this kinase is not present in sperm from the caput epididymis. This cSrc is incorporated into spermatozoa (Krapf *et al*. 2012) during their transit from the corpus to the cauda. Normal tyrosine phosphorylation is displayed in *cSrc*-null mice, but forward sperm motility is significantly reduced (Krapf *et al*. 2012). However, there are differences between mature mouse sperm

and human sperm for such effects of SFK activity on motility (Varano *et al*. 2008, Krapf *et al*. 2010, Battistone *et al*. 2013). The hypothesis that this kinase is important during epididymal sperm maturation is strong, but needs further investigation.

The involvement of the 'endocannabinoid system' in the development of sperm motility in the epididymis has recently been proposed (Cobellis *et al*. 2010). The low levels of cAMP production induced by sAC from immature sperm may be related to the inhibitory effect on sAC exerted by the activated cannabinoid receptor CNR1 present on the sperm cell membrane. The inhibitory activity of the CNR1 receptor during epididymal transit may be induced by the decrease in 2-arachidonoylglycerol (2-AG) levels from the caput to the cauda. Such a decrease in 2-AG levels may hypothetically be associated with certain sperm membrane modifications that remain to be identified.

Another pathway, PI3K–AKT, has also been found to be involved in the phosphorylation of target proteins associated with mature sperm motility (Luconi *et al*. 2011, Sagare-Patil *et al*. 2013), but its activity during epididymal transit is yet to be demonstrated.

Epididymal surroundings and sperm proteins involved in egg-binding components

The development of sperm motility during transit in the epididymis is the first essential step for the spermatozoa to acquire fertility, but motile activated testicular spermatozoa are never fertile. The second important maturation step is the gradual acquisition of the ability to bind to the ZP and the oocyte membrane. The plasma membrane, i.e. the inner acrosomal membrane and the equatorial segment exposed to the sperm surface after the acrosome reaction, participates in interactions with the egg plasma membranes. The interactions between the male and female gametes are the result of successive sperm surface remodelling initiated in the testis, during epididymal transit and ending in the female tract after capacitation. The sperm plasma membrane proteins involved in ZP binding have been the most studied ones.

Different types of methodologies have been used to identify sperm and epididymal proteins involved in sperm–egg membrane interactions. Most of them have been developed using specific sperm antibodies (competitor or inhibitor) to prevent the binding of a candidate protein to the ZP or oocyte membrane. More global approaches have also been developed such as phage display technology (Naz 2005, Samoylova *et al*. 2012), and the ZP affinity properties of sperm proteins have been analysed using different global and selective proteomic approaches (Stein *et al*. 2006, Brewis & Gadella 2010, Belleannee *et al*. 2011*a*, Guyonnet *et al*. 2012, Petit *et al*. 2013). Many protein candidates have been proposed following these *in vitro* studies, and more than 50 proteins have already been reported to be involved in sperm–egg interactions (Supplementary Table 1). However, the effective role of several of them in male fertility has not been confirmed, as no significant modification has been observed after their gene deletion. More surprising is the fact that when infertility was induced after gene KO for some of these proteins, sterility was found to be not linked to the lack of sperm binding to the oocyte components, as expected, but to a modification of sperm motility or to an unknown sperm surface component preventing the gamete from passing through the uterotubal junction (Supplementary Table 1). Furthermore, the gene deletion technique provided the opportunity to identify several other sperm proteins (ACE, CLG, HSPA2, PDILT or TPST2) (Supplementary Table 1) not directly involved in sperm–egg interactions, but linked to male sterility, although the sperm production, morphology and motility of the KO male mice were normal.

The most important role of the epididymis in the development of the sperm–egg interactions may be linked to the control of the processing and domain redistribution of several testicular sperm surface proteins, particularly for different members of the ADAM protein family (e.g. ADAM1B, ADAM2, ADAM3, ADAM5 and ADAM32) and also for other proteins such as tACE, basigin and TEX101 (Supplementary Table 1). Most of these surface changes, occurring principally in the first part of the epididymis, are linked to the local activation of proteolytic activities either on the sperm surface itself or from luminal components. However, no luminal protease activities already identified in the epididymal tubule have formally been reported to be linked to such sperm surface processes (Metayer *et al*. 2002). These testicular sperm surface modifications may be associated with the development of the binding properties of the spermatozoa, but synchronization between these two events during epididymal transit is not obvious. The development of egg–sperm binding may also be induced by the adsorption or fusion of several epididymal proteins to the sperm membrane (e.g. ARSA, CD52, CRISP1, CRISP4, CRISP7, DCX, EPPIN, MFGE8, SPAM1 and SPINK13). The contribution of each of these epididymal proteins to the egg-binding properties of mature epididymal sperm is difficult to evaluate, as no gene KO of these proteins has induced a severe reduction in male fertility to date (Supplementary Table 1).

Nevertheless, among the many proteins reported to have binding affinity to the ZP and oocyte membrane, two appear to be more strongly associated with sperm fertility. The first, IZUMO, is a newly discovered member of the immunoglobulin superfamily but apparently not involved in the epididymal sperm maturation process, since it is detectable on the mature sperm surface only after the acrosome reaction. The complete sterility of *Izumo1*$^{-/-}$ male mice is induced by a defect in sperm–egg fusion, although the sperm is able to bind

to and penetrate the ZP and also to bind to the egg plasma membrane (Inoue *et al*. 2005). The egg partners of IZUMO and its role in the egg fusogen function remain to be determined.

The second is ADAM3 (cyritestin), a 42 kDa surface protein of epididymal sperm resulting from the processing of a 110 kDa testicular precursor in the first part of the epididymis. The sterility of *Adam3*-deficient male mice is mainly linked to a lack of sperm migration from the uterus to the oviduct. *Adam3*$^{-/-}$ mouse sperm have poor binding affinity to the ZP and egg plasma membrane *in vitro*, but are fertile after oviduct AI or IVF with cumulus-intact and ZP-intact eggs. The same phenotype of *Adam3*$^{-/-}$ male sterility is also obtained after gene KO for at least ten sperm proteins (i.e. ACE, CLGN, ADAM2, ADAM1A, CALR3, TPST2, PDILT, PMIS2, PRSS37, and TEX101) (Supplementary Table 1). Among all these KO genes in male mice, *Adam3* is strongly reduced or incorrectly located on the mature epididymal sperm surface. The loss of ADAM3 from the mature sperm surface was also observed after *Rnase10* gene KO, a protein secreted only in the initial segment of the epididymis and without any sperm membrane affinities (Krutskikh *et al*. 2012). Such results show that the stability, integrity, and location of ADAM3 at the sperm surface are linked to the presence and/or activities of several other proteins. In the testis, several chaperone proteins (e.g. calmegin, CALR3, and PDILT), different ADAM proteins associated in a complex with ADAM3 (ADAM1b, ADAM2 and ADAM6), and other proteins such as TPST2 and ACE (see review by Cho (2012)) are essential for the location of ADAM3 on the sperm surface. In the epididymis, the activity of unknown proteases is needed to cleave the prodomain and MMP domains of ADAM3, which is also dependent on the removal of TEX101 from the sperm surface in the epididymis (Fujihara *et al*. 2013).

Thus, when one or more of these essential proteins for ADAM3 are missing, all or a great number of the ADAM3 molecules (Cho 2012) are specifically removed from the sperm surface during epididymal transit by the activity of an unknown protease. Therefore, ADAM3 seems to be a key protein in male fertility, but its removal from the sperm surface only prevents the sperm from passing through the uterotubal junction but not from fertilizing cumulus-intact eggs after oviduct insemination. Furthermore, such an ADAM protein is not present in human sperm, since human *ADAM3* genes are not found to be functional (Grzmil 2001).

The mechanisms involved in sperm–egg interactions in mammals (including humans) are still unclear. The interactions between male and female gametes appear to be more complicated than a simple ligand–receptor model. Most of the sperm proteins involved in egg–membrane interactions, such as the ADAM protein family, are present in very low quantities at the sperm surface and can only be visualized at the sperm surface by sensitive techniques such as immunodetection. Therefore, it is realistic that the binding force between the sperm and the egg generated by only one of these proteins would not be enough to maintain such an interaction *in vivo*. It is now accepted that more than one protein is involved and that these proteins could be combined together in a protein complex (Reid *et al*. 2011), potentially included in the lipid raft domains of the sperm membrane. Such a protein complex might be formed by several ADAMs and also by other known or as-yet-unknown sperm components such as HSPA2, HSPA5, ARSA, SPAM1, or CCT/TRiC complex and ITM2B, already identified as major sperm surface proteins (Belleannee *et al*. 2011*a*, Bromfield & Nixon 2013; Fig. 2). In the epididymis, an abnormal composition of such a sperm surface complex might induce abnormal epididymal protease activity towards some of these sperm surface proteins such as ADAM3.

The identification of sperm proteins and the exact role of the epididymis in the molecular mechanisms really involved in gamete interactions is still a challenge. Despite the complexity, the role of epididymal maturation in the development and/or the control of the egg–sperm binding properties is indisputable.

Conclusion

The epididymal maturation of spermatozoa is an essential post-testicular stage in the acquisition of fertility by the male gamete. During the 1 or 2 weeks of transit of the sperm in this organ, subtle sequential changes occur successively in several subdomains of the gamete. In spite of the significant improvement in the understanding of the protein composition of the spermatozoa and the molecular mechanisms involved in both the motility process and egg binding during the last 10 years, the role of the epididymis is still not fully explained. Different signalling pathways between immature and mature spermatozoa are now well described, but their targets, such as the motility process, initiation of capacitation, or other metabolism changes, have not been fully identified. The role of the epididymal surroundings in the activation or inactivation of several of these sperm signalling pathways is undeniable, but the mechanisms by which several luminal components such as ionic components (calcium, pH, and HCO_3^-) exert either inhibitory or stimulatory effects according to the maturation stage of the gamete remain to be fully elucidated. Furthermore, the fact that the sperm modifications occurring during epididymal transit have a physiological effect only when the gametes are in the female tract make the *in vitro* evaluation of such modifications more complicated and perhaps liable to misinterpretation. This may be the case for the sperm surface proteins involved in the egg-binding components.

With the recent increase in the understanding of the protein composition of spermatozoa and the epididymal

surroundings, it is now evident that there are considerable differences between species. The post-testicular maturation of the spermatozoon is a general process in mammals, but most of the recent results, in particular, those obtained using KO genes, have been obtained from rodent studies. The study of epididymal sperm maturation in other species will be an important step in the understanding of the species specificity of sperm fertility, particularly for humans.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-13-0420.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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