The impact of epididymal proteins on sperm function

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Abstract

The epididymis is necessary for post-testicular sperm maturation as it provides the milieu required for spermatozoa to gain the ability for progressive movement and fertilization. In the epididymis the sperm protein, lipid and small RNA content are heavily modified due to interaction with luminal proteins secreted by the epididymal epithelium and extracellular vesicles, epididymosomes. This review focuses on epididymal proteins demonstrated to have an effect on sperm functions, such as motility, capacitation, acrosome reaction, sperm-zona pellucida binding and sperm-egg binding, as well as on embryonic development.

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Introduction

After the immature spermatozoa leave testis they acquire the ability to move progressively forward and fertilize the oocyte while being transported through the epididymis. During epididymal sperm maturation, the sperm membrane is under constant remodeling, with attachment and shedding of molecules in a sequential manner. Many of the proteins affecting sperm maturation are secreted into the epididymal lumen where they come into contact with sperm. Another means to deliver proteins as well as other cargo to sperm is the use of extracellular vesicles called epididymosomes. Epididymosomes contain proteins (Nixon *et al.* 2019), small non-coding RNAs (Reilly *et al.* 2016, Sharma *et al.* 2016) and lipids (Girouard *et al.* 2011) that are delivered to maturing sperm. The exact mechanism by which the epididymosomes deliver their cargo to sperm is still unclear, but it has been suggested that after GPI-anchor-mediated docking, the membranes of epididymosomes and sperm fuse. The other putative, and potentially bi-directional, mechanism of delivering cargo is a formation of transient fusion pores between epididymosomes and sperm (reviewed by Sullivan 2015, Zhou *et al.* 2018). As a consequence, the sperm proteome (Belleannee *et al.* 2011, Ijiri *et al.* 2011), lipid composition (Gervasi & Visconti 2017) and small RNAs (Hutcheon *et al.* 2017) change during the transit from the proximal initial segment (IS) to the distal cauda.

The elegant work by Bedford and Orgebin-Crist demonstrated the importance of epididymal transit for acquisition of sperm progressive motility and fertilizing capacity already in the 1960's (Bedford 1963,

Orgebin-Crist 1967). The significance of epididymal transit was further confirmed by using genetically modified (GM) mouse models. Knockout (KO) models of *Ros1* (also known as c-ros, (Sonnenberg-Riethmacher *et al.* 1996, Yeung *et al.* 1999), leucine-rich repeat-containing G protein-coupled receptor 4 (LGR4, Hoshii *et al.* 2007) and *Pten* (Xu *et al.* 2014) as well as the transgenic GPX5-Tag2 mice (Sipilä *et al.* 2002) demonstrated that a lack or dysfunction of the proximal epididymal epithelium leads to male infertility. Many of the above mentioned models exhibited a hairpin bend of the sperm tail caused by a failure of the sperm to regulate the intracellular osmotic pressure. Another frequently seen defect in these models is a change in sperm motility.

GM models in which the gene regulatory programs are disturbed have demonstrated several regulatory pathways necessary for proper epididymal functions and subsequent male fertility. The importance of androgens for epididymal gene expression have been long known, however, as those early experiments involved removal of testes as a source of androgens, the importance of androgen regulation of the given genes for male fertility could not be proven. GM models with conditional androgen receptor (AR) deletion either in developmental precursors of the epididymis, Wolffian duct or IS or caput epididymidis resulted in dedifferentiation of the epithelium, obstruction of the duct and male infertility (Krutskikh *et al.* 2011, Murashima *et al.* 2011, O'Hara *et al.* 2011). AR mutations affecting its functions further confirmed the necessity of AR to epididymal sperm maturation as both, Specificity-affecting AR knockin (SPARKI, Schauwaers *et al.* 2007, Kerkhofs *et al.* 2012)

and SUMOylation-deficient AR (ArKI, Zhang *et al.* 2019) mice, were present with subfertility or infertility, respectively. Although there were only subtle changes in the epididymal epithelium, both models displayed similar defects in sperm motility. Despite phenotypic similarities, the underlying molecular mechanisms for these defects appear to be different in these mouse models, as the mutations resulted in distinct changes in the gene expression in the epididymides with only a handful of genes changed in both models (Sahu *et al.* 2014, Zhang *et al.* 2019).

The miRNA pathway is a well-known post-transcriptional regulator of protein levels. Conditional deletion of *Dicer1*, a necessary RNaseIII enzyme in miRNA processing, from the proximal epididymis resulted in dedifferentiation of the epididymal epithelium demonstrating the importance of miRNA-mediated regulation for epididymal maintenance (Björkgren *et al.* 2012). Interestingly, lack of *Dicer1* also caused problems in lipid synthesis in the epididymal epithelium and a consequent imbalance of cholesterol and long chain polyunsaturated fatty acids (PUFAs) in the sperm membrane, detachment of the sperm head from the tail and breakage of the acrosome region (Björkgren *et al.* 2015). Whereas individual epididymal miRNAs have not been linked to sperm dysfunctions, a recent paper described a correlation between unexplained asthenozoospermia (UA) and dysregulation of five members of the X-linked primate-specific epididymal miRNA cluster. miRNA analysis of semen showed that 13% of UA patients had lower levels of the miRNAs in question compared to normospermic controls and further, the levels of miRNAs correlated with sperm progressive motility (Qing *et al.* 2017). Intriguingly, epididymally produced small RNAs, such as tRNA fragments and miRNAs are trafficked from the epididymal epithelium to maturing sperm. They have been shown to transfer epigenetic information to offspring and are essential for normal embryonic development in mice (Sharma *et al.* 2016, 2018, Conine *et al.* 2018).

In all the above mentioned models, the epididymal environment changes so drastically that problems in several signaling pathways are likely to contribute to the loss of fertility and thus it is impossible to pinpoint any individual molecule behind defects in sperm function. In this review, we focus on individual epididymal proteins that, although not all epididymis specific, have been shown to affect sperm functional parameters, such as motility and capacitation, and subsequently male fertility (Fig. 1). In addition to the proteins mentioned in this review, there is a large group of proteins that are known to be expressed both in the testis and in the epididymis which are required for sperm function. However, as it is not clear how the epididymal expression of those proteins per se contribute to sperm function, those genes were not included in this review.

Figure 1 Epididymal proteins affecting different sperm functions during fertilization. I. Epididymal proteins affecting sperm calcium signaling, II. Epididymal proteins modifying sperm proteins, III. Epididymal proteins affecting sperm membrane lipid composition, IV. Epididymal proteins involved in cell–cell interactions.

Epididymal proteins affecting sperm calcium signaling

The activation of sperm cells in the female reproductive tract mainly relies on an increased influx of calcium ions. The signaling pathway elicited by Ca^{2+} uptake leads to capacitation of sperm cells with marked increase in protein phosphorylation and the ability of sperm to display hyperactive motility (Yanagimachi 1994, Kirichok *et al.* 2006). Hyperactivation is characterized as an increase in flagellar bend amplitude and asymmetry which gives the sperm the powerful tail strokes required to reach the egg. In close proximity to the egg, the increased calcium signaling causes the sperm to go through the acrosome reaction (Yanagimachi 1994). The release of the acrosome content reveals egg-binding proteins on the inner acrosome membrane and allows the gametes to fuse. As Ca^{2+} signaling drives sperm motility and fertility in the female reproductive tract, ion influx and efflux is highly regulated during sperm maturation. For example, increased levels of the epididymal Ca^{2+} -ATPase isoform 4 (PMCA4a) was detected in bull caudal sperm where it contributes to maintaining the low intracellular Ca^{2+} levels needed for proper sperm activation (Brandenburger *et al.* 2011). Furthermore, several proteins secreted by the epididymal epithelium bind to sperm and regulate Ca^{2+} channels in the sperm membrane. This chapter will describe the proteins/protein families whose main function is to regulate Ca^{2+} channels in more detail.

CRISP1 and CRISP4

Members of the CRISP protein family are known to function as ion channel blockers in snake venom and are thought to serve a similar function when expressed

in the male reproductive tract of mammals (Yamazaki & Morita 2004). In support of this, the incubation of sperm cells with CRISP1 resulted in reduced activation of the calcium ion channel CATSPER1 (Ernesto *et al.* 2015) while CRISP4 was shown to inhibit the transient receptor potential M8 (TRPM8) ion channel in mouse sperm (Gibbs *et al.* 2011). CRISP1 was first identified in the principal cells and lumen of rat cauda (Cameo & Blaquier 1976). Here it binds to sperm in two isoforms; D, which is only transiently bound to sperm and released during capacitation, and the smaller isoform E, which is more tightly bound to sperm (Roberts *et al.* 2008). CRISP1 D is considered to be a decapacitating factor as incubation of rat sperm with exogenous CRISP1 led to reduced protein phosphorylation and inhibition of the acrosome reaction (Roberts *et al.* 2003). However, this effect seems to be species specific as sperm of *Crisp1*-knockout mice displayed lower levels of protein tyrosine phosphorylation, although the sperm were able to acrosome react and the mice were fully fertile in normal breeding (Da Ros *et al.* 2008). Interestingly, the genetic background of the animals also play a role in the severity of the phenotype. While mice of a mixed (129/SvEv and C57Bl/6) background showed reduced levels of tyrosine phosphorylation, a pure C57Bl/6 background led to defects in both motility and in the induction of the acrosome reaction, although the levels of phosphorylated proteins in capacitated sperm were similar to those of WT mice (Weigel Muñoz *et al.* 2018).

The phenotype of *Crisp1*-knockout sperm can partially be compensated for by the presence of CRISP4, which is bound to rodent sperm during transit through the proximal epididymis. Deletion of *Crisp4* in mouse led to a reduced induction of the acrosome reaction (Gibbs *et al.* 2011, Turunen *et al.* 2012, Hu *et al.* 2018). The altered acrosome reaction was also thought to affect the interaction of sperm with ZP, as one study showed reduced ZP binding and fertility of knockout sperm (Turunen *et al.* 2012). In addition, a study by Hu *et al.* (2018) indicated a role for CRISP4 in capacitation and sperm motility (Hu *et al.* 2018). The discrepancy between the *Crisp4* KO phenotype in the different studies could, like CRISP1, be due to different genetic background of the mice (Weigel Muñoz *et al.* 2018). This has also been observed in *Crisp1/Crisp4*-double mutants which show a more prominent, but variable decrease in sperm fertility parameters compared to the single knockout animals. The double KO generated by Carvajal *et al.* (2018) led to a subset of mice displaying an inflammation phenotype of the epididymal epithelium which caused decreased viability of sperm. Even without signs of epididymo-orchitis, there was a significantly increased pH of the epididymal lumen, which could give rise to a multitude of defects during sperm maturation (Carvajal *et al.* 2018). On the other hand, the double KO generated by Hu *et al.* only showed

signs of epididymal inflammation in older animals, with younger males displaying similar litter sizes as WT mice (Hu *et al.* 2018). It is interesting to note the significant effect of genetic predisposition to inflammation when considering how the human epididymis only expresses CRISP1, the functional ortholog of both rodent CRISP1 and CRISP4 (Jalkanen *et al.* 2005, Nolan *et al.* 2006). Studies of the genetic differences that give rise to the non-infectious epididymitis could therefore also give us new information on human disease and fertility problems.

Beta-defensins

Similar to the CRISP protein family, DEFB family members are also thought to regulate ion channel activity. Beta-defensins belong to a large family of antimicrobial peptides predominantly expressed in the male reproductive tract, and more precisely, in the epididymis (reviewed in Dorin & Barratt 2014). In addition to host-defense function, several beta-defensins have been shown to have a role in sperm maturation. SPAG11E (also known as Bin1b) and DEFB15 bind to the sperm head during epididymal transit and induce sperm progressive motility *in vitro* in rat (Zhou *et al.* 2004, Zhao *et al.* 2011). Deletion of mouse *Defb41* did not affect male fertility *in vivo*, but advanced *in vitro* imaging and analyzing techniques revealed an altered flagellar beat pattern of capacitated *Defb41*-knockout sperm and consequently altered sperm velocity and a reduction in oocyte binding (Björkgren *et al.* 2016). The mild phenotypic changes in the mouse and rat models introduced above might be due to compensatory functions of other beta-defensins present in the epididymal fluid. Indeed, a series of knockouts of rat beta-defensins, namely *Defb23*, *Defb26* and *Defb42*, demonstrated that as single gene knockouts, they did not cause any change in sperm motility or male fertility, whereas animals carrying double, *Defb23/26*, or triple, *Defb23/26/42*, gene deletions were subfertile and demonstrated decreased sperm motility, precocious capacitation and increased spontaneous acrosome reaction (Zhang *et al.* 2018). Similarly, a concurrent deletion of nine mouse beta-defensins, *Defb1*, *Defb50*, *Defb2*, *Defb10*, *Defb9*, *Defb11*, *Defb15*, *Defb35* and *Defb13* from the beta-defensin gene cluster on chromosome 8, caused reduced sperm motility and increased fragility with disintegration of the microtubule structure of *DefbΔ9* sperm. Furthermore, *DefbΔ9* sperm displayed precocious capacitation, increased spontaneous acrosome reactions and reduced ability to bind to oocytes (Zhou *et al.* 2013). Moreover, in contrast to *Defb1*-knockout mice which do not have a reproductive phenotype (Morrison *et al.* 2002), significantly reduced levels of human DEFB1 were found in sperm of infertile men with low sperm motility (Diao *et al.* 2014). This suggests that the larger rodent

beta-defensin family have more overlapping functions compared to their human homologs.

The antimicrobial properties of beta-defensins are due to the ability of these cationic molecules to bore holes into phospholipid membranes (Hall *et al.* 2007). This mechanism has also been shown to activate L-type Ca^{2+} channels (Bateman *et al.* 1996). Studies with SPAG11E indicate that it is activating sperm Ca^{2+} channels, leading to Ca^{2+} influx into sperm (Zhou *et al.* 2004). In addition, DEFB1 was shown to interact with chemokine receptor type 6 (CCR6) and thereby triggering a rise in intracellular Ca^{2+} (Diao *et al.* 2014). In contrast, both animal models with several beta-defensins deleted, showed increased intracellular Ca^{2+} content of the sperm, which was suggested to lead to premature hyperactivation and spontaneous acrosome reaction of the knockout sperm (Zhou *et al.* 2013, Zhang *et al.* 2018). Thus, the role of DEFBs in controlling the function of Ca^{2+} channels is still unclear. It is however possible that different DEFBs regulate different Ca^{2+} channels with varying outcomes. Moreover, the molecular mechanism of sperm Ca^{2+} channel regulation is unsolved and whether the hole-boring ability of beta-defensins has a role here remains to be studied.

A distinctive mode of action has been described for DEFB126 (mouse and rat homolog DEFB22). DEFB126 is produced in the corpus epididymidis, from where it is secreted, binds to the sperm surface and forms an integral part of the sperm glycocalyx (Tollner *et al.* 2012). It has a highly glycosylated C-terminal tail that provides a negatively charged coating for sperm, necessary for spermatozoa to swim efficiently in the cervical mucus in macaques (Tollner *et al.* 2008a). When sperm reaches the oviduct, DEFB126 is critical for attachment of the sperm to the oviductal epithelium. The removal of DEFB126 from the sperm membrane was shown to be necessary for sperm release from the oviductal reservoir at the time of ovulation, and conditions simulating periovulatory oviductal fluid quickly induced both shedding of DEFB126 from the sperm membrane and capacitation (Tollner *et al.* 2008b). In addition, experimental data suggest that the sialylated oligosaccharides of DEFB126 on the sperm glycocalyx effectively mask other protein components on the sperm surface, protecting sperm from immune surveillance in the female reproductive tract (Tollner *et al.* 2012). Interestingly, two human frame-shift mutations of DEFB126 have been shown to affect male fertility. Mutation rs11468374 described by Tollner *et al.* affected the ability of sperm to penetrate the cervical mucus, whereas other sperm parameters, including motility were unchanged. Men homozygous for this frame-shift mutation were found to have a reduced chance of successful conception (Tollner *et al.* 2011). In addition, another described DEFB126 mutation, rs11467497, has significant association with male infertility, without affecting sperm motility (Duan *et al.* 2015).

LCN6

The epididymis of rodents and humans express several lipocalins (LCNs) belonging to a highly conserved gene cluster (Suzuki *et al.* 2004). Lipocalins are extracellular proteins that are able to transport small hydrophobic molecules such as steroids and lipids (Flower 1996). Although LCN proteins are hypothesized to be important for sperm maturation, so far only LCN6 is known to play a role in sperm function. Expression of *Lcn6* in the proximal epididymis leads to binding of the protein to the postacrosomal region in human and mouse sperm cells (Hamil *et al.* 2003, Yin *et al.* 2018). Studies of an *Lcn6*-knockout mouse model showed an important function in regulating Ca^{2+} influx of sperm cells. Although *Lcn6* ablation led to Ca^{2+} overload of sperm cells and, consequently, an increased spontaneous acrosome reaction, the knockout males were fertile and the sperm were motile and able to capacitate in a similar manner to WT sperm (Yin *et al.* 2018). An explanation for this could be that a majority of sperm cells were still acrosome intact after *in vitro* capacitation which would indicate either a minor role for LCN6 or a functional substitution of the protein with other lipocalins still present in the epididymis. Thus, further studies are needed to show how different lipocalins interact during sperm maturation and what the mechanism of LCN6 regulation of ion channel(s) entails.

Epididymal proteins modifying sperm proteins

Several posttranslational modifications (PTMs), such as phosphorylation, glycosylation (Baker 2016) and O-GlcNAcylation (Tourzani *et al.* 2018) of sperm proteins occur during epididymal sperm maturation. The importance of the PTMs is still unclear, but as sperm are transcriptionally silent cells, PTMs provide a means of controlling the protein activity in sperm. As an example of changes in protein phosphorylation status during epididymal transit, the cytoplasmic domain of IZUMO1, a protein necessary for sperm-egg fusion, becomes heavily phosphorylated in the epididymis. It has been postulated that in the case of IZUMO1, phosphorylation would play a role in re-location of the protein in the sperm membrane or during sperm-oocyte fusion (Baker *et al.* 2012). Another example of a protein modified during epididymal transit is ADAM3 (also known as cyritestin), which is required for gamete interaction (Shamsadin *et al.* 1999, Inoue *et al.* 2005). A lack of epididymal cleavage of sperm surface ADAMs (ADAM3, -2, and -6) does not only reduce the activity of the enzymes but also the attachment of the protein complex to the sperm surface (Nishimura *et al.* 2007, Han *et al.* 2009, Krutskikh *et al.* 2012). In the following chapter we will describe proteins known to affect sperm function via PTMs during epididymal maturation, for more information about the PTMs the reader is directed to the following excellent reviews (Baker 2016, Brohi & Huo 2017).

RNase10

The cleavage of ADAMs in the epididymis is segment specific, as seen in the case of *RNase10*, an enzyme expressed in the most proximal region of the mouse and boar epididymides (Penttinen *et al.* 2003, Castella *et al.* 2004). Although *RNase10* lacks ribonuclease activity, it was shown to cleave the immature form of ADAM6 and thereby retain the protein in complex with ADAM3 (Krutskikh *et al.* 2012). In *RNase10*-knockout mice, the consequent loss of ADAM3 from spermatozoa led to a significantly reduced fertility although the sperm cells displayed normal motility, capacitation and acrosome reaction. However, they were not able to fully adhere to ZP although they showed increased ability to fuse with and fertilize eggs *in vitro*. The study by Krutskikh *et al.* (2012) also propose that the IS-specific expression and secretion of the enzyme could lead to cleavage of additional sperm proteins (Krutskikh *et al.* 2012).

INPP5b

Another protein important for sperm maturation and ADAM cleavage is the inositol polyphosphate 5-phosphatase *INPP5b*. Instead of a direct proteolytic activity on sperm cells, *INPP5b* was hypothesized to regulate the availability of proteases for ADAM2 and ADAM3 cleavage in the epididymis (Hellsten *et al.* 2001). Although the phosphatase is expressed in the testis as well as the epididymis, a spermatid-specific knockout of *Inpp5b* did not cause similar defects as those observed in the full knockout animals where spermatozoa displayed both reduced motility and an inability to bind to ZP and fuse with the egg (Hellsten *et al.* 2001). The phenotype was linked to a defect in ADAM2 cleavage during transit of sperm from caput to cauda. However, mouse of different background display phenotypes of varied severity and a later study by Marcello and Evans (Marcello & Evans 2010) showed little correlation between ADAM2 and ADAM3 processing and the fertility phenotype, indicating that *INPP5b* is able to regulate the cleavage of additional proteins during sperm maturation. In addition, since neither the *Inpp5b* nor the *RNase10*-knockout mice showed complete inhibition of ADAM cleavage, other, still unknown, proteins are probably also utilized for this process.

Wnt signaling

An elegant example of how modification of sperm proteins during epididymal transit affect sperm motility came from studies by Koch and colleagues who demonstrated that epididymal Wnt is an important regulator of sperm motility through inhibition of glycogen synthase kinase-3 (GSK3) (Koch *et al.* 2015). It has been known for over 20 years that inhibition of Ser/Thr-protein phosphatase I catalytic subunit gamma 2, PPP1CC2 (previously

known as PP1 γ 2), activity in caput sperm induces motility (Vijayaraghavan *et al.* 1996). The early studies with bovine sperm suggested that in caput sperm, a high activity of GSK3 inhibits the activity of the PPP1CC2 inhibitor, protein phosphatase 1, regulatory inhibitor subunit 2, PPP1R2 (previously known as I2). In cauda sperm, the reduced activity of GSK3 results in activation of PPP1R2, subsequent inactivation of PPP1CC2 and induction of sperm motility (Vijayaraghavan *et al.* 1996). A more recent study in mouse further demonstrated that three PPP1CC2 inhibitors, PPP1R2, -R7 and -R11, are present in mouse sperm where they co-localize with PPP1CC2 in the head and the principal piece. In immotile caput sperm, PPP1R2 and PPP1R7 are not bound to PPP1CC2, whereas in motile caudal sperm, all three inhibitors are bound as heterodimers or heterotrimers. The binding of the inhibitors to PPP1CC2 is affected by their phosphorylation, and it is known that GSK3 is the protein kinase phosphorylating PPP1R2. Moreover, in infertile GSK3-knockout mice, PPP1CC2 is not associated with the inhibitors (Goswami *et al.* 2019).

How does the epididymis regulate this sperm intrinsic signaling pathway? Here the epididymal Wnt signaling steps in. Multiple Wnt ligands are expressed in the epididymis and Wnt2b was also found in epididymosomes (Koch *et al.* 2015). Wnts signal through two co-receptors, Frizzled (FZD) and low-density lipoprotein receptor-related 6 (LRP6), and epididymosomes were found to activate LRP6 at the sperm membrane *ex vivo*. The Wnt regulator cyclin Y-like 1 (*Ccnyl1*) is highly expressed in germ cells, and *Ccnyl1*-knockout male mice are infertile due to immotile and malformed spermatozoa. *Ccny*-dependent Wnt signaling is known to regulate GSK3 activity. The phosphorylation of inhibitor PPP1R2 was shown to be greatly increased in *Ccnyl*-knockout sperm, likely leading to higher PPP1CC2 activity and hence reduced protein phosphorylation. Indeed, it was shown that total phospho-serine was markedly reduced in *Ccnyl1*-knockout sperm (Koch *et al.* 2015). A recent study, where *Wntless*, a membrane protein required for all WNT protein secretion, was conditionally deleted from mouse caput epididymidis failed to demonstrate any effects on sperm motility and male fertility even though WNT10A and WNT2b proteins were significantly reduced in the epididymal luminal fluid (Cheng *et al.* 2018). This difference might have multiple explanations; incomplete recombination by the Cre-recombinase used, expression of WNTs and WNTLESS in other epididymal segments and most of all, presence of WNTs in the epididymosomes that deliver their cargo directly to sperm thus bypassing the need of secretion of WNTs to the epididymal fluid of caput. Altogether these findings suggest the following mechanism controlling epididymal sperm motility: epididymal Wnts in epididymosomes regulate sperm GSK3 activity via LRP6 and *Ccnyl1*. In the presence of inactive GSK3, the PPP1CC2 inhibitors, PPP1R2 and -R7, bind to PPP1CC2

Figure 2 Schematic of epididymal Wnt-mediated regulation of sperm motility. Epididymal epithelial cells secrete epididymosomes that contain Wnt proteins. In the absence of Wnt signaling, sperm glycogen synthase kinase-3 (GSK3) is active and phosphorylates the PPP1CC2 inhibitors PPP1R2, -R7 and -R11 and thus PPP1CC2 remains active. When epididymal WNTs activate the receptors Frizzled (FZD) and low-density lipoprotein receptor-related 6 (LRP6) primed by cyclin Y-like 1 (CCNYL1), GSK3 is inactivated allowing PPP1R2 and -R7 binding to PPP1CC2 and thus rendering the PPP1CC2 inactive. When PPP1CC2 is inactivated, total phospho-serine levels increase and the spermatozoon becomes fully motile.

rendering it inactive, hence leading to increases in total phospho-serine in sperm and increased motility (Fig. 2).

SPINK13

The epididymis-specific SPINK13 (serine peptidase inhibitor; Kazal type 13) is a protease inhibitor needed for proper sperm maturation. Several members of the SPINK protein family are highly expressed in the epididymis (Jalkanen *et al.* 2006) and in rats *Spink13* is mainly found in the IS and with lower levels in more distal segments (Ma *et al.* 2013). The protein was secreted into the epididymal lumen where it localized to the acrosome of maturing sperm cells. Analyses of sperm after RNAi knockdown of *Spink13* showed increased spontaneous acrosome reaction, although no difference in capacitation was detected (Ma *et al.* 2013). Interestingly, comparison between SPINK13 and LCN6 models revealed that *Lcn6*-knockout sperm displayed much higher levels of spontaneous acrosome reaction compared to sperm lacking SPINK13. However, *Spink13* downregulation gave rise to a significant reduction in male fertility both *in vitro* and *in vivo*, while the *Lcn6* KO did not show any fertility phenotype (Ma *et al.* 2013,

Yin *et al.* 2018). This could of course be due to the different rodent species used for the studies, but could also indicate an additional function for SPINK13 in sperm-egg interaction. A number of serine proteases have been identified on sperm acrosome, however, *in vivo* target proteases of SPINK13 have not yet been identified.

ERp29

The sperm endoplasmic reticulum protein 29 (ERp29) in rodents is hypothesized to serve a role in gamete interaction. ERp29 belongs to the protein disulfide isomerase (PDI) family which causes a conformational change in proteins and thereby promotes cell-cell interaction. Among others, ERp29 can facilitate polyomavirus infection by changing the viral protein structure and stimulate penetration of the virus into the host cells endoplasmic reticulum (Magnuson *et al.* 2005). In both mice and rats, ERp29 is detected in the epididymal epithelium with increased expression from caput to cauda and a subsequent increase in protein levels of caudal sperm (Guo *et al.* 2007, Ying *et al.* 2010). The motility and acrosome reaction of mouse sperm cells were not affected by incubation with an antibody against ERp29. However, the fertilization capacity of sperm was significantly reduced with increased concentration of the antibody (Ying *et al.* 2010). To further support the role of ERp29 in sperm-egg interaction the protein was relocated to the equatorial segment, the initial site of gamete fusion, after the acrosome reaction. Thus, similar to its role in viral infections, ERp29 was hypothesized to cause thiol-disulfide exchange in proteins on the sperm surface and thereby trigger binding to receptors on the egg (Ying *et al.* 2010).

Epididymal proteins affecting sperm membrane lipid composition

When spermatozoa enter the female reproductive tract, an efflux of cholesterol from the sperm membrane precedes the influx of Ca^{2+} required for capacitation. The change gives rise to an increased fluidity of the sperm membrane which is needed to prepare the cells for fertilization of the egg (Travis & Kopf 2002). However, already during maturation in the epididymis the sperm membrane is modified by incorporation of unsaturated lipids and a gradual removal of cholesterol. Sperm from most mammalian species experience an approximate 50% reduction in cholesterol levels when moving from the proximal epididymal segments to cauda (Parks & Hammerstedt 1985, Hall *et al.* 1991, Awano *et al.* 1993, Rejraji *et al.* 2006). Although this results in a more fluid membrane, the epididymal environment keeps the sperm in a quiescent state throughout transit and storage. This is achieved, in part, by the acidic pH of the luminal fluid but also by binding of so called decapacitation

factors to the sperm surface (Nixon *et al.* 2006, Shum *et al.* 2011). When in transit to the fallopian tube, the inhibiting factors are released and the sperm is able to achieve capacitation.

Binder of Sperm protein homolog 1

A protein that influences the lipid content of the sperm membrane and in turn capacitation is Binder of Sperm protein homolog 1 (BSPH1). The protein was first discovered in the seminal plasma of bulls (Esch *et al.* 1983, Manjunath 1984) and, later, homologous genes were found to be expressed in the bovine (BSPH1, Han *et al.* 2009), mouse (*Bsph1*) and human (BSPH1) epididymis (Fan *et al.* 2006, Lefebvre *et al.* 2007). During epididymal transit and while still bound to sperm, BSPH1 is said to prevent movement of lipids in the sperm membrane and thereby protect the sperm from premature capacitation. When the sperm cell reaches the oviduct it comes in contact with high-density lipoproteins (HDLs) which interact with BSPH1 and can cause release of phospholipids and cholesterol from the sperm membrane (Plante & Manjunath 2015). This was shown for both mouse and human sperm, which displayed increased protein tyrosine phosphorylation after incubation with recombinant BSPH1. However, no change in sperm acrosome reaction or motility was detected (Plante *et al.* 2012, 2014).

CES5A

One factor that is hypothesized to play a role in maturation of the sperm lipid membrane is the carboxylesterase CES5A, previously known as CES7. The protein was first detected in rat epididymis where it is secreted into the lumen of corpus and cauda (Zhang *et al.* 2009). Knockdown of *Ces5a* by RNAi injection into rat cauda, caused reduced levels of protein tyrosine phosphorylation during capacitation and a reduced fertility both *in vitro* and *in vivo* (Ru *et al.* 2015). The function of CES5A in sperm capacitation is not fully understood as it does not seem to have a direct interaction with spermatozoa in the epididymal lumen but is instead thought to alter the lipid content of the luminal fluid and then indirectly that of the sperm membrane (Zhang *et al.* 2009, Ru *et al.* 2015). Expression of CES5A is tightly regulated by the epididymis specific miRNA-like small RNA *HongrES2* (*mil-HongrES2*, Ni *et al.* 2011). Considering this study and how the ablation of miRNA processing in the proximal segment of the mouse epididymis caused a significant change in epididymal lipid production and animal fertility (Björkgren *et al.* 2015), it would be interesting to see if miRNAs of the distal epididymis have a similar effect on the lipid content of the sperm membrane.

SERPINA16

A decapacitating factor expressed by the principal cells of mouse, rat and guinea pig cauda is *Serpina16*, also known as *HongrES1* in rat and *mHong1* in mouse (Hu *et al.* 2002, 2012, Ni *et al.* 2009). It is secreted into the epididymal lumen and bound to sperm, where the protein is hypothesized to inhibit the action of cholesterol acceptors. Incubation of *HongrES1*-knockdown sperm with bovine serum albumin (BSA) caused an increased membrane fluidity and premature protein tyrosine phosphorylation (Zhou *et al.* 2008). In addition, the RNAi knockdown of *HongrES1* led to a reduced number of progeny and, interestingly, an increased number of fetuses that displayed developmental defects (Zhou *et al.* 2008). *Serpina16* belongs to the Serpin family of protease inhibitors. However, its amino acid sequence differs from that of traditional inhibitory serpins and the protein function and its role in sperm capacitation remains unknown (Hu *et al.* 2002).

CLPSL2

Colipase-like 2 (*Clpsl2*) is specifically expressed in human (Li *et al.* 2008) and mouse (Oh *et al.* 2006) caput epithelium from where it is secreted and binds to sperm cells (Lu *et al.* 2018). Localization of the protein to the acrosome region and principal piece of mouse sperm is important for the integrity of the acrosome region and the progressive motility of spermatozoa (Lu *et al.* 2018). In addition, the injection of shRNA lentivirus particles against *Clpsl2* caused a significant decrease in sperm number as well as reduced fertility of male mice, both *in vitro* and in normal breeding (Lu *et al.* 2018), which was mainly accredited to the reduced integrity of the sperm membrane. Unlike its relative, the pancreatic colipase, CLPSL2 is not thought to function in lipid hydrolysis but instead in remodeling of the sperm membrane lipid profile (Lu *et al.* 2018). This could also affect additional sperm functions, for example capacitation, which also requires changes in the lipid content of the membrane.

GPX5

The glutathione peroxidase GPX5 is highly expressed in the epididymis of mammals, where it is secreted into the lumen and able to protect sperm from lipid peroxidation. When *Gpx5* was ablated from the mouse epididymis, it did not lead to any fertility phenotype at younger age, but breeding of 1-year-old males and older resulted in more miscarriages and developmental defects than for WT males (Chabory *et al.* 2009). This was hypothesized to be the result of increased DNA fragmentation in *Gpx5* KO sperm. As a result of *Gpx5* ablation, the mice displayed increased expression of other glutathione peroxidases and catalase in cauda, which could partially compensate for the observed phenotype (Chabory *et al.* 2009). Interestingly,

in studies of a double knockout for *Gpx5* and the sperm nuclear *Gpx4*, the male mice showed a much higher compensatory expression of ROS scavengers, which allowed them to sire the same number of pups as wild type mice, regardless of age (Noblanc *et al.* 2012). Similar to mice, boars with lower levels of GPX5 in their seminal plasma showed reduced farrowing rates due to increased oxidative stress (Barranco *et al.* 2016). However, unlike mice, *Gpx5* is expressed throughout the genital tract of boars and is also required for proper motility of sperm cells, which could further influence the observed difference in breeding (Barranco *et al.* 2016). Overall, larger mammals display an expression pattern of *Gpx5* that differs from that of rodents (Grignard *et al.* 2005), with humans having much lower expression levels than other species (Hall *et al.* 1998). Further studies are thus needed to clarify if and how the antioxidant affect the fertility of these males.

Epididymal proteins involved in cell–cell interactions

The epididymis secretes several proteins involved in cell–cell interaction that, after binding to spermatozoa, allow the sperm to recognize and fertilize the egg. The process of sperm–egg fusion share common features with that of fusion between a virus and its host cell, where an initial binding of viral proteins with receptors on the cell surface is required before the two membranes can merge (Stein *et al.* 2004). Similarly to viral infections, sperm cells are known to utilize a number of proteins to facilitate the recognition and binding of gametes. Although blocking their function does not prevent fertilization completely, these proteins work synergistically to promote sperm–egg membrane fusion.

CRISP1

In addition to its role in calcium signaling, CRISP1 is also required for gamete interaction. Incubation with recombinant human CRISP1 completely blocked sperm binding to zona pellucida (ZP), as the recombinant protein was hypothesized to occupy the CRISP1 epitopes of ZP3 (Maldera *et al.* 2014). The sperm cells were already capacitated and only exposed to the protein during the short time of ZP interaction, thus excluding any effect the inhibitor could have on the role of CRISP1 during sperm capacitation (Da Ros *et al.* 2008, Maldera *et al.* 2014). After the sperm binds to ZP and goes through the acrosome reaction, rat CRISP1 is relocated from the dorsal part of the acrosome to the region destined for gamete fusion, the equatorial segment of the sperm cell (Rochwerger & Cuasnicu 1992). Complementary sites across the murine and human egg membrane are able to directly bind CRISP1. However, CRISP1 does not affect the initial binding

of sperm, as incubation of eggs with recombinant CRISP1 protein did not lead to a reduced number of bound sperm cells but only prevented the penetration of the egg by spermatozoa (Cohen *et al.* 2000, 2001, Busso *et al.* 2007). Because CRISP1 does not contain any hydrophobic domains (Brooks 1987), the protein was hypothesized to provide the interaction needed for membrane fusion instead of having a direct role in the process (Cohen *et al.* 2000).

DCXR

Dicarbonyl/L-xylulose reductase (DCXR, also known as sperm surface protein P34H) is, as the name implies, an enzyme able to catalyze the reduction of several different aromatic dicarbonyl compounds and sugars (Wang & Van Eys 1970, Nakagawa *et al.* 2002). However, it can also serve in a non-catalytic fashion by interacting with proteins such as cadherins and catenins, and thereby influence cell–cell adhesion (Cho-Vega *et al.* 2007). In humans, the expression of DCXR in male reproductive tissues increases from testis to the distal segments of the epididymis, with the highest expression observed in corpus epididymis. Similarly, DCXR protein levels increase on the acrosome of spermatozoa during epididymal transit (Légaré *et al.* 1999). Incubation of sperm with an antibody against DCXR greatly inhibited binding to ZP, although the ability to bind to and fuse with ZP-free eggs was not reduced (Boué *et al.* 1994). The molecular mechanism of DCXR during sperm–ZP interaction is not known, but as it is bound to the sperm surface, it may have a similar function as that observed in epithelial cell–cell interaction. Interestingly, sperm samples from infertile patients often show lower levels of DCXR (Boué & Sullivan 1996, Moskovtsev *et al.* 2007) and a study using DCXR-negative sperm cells in IVF did not lead to any fertilized eggs (Sullivan *et al.* 2006), a strong indication of the importance of this protein in human reproduction.

The hamster and bovine epididymides express another member of the dehydrogenase/reductase family, carbonyl reductase 2 (*Cbr2*), a protein not found in primates or humans (Sullivan & Robitaille 1989, Frenette & Sullivan 2001). Similar to DCXR, hamster CBR2 (also known as P26h) and bovine CBR2 (also known as P25b) are tethered to spermatozoa by a GPI-anchor during sperm maturation and are known to be involved in sperm–ZP binding (Bérubé & Sullivan 1994, Légaré *et al.* 1999, Parent *et al.* 1999, Frenette & Sullivan 2001). Especially, inhibition of the enzymatic activity of hamster CBR2 was shown to reduce binding capacity of sperm to ZP by almost 50% (Montfort *et al.* 2002). However, the enzyme does seem to serve species specific functions, as the mouse CBR2 (also known as MLCR or AP27) has not yet been shown to participate in any fertilization event (Bégin *et al.* 1995).

SOB2 and FLB1

In a valiant effort to identify additional proteins involved in sperm–oocyte interaction, Boué *et al.* incubated human sperm with monoclonal antibodies against their surface proteins (Boué *et al.* 1992). This led to the discovery of the so-called sperm–oocyte-binding antigen 2 (SOB2) and FLB1, which are specifically expressed in caput and corpus of human epididymis, with additional expression of FLB1 in the deferens (Boué *et al.* 1995, Lefèvre *et al.* 1997). During maturation, SOB2 first displays a diffuse localization over the sperm head which becomes restricted to the post-acrosomal and neck area in corpus (Lefèvre *et al.* 1997). FLB1, in turn, was shown to be secreted by hamster epididymal cells *in vitro*, from where it is thought to be transferred to the equatorial region of sperm cells (Boué *et al.* 1995). When incubating spermatozoa with antibodies against SOB2 or FLB1, the sperm showed a significantly reduced ability to bind to zona-free hamster eggs even though sperm motility and acrosome reaction were unaffected (Boué *et al.* 1995, Lefèvre *et al.* 1997). Although the SOB2 antibody detected proteins in rat, hamster and rabbit sperm (Lefèvre *et al.* 1997), a similar function to that of human SOB2 has not to our knowledge been detected in these species.

Conclusions

The essential role of the epididymis in male fertility, as the place where sperm obtain their full capacity for fertilization, has already been known for a long time. However, many of the earlier models used to demonstrate this effect were so crude that the function of individual molecules in the maturation process could not be discerned. During recent years, several elegant studies have made more detailed analyses of the important role of the epididymis in attaining proper sperm function. In this review, we have introduced studies of 16 such proteins, protein families and signaling pathways to the reader. The CRISPR/Cas9 gene-editing technology is expected to speed up the identification and functional validation of epididymal proteins involved in male fertility. Epididymis-specific conditional models are further anticipated to reveal the importance of numerous genes with expression in both the epididymal epithelium and in testicular germ cells. These studies will be of utmost importance for understanding the molecular basis of sperm fertilizing ability, especially in regards to the alarming decline in male fertility observed in Western countries.

Declaration of interest

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

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