Novel epididymal proteins as targets for the development of post-testicular male contraception

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Abstract

Apart from condoms and vasectomy, modern contraceptive methods for men are still not available. Besides hormonal approaches to stop testicular sperm production, the post-meiotic blockage of epididymal sperm maturation carries lots of promise. Microarray and proteomics techniques and libraries of expressed sequence tags, in combination with digital differential display tools and publicly available gene expression databases, are being currently used to identify and characterize novel epididymal proteins as putative targets for male contraception. The data reported indicate that these technologies provide complementary information for the identification of novel highly expressed genes in the epididymis. Deleting the gene of interest by targeted ablation technology in mice or using immunization against the cognate protein are the two preferred methods to functionally validate the function of novel genes *in vivo*. In this review, we summarize the current knowledge of several epididymal proteins shown either *in vivo* or *in vitro* to be involved in the epididymal sperm maturation. These proteins include CRISP1, SPAG11e, DEFB126, carbonyl reductase P34H, CD52, and GPR64. In addition, we introduce novel proteinases and protease inhibitor gene families with potentially important roles in regulating the sperm maturation process. Furthermore, potential contraceptive strategies as well as delivery methods will be discussed. Despite the progress made in recent years, further studies are needed to reveal further details in the epididymal sperm maturation process and the factors involved, in order to facilitate the development of new epididymal contraceptives.

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Introduction

The large number of unintended pregnancies worldwide (nearly 50% of pregnancies in USA are unintended and 22% of all pregnancies end in abortion, [Finer & Henshaw 2006](#)) indicates the burning need for new contraceptive methods. While there are multiple contraceptive methods for women, condoms are the only reversible method available for men. Vasectomy is another option for men, but it requires surgery and is not intended to be reversible. However, despite these shortcomings, it has been estimated that male methods account for 37% of all contraceptive usage ([Lye *et al.* 2004](#)), indicating a need for novel male methods, as well as the willingness of men to use them if available. An ideal male contraceptive would allow normal hormone production and spermatogenesis in the testis but would prevent the post-testicular sperm maturation, thus, blocking the ability of spermatozoa to fertilize the oocytes. There is ample clinical and experimental information that sperm are unable to fertilize if the epididymal phase of their maturation does not occur, indicating the feasibility of the epididymis as the target of

a male contraceptive. An ideal method should be rapid, fully and quickly reversible, and without side effects. In order to be regulatable by pharmacological means the ideal drug target would be an enzyme, ion channel or receptor, because small molecular weight pharmacological inhibitors are more feasible to be developed for such proteins. Furthermore, the target should be expressed in a limited number of tissues, preferably only in the epididymis. Owing to the final maturation of spermatozoa in the epididymis, this organ provides a promising target for the development of new male contraceptives.

Epididymal sperm maturation

When the maturing spermatozoa leave the testis, they are non-motile and unable to fertilize the oocyte *in vivo*, while their full maturation, including potential to display motility, takes place during transit through the epididymis. The maturational changes in spermatozoa are caused by changes in the luminal ion concentration and the proteins secreted into the lumen by the epididymal epithelium. The maturation includes the

acquisition of progressive and sustained motility, while the pattern of movement changes from circular (rat) or slightly vibrating (human) into rapid straightforward progression. The flagellum of spermatozoa becomes stiffened, and the neck region less flexible, enabling more rigid movement of the spermatozoa (for review, see Yeung & Cooper 2002). Furthermore, during epididymal transit the cytoplasmic droplet slides from the base of the sperm head to the end of the midpiece of the flagellum. There are contradictory reports on whether or not the cytoplasmic droplet is shed in the epididymis; however, it seems that it is shed later, during ejaculation (Cooper & Yeung 2003). A failure in migration of the droplet is often related to subfertility (for review, see Gatti *et al.* 2004).

During epididymal transit, the spermatozoa also undergo a change in size, shape, and internal structure of the acrosome (Olson *et al.* 2002). In addition, the sperm cell membrane is under constant remodeling, with attachment and shedding of molecules in a sequential manner (for review, see Cuasnicu *et al.* 2002, Jones 2002). It has been postulated, that the factors involved in sperm–egg fusion are produced in the proximal epididymis, whereas proteins involved in the sperm–zona binding as well as the decapacitation factors are produced more distally (Cooper 1999).

Methods for identifying putative epididymal target genes

The rapid improvement of the techniques of analyzing global gene expression in the tissues of interest has provided new tools for novel gene identification. Several groups, including ours, have extensively used microarrays to identify genes expressed in the various epididymal segments and under different regulatory factors in the mouse, rat, and human (Ezer & Robaire 2003, Hsia & Cornwall 2004, Sipilä *et al.* 2006, Zhang *et al.* 2006, Turner *et al.* 2007). In addition, proteomics has been utilized by many groups to reveal total protein profiles (proteome) or the secretory proteins (secretome) in the epididymides of rat, stallion, boar, and human (Syntin *et al.* 1996, Fouchecourt *et al.* 2000, Dacheux *et al.* 2006, Yuan *et al.* 2006). It is currently also possible to use imaging mass spectrometry with which one can investigate the protein content of biological tissue sections at the cellular level in direct correlation with the underlying histology. Such an analysis was recently performed on mouse epididymal samples, and on the basis of this study about 50 proteins were suggested to be expressed in a region-specific manner (Chaurand *et al.* 2003). However, the technology is in its infancy at present, and still in need of validation by other methods.

Gene discovery and analysis *in silico* has become a powerful tool of modern biotechnology. Libraries of expressed sequence tags (ESTs) have been used successfully to identify genes expressed in the various epididymal

segments of the mouse (Penttinen *et al.* 2003, Jalkanen *et al.* 2006a). The UniGene database (<http://www.ncbi.nlm.nih.gov/uniGene>) is a collection of sequences of individual clones of the cDNA libraries of various tissues. These sequences are organized into clusters in such a way that each cluster contains ESTs that overlap in their sequence with at least one other EST of the cluster but not with ESTs of other clusters. Thus, each cluster is likely to contain sequence information of a single gene (Pontius *et al.* 2003). Among various species, the highest number of EST sequences is available for the human and mouse. The number of ESTs of a specific gene in the library is likely to predict the level of its expression. Furthermore, as EST libraries are available from various tissues, developmental, and diseases stages, the EST database also provides valuable information about the putative tissue distribution of gene expression. By using the digital differential display (DDD) tool at the NCBI (<http://www.ncbi.nlm.nih.gov/UniGene/ddd.cgi>) or on the TIGR database (<http://www.jcvi.org/cms/research/software/>) one can search genes present exclusively in the EST library of a certain tissue but not among EST libraries of other tissues. For example, DDD has been used recently to identify two novel members of the Cres subgroup of family two cystatins expressed in the mouse testis, epididymis, cerebrum, and pituitary (Xiang *et al.* 2008). Other useful databases for obtaining gene expression data from tissues of interest include SAGEmap (www.ncbi.nlm.nih.gov/projects/SAGE/), GUDMAP (<http://www.gudmap.org/>), and GEO database (<http://www.ncbi.nlm.nih.gov/geo/>). The strategy used to identify and characterize novel epididymal proteins as putative targets for a male contraceptive is shown in Fig. 1.

EST analysis and proteomic methods provide an unbiased and effective way for the identification of novel, unknown, and epididymis-specific genes. By contrast, the current microarray strategies are based on pre-defined probes for known or predicted transcripts. However, the rapid evolution of the sequencing and array technologies is likely to overcome this major disadvantage of the microarrays in the near future. The problems with traditional two-dimensional proteomics include poor detection of low abundance proteins and problems in definitive identification of proteins. However, when two dimensions are combined with LC-MS/MS identification of proteins, it is possible to achieve high-confidence identifications owing to the sequence dependency of the data. The major advantage of the cDNA microarray and proteomics approaches is the possibility to analyze gene expression at various experimental stages, while the EST libraries provide static information from limited tissues and experimental conditions. Concerning the epididymis, the number of EST sequences in the UniGene RIKEN epididymal library is also low, further limiting the chances of novel gene identification. The benefits and drawbacks of the EST, cDNA microarray, and proteomics approaches are

Figure 1 The strategy to identify and characterize novel epididymal proteins as putative targets for a male contraceptive.

described in Table 1. In summary, the various technologies available provide complementary information about novel highly expressed genes in the epididymis.

Although the methods used to identify new epididymal genes and proteins are useful, the caveat in these techniques is that they do not provide evidence for the role of the identified proteins in the sperm maturation process. An opposite strategy applies to the phenotype-based gene discovery process. The *N*-ethyl-*N*-nitrosourea (ENU)-induced chemical mutagenesis strategy is one of the available methods to generate point mutations in male mouse (and to lesser extend female) germ cells (Kennedy *et al.* 2005, Handel *et al.* 2006, Lessard *et al.* 2007). Mice harboring the mutations are bred to homozygosity and reproductive tests are performed to identify mutations that cause infertility. The final step of the process is the mapping and positional cloning of such a mutation. The Jackson Laboratory provides phenotype description of each mutation generated on their ReproGenomics program (<http://reprogenomics.jax.org>), and all the mutant mice are available to the scientific community. The reprogenomics database mainly contains models for male infertility caused by testicular defects. However, the database also includes mutant male mice with

post-testicular defects. Upon identification of the mutated genes behind the infertility, new candidates for contraceptive targets are expected to arise.

Functional characterization of novel genes

The methods used to validate novel genes functionally *in vivo* have also developed rapidly. Both human and mouse genomes are well characterized, and genomic clones for any gene are available in the form of bacterial artificial chromosomes. This, together with novel cloning techniques available (Angrand *et al.* 1999, Rivero-Muller *et al.* 2007) provide us with tools to generate efficiently gene targeting vectors for knockout (KO) mouse production. In addition, the gene-trap libraries (for example www.genetrap.org) with numerous embryonic stem cell clones with a disrupted gene are currently available for the fast generation of mouse models. However, to date, there are only a few genetically modified (GM) mouse models from epididymal genes presenting with defects in sperm maturation and/or function. Nevertheless, such models have revealed the importance of the epididymis for sperm maturation. For example, the *Ros1* proto-oncogene (also known as *c-ros*, Sonnenberg-Riethmacher *et al.* 1996) and apolipoprotein E receptor-2 (LRP8, Andersen *et al.* 2003) KO male mice as well as mice carrying a natural mutation of SHP1 phosphatase gene (Keilhack *et al.* 2001) lack the most proximal region of the epididymis, initial segment (IS), and are infertile. In addition, transgenic mice expressing the SV40 virus T-antigen under the *Gpx5* promoter in the epididymis (GPX5-Tag2 mice, Sipilä *et al.* 2002) have dysfunctional IS and male infertility. In all of these GM mouse models, the differentiation of IS is disrupted, resulting in disturbed osmolyte balance of the epididymal fluid, angulation of sperm tails and infertility (Cooper & Barfield 2006). However, the exact molecular mechanism underlying this defect is still not known.

In addition to the GM mouse models, immunological techniques have been used to study the role of proteins in sperm–zona pellucida (ZP) binding *in vivo*. In this

Table 1 Benefits and drawbacks of the expressed sequence tag, microarray and proteomics approaches.

Approach	Benefits	Drawbacks
Microarray	Experimentation possible	Poor coverage of novel genes
	Over 39 000 transcripts	Huge amount of data to analyze
Proteomics	Experimentation possible	Poor detection of low abundance proteins
	An unbiased way for the identification of novel genes	Definite identification of proteins difficult
EST library	Thirty percent of clusters represent novel genes	Limited number of genes in the library
	Tissue distribution and expression level can be estimated	No experimentation

approach, the experimental animals are immunized against a protein specifically present on sperm surface, and the antibodies produced are used to block the sperm motility or ability of sperm to bind to the complementary sites of the oocyte. The epididymal genes with role in fertilization that have been studied using the immunological approach include cysteine-rich secretory protein 1 (*Crisp1*; Ellerman *et al.* 2008), carbonyl reductase P34H (Sullivan 2004), CD52 (also known as *HE5*; Koyama *et al.* 2007), and serine protease inhibitor-like, with Kunitz and whey acidic protein (WAP) domains 1 (*Spinlw1*, also known as *Eppin*; O'Rand *et al.* 2006).

An intriguing option to study the functions of epididymal genes is to apply *in vivo* electroporation. In that technique, a DNA construct is injected into the interstitium or lumen of the epididymis using electric pulses that are provided by electric tweezers. After a recovery period, tissue is analyzed. This method has been applied successfully in promoter analysis *in vivo* (Kirby *et al.* 2004), and in studying the Ets variant 4 (ETV4, also known as *PEA3*) transcription factor family and fibroblast growth factor receptor 1 (FGFR1) pathways (Yang *et al.* 2006). An apparent problem in identifying putative contraceptive drug targets is that many genes have important functions in a variety of different tissues which makes them unattractive drug targets. However, when a signaling pathway necessary for male fertility is identified, this technique in a combination with short hairpin RNA (shRNA) techniques could well provide an efficient strategy to screen epididymal pathway members that would be more suitable drug targets to be further validated in studies in GM animals.

Putative epididymal target genes

The cysteine-rich secretory protein (*Crisp*) family

The family of CRISP proteins consists of four members: *Crisp1* (also known as DE), *Crisp2* (also known as *Tpx1*), *Crisp3* and *Crisp4*. Of these family members, mouse *Crisp1* and *Crisp4* are expressed in the epididymis (Eberspaecher *et al.* 1995, Jalkanen *et al.* 2005), *Crisp2* in developing spermatocytes (Mizuki *et al.* 1992) and *Crisp3* mainly in the salivary glands, pancreas, and prostate (Haendler *et al.* 1993). Human homologues have been identified for *Crisp2*, 3, and 4 (Kratzschmar *et al.* 1996). Surprisingly, it has been recently reported that *CRISP3* is highly expressed in the human cauda epididymidis and ampulla of vas deferens (Udby *et al.* 2005). Both the mouse and the human CRISP1 proteins are secreted into the epididymal lumen and have been detected on the surface of epididymal spermatozoa. Mouse CRISP1 has been localized in the dorsal region of the acrosome and human CRISP1 in the post-acrosomal region of the sperm head (for review, see Cohen *et al.* 2007). Two different populations of rat and human

CRISP1 are bound to sperm; a major loosely associated population and a minor tightly bound population. Dissociation of the major population seems to be necessary for the process of capacitation (Roberts *et al.* 2003). Thus, CRISP1 could be involved in the prevention of precocious capacitation. The minor strongly bound population remaining on sperm surface after capacitation migrates to the equatorial segment during acrosome reaction, and has been suggested to participate in sperm–ZP interaction (Busso *et al.* 2007) and gamete fusion (for review, see Cohen *et al.* 2007). Interestingly, immunization against rat CRISP1 protein leads to significant reduction in fertility (Ellerman *et al.* 2008), postulating that the CRISP family proteins might make putative targets for post-testicular male contraception. However, the recently published CRISP1 KO mouse model was shown to be fully fertile in natural matings and in IVF with cumulus-intact oocytes (Da Ros *et al.* 2008). Nevertheless, KO sperm exhibited a reduced ability to fertilize ZP-intact oocytes devoid of cumulus cells as well as ZP-free oocytes. As mouse CRISP2 protein was able to bind the mouse oocytes and further inhibit fertilization by CRISP1 KO sperm, it is likely that other Crisp-family members can compensate for the loss of CRISP1 (Da Ros *et al.* 2008). KO models from CRISP2 and -4 and double/triple KO mice would provide the ultimate proof for the importance of the CRISP family proteins in fertilization. Nevertheless, since it seems that the protein domain needed for the CRISP–oocyte binding, namely Signature 2 (S2) domain, is evolutionarily conserved within the CRISP family (Cohen *et al.* 2008), it might be possible to interfere with the functions of all family members at once by targeting that particular domain.

The beta-defensin family

Beta-defensins belong to a large family (a total of 52 in the mouse) of small antimicrobial peptides. In mammals, beta-defensins are organized into four to five dense clusters on the genome (Patil *et al.* 2005), and the organization of the clusters is highly similar between the mouse, rat, dog, and human. Furthermore, almost all mouse beta-defensins have been reported to have a human homologue. Recent studies have revealed that many of the beta-defensins are mainly expressed in the male reproductive track, particularly in the epididymis and testis (Patil *et al.* 2005). In addition to antimicrobial and host defense activities, a role in sperm maturation has been suggested for them on the basis of the localization of several defensins on sperm surface (Liu *et al.* 2001, Rao *et al.* 2003). However, only the rat SPAG11e and macaque DEFB126 have been reported to be involved in reproductive function. The rat SPAG11e induces progressive sperm motility by increasing the uptake of calcium by spermatozoa (Zhou *et al.* 2004) and the macaque DEFB126 has been reported to be involved in

sperm penetration through the cervical mucus (Tollner *et al.* 2008), and sperm-ZP recognition and binding (Tollner *et al.* 2004). However, more studies are needed to confirm the role of this gene family in sperm maturation.

Carbonyl reductase

Human carbonyl reductase P34H is a member of the short chain dehydrogenase/reductase superfamily, and it is expressed in the epididymis with the highest level in the corpus region. During the epididymal transit P34H progressively accumulates in the sperm acrosomal region (reviewed by Sullivan 2004). It has been suggested that P34H is involved in sperm-ZP binding. This is supported by the results indicating that immunization against P34H inhibits sperm-zona binding *in vitro* (Boué *et al.* 1994). Furthermore, the loss of P34H from sperm surface has been associated with idiopathic infertility in men (Boué & Sullivan 1996, Moskovtsev *et al.* 2007), and human spermatozoa lacking P34H have been shown to be unable to bind to the extracellular matrix of oocyte (Boué & Sullivan 1996). However, the usefulness of P34H as a contraceptive target should be critically considered owing to the wide tissue distribution (Felsted & Bachur 1980) of its expression and the general metabolic role of carbonyl reductases in converting various carbonyl compounds to corresponding alcohols (Wermuth 1981).

CD52 (also known as HE5)

Human CD52 is a small, highly glycosylated, glycosylphosphatidyl inositol (GPI)-anchored cell membrane glycopeptide expressed in the lymphocytes, distal epididymis, and vas deferens. Detailed structural analysis of the CD52 protein in the lymphocytes and genital tract revealed that these two populations are distinct in the attached *N*-linked glycans and GPI-anchor domains (reviewed in Kirchhoff 2002). CD52 is incorporated into the sperm membrane with a GPI-anchor, but the function of this protein in sperm surface remains unknown. Antibodies directed against CD52 yielded in sperm immobilization and inhibition of zona binding when applied in IVF conditions (Mahony *et al.* 1991, Koyama *et al.* 2007), and further, antibodies against CD52 occur naturally in some women (Koyama *et al.* 2007), which leads to difficulties in conceiving. Disappointingly, it was recently shown that CD52 KO male mice are fully fertile both *in vivo* and *in vitro* (Yamaguchi *et al.* 2008). Whether this reflects a species difference between mouse and human remains to be solved.

Gpr64 (also known as HE6)

Gpr64 is an efferent duct and IS specific member of the LNB-7TM subfamily of G-protein coupled receptors

(Osterhoff *et al.* 1997). Disruption of the gene in mice results in dysregulation of fluid reabsorption within the efferent ductules, leading to fluid accumulation in the testis, subsequent stasis of spermatozoa within the efferent ducts, and finally to infertility (Davies *et al.* 2004). Currently, *Gpr64* is one of the most promising targets for a post-testicular male contraceptive. It remains to be assured that long-term inhibition of *Gpr64* action does not lead to complete atrophy of the germinal epithelium due to fluid accumulation and back pressure in the testis. However, the studies so far carried out with the *Gpr64* KO mouse model do not indicate such effects (Davies *et al.* 2004).

Proteases

The modification of sperm surface proteins is important for epididymal sperm maturation. These modifications lead to complete removal of proteins or redistribution of modified proteins in the sperm membranes. Examples of such proteolytically processed proteins include the germinal form of angiotensin I converting enzyme (*gACE*) and the disintegrin and metalloproteinase domain 2 (*Adam2*). *gACE* is released from the surface of spermatozoa during their transit through caput epididymidis, whereas *Adam2* is degraded by two successive cleavages during the caput transit, leaving the protein to the post-acrosomal domain of spermatozoa (Gatti *et al.* 2004). Other sperm membrane proteins known to be modified during epididymal transit include sperm adhesion molecule 1 (*Spam1*, also known as PH-20 hyaluronidase), basigin (*Bsg*, also known as CE9, CD147, EMMPRIN, gp42, HT-7, neurothelin), and mannosidase 2 alpha B2 (*Man2b2*, also known as α -D-mannosidase; Gatti *et al.* 2004). However, to date none of the protease(s) responsible for these modifications is known.

The ADAM family

One of the most studied proteinase families in the epididymis is the disintegrin and metalloprotease (ADAM) gene family, which currently contains about 40 members (http://people.virginia.edu/~jw7g/Table_of_the_ADAMs.html). The ADAM proteins are considered to have two distinct functions, while some of them act as cell adhesion molecules, and some act as proteases (Primakoff & Myles 2000). At least 16 ADAM family members are expressed in the testis, and the expression of five has been reported in the epididymis. Of the epididymal ADAM proteins, *Adam7* is expressed uniformly in the all epididymal regions and it has been shown to bind to sperm surface, and to be redistributed in the sperm head during acrosome reaction (Oh *et al.* 2005). Furthermore, on the basis of *Adam* KO mouse models, it has been suggested that ADAM1a and

ADAM2 form a protein complex, which in turn is involved in maintaining the stability of other sperm surface proteins including ADAM3 (Nishimura *et al.* 2004). Studies on the *Adam2* and *Adam3* KO spermatozoa led to the hypothesis that ADAM7 also associates with the ADAM1a/2-ADAM3 heterodimers on the sperm membrane. On the basis of these results, a putative role for ADAM7 in sperm – oocyte binding was suggested (Kim *et al.* 2006b). Furthermore, ADAM7 does not seem to contain the enzymatically active metalloprotease domain (Lin *et al.* 2001), and thus it probably acts only as a cell adhesion molecule.

The other epididymal ADAM family member, *Adam28*, is expressed in the IS and is regulated by testicular factors other than testosterone (Sipilä *et al.* 2006). There are two splice variants of *Adam28* in the epididymis, a membrane bound and a secreted form. Unlike ADAM7, ADAM28 possesses an active metalloprotease domain, and is therefore, suggested to perform proteolytic functions within the epididymis (Jury *et al.* 1999). In conclusion, the putative role of these epididymal ADAM proteins in sperm maturation process and fertility is yet to be resolved.

KO mice are available for *Adam1a* (Nishimura *et al.* 2004) and *-1b* (also known as fertilin α , Kim *et al.* 2006a), *Adam2* (also known as fertilin β , Cho *et al.* 1998) and *Adam3* (also known as cyritestin, Shamsadin *et al.* 1999, Nishimura *et al.* 2001). Interestingly, males of all these mouse models are infertile owing to either defects in the ability of sperm to migrate from the uterus into the oviduct (*Adam1* and *-2*), and/or to defects in ZP binding (*Adam2* and *-3*). These findings provide strong evidence for the importance of the ADAM family proteins for fertility.

Protease inhibitors

It has been reported that the loss of certain ADAM proteins from the sperm surface results in the concomitant disappearance of the other ADAMs (Nishimura *et al.* 2004, 2007). This suggests that the sperm surface architecture depends on correct localization of its components, and highlights the importance of the sequence of the proteolytic processing of the sperm surface in the epididymal duct. Proteolysis in the epididymal lumen thus needs to be well controlled, and several protease inhibitors expressed in the certain epididymal regions are expected to be responsible for this control. There are several protease inhibitor families present in the epididymis including cystatin (Cornwall & Hsia 2003), Kunitz (Richardson *et al.* 2001, Penttinen *et al.* 2003), Kazal (Moritz *et al.* 1991, Jalkanen *et al.* 2006b) and WAP, (Jalkanen *et al.* 2006b), and serpin families (Laurell *et al.* 1992, Hu *et al.* 2002).

From the cystatin family of cysteine protease inhibitors, *Cst8* (also known as *Cres*) and *Cst12* (also known as *Cres3*) are expressed mainly in the epididymis

(Cornwall & Hsia 2003), whereas *Cst11* (also known as *Cres2*) has been reported to be epididymis-specific (Cornwall & Hsia 2003). Classical cystatins are potent cysteine protease inhibitors. However, studies *in vitro* have shown that *Cst8* inhibits the serine protease (prohormone convertase 2) instead of cysteine proteases (Cornwall & Hsia 2003).

Kazal protease inhibitors also belong to the group of serine protease inhibitors, and they inhibit trypsin-, chymotrypsin-, and elastase-like proteases, including acrosin (for review, see Rawlings *et al.* 2004). Five members of the Kazal family have been detected in the epididymis, namely *Spink2* (serine protease inhibitor, Kazal type 2; Moritz *et al.* 1991), *Spink8*, *Spink10*, *Spink11*, and *Spink12* (Jalkanen *et al.* 2006b). Analyses of the testicular gene expression of azoospermic infertile men revealed that they were characterized by a 4.2-fold decrease in *Spink2* (previously known as human acrosin-trypsin inhibitor HUSI-II) expression compared with fertile men (Rockett *et al.* 2004). Nevertheless, further studies are required to investigate the significance of this finding, as well as the functions of SPINKs in the epididymis.

The Kunitz protease inhibitors are a large protein family whose members are able to inhibit serine proteases (Pritchard & Dufton 1999). To our knowledge, *Spint4* (Penttinen *et al.* 2003) is the only pure Kunitz protease inhibitor so far identified in the epididymis. In addition, there are some protease inhibitors with Kunitz and WAP domains present in the epididymis, those including serine protease inhibitor-like, with Kunitz and WAP domains 1 (*Spinlw1*, also known as *Eppin*) and (WAP)-type four sulfide core (WFDC) genes – 8 and 10 (Jalkanen *et al.* 2006b, O'Rand *et al.* 2006). A role in male fertility has been suggested for *Spinlw1*, since active immunization against *Spinlw1* resulted in reversible contraception in five out of nine male monkeys (O'Rand *et al.* 2004). Because *Spinlw1* belongs to the large group of genes expressed both in the testis and epididymis, the role of the epididymal expression in the maintenance of fertility remains unclear. However, the data on *Spinlw1* emphasize the role of protease inhibitors in male fertility.

The presence of several proteinase inhibitors from different families is consistent with the hypothesis that correctly timed epididymal proprotein processing is essential for proper sperm maturation. Furthermore, the fact that many of these enzymes have a human homologue, and are highly epididymis-specific (Table 2), indicates that they are putative targets for post-testicular male contraception.

Genes expressed in the testis and epididymis

KO mouse models are available for several genes expressed both in the testis and epididymis, and interestingly, many of these models are characterized by defects in male fertility. Such genes include sperm

Table 2 Epididymal proteinase inhibitors.

Gene	Gene family	Expressed in	Human homolog	References
<i>Spint4</i> (serine protease inhibitor, Kunitz type 4)	Kunitz	Epididymis	<i>SPINT4</i>	Penttinen <i>et al.</i> (2003)
<i>Spink2</i> (serine protease inhibitor, Kazal type 2)	Kazal	Epididymis, testis, sem. ves.	<i>SPINK2</i>	Moritz <i>et al.</i> (1991)
<i>Spink8</i> (serine protease inhibitor, Kazal type 8)	Kazal	Epididymis, vas deferens	<i>SPINK8</i>	Jalkanen <i>et al.</i> (2006b)
<i>Spink10</i> (serine protease inhibitor, Kazal type 10)	Kazal	Epididymis, brain	nf	Jalkanen <i>et al.</i> (2006b)
<i>Spink11</i> (serine protease inhibitor, Kazal type 11)	Kazal	Epididymis, seminal vesicle	nf	Jalkanen <i>et al.</i> (2006b)
<i>Spink12</i> (serine protease inhibitor, Kazal type 12)	Kazal	Epididymis	nf	Jalkanen <i>et al.</i> (2006b)
<i>Spinlw1</i> (serine protease inhibitor-like with Kunitz and WAP domains 1)	WAP & Kunitz	Epididymis, testis	<i>WFDC7</i>	O'Rand <i>et al.</i> (2006)
<i>Wfdc2</i> (WAP-type four disulfide core 2)	WAP	Several tissues	<i>WFDC2 (HE4)</i>	Galgano <i>et al.</i> (2006)
<i>Wfdc8</i> (WAP-type four disulfide core 8)	WAP & Kunitz	Epididymis, testis	<i>WFDC8</i>	O'Rand <i>et al.</i> (2006)
<i>Wfdc10</i> (WAP-type four disulfide core 10)	WAP	Epididymis	<i>WFDC10</i>	Jalkanen <i>et al.</i> (2006b)
<i>Wfdc15</i> (WAP-type four disulfide core 15)	WAP	Epididymis, kidney	nf	Hagiwara <i>et al.</i> (2003)
<i>Cst8</i> (cystatin 8)	Cystatin	Epididymis, testis, pituitary	<i>CST8</i>	Cornwall & Hsia (2003)
<i>Cst12</i> (cystatin 12)	Cystatin	Epididymis, testis	nf	Cornwall & Hsia (2003)
<i>Cst11</i> (cystatin 11)	Cystatin	Epididymis	<i>CST11</i>	Cornwall & Hsia (2003)

nf, not found; sem. ves., seminal vesicle.

adhesion molecule 1 (*Spam1*, also known as PH-20) and milk fat globule-EGF factor 8 (*Mfge8*, also known as SED1 and lactadherin). For example, SPAM1 has been shown to have a role in cumulus cell dispersion along with two other testicular hyaluronidases (HYAL1 and HYALP1, Lin *et al.* 1994, Martin-DeLeon 2006), whereas the MFGE8 KO males were found to be subfertile, owing to a defect in sperm-ZP binding (Ensslin & Shur 2003). Since these proteins are present on the sperm surface already in the testis, the role of epididymal dysfunction as a cause of the phenotype is not clear. Thus, to distinguish the protein function in the testis and epididymis, the conditional Cre-loxP technique should be applied. However, it is worth noticing that many such proteins are likely to be potential contraceptive drug targets.

From epididymal genes to contraceptives

For the time being, inhibition of sperm maturation or sperm-oocyte binding by blocking epididymal proteins has only been achieved using immunological approach (Sullivan 2004, O'Rand *et al.* 2006, Koyama *et al.* 2007, Ellerman *et al.* 2008). Reports from immunological studies have raised several concerns such as the possibility of provoking autoimmunity, and the variability in both the degree and duration of response among individuals and reversibility. Contraceptives available for women nowadays are well defined and highly efficient, thus setting the criteria for novel male contraceptives high. Therefore, the above-mentioned concerns for the use of immunological approach have reduced the attractiveness of the approach. A more attractive choice would be the use of small molecule pharmacological inhibitors to target epididymal proteins necessary for the sperm maturation process. Traditionally, small molecule inhibitors of enzymes (for example tyrosine kinases) and receptors (particularly, G-protein coupled receptors)

have been used successfully as pharmaceuticals, and more recently progress has been made in the development of inhibitors of protein-protein interaction as well (Gadek & Nicholas 2003). Small molecule inhibitors are often end products of rational design, on the basis of well characterized targets. Interestingly, cell-based drug screening strategies, which have applied the available small molecule libraries, have identified several potential drugs, for example for cancer therapy, over the last few years (Gudkov 2004, Vita & Henriksson 2006). Importantly, this means that when a suitable target protein is identified, potential inhibitors can be quickly identified, before the target's interactions with other proteins are fully characterized. Nevertheless, even though a number of novel potential drug targets are emerging, epididymal male contraceptives are still years away from clinical use, since the appropriate lead compounds need first to be identified and developed further *in vitro*, in preclinical *in vivo* models, and finally in clinical trials.

The presence of the blood-epididymis barrier (Cyr *et al.* 2002) presents a major challenge in delivering potential contraceptive drugs to the epididymis. Drug delivery through the blood-brain and blood-testis barriers have been studied in more detail, and solutions from those systems are expected to apply for the epididymis as well. For example, there are already potential contraceptive drugs (for example Miglustat, *N*-butyldeoxynojirimycin, NB-DNJ, Amory *et al.* 2007) that appear to traverse through the blood-testis barrier by passive diffusion. Another strategy to cross the blood-epididymis barrier could be the use of endogenous transport systems including glucose, amino acid carriers, and receptor-mediated transcytosis. For example, another putative non-hormonal contraceptive, Adjudin (1-(2,4-dichlorobenzyl)-1*H*-indazole-3-carbohydrazide), was found to be unacceptable as a male contraceptive in humans owing to liver inflammation and

muscle atrophy (Mruk *et al.* 2006). To circumvent the systemic side effects adjuvin was conjugated to a modified FSH mutant protein, which lacked hormonal activity but retained its receptor-binding ability (Mruk *et al.* 2006). Thus, the use of such shuttle molecules would enable both drug delivery through the blood-epididymis barrier as well as to target drug molecules to specific tissues/cells. However, possible epididymal shuttle molecules are yet to be discovered. Over the past few years, considerable effort has been put to developing nanoparticles loaded with liposomes for delivery of drugs into the brain. For example, doxorubicin bound to polysorbate-coated nanoparticles crosses the blood-brain barrier and results in better treatment response than doxorubicin alone (Steiniger *et al.* 2004). However, nanoparticles are given as injections, which is one of the least accepted contraceptive methods among men (Martin *et al.* 2000), and they have to be coated with suitable surfactant, which makes them expensive. These disadvantages are acceptable for cancer drugs, but not for contraceptives meant for every day use.

In addition to epididymal protein targets, there are several other aspects in epididymal functions that could serve as contraceptive targets. They have been reviewed recently by Cooper (2002) and will not be discussed in this review.

Conclusions

There is a large body of information on the modifications occurring on sperm surface during their epididymal transit. However, only few of these modifications have been shown to have direct effects on sperm function. Furthermore, in most cases, the molecular events leading to the modifications on sperm surface are not known. However, several epididymal proteins have been shown to be involved in epididymal sperm maturation, and several proteins and protein families expected to be important in regulating the sperm maturation process have been identified, though not fully characterized. Many of these proteins have been described previously in several review articles (Kirchhoff 1999, Cornwall *et al.* 2002). The advanced high-throughput methods such as expression profiling and *in silico* analyses together with targeted mutagenesis in mice are expected to speed up the identification and functional validation of the key players in the sperm maturation process. Understanding the molecular mechanisms essential for epididymal sperm maturation will not only provide new targets for post-testicular male contraceptives, but will also further increase our understanding of reasons of idiopathic male infertility.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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