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REVIEW

The role of cysteine-rich secretory proteins in male fertility

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The cysteine-rich secretory proteins (CRISPs) are a subgroup of the CRISP, antigen 5 and Pr-1 (CAP) protein superfamily, and are found only in vertebrates. They show a strong expression bias to the mammalian male reproductive tract and the venom of poisonous reptiles. Within the male reproductive tract CRISPs have been implicated in many aspects of male germ cell biology spanning haploid germ cell development, epididymal maturation, capacitation, motility and the actual processes of fertilization. At a structural level, CRISPs are composed of two domains, a CAP domain, which has been implicated in cell–cell adhesion, and a CRISP domain, which has been shown to regulate several classes of ion channels across multiple species. Herein, we will review the current literature on the role of CRISPs in male fertility, and by inference to related non-mammalian protein, infer potential biochemical functions.
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INTRODUCTION

Mammalian fertilization encompasses a series of complex events required to achieve pregnancy. Morphologically complete spermatozoa are produced during spermatogenesis in the testis; however, the potential for fertilization cannot be achieved until spermatozoa undergo two post-testicular maturation events: epididymal maturation and capacitation. Epididymal maturation is a progressive process that occurs during passage through the epididymis that ultimately confers on sperm the capacity for motility, and the full suite of proteins required for oocyte recognition and fertilization.[1,2] Capacitation, on the other hand, occurs in the female reproductive tract and includes the release of suppressive molecules called 'decapacitation factors', the translocation of molecules within the membrane and the initiation of various signal transduction processes. Capacitation leads to sperm hyperactivated motility, their ability to interact with the zona pellucida of the oocyte and undergo the acrosome reaction.

The cysteine-rich secretory proteins (CRISPs) are a group of proteins found in vertebrates that show a strong expression bias in the male reproductive tract in mammals, to the venom of poisonous reptiles and the saliva of the lamprey.[3] Most mammals produce three CRISPs; however, mice produce four CRISPs. Within mammals, CRISPs are also expressed in lower levels within non-reproductive tissues, including secretory glands, skeletal muscle, the spleen and the thymus.[4]

Within mammals, male germ cells encounter CRISPs at virtually every phase of development and maturation. CRISP2 forms an integral component of the sperm head and tail; sperm are bathed in CRISPs within the epididymis, then later mixed with CRISPs from both the seminal vesicles and the prostate and finally at least two CRISPs remain present at, and have been implicated in, the actual processes of fertilization. Not surprisingly, therefore, the CRISP genes have been nominated as infertility candidate genes and proposed as targets for contraceptive action.[5–7] This article will review current literature on the role of CRISPs in male fertility; it will draw upon the function of related non-mammalian CRISPs and other related proteins to infer function and finally will summarize the actual evidence for the role of CRISPs in mammalian male fertility.

CRISP STRUCTURE

CRISPs are proteins defined by the presence of 16 absolutely conserved cysteines that fold into two domains: a N-terminal CRISP, antigen 5 and Pr-1 (CAP) domain (~21 kDa) that contains six conserved cysteine residues, and the smaller C-terminal CRISP (or cysteine-rich) domain (~6 kDa) that contains 10 conserved cysteine residues.[8] In turn, the CRISP domain is composed of two regions: a hinge and an ion channel regulatory (ICR) region. Structural data indicate that all cysteines are involved in intradomain disulfide bonding[9–12] and that the CAP domain contains an active site-like pocket.[13] Although the hinge region is rigidly attached to the CAP domain,[9] there is a relatively high degree of rotational freedom between the hinge region and the ICR region in the CRISP domain.[14]

From an evolutionary perspective, CRISPs are only found in vertebrates.[15] The CAP protein superfamily, within which the CRISPs form one of nine subfamilies, includes 437 known proteins across the Kingdoms *Archaea*, *Bacteria* and *Eukaryota*.[8] Humans express 31 CAP proteins, and mice 33, of which 3 and 4, respectively, are CRISPs. As the name suggests, all CAPs contain a related N-terminal CAP domain. Despite this wide and evolutionarily conserved expression, no single unifying biochemical role for the CAP domain has been elucidated. CAP domains have, however, been implicated in a range of functions spanning immunity, chemoattraction, fertility, carcinogenesis, as a protease and as a protease inhibitor.[13,16–18] For a detailed review of CAP expression and function, readers are referred to Gibbs *et al.*[8]

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Table 1 Summary of CRISP nomenclature

Mouse CRISP name	*Other known names*
CRISP1	Protein DE,[47,49,81] AEG,[82] sialoprotein,[83] SEP,[48] protein IV,[84] 32-kDa protein,[85] MEP7[86]
CRISP2	AA1,[87] TPX1[88]
CRISP3	Specific granule protein 28,[89] horse seminal plasma protein-3[60]
CRISP4	No other known names

Abbreviations: AA1, autoantigen 1; AEG, acidic epididymal glycoprotein; CRISP, cysteine-rich secretory protein; MEP7, mouse epididymal protein 7; SEP, specific epididymal protein.

A consensus on the function of the CRISP domain is, however, beginning to form, in which data strongly suggest that most (if not all) CRISP domains function to regulate ion channels. ICR activity has been most extensively explored using reptile venoms, but emerging data suggest that this is also likely to be the case for non-reptile CRISPs. The ability to regulate ion channels was first demonstrated by Mochca-Morales *et al.*[19] using the Mexican Bearded Lizard CRISP helothermine. Since then reptilian CRISPs have been shown to regulate many classes of ion channels, including voltage-gated ion channels, ryanodine receptors (RyRs) and cyclic nucleotide-gated ion channels A1–3.[20–25]

Of note, it has been shown that the ability of the CRISP domain to regulate ion channel gating, for the King Brown snake CRISP pseudechetoxin, is enhanced by the presence of the CAP domain.[26] Specifically full-length pseudechetoxin at 100 nmol l^{-1} inhibited the cyclic nucleotide-gated ion channel A2, 30 times more potently than the ICR region of pseudechetoxin at 12 μmol l^{-1}.[26]

CRISP nomenclature

CRISP nomenclature can be very confusing. The reasons for this are related to differences in the order within which CRISPs were discovered between species, multiple groups bestowing different names on the same protein (Table 1) and the mouse containing four CRISPs as opposed to the usual three observed in other mammals. This will be briefly clarified to allow an effective assessment of the literature.

The inconsistencies within the CRISP nomenclature are highlighted in the phylogenetic tree constructed by Gibbs *et al.*,[8] which in some instances reveals as much interspecies variation between apparent orthlogous, as within a species between homologs of CRISPs.[8] Therefore, care must be taken when comparing inter- and intraspecies CRISP data; for example, the mouse CRISP1 primary sequence is more closely related to human CRISP3 than human CRISP1 (Table 2). Similarly, comparisons reveal that mouse CRISP4, rather than mouse CRISP1, is the likely orthologous protein to human CRISP1.[27,28] To aid data interpretation, a table comparing CRISP identity and similarity (homology), including the human, mouse, rat and horse, is shown in Table 2.

Of potential relevance, several years ago reproductive proteins were identified as among the 10% most evolutionary divergent proteins.[29] The high level of divergence and interspecies variation is an indicator of rapid evolution, and as proposed by Swanson and Vacquire[30] is a means to drive speciation, that is, biochemical barriers blocking interspecies fertilization. The list of most divergent proteins included CRISP2.

CRISPS IN MALE FERTILITY

Developing germ cells come into contact with CRISPs during every phase of development in the adult male mammal (Figure 1). They are incorporated into the developing sperm acrosome and tail during spermatogenesis, they bathe sperm within the epididymis, and they are mixed with sperm upon ejaculation and present and potentially involved, at the time of fertilization. Although much of the precise biochemistry surrounding CRISP function during these critical periods remains to be elucidated, their presence is surely more than coincidence. Herein, we will summarize the current state of knowledge regarding CRISP localization and where possible speculate on function. A reoccurring theme within this description will be the potential for functional redundancy between CRISPs. It should also be remembered that there are several non-CRISP CAP proteins produced in the male reproductive tract. These include GLIPR1, GLIPR1L1 and GLIPR1L2.[31,32] As such, there is also the potential for functional redundancy between CAP domains. Because of the relatively low state of knowledge on these proteins, however, this potential will not be considered in depth herein.

CRISPS DURING SPERMATOGENESIS

On the basis of published data, *Crisp2* is the only CRISP normally expressed in the mammalian testis. *Crisp2* transcription initiates in

Table 2 Sequence identity (white boxes) and similarity (homology) (gray boxes) between human, horse, mouse and rat CRISPs expressed as percentages

			Identity												
			Mouse				*Human*			*Rat*			*Horse*		
			CRISP1	*CRISP2*	*CRISP3*	*CRISP4*	*CRISP1*	*CRISP2*	*CRISP3*	*CRISP1*	*CRISP2*	*CRISP4*	*CRISP1*	*CRISP2*	*CRISP3*
Similarity	Mouse	CRISP1	—	54	78	42	41	57	55	70	53	42	39	58	57
		CRISP2	71	—	48	39	41	69	59	56	85	40	41	72	60
		CRISP3	82	67	—	38	38	49	50	64	48	37	37	50	50
		CRISP4	54	56	50	—	62	42	44	43	38	89	59	42	42
	Human	CRISP1	54	59	50	77	—	44	43	40	39	63	64	45	43
		CRISP2	71	86	65	57	58	—	70	54	66	41	43	83	71
		CRISP3	72	76	67	58	54	82	—	55	61	44	43	69	66
	Rat	CRISP1	83	71	75	55	53	71	71	—	56	41	37	56	57
		CRISP2	69	92	65	56	58	83	75	69	—	39	39	69	62
		CRISP4	54	56	49	94	77	57	56	55	55	—	61	42	42
	Horse	CRISP1	56	60	54	74	80	58	56	54	59	74	—	44	43
		CRISP2	71	86	68	58	59	90	81	72	84	59	61	—	73
		CRISP3	69	78	63	56	55	82	79	71	78	56	56	84	—

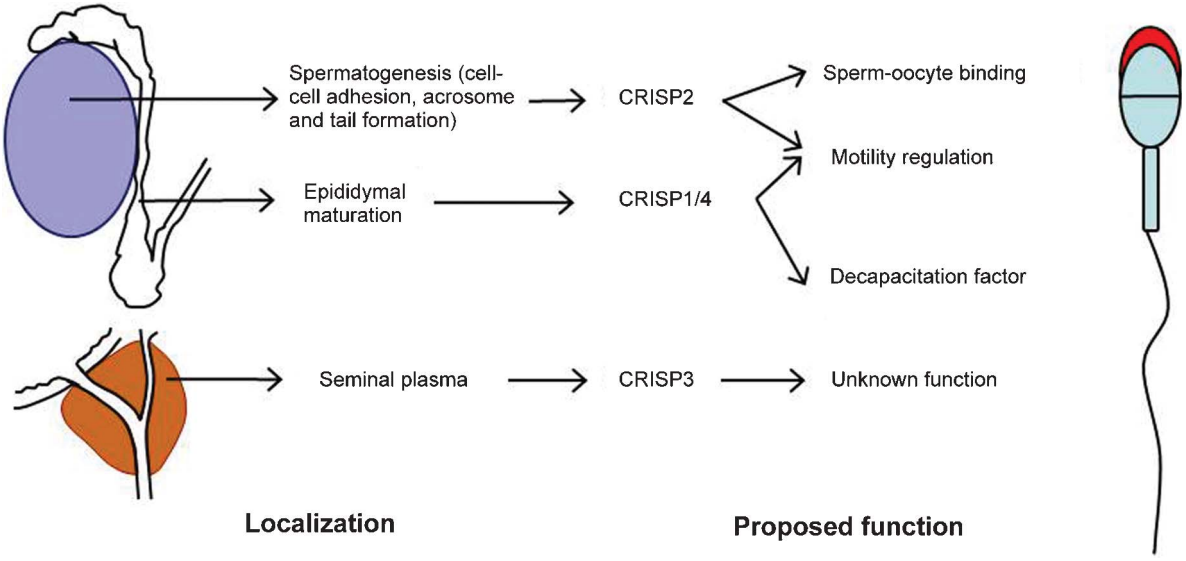

Figure 1 Diagrammatic representation of CRISP expression and function in male fertility. CRISP2 is expressed in haploid germ cells, wherein it is incorporated into the growing acrosome and sperm tail. Within the testis, it has been proposed that CRISP2 is involved in germ cell–Sertoli cell adhesion, and in the tail, it has been proposed to be involved in regulating flagellar beating *via* its ability to regulate ryanodine receptors. CRISP2 remains associated with the fusogenic region of the sperm head after the acrosome reaction. CRISP1 and 4 are both expressed by the principal cells of the epididymis and become incorporated into the maturing spermatozoa and have been implicated as a decapacitation factor. CRISP3 is excreted from the prostate and seminal vesicles and forms part of the seminal plasma. Although variations in CRISP3 sperm content and sequence have been correlated with fertility, no defined role is currently known. CRISP, cysteine-rich secretory protein.

pachytene spermatocytes; however, *via* the activity of the RNA-binding protein DAZL, *Crisp2* mRNA undergoes a period of translational delay before the initiation of translation in round spermatids.[33–37] As shown by the staining (Figure 2), mouse CRISP2 was localized in round spermatids through to elongated spermatids, wherein the protein becomes included into the developing acrosome and the connecting piece and outer dense fibers of the sperm tail.[34–36,38] The potential function of CRISP2 in the connecting piece is further discussed in relation to sperm motility later in this review. Unlike the other CRISP genes, *Crisp2* is not induced by androgens.[39]

The first hint at CRISP2 function came from a study by Maeda *et al.*[40] who used a Jurkat cell transfection assay to identify CRISP2 as having the potential to promote fusion between Jurkat and Sertoli cells. Further, they showed that CRISP2 antisera were able to interfere in the cell adhesion between germ and Sertoli cells. Using deletion studies, the adhesive sequence was narrowed to the N-terminal most

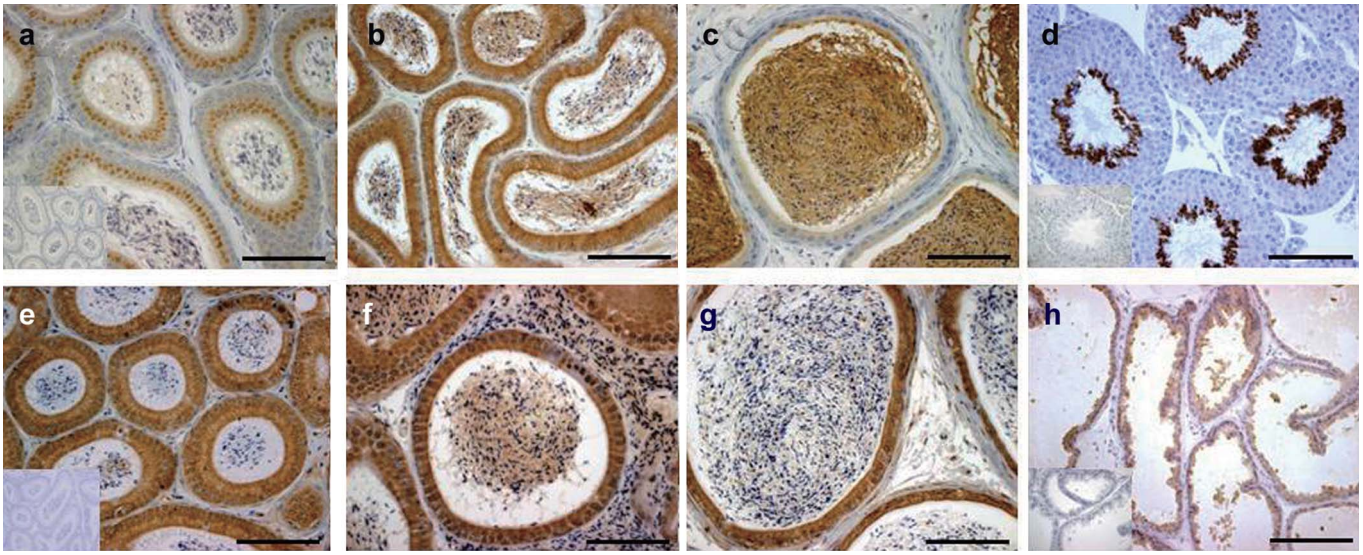

Figure 2 Localization of murine CRISP proteins in male reproductive tissues. (**a**–**c**) CRISP1 staining of the caput, corpus and cauda epididymis, respectively. Staining present in the cytoplasm and stereocilia of the principal cells and spermatozoa within the epididymal lumen. (**d**) CRISP2 staining of the testis shows protein present within the round and elongated spermatids. (**e**–**g**) CRISP4 staining of the caput, corpus and cauda epididymis, respectively. Staining present within the cytoplasm of the principal cells, within the stereocilia and in the epididymal lumen, including within epididymosomes. (**h**) CRISP3 staining of the prostate gland within the apical aspect of the epithelium and in luminal secretory products. Scale bars=100 μm. CRISP, cysteine-rich secretory protein.

101 amino acids.[33] Although this observation is at odds with reports on CRISP2 localization within the testis, it does corroborate observations on the localization of CRISP2 at the site of fertilization and the involvement of CAP domain sequences in sperm–egg fusion (see later).

In addition to the localization of CRISP2 within testis, three CRISP2-binding partners have been identified: mitogen-activating protein kinanse kinase kinase 11, gametogenetin 1 and sperm head and tail-associated protein.[41–43] All three CRISP2-binding proteins require the CRISP domain for binding; however, each has a specific region of colocalization with CRISP2. Mitogen-activating protein kinanse kinase kinase 11 and CRISP2 colocalize in the developing and mature sperm acrosome,[41] gametogenetin 1 and CRISP2 colocalize in the principal piece of the sperm tail,[42] and sperm head and tail-associated protein and CRISP2 colocalize in the sperm head and principal piece of the sperm tail.[43]

CRISPS IN THE EPIDIDYMIS AND DURING EPIDIDYMAL SPERM MATURATION

On leaving the testis, sperm enter the epididymis and begin the process of epididymal maturation. During this time within the seminal plasma, spermatozoa are surrounded by huge concentrations of CRISPs produced by the principal cells of the epididymal epithelium. In the mouse, CRISP1 is produced by all regions of the epididymis, but is preferentially expressed in the cauda.[4,28] In contrast, the second mouse epididymal CRISP, CRISP4, is preferentially produced in the caput, incorporated into epididymosomes and binds to the sperm surface.[4,27,28]

Humans, however, appear to produce only one CRISP in the epididymis, CRISP1. CRISP1 is produced by principal cells throughout the epididymis, whereupon it is incorporated into epididymosomes and presumably transferred onto the sperm surface.[44–46] Collectively, these data raise the possibility that at a functional level, the function of human CRISP1 may be equivalent to the sum of the activities of mouse CRISP1 plus CRISP4.

Rats also contain only one epididymal CRISP, CRISP1. However, CRISP1 is produced as two isoforms, proteins D and E, which are differentially localized within the rat epididymis[47] Protein D is more abundant than E (70% of total rat CRISP1), and is synthesized by the principal cells of all regions of the epididymis and localizes to the sperm head.[47–50] Protein E is produced only in the corpus and proximal cauda epididymis.[47] Although originally localized in the dorsal region of the sperm head, as rat spermatozoa undergo the acrosome reaction, protein E migrates to the equatorial segment.[51,52] Protein estimates indicate that CRISP1 has a concentration of 1.6 mg ml^{-1} within rat epididymal secretions.[53] The two distinct isoforms of CRISP1 in rats has led to the hypothesis of two different functions. Most simplistically data suggest that the loosely associated D form may function as a decapacitation factor (see below) and that the E form may function during fertilization (see below).

CRISPS AS A DECAPACITATION FACTOR

Although the precise function of the CRISPs in the epididymis remains enigmatic, data describing their properties once they are diluted in the fluids of the female reproductive tract, suggest that they may function as decapacitation factors. Decapacitation factors function to keep sperm within the epididymis in a quiescent state. Their diffusion off sperm initiates (at least some of) the pathways leading to capacitation.[54]

Several pieces of data suggest that CRISP1, in particular, may be a decapacitation factor. The rat D isoform of CRISP1 interacts with spermatozoa transiently and has a reversible association with the sperm surface.[50] The majority of human CRISP1 is also weakly attached to ejaculated sperm, is easily removed by washing and thus likely to rapidly diffuse off the sperm in the female reproductive tract.[45] Mouse CRISP1 was identified in the decapacitation factor containing fraction of mouse epididymal fluid.[55] Furthermore, the addition of purified native CRISP1 to rat spermatozoa *in vitro* was able to inhibit the manifestation of tail protein tyrosine phosphorylation, a robust marker of capacitation, and the number of sperm undergoing the acrosome reaction in response to cholesterol removal from the sperm plasma membrane.[56] Collectively, these, and other data, suggest that CRISP1 binds to sites on the sperm surface resulting in a suppressed (or quiescent) state of storage in the cauda epididymis.[50] The mechanism by which CRISP1 inhibits capacitation is currently unknown, but may logically involve the inhibition of ion channels similar to that observed for reptile CRISPs or mouse CRISP2.[14]

In apparent conflict with the rat data, *Crisp1* knockout male mice were fertile. They did, however, manifest a subtle form of subfertility characterized by lower levels of tyrosine phosphorylation after *in vitro* capacitation.[57] Although the reason for this apparent conflict remains to be resolved, it may be the result of species variation in the function of CRISP1 between the mouse and the rat; the absence of CRISP1 may alter epididymal sperm maturation, or the presence of CRISP4 in the mouse may compensate for the absence of CRISP1.

CRISPS AT THE EJACULATE AND IN THE ACCESSORY SEX ORGANS

CRISP3 is produced by the normal human vas deferens and the prostate.[58] CRISP3 is produced by the mouse prostatic epithelium and is present in secretions within the lumen (Figure 2).[4] Similarly, CRISP3 is produced in horse seminal vesicles[59] and is present in both horse and human seminal plasma at concentrations of 0.3–1.3 mg ml^{-1} and 14.8 μg ml^{-1}, respectively.[58,60] Despite its presence in such high concentrations, the function of these CRISPs within the ejaculate remains virtually unknown. As mentioned below, although the localization of CRISP3 on horse sperm has been correlated with relative fertility, the crude concentration of CRISP3 in the ejaculate has no relationship with fertility status.[61]

Regardless of the above, we have some insight of the biochemistry of CRISP3 thanks to some excellent research on CRISP3 in the immune systems and its association with various pathological conditions.[58,62,63] Within human serum and seminal plasma, CRISP3 binds to β-microseminoprotein and α1B-glycoprotein in a 1 : 1 stoichiometry.[63,64] A three-dimensional nuclear magnetic resonance spectrum for the interaction between CRISP3 and α1B-glycoprotein has been determined.[65] By analogy to snake CRISPs that become bound to snake serum proteins to limit the chances of self-envenomation,[66] it has been proposed that the binding of CRISP3 to β-microseminoprotein in seminal plasma serves to limit CRISP3 bioactivity.[63] The challenge remains, however, to determine the bioactivity that must be limited.

Although CRISP3 is expressed by the normal prostate, several studies have revealed an association between increased CRISP3 production and malignant prostate disease.[67] CRISP3 expression is increased more than 50-fold in pre-malignant prostate lesions and in primary tumors compared with normal prostatic epithelium.[68,69] The significance of this observation is yet to be realized; however, CRISP3 shows promise as a marker for prostate disease.[68,69]

CRISP2 IN ION CHANNEL REGULATION AND SPERM MOTILITY

A role for CRISP2 in ICR activity was first intimated through the nuclear magnetic resonance structure of the CRISP2 CRISP domain, and then proven using recombinant CRISP2 in *in vitro* assays.[14] This study showed that the CRISP2 CRISP domain was capable of inhibiting Ca^{2+} flow through RyR2 in a non-voltage-dependent manner and activated RyR1 opening in a weakly voltage-dependent manner.[14] As an extension of this work, studies in humans have shown that RyRs are localized over the sperm head, including the head/mid-piece junction (connecting piece), in which they are hypothesized to mediate Ca^{2+} release from internal store and to modulate flagellar activity.[70,71]

CRISPS IN SPERM–OOCYTE BINDING

Several lines of evidence suggest a role for CRISPs in the interaction between the sperm and the oocyte at fertilization. Specifically, rat (protein E) and mouse CRISP1 irreversibly associate with the sperm plasma membrane during epididymal transit.[56,72,73] and remain localized to the fusogenic region of the sperm head after the acrosome reaction.[52] A similar localization was observed on human sperm,[44,74] and CRISP1 antibodies were capable of significantly reducing the number of penetrating human sperm in the hamster oocyte penetration assay.[75] CRISP1 antisera have been shown to interfere with sperm–oocyte binding[76] and the coincubation of peptides derived from the CAP signature motifs in the CAP domain (amino acids 114–158) of rat CRISP1-inhibited sperm–zona pellucida binding and sperm–egg fusion in *in vitro* fertilization.[74] Furthermore, although *Crisp1* knockout mice produced litters of normal size, their sperm showed a reduced ability to penetrate the zona pellucida and to adhere to the oocyte plasma membrane.[57]

It also has been shown that mouse CRISP2 remains associated in the equatorial region of the head after the acrosome reaction and that the equivalent CRISP2 CAP domain peptides to those mentioned above, reduces sperm–oocyte binding.[77] *In vitro* sperm competition assays suggested that both CRISP1 and CRISP2 have common binding sites on the oocyte surface.[77] These data, and associated data showing the presence of at least one other CAP at the site of fertilization,[31] leave little doubt that CAP proteins are involved in fertilization. The precise number of CAPs and their mode of action, however, remain to be elucidated.

CRISPS AND MALE INFERTILITY

Not surprisingly, given the widespread and apparently multilayered production of CRISPs in the male reproductive tract, aberrant CRISP function has been proposed as a cause of both human and animal infertility. As implied by in the evolutionary study from Swanson and Vacquier,[30] CRISPs tend to have a high degree of intraspecies sequence variation. To date, however, only variations in the horse *Crisp3* gene have been identified as a statistically significant cause of reduced fertility. Specifically, Hamann *et al.*[78] identified a single polymorphism in *Crisp3* of the Hanoverian warm blood horse that were related to stallion fertility.[78] The E208K polymorphisms when present in a heterozygous state compromised fertility by an average of 7% compared with animals carrying the more common allele in a homozygous state. The E208K polymorphism sits within the hinge region of the CRISP domain.

A similar study using human samples identified a large number of single nucleotide polymorphisms in human *CRISP2*.[79] Although none of the single nucleotide polymorphisms were definitively associated within infertility, at least one (C196R) is of biochemical significance by virtue of its ability to interfere with binding to gametogenetin 1. This substitution also occurs within the hinge region and is proposed to destabilize the cross-disulfide bonds.[79] Similarly, several studies have shown that the human *CRISP2* gene occurs in a region frequently associated with translocations and male infertility.[80]

NOVEL CONTRACEPTIVE TARGET

Equally obviously, CRISPs, by virtue of the reproductive tract enriched expression, have been proposed as potential contraceptive targets, and considerable resources have been devoted to testing this hypothesis. The use of CRISP1 for immunocontraception has been shown to result in the production of anti-CRISP1 immunoglobulinin and their adherence to sperm in the rat and the macaque. Although some studies show reduced sperm–zona binding *in vitro*, males retained fertility.[5–7] Although these studies, and the *Crisp1* knockout mouse, suggest that the collective inhibition of all CRISPs may form the basis of a contraceptive, immunocontraceptives are unlikely to be an effective and acceptable mode of action.

CONCLUSION

Collectively, the CRISPs are a fascinating group of proteins. They show a notable expression bias to the male reproductive tract and are present at virtually every phase of adult germ cell development and maturation. It is hard, therefore, to believe that they do not have an important role. Defining these roles has, however, proved elusive and is exacerbated by a number of factors, including their protein structure and the limited availability of precise analytical tools. Considerable progress has, however, been made in defining their function within the last few years and a picture of function in cell–cell adhesion and ion channel regulation is beginning to emerge.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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