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Evaluation of Testicular Sperm CRISP2 as a Potential Target for Contraception

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ABSTRACT: Cysteine-rich secretory protein 2 (CRISP2) is a testicular sperm protein proposed to be involved in fertilization. With the aim of examining the relevance of CRISP2 for fertility and its potential use as a target for contraception, in the present work, male and female rats were immunized with recombinant CRISP2 coupled to maltose-binding protein (MBP) and evaluated for their subsequent fertility. As controls, animals were injected with either MBP or recombinant CRISP1. Enzyme-linked immunosorbent assay of sera collected at different intervals after immunization indicated that CRISP2 immunization raised specific antibodies in both sexes, with levels that increased as a function of time. Western blot studies revealed that anti-CRISP2 sera were capable of recognizing CRISP2 in testicular, epididymal, and sperm extracts, whereas histological studies showed no evidence of autoimmune orchitis or epididymitis. Indirect immunofluorescence experiments revealed the ability of anti-CRISP2 sera to recognize the native sperm protein in fresh, capacitated, and ionophore-induced acrosome–reacted cells. Moreover, anti-CRISP2 sera significantly inhibited the sperm ability to penetrate zona-free eggs, confirming the role of CRISP2 in rat gamete fusion. In spite of the presence of circulating anti-CRISP2 antibodies capable of inhibiting the sperm fertilizing ability, mating studies revealed no effects of CRISP2 immunization on male or female fertility, in contrast to the significant inhibition observed in both sexes in animals injected with CRISP1. Together, these observations indicated the immunogenic properties of testicular CRISP2 but do not support CRISP2 as a target for immunocontraception or as a molecule responsible for generating autoimmune orchitis or immunoinfertility.

Key words: Fertility, fertilization, contraception sperm.
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The cysteine-rich secretory protein (CRISP) family is a large group of secreted proteins with molecular weights of about 20–30 kd, characterized by the presence of 16 conserved cysteine residues, 10 of which are clustered in the C-terminal portion of the molecule (Gibbs et al, 2008). The first member of this family was originally described by our laboratory in the rat epididymis (Cameo and Blaquier, 1976), and it is known as CRISP1. Since then, other members of the family have been identified in different mammalian tissues: CRISP2, also known as Tpx-1, which is expressed predominantly in the testis (Hardy et al, 1988; Kasahara et al, 1989); CRISP3, with a wider tissue distribution, including reproductive and nonreproductive organs (Haendler et al, 1993; Kjeldsen et al, 1996; Udby et al, 2005); and CRISP4, which is primarily expressed in the epididymis (Jalkanen et al, 2005). Nonmammalian proteins belonging to the CRISP family have also been described in venoms from lizards and snakes and in oocytes and embryos from *Xenopus* (Gibbs et al, 2008). Significant molecular similarities have been found between CRISP proteins, allergens from insect venoms, and pathogenesis-related proteins from plants, comprising altogether the widely distributed and evolutionarily conserved CRISP/antigen 5/pathogenesis–related (CAP) superfamily.

Mammalian CRISP1 is a surface protein mainly localized in the dorsal region of the sperm head and proposed to mediate both sperm–zona pellucida (ZP) interaction and gamete fusion through complementary sites localized in the egg (Rochwerger et al, 1992; Cohen et al, 2000; Busso et al, 2007a). These functional roles were confirmed by the observation that CRISP1 knockout mice generated in our laboratory exhibited a significantly impaired ability to interact with the ZP and oolema (Da Ros et al, 2008). Moreover, our results indicated that CRISP1 is relevant for fertility, in that immunization of male and female rats with the native or recombinant protein produced a specific immune response and a significant inhibition of fertility through a mechanism involving the entry of the antibodies to the

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reproductive tract and their specific interference with the sperm fertilizing ability (Cuasnicú et al, 1990; Perez Martinez et al, 1995; Ellerman et al, 1998, 2008).

CRISP2 was originally described as one of the primary autoantigens abundantly present within the guinea pig acrosome (Hardy et al, 1988) and as a possible causative agent for autoimmune orchitis (Hardy et al, 1988; Foster and Gerton, 1996). It was found to exist as a component of both the acrosome and the outer dense fibers of the tail (O'Bryan et al, 1998, 2001), where several binding partners have been described (Gibbs et al, 2007; Jamsai et al, 2008, 2010), and as a surface molecule responsible for the specific interaction between spermatogenic and Sertoli cells (Maeda et al, 1998, 1999). Subsequent studies showed that the C-terminal domain of recombinant mouse CRISP2 is able to regulate ryanodine Ca^{2+} channels (Gibbs et al, 2006) in an analogous manner to several CRISPs isolated from reptile venoms (Yamazaki and Morita, 2004).

Results from our group supported that, in both human and mouse sperm, CRISP2 participates in gamete fusion through its interaction with complementary sites in the egg (Busso et al, 2005, 2007b). Moreover, experiments with CRISP1 and CRISP2 proteins in competition studies revealed that CRISP2 interacts with the same egg-binding sites as CRISP1 (Busso et al, 2007b). In this regard, it is interesting to note that the egg-binding site of CRISP1 resides in a region of only 12 amino acids, corresponding to 1 of the 2 consensus motifs of the CRISP family, being only 2 amino acids different from that corresponding to CRISP2 (Ellerman et al, 2006). These observations suggest that CRISP1 and CRISP2 might act in gamete fusion through a similar molecular mechanism and cooperate with each other during fertilization, CRISP2 being a good candidate to partly compensate for the absence of CRISP1 in the knockout mice.

In addition to its role in fertilization, CRISP2 seems to be relevant for fertility, as judged by results showing alterations in protein levels and localization in sperm from patients with spermatogenic arrest and in infertile men with oligoasthenoteratospermia syndrome (Du et al, 2006). In agreement with this, a recent study reported a down-regulation of CRISP2 mRNA and protein expression in asthenospermic patients, suggesting that CRISP2 modifications might be related to the decreased sperm motility observed in these patients (Jing et al, 2011). In addition to this, anti-CRISP2 antibodies have been detected in antisperm antibody (ASA)–positive seminal plasma of immune infertile men (Domagala et al, 2007). Together, these observations in men open the possibility that CRISP2 might serve as a potential target for the development of both diagnostic assays and methods for fertility regulation.

With the aim of exploring both the relevance of CRISP2 for fertility and its potential use for the development of a contraceptive approach, in the present study, we examined whether immunization of male and female rats with CRISP2 was able to elicit an immune response that inhibits animal fertility.

Materials and Methods

Animals

Young adult (70–90 day-old) male and female Wistar rats and adult (70–120 day-old) male and prepubertal (26–29 day-old) female Sprague Dawley rats were used. Animals were maintained with food and water ad libitum in a temperature-controlled room with a 12:12 hour light:dark cycle. Experiments were conducted in accordance with the *Guide for Care and Use of Laboratory Animals* published by the National Institutes of Health (2011).

Production and Treatment of Proteins

Escherichia coli BL21 bacteria were transformed with the expression plasmid p-MAL for expression of maltose-binding protein (MBP), or with p-MAL containing the expression sequences for CRISP2 [p-MAL-CRISP2] or CRISP1 [p-MAL-CRISP1]. Recombinant MBP and recombinant CRISP2 (recCRISP2) and CRISP1 (recCRISP1) proteins fused to MBP were expressed by induction with isopropyl-1-thio-b-galactoside (Promega, Madison, Wisconsin) as previously described (Ellerman et al, 2002). The 3 proteins were then purified by affinity chromatography through an amylose (maltose polymer) resin column (New England BioLabs Inc, Beverly, Massachusetts). The presence of each protein in bacterial extracts was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining, and the purity of the protein was analyzed by SDS-PAGE followed by silver staining and Western blot. Native CRISP1 was purified from rat epididymal homogenates according to the protocol described by Garberi et al (1979).

Immunization and Collection of Sera

Animals received 4 injections of 100 μg of recCRISP2 (10 males and 10 females) or equimolar concentrations of MBP (9 males and 9 females) or recCRISP1 (6 males and 6 females) according to the immunization schedule shown in Figure 1. The first injection consisted of 300 μL of antigen emulsified with 300 μL of Freund complete adjuvant (Sigma-Aldrich, St Louis, Missouri). For subsequent injections, Freund incomplete adjuvant (Sigma-Aldrich) was used. Each animal received 300 μL of the emulsion intramuscularly in a rear leg and 300 μL subcutaneous (SC) in the back. At various times during the study (Figure 1), animals were anesthetized with ketamine hydrochloride and bled from the jugular vein as previously

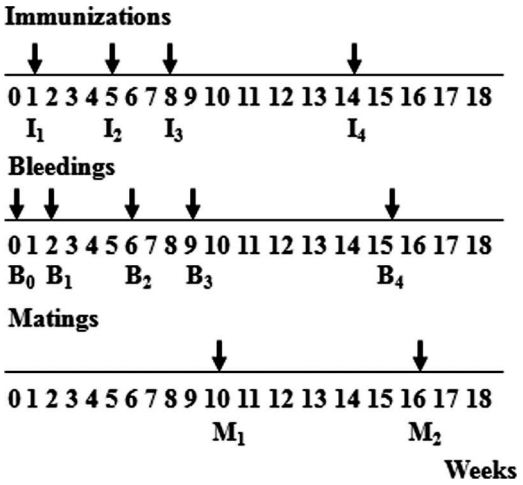

Figure 1. Experimental time course of immunizations (I), bleedings (B), and matings (M) for Wistar rats. B0 indicates preimmunization bleeding.

described (Ellerman et al, 1998). Samples were incubated for 30 minutes at 37°C, overnight at 4°C, and centrifuged for 15 minutes at 600 × g. Sera were separated from the pellet and stored at −20°C until use.

Enzyme-Linked Immunosorbent Assay

Purified recCRISP2, MBP, or native CRISP1 diluted in 0.3 M $NaHCO_3$, pH 9.6 (50 ng/mL), was coated in each well of a 96-well microtiter plate (Nunc A/S, Roskilde, Denmark) and incubated overnight at 4°C. Nonspecific binding sites were blocked for 90 minutes at room temperature (RT) with a solution of 20 mg/mL of powdered skim milk in phosphate-buffered saline (PBS). Sera diluted 1:5000 in PBS containing 0.1% bovine serum albumin (BSA; PBS-BSA1) were placed in duplicate wells and incubated for 90 minutes at 37°C. Wells were then washed and incubated for 1 hour at 37°C with biotin-conjugated anti-rat immunoglobulin G (IgG) antibody (Sigma-Aldrich; 1:500 in PBS-BSA1). After washing, ExtrAvidin-alkaline phosphatase (Sigma-Aldrich; 1:1000 in PBS-BSA1) was added, and incubation was continued for 30 minutes at RT. Wells were washed, and color reaction was allowed to develop by addition of *p*-nitro-phenyl-phosphate (1 mg/mL in 1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8). The absorbance at 405 nm was determined with a microplate reader (Cambridge Technology Inc, Watertown, Massachusetts).

Electrophoresis and Western Blot

For preparation of protein extracts, tissues were homogenized in 1.5 volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.4) containing 0.2 mM phenylmethylsulfonyl fluoride. The homogenates were then centrifuged twice at 10 000 × *g* for 20 minutes at 4°C, and the supernatants were dialyzed against 50 mM Tris–HCl buffer (pH 6.8). For preparation of sperm extracts, sperm samples were incubated in Laemmli buffer (Laemmli, 1970; without 2-β-mercaptoethanol) for 5 minutes, boiled, and centrifuged at 5000 × *g* for 5 minutes at RT. All protein samples were separated by SDS–PAGE (15%) along with broad-range protein markers (SeeBlue Plus 2; Invitrogen Corporation, Carlsbad, California) and transferred onto nitrocellulose membranes (Towbin et al, 1979). After blocking with 20 mg/mL of powdered skim milk in PBS, the membranes were probed with 1:100 of either preimmune sera or sera from animals injected with recCRISP2 or MBP. Membranes were thoroughly washed before incubation for 1 hour with biotin-conjugated anti-rat IgG (1:500 dilution, Sigma-Aldrich). After extensive washing, the membranes were incubated for 1 hour with ExtrAvidin-horseradish-peroxidase (1:1000 dilution, Sigma-Aldrich), and reactive bands were visualized with 3,3′-diaminobenzidine (40 μg/mL in Tris 0.1 M, pH 7.5, 0.01% H_2O). All incubations were carried out at RT.

Histology

Animals immunized with either recCRISP2 or MBP (4 males and 4 females of each group) were used for histological examination 1 month after the second mating. Rats were anesthetized, and the testes, epididymis, and ovaries were removed and fixed for at least 24 hours by immersion in Bouin solution diluted 1:1 with deionized water. Tissues were then processed for paraffin embedding and sectioning by routine methods. Sections were stained with hematoxylin and eosin solutions and examined by light microscopy.

Recovery and Treatment of Gametes

Spermatozoa—Sperm collected by puncturing the cauda epididymidis of adult Sprague Dawley rats were placed in a conical tube, covered with 1.5 mL of rat fertilization medium (RFM; Kaplan and Kraicer, 1978), and allowed to swim up at 37°C (Rochwerger and Cuasnicu, 1992). For capacitation, aliquots of the upper sperm layer were added to 400 μL of media previously placed in tissue culture wells (16 mm; Nunc Brand Products), at a final concentration of 0.5–1.0 × 10^6 sperm/mL. Sperm suspensions were then incubated under paraffin oil at 37°C for 5 hours in an atmosphere of 5% CO_2 in air.

For induction of the acrosome reaction (AR), ionophore A23187 (Sigma-Aldrich) was added (10 μM final concentration) to capacitated sperm 30 minutes before the end of the capacitation period. The occurrence of the AR was evaluated by Coomassie brilliant blue staining (Bendahmane et al, 2002).

Eggs—Prepuberal Sprague Dawley female rats were superovulated by injection (SC) of equine chorionic gonadotropin (20 IU; Syntex, Buenos Aires, Argentina) followed by the administration (SC) of human chorionic gonadotropin (hCG, 25 IU; Sigma-Aldrich) 48 hours later. Metaphase II oocytes were collected from the oviducts of superovulated females 15 hours after hCG injection. Cumulus cells were removed by incubating the egg-cumulus complex for 3 minutes in RFM containing 0.1% hyaluronidase (Type IV; Sigma-Aldrich), and ZP were dissolved by treating the cumulus-free eggs with acid Tyrode solution (pH 2.5) for 10–20 seconds (Rochwerger and Cuasnicu, 1992). Zona-free eggs were thoroughly washed before their use in fertilization or immunofluorescence studies.

Indirect Immunofluorescence

Spermatozoa—Rat caudal epididymal sperm were fixed for 10 minutes in 2% paraformaldehyde in PBS at RT, extensively washed with PBS, and placed on slides. For permeabilization, the slides were immersed serially in decreasing percentages of ice-cold methanol in PBS for 5 minutes. Sperm were then incubated in 0.4% BSA in PBS (PBS-BSA4), for 30 minutes at 37°C and exposed to anti-CRISP2 or anti-MBP sera 1:50 in PBS-BSA4 overnight at 4°C. After washing 3 times in PBS, sperm were incubated for 30 minutes at 37°C with fluorescein isothiocyanate (FITC)–conjugated goat anti-rat IgG (1:100 in PBS, Sigma-Aldrich), washed, and examined with a Nikon Optiphot microscope (Tokyo, Japan) equipped with epifluorescence optics. In those cases in which indirect immunofluorescence (IIF) was carried out on live cells, sperm aliquots were washed twice in PBS-BSA4 incubated with anti-CRISP2 or anti-MBP sera 1:50 for 2 hours at 37°C, and then with FITC-conjugated anti-rat IgG for 1 hour at 37°C. After washing with 10 volumes of PBS-BSA4, sperm were mounted on slides at 37°C and observed under an epifluorescence microscope.

Eggs—Zona-free rat eggs exposed to recCRISP2 or MBP were fixed in 2% paraformaldehyde in PBS for 40 minutes at RT and then washed several times with PBS-BSA4. Eggs were then incubated in 5% normal goat serum in PBS for 30 minutes at 37°C and exposed to anti-MBP antibody for 1 hour at 37°C. After washing 3 times in PBS-BSA4, the oocytes were incubated for 30 minutes at 37°C in FITC-conjugated anti-rabbit antibody (1/100 in PBS-BSA4, Sigma-Aldrich). After 3 washings, oocytes were mounted in 90% glycerol in PBS, and observed under an epifluorescence microscope.

Sperm-Egg Fusion Assays

Capacitated sperm were added (final concentration 0.5–10 $\times$ 10^5 cells/mL) to zona-free eggs, and the gametes were coincubated for 3 hours at 37°C in an atmosphere of 5% CO_2 in air. Eggs were then washed and analyzed for evidence of sperm penetration under phase-contrast microscopy. Eggs were considered penetrated if a decondensing sperm head or 2 pronuclei and a sperm tail were present in the ooplasm. For experiments examining the effect of the sera on sperm-egg fusion, sperm were incubated for 30 minutes in 50 μL drops (under oil) of RFM containing 1/100 dilution of either anti-CRISP2 or anti-MBP sera before the addition of the eggs. For those cases in which the effect of the protein on gamete fusion was examined, zona-free eggs were exposed to 0.6–18 μM of recCRISP2 or MBP for 30 minutes before insemination.

Mating

Fertility of Wistar rats was analyzed by natural mating 10 and 16 weeks after initial immunization. For evaluation of male fertility, each male was housed with 3 females of proven fertility for 1 week. Female fertility was evaluated by caging 2 females (from 2 different experimental groups) with 1 male of proven fertility for 1 week. In all the cases, females were examined thereafter, and those showing evidence of pregnancy were removed from the cage and allowed to deliver in a separate cage. Fertility was expressed as both fertility rates and average litter size. Fertility rates for each male were expressed as the ([number of pregnant females]/[total number of females in the cage {3}] $\times$ 100), whereas female fertility rate was calculated as ([the number of pregnant females])/([total number of mated females]) $\times$ 100.

Statistical Analysis

Results are expressed as $\bar{x} \pm$ SD for each series of experiments. The enzyme-linked immunosorbent assay (ELISA) data was determined using 1-way analysis of variance. The percentages of sperm exhibiting different immunofluorescent patterns, as well as the percentages of fertilized eggs, were analyzed by the chi-squared test. Male and female fertility data were analyzed by unpaired t test. Values with a confidence level of $P < .05$ were considered statistically significant.

Results

To investigate whether CRISP2 was able to elicit an immune response that inhibits animal fertility, male and female rats were immunized with recCRISP2 according to the schedule shown in Figure 1 using MBP as control. On the basis of our previous results showing that immunization of rats with recCRISP1 produced an inhibition of fertility (Ellerman et al, 2008), a third group was injected with the recombinant epididymal protein.

To determine the time course of the immune response, sera from recCRISP2- immunized animals were used in ELISA against the protein. All sera were diluted in the presence of an excess of soluble MBP (300-fold compared with MBP bound to the plate) to preabsorb the anti-MBP antibodies. Under these conditions, no detectable antibodies were observed when MBP-coated plates were used. Results showed that the antibodies against recCRISP2 increased in male and females as a function of time, reaching values significantly higher than those corresponding to preimmune sera at 2 weeks after initial immunization (Figure 2A). No differences were observed in final antibody levels between recCRISP2- and recCRISP1-immunized animals (Figure 2B).

To study the ability and specificity of the raised antibodies to detect CRISP2, immune sera were used as first antibodies in Western blot assays. Results indicated that sera from male and female CRISP2-immunized animals were able to recognize a band corresponding to CRISP2 in testis, epididymis, and sperm, giving a negative reaction when tested against a series of essential and reproductive organs (Figure 3A). Anti-CRISP2 antibodies detected a band in both epididymal and ovarian extracts with a molecular weight different from CRISP2 and did not cross-react with purified native CRISP1. Neither the preimmune sera nor the sera from

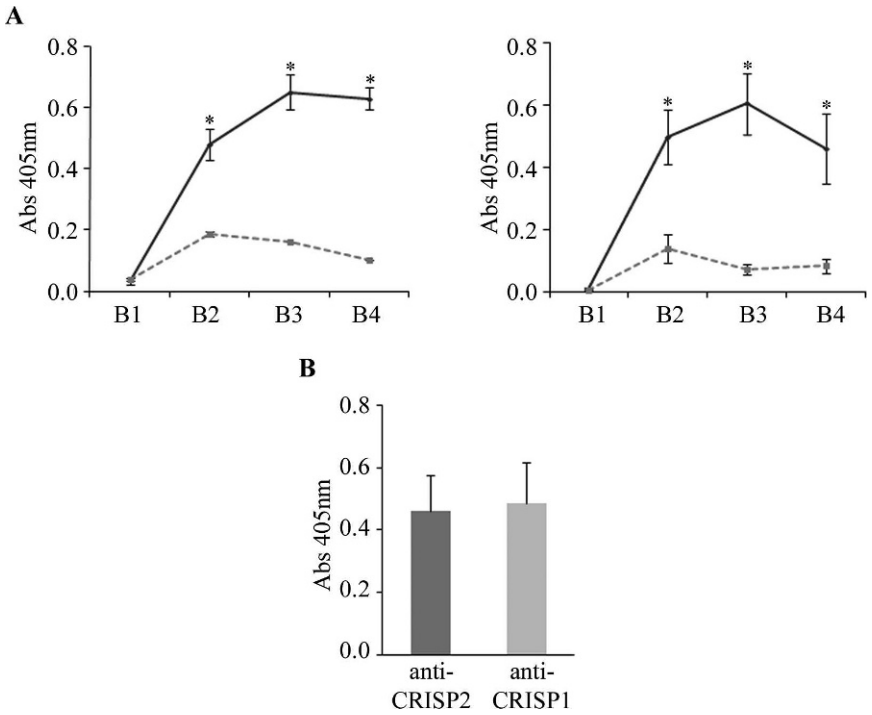

Figure 2. Kinetics of the immune response against recombinant cysteine-rich secretory protein 2 (recCRISP2). **(A)** Sera from CRISP2-immunized male (left) and female (right) animals were preabsorbed with maltose-binding protein (MBP) and used as primary antibodies in enzyme-linked immunosorbent assay (ELISA) against recCRISP2 (——) or MBP (- - - -) at a 1:5000 dilution. B indicates bleedings. **(B)** MBP-absorbed anti-CRISP2 sera and anti-CRISP1 sera (1:5000 dilution) were used as a first antibody in ELISA against recCRISP2 and native CRISP1, respectively. Values represent the mean absorbances (Abs) corresponding to each experimental group. $*P < .05$ vs preimmune sera, and vs anti-recCRISP2 sera absorbed with MBP.

MBP-immunized animals were able to recognize CRISP2 or other proteins in testicular or sperm extracts (Figure 3A).

The ability of anti-CRISP2 sera to recognize bands in the testis, epididymis, and ovary led us to explore whether immunization with CRISP2 produced a deleterious effect on these organs. For this purpose, the testes, epididymides, and ovaries from the immunized animals were subjected to histological examination. Male CRISP2-immunized rats exhibited normal seminiferous and epididymal tubules with presence of spermatids and spermatozoa, respectively, whereas the ovaries from CRISP2-immunized rats showed the presence of corpora lutea and growing follicles without evidence of degeneration or atresia (Figure 3B). No signals of leukocyte infiltration were observed in any of the tissues analyzed, excluding a possible pathological effect of the raised antibodies on these organs.

To investigate whether anti-CRISP2 sera were capable of recognizing the native sperm protein, fresh, capacitated, and ionophore-induced acrosome–reacted cells were subjected to IIF using the immune sera as first antibodies. Results revealed that whereas both live and fixed nonpermeabilized fresh spermatozoa incubated with anti-CRISP2 sera showed no staining or a very faint labeling in the head, respectively (data not shown), those permeabilized with cold methanol exhibited 2 clear staining patterns: 1 showing labeling in the dorsal region of the acrosome accompanied by labeling in the concave region of the head (pattern I; Figure 4A, a and b), and 1 showing fluorescent staining only in the concave region of the head (pattern II; Figure 4A, c and d). In both cases, fluorescent staining was also observed in the tail. Although pattern I was detected in most (67% ± 3%) fresh sperm, the percentages of cells with pattern II were significantly ($P < .0001$) higher in capacitated (52% ± 8%) and acrosome reacted (72% ± 3%) populations compared with fresh sperm. No labeling was observed in any of the cells when anti-MBP was used as first antibody (Figure 4A, e and f). Sperm CRISP2 was not affected by capacitation, the acrosome reaction (AR), or both as judged by Western blot results revealing the presence of only 1 band of 25 kd in sperm extracts from the 3 populations (Figure 4B).

To examine whether the anti-CRISP2 sera were capable of inhibiting the sperm fertilizing ability, zona-free rat eggs were coincubated with capacitated sperm in the presence of either anti-CRISP2 or anti-MBP sera. Results showed that anti-CRISP2 sera (1:100) produced a significant decrease in the percentage of fertilized eggs compared with controls (Figure 5A).

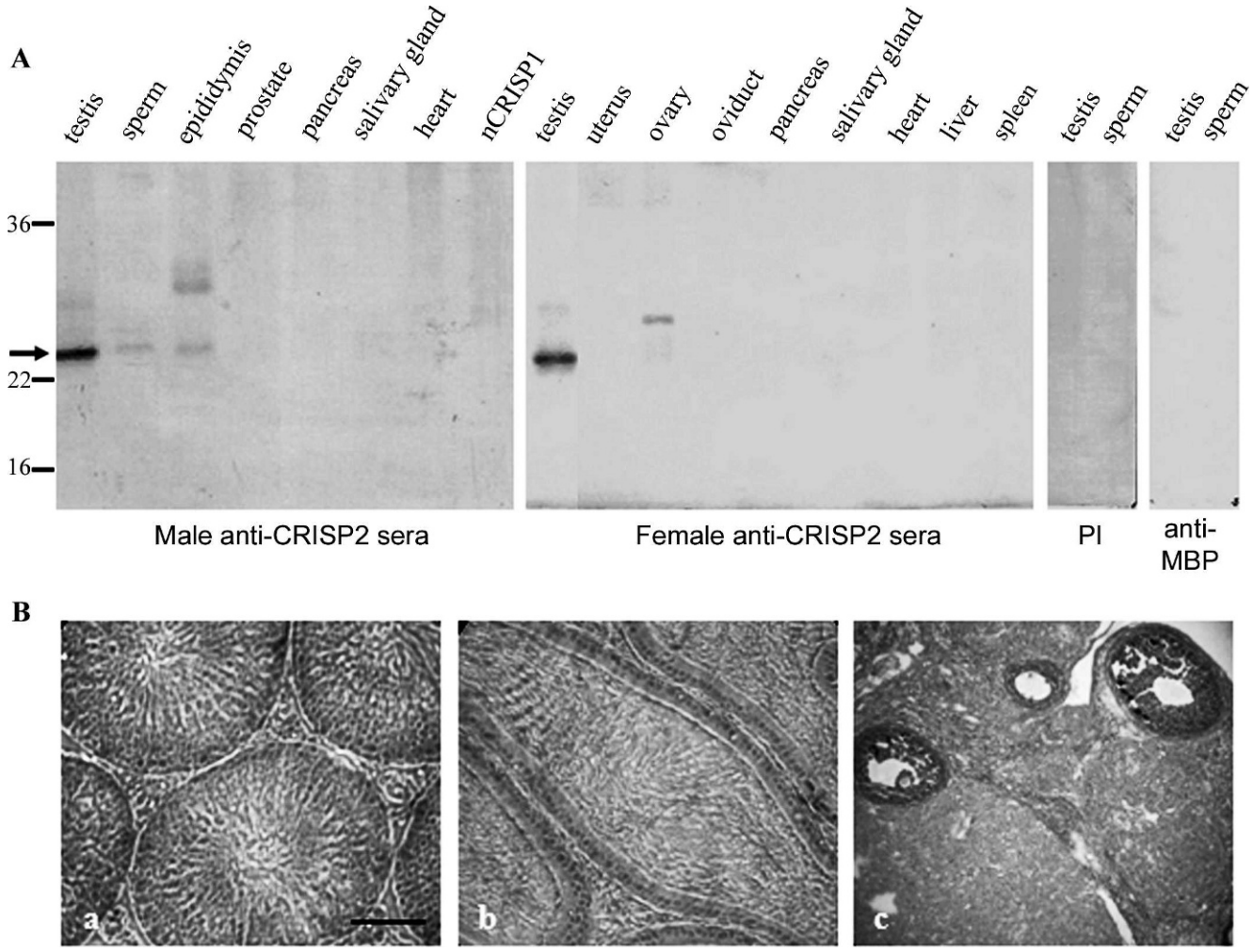

Figure 3. Specificity of the immune response. **(A)** Sera from recombinant cysteine-rich secretory protein 2 (recCRISP2)- or maltose-binding protein (MBP)–immunized rats as well as preimmune (PI) sera were used as primary antibodies in Western blots of different male and female protein extracts. Arrow indicates protein CRISP2. **(B)** Testicular **(a)**, epididymal **(b)**, and ovarian **(c)** sections from CRISP2-immunized rats subjected to histological examination (hematoxylin and eosin staining). **(a, b)** Magnification ×400. **(c)** Magnification ×100. Bar = 60 μm.

No further inhibition was observed when higher antibody concentrations were used. Control experiments indicated that anti-CRISP2 sera neither produce sperm agglutination nor affected sperm viability or motility (data not shown). These results showing the ability of anti-CRISP2 to inhibit sperm fertilizing ability indicated the involvement of rat CRISP2 in sperm-egg fusion. As another approach to confirm this conclusion, zona-free rat eggs were coincubated with capacitated sperm in the presence of purified recCRISP2. Under these conditions, there was a significant and concentration-dependent decrease in the percentage of fertilized eggs (Figure 5B) indicating that CRISP2 mediates gamete fusion through its binding to complementary sites in the egg. To examine the presence and localization of these egg binding sites, zona-free rat eggs were incubated with recCRISP2 or MBP and then subjected to IIF using anti-MBP as first antibody. Results showed that eggs incubated with recCRISP2 exhibited a fluorescent staining over the surface not observed in the MBP-incubated cells (Figure 5B).

Having confirmed the involvement of CRISP2 in gamete fusion and the ability of anti-CRISP2 sera to inhibit the sperm fertilizing ability, we evaluated the effect of CRISP2 immunization on male and female fertility. CRISP2-, CRISP1-, and MBP-immunized rats were mated with nontreated animals at 2 intervals of high antibody levels (see Figure 1). Although no differences in fertility among groups were observed after the first mating, the results of the second mating revealed a significant decrease in fertility in those animals injected with epididymal CRISP1 but not in those injected with the testicular CRISP2 protein (Table).

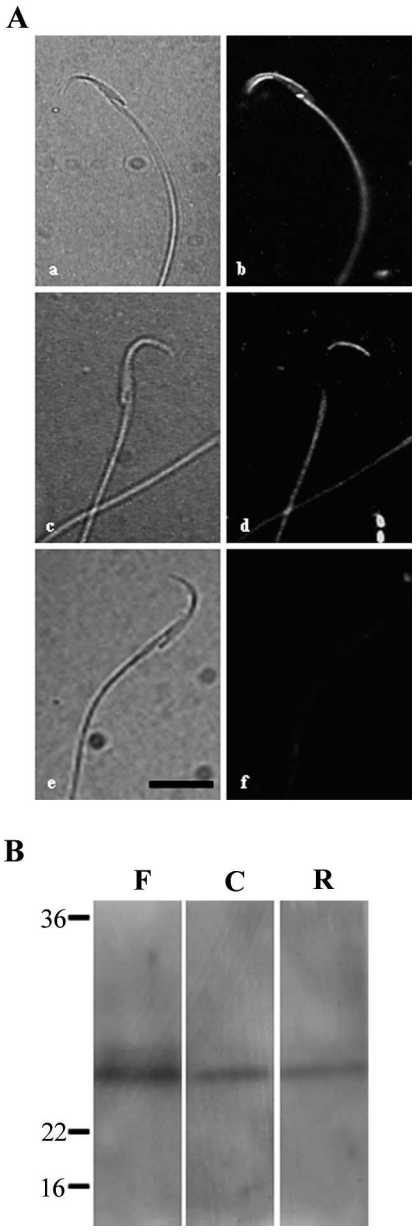

Figure 4. Behavior of cysteine-rich secretory protein 2 (CRISP2) during capacitation and acrosome reaction. **(A)** Photographs corresponding to phase contrast (left) and immunofluorescent (right) images of permeabilized fixed sperm subjected to indirect immunofluorescence using anti-CRISP2 **(a, b, c, d)** or anti–maltose-binding protein **(e, f)** sera showing labeling in both the dorsal and concave region of the head (pattern I) or only in the concave region of the head (pattern II). Bar = 10 μm. **(B)** Protein extracts from fresh (F), capacitated (C), and ionophore-acrosome reacted (R) sperm were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and subjected to Western blot analysis using sera from CRISP2-immunized rats as primary antibodies.

Discussion

Immunization of animals with a protein and evaluation of their subsequent fertility constitutes a straightforward strategy to investigate both the relevance of the protein for fertility and its potential use for contraceptive development. On the basis of this premise, and considering previous evidence proposing testicular CRISP2 as a candidate molecule to be involved in fertility (Du et al, 2006), in the present work, we investigated whether immunization of rats with CRISP2 was capable of eliciting a specific immune response that inhibits animal fertility. In this regard, this is the first report in which purified CRISP2 has been injected to evaluate its immunogenic and immunocontraceptive properties.

ELISA results showed that immunization with recCRISP2 produced a specific immune response in male and female animals that increased as a function of time and exhibited the same level (this study) and time course behavior as that corresponding to CRISP1 immunization (Ellerman et al, 2008). These results confirmed the immunogenicity of rat CRISP2 consistent with a previous report showing the presence of anti-CRISP2 antibodies in sera from vasectomized men as well as in the seminal plasma of patients with ASA (Domagala et al, 2007). The immunogenicity of recCRISP2 likely might be increased by the presence of MBP, a bacterial protein known to be highly immunogenic (Martineau et al, 1996).

The specificity of the immune response was indicated by Western blot results showing that sera against recCRISP2 recognized CRISP2 protein in the testis, epididymis, and sperm but not in any of the essential or reproductive organs tested. Anti-CRISP2 sera did not cross-react with purified CRISP1 in agreement with previous observations showing that an anti-rabbit CRISP2 antibody was not capable of recognizing mouse CRISP1 in epididymal tissue (Busso et al, 2007b). Although anti-CRISP2 sera did not detect any band at the expected molecular weight for CRISP2 (25 kd) in ovarian extracts, recent observations from our laboratory revealed the ability of these sera to detect CRISP2 when higher amounts of rat ovarian extracts were used, consistent with the reported presence of CRISP2 in the mouse ovary (Reddy et al, 2008). In spite of these observations, histological examination of the ovaries from CRISP2-immunized rats revealed the lack of pathological effects on this organ.

CRISP2 has been originally described as one of the primary autoantigens abundantly present within the guinea pig acrosome (Hardy et al, 1988) and also proposed as a good candidate for generating autoimmune orchitis in this species (Foster and Gerton, 1996).

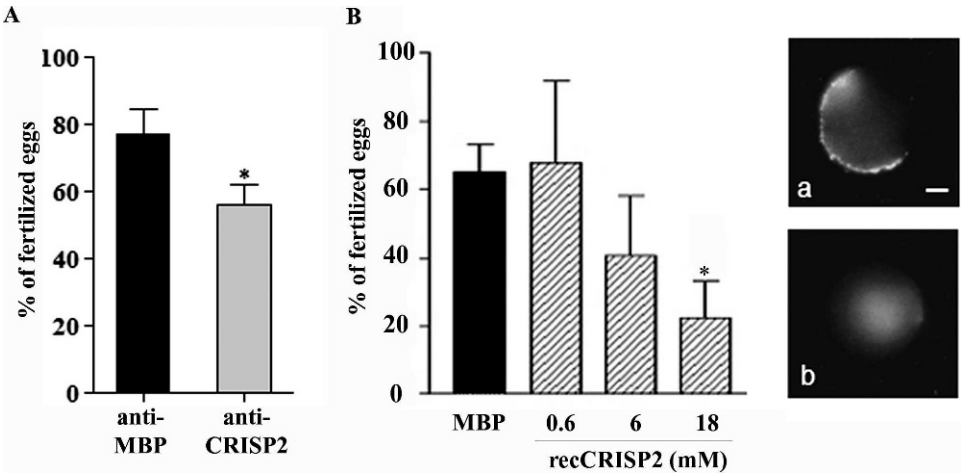

Figure 5. Participation of cysteine-rich secretory protein 2 (CRISP2) in fertilization. **(A)** Zona-free rat eggs were inseminated with capacitated sperm in the presence of (1:100) anti-CRISP2 or anti–maltose-binding protein (anti-MBP) sera and the percentages of fertilized eggs were determined. **(B)** Zona-free rat eggs were incubated with recombinant CRISP2 (recCRISP2) or MBP and either inseminated for evaluation of the percentages of fertilization (left panel) or subjected to indirect immunofluorescence using anti-MBP as primary antibody (right panel) **(a)** recCRISP2; **(b)** MBP. * $P < .05$. Bar = 20 μm.

In this regard, the gene for CRISP2 has been mapped to mouse chromosome 17 in a region that contains Orch-1, a gene controlling susceptibility to experimental autoimmune orchitis in mice (Kasahara et al, 1989; Himmelbauer et al, 1993). These observations raised the possibility that immunization of male rats with recCRISP2 could produce an autoimmune orchitis that compromises spermatogenesis. Moreover, on the basis of the presence of CRISP2 in the epididymis, the detrimental effects of immunization with CRISP2 could also be extended to this organ. Histological observations revealed, however, that recCRISP2-immunized males exhibited normal testicular and epididymal tissues, indicating that it is unlikely that CRISP2 is one of the molecules responsible for generating autoimmune orchitis in the rat. This conclusion is supported by a report indicating that CRISP2 is not among the identified immunodominant autoantigens responsible for autoimmune orchitis in the rat (Fijak et al, 2005). Moreover, considering the ease of orchitis induction in guinea pigs by immunization with homologous testicular antigens (Itoh et al, 1991), the lack of effect observed in our studies opens the possibility that CRISP2 is not a causative agent of orchitis in other mammalian species either. This idea seems to be supported by a recent report showing that sera from mice with spontaneous or induced autoimmune orchitis did not detect bands in the molecular weight range corresponding to CRISP proteins (20–30 kd) but strongly reacted with another intra-acrosomal protein, zonadhesin, described as the first murine orchitogenic antigen identified (Wheeler et al, 2011).

IIF results confirmed the ability of anti-CRISP2 sera to recognize native CRISP2 in fresh, capacitated, and acrosome-reacted sperm. Our observations revealed the presence of labeling in both the head and the tail only in permeabilized cells, consistent with an intracellular localization of CRISP2 (Hardy et al, 1988; O'Bryan et al, 2001; Busso et al, 2005, 2007b). Although a surface CRISP2 population was not detected in mature sperm, previous results suggested that rat germ cells are capable

Table. *Fertility of male and female immunized rats*

Antigen	No. of Animals	No. of Mated Females	No. of Pregnant Females	% of Fertility	Litter Size, No. of Pups
Male					
MBP	5	15	11	73 ± 12	7.1 ± 0.8
recCRISP2	7	21	17	81 ± 10	5.6 ± 0.7
recCRISP1	5	15	7	47 ± 13[a]	5.2 ± 1.7
Female					
MBP	6	6	5	83	8.3 ± 2.3
recCRISP2	10	10	9	90	5.7 ± 1.3
recCRISP1	5	5	2	40[a]	1.6 ± 1.2[a]

Abbreviations: MBP, maltose-binding protein; recCRISP1, recombinant cysteine-rich secretory protein 1; recCRISP2, recombinant cysteine-rich secretory protein 2.

[a] $P < .05$.

of secreting CRISP2 to the media and that the secreted protein bind to the surface of spermatogenic cells to participate in their specific interaction with Sertoli cells during spermatogenesis (Maeda et al, 1998, 1999). The different localizations of CRISP2 (ie, surface/internal) might be reflecting different functional roles of the protein during germ cell differentiation. Most fresh sperm showed labeling in the dorsal region of the head accompanied by staining in the concave side, a region that might correspond to the perinuclear theca (O'Bryan et al, 2001) or acroplaxoma (Rivkin et al, 2009) located between the acrosomal and nuclear membranes. Interestingly, this region has been implicated in the interaction between germ and Sertoli cells (Kierszenbaum et al, 2003), consistent with the proposed role of CRISP2 as a bridging molecule between these 2 cells (Maeda et al, 1998, 1999). When capacitated sperm were exposed to calcium ionophore, most of the cells exhibited CRISP2 only in the concave region of the head, indicating the release of the dorsal CRISP2 protein as a consequence of the occurrence of the AR. The finding that the electrophoretic mobility of the protein remained unchanged after capacitation and AR, indicates that rat CRISP2 does not undergo posttranslational modifications as a consequence of these functional events.

In vitro fertilization experiments revealed that anti-CRISP2 sera were not only capable of recognizing CRISP2 in capacitated and acrosome-reacted sperm, but also capable of inhibiting the sperm ability to penetrate zona-free eggs. This inhibition cannot be attributed to a blocking effect of the antibodies on epididymal CRISP1 because Western blot studies revealed the lack of cross-reactivity between anti-CRISP2 sera and sperm CRISP1. These results confirmed the involvement of rat CRISP2 in gamete fusion, in agreement with our previous reports in mouse and human (Busso et al, 2005, 2007b). By contrast, our observations differ from those reported by Hardy et al (1988) showing that the antibodies against CRISP2 did not inhibit fertilization in vitro. This discrepancy might be due to the different animal models used in each case. In this regard, it is known that guinea pig sperm have very big acrosomes that might need higher antibody concentrations to significantly inhibit the intra-acrosomal CRISP2 concentrations. Moreover, our observations revealed that CRISP2 participates in rat gamete fusion by its interaction with complementary sites in the egg, as judged by both the inhibition in fertilization observed when the gametes were exposed to recCRISP2 and the presence of fluorescent labeling in CRISP2-incubated zona-free eggs. The ability of CRISP2 to bind to complementary sites in the oolema is consistent with the role of CRISP2 in another cell-cell interaction event, such as the association between rat spermatogenic and Sertoli cells (Maeda et al, 1998, 1999). We could not evaluate a possible role of CRISP2 in the previous stage of sperm-ZP because of the known difficulties of fertilizing rat zona–intact eggs in vitro. However, our previous observations in mouse showing that an anti-CRISP2 antibody did not interfere with ZP penetration and that recCRISP2 did not bind to the ZP (Busso et al, 2007b) support a specific participation of rat CRISP2 in gamete fusion.

It is known that sperm become fusion competent only after the occurrence of the AR. In this regard, several reports support the involvement of intra-acrosomal proteins in the development of sperm fusion ability, such as acrosin, metalloproteinases, SLLP1, ESP, etc (Diaz-Perez and Meizel, 1992; Takano et al, 1993; Herrero et al, 2005; Lv et al, 2010). The strongest evidence comes from a study reporting the infertility of knockout mice for the intra-acrosomal protein Izumo, which is exposed only after the AR (Inoue et al, 2005). In these mutant animals, sperm are able to bind to and penetrate the ZP but incapable of fusing with the oolema. Our present observations in the rat, together with our reports in mouse and human (Busso et al, 2005, 2007b), support CRISP2 as another example of intra-acrosomal proteins involved in gamete fusion.

In spite of the presence of circulating anti-CRISP2 antibodies capable of both recognizing sperm CRISP2 and inhibiting its role in fertilization, mating experiments showed no inhibition of fertility in CRISP2-immunized male and female rats, even after the second mating when a significant decreased in fertility was observed for male and female CRISP1-immunized rats. A possible explanation for the lack of fertility inhibition by CRISP2 immunization could be that insufficient anti-CRISP2 antibodies are present within the male and female reproductive tracts. ELISA results showed, however, similar anti-CRISP2 and anti-CRISP1 circulating antibody levels. In addition, although anti-CRISP2 sera might not enter the testes because of the blood-testis barrier, we have previously shown that the antibodies are able to enter into the epididymis, as observed in CRISP1-immunized rats (Ellerman et al, 1998). Another explanation for the lack of fertility inhibition after recCRISP2 immunization could be that the bacterially expressed protein lacks the immunogenic epitopes present in the native CRISP2 molecule. Our results revealed, however, that anti-CRISP2 sera are capable of both recognizing native CRISP2 in sperm by IIF and inhibiting the sperm fertilizing ability in vitro. All these observations support the idea that the lack of fertility inhibition in CRISP2-immunized rats would be due to the low accessibility of the protein to the circulating antibodies. In this regard, the fact that both recCRISP1 and recCRISP2 are capable of generating specific

immune sera that inhibit sperm-egg fusion in vitro (Busso et al, 2007a; this study) supports in vivo antigen accessibility as one of the main reasons for the different fertility results observed for these 2 highly homologous proteins. In this regard, whereas CRISP1 is a surface protein located in the plasma membrane covering the acrosomal cap or equatorial segment of the sperm head, CRISP2 is intra-acrosomal in mature sperm, eventually located in the surface of germ cells, and exposed only after the AR in females. Thus, the specific antibodies need to be present at a particular time and space in both males and females for binding to native CRISP2 to affect its biological function. This narrow window for antibody-antigen interaction could also explain the lack of fertility inhibition observed in male and female mice immunized with the intra-acrosomal protein Izumo (Wang et al, 2008). In view of this, it could be speculated that higher anti-CRISP2 antibody levels in both the male and female reproductive tract are required to block CRISP2 effectively in vivo. Besides the low accessibility of CRISP2 to the antibodies, we cannot exclude the possibility that the additional involvement of CRISP1 in the previous stage of sperm-ZP interaction constitutes another factor contributing to the different fertility results obtained for the 2 proteins. All these observations suggest that, although present in ASA-positive seminal plasma of immune infertile men (Domagala et al, 2007), anti-CRISP2 antibodies might not be responsible for the immunoinfertility of these patients.

The finding that CRISP2 immunization did not affect animal fertility does not preclude the idea that testicular CRISP2 might be relevant for fertility and, thus, a good marker for infertility diagnosis, as suggested by recent studies in asthenospermic patients (Jing et al, 2011). The generation of knockout mice for CRISP2 will contribute to elucidate the relevance of the protein for fertility.

In conclusion, our results indicate that CRISP2 is capable of generating a specific immune response in male and female rats that neither generates autoimmune orchitis nor compromises fertility. Although not excluding the potential relevance of CRISP2 for fertility, these observations do not support CRISP2 as a target for immunocontraception or as a molecule responsible for generating autoimmune orchitis or immunoinfertility.

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References

Bendahmane M, Zeng HT, Tulsiani DR. Assessment of acrosomal status in rat spermatozoa: studies on carbohydrate and non-carbohydrate agonists. *Arch Biochem Biophys*. 2002;404:38–47.

Busso D, Cohen DJ, Hayashi M, Kasahara M, Cuasnicu PS. Human testicular protein TPX1/CRISP-2: localization in spermatozoa, fate after capacitation and relevance for gamete interaction. *Mol Hum Reprod*. 2005;11:299–305.

Busso D, Cohen DJ, Maldera JA, Dematteis A, Cuasnicu PS. A novel function for CRISP1 in rodent fertilization: involvement in sperm-zona pellucida interaction. *Biol Reprod*. 2007a;77:848–854.

Busso D, Goldweic NM, Hayashi M, Kasahara M, Cuasnicu PS. Evidence for the involvement of testicular protein CRISP2 in mouse sperm-egg fusion. *Biol Reprod*. 2007b;76:701–708.

Cameo MS, Blaquier JA. Androgen-controlled specific proteins in rat epididymis. *J Endocr*. 1976;69:317–324.

Cohen DJ, Rochwerger L, Ellerman DA, Morgenfeld M, Busso D, Cuasnicu PS. Relationship between the association of rat epididymal protein DE with spermatozoa and the behavior and function of the protein. *Mol Reprod Dev*. 2000;56:180–188.

Cuasnicú PS, Conesa D, Rochwerger L. Potential contraceptive use of an epididymal protein that participates in fertilization. In: Alexander NJ, Griffin D, Spieler JM, Waites GMH, eds. *Gamete Interaction. Prospects for Immunocontraception*. New York, NY: Wiley-Liss; 1990:143–153.

Da Ros VG, Maldera JA, Willis WD, Cohen DJ, Goulding EH, Gelman DM, Rubinstein M, Eddy EM, Cuasnicu PS. Impaired sperm fertilizing ability in mice lacking Cysteine-RIch Secretory Protein 1 (CRISP1). *Dev Biol*. 2008;320:12–18.

Diaz-Perez E, Meizel S. Importance of mammalian sperm metalloendoprotease activity during the acrosome reaction to subsequent sperm-egg fusion: Inhibitor studies with human sperm and zona-free hamster eggs. *Mol Reprod Dev*. 1992;31:122–130.

Domagala A, Pulido S, Kurpisz M, Herr JC. Application of proteomic methods for identification of sperm immunogenic antigens. *Mol Hum Reprod*. 2007;13:437–444.

Du Y, Huang X, Li J, Hu Y, Zhou Z, Sha J. Human testis specific protein 1 expression in human spermatogenesis and involvement in the pathogenesis of male infertility. *Fertil Steril*. 2006;85:1852–1854.

Ellerman DA, Brantua VS, Martinez SP, Cohen DJ, Conesa D, Cuasnicu PS. Potential contraceptive use of epididymal proteins: immunization of male rats with epididymal protein DE inhibits sperm fusion ability. *Biol Reprod*. 1998;59:1029–1036.

Ellerman DA, Busso D, Maldera JA, Cuasnicu PS. Immunocontraceptive properties of recombinant sperm protein DE: implications for the development of novel contraceptives. *Fertil Steril*. 2008;89:199–205.

Ellerman DA, Cohen DJ, Da Ros VG, Morgenfeld MM, Busso D, Cuasnicu PS. Sperm protein "DE" mediates gamete fusion through an evolutionarily conserved site of the CRISP family. *Dev Biol*. 2006;297:228–237.

Ellerman DA, Da Ros VG, Cohen DJ, Busso D, Morgenfeld MM, Cuasnicu PS. Expression and structure-function analysis of DE, a sperm cysteine-rich secretory protein that mediates gamete fusion. *Biol Reprod*. 2002;67:1225–1231.

Fijak M, Iosub R, Schneider E, Linder M, Respondek K, Klug J, Meinhardt A. Identification of immunodominant autoantigens in rat autoimmune orchitis. *J Pathol*. 2005;207:127–138.

Foster JA, Gerton GL. Autoantigen 1 of the guinea pig sperm acrosome is the homologue of mouse Tpx-1 and human TPX1 and is a member of the cysteine- rich secretory protein (CRISP) family. *Mol Reprod Dev*. 1996;44:221–229.

Garberi JC, Kohane AC, Cameo MS, Blaquier JA. Isolation and characterization of specific rat epididymal proteins. *Mol Cell Endocrinol*. 1979;13:73–82.

Gibbs GM, Bianco DM, Jamsai D, Herlihy A, Ristevski S, Aitken RJ, Kretser DM, O'Bryan MK. Cysteine-rich secretory protein 2 binds to mitogen-activated protein kinase kinase kinase 11 in mouse sperm. *Biol Reprod*. 2007;77:108–114.

Gibbs GM, Roelants K, O'Bryan MK. The CAP superfamily: cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins—roles in reproduction, cancer, and immune defense. *Endocr Rev*. 2008;29:865–897.

Gibbs GM, Scanlon MJ, Swarbrick J, Curtis S, Gallant E, Dulhunty AF, O'Bryan MK. The cysteine-rich secretory protein domain of Tpx-1 is related to ion channel toxins and regulates ryanodine receptor Ca2+ signaling. *J Biol Chem*. 2006;281:4156–4163.

Haendler B, Kratzschmar J, Theuring F, Schleuning WD. Transcripts for cysteine-rich secretory protein-1 (CRISP-1; DE/AEG) and the novel related CRISP-3 are expressed under androgen control in the mouse salivary gland. *Endocrinology*. 1993;133:192–198.

Hardy DM, Huang TTF, Driscoll WJ, Tung KSK, Wild GC. Purification and characterization of the primary acrosomal autoantigen of guinea pig epididymal spermatozoa. *Biol Reprod*. 1988;38:423–437.

Herrero MB, Mandal A, Digilio LC, Coonrod SA, Maier B, Herr JC. Mouse SLLP1, a sperm lysozyme-like protein involved in sperm-egg binding and fertilization. *Dev Biol*. 2005;284:126–142.

Himmelbauer H, Artzt K, Barlow D, Fischer-Lindahl K, Lyon M, Klein J, Silver LM. Encyclopedia of the mouse genome III. October 1993. Mouse chromosome 17. *Mamm Genome*. 1993;4 spec no:S230–S252.

Inoue N, Ikawa M, Isotani A, Okabe M. The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. *Nature*. 2005;434:234–238.

Itoh M, Hiramine C, Hojo K. A new murine model of autoimmune orchitis induced by immunization with viable syngeneic testicular germ cells alone. I. Immunological and histological studies. *Clin Exp Immunol*. 1991;83:137–142.

Jalkanen J, Huhtaniemi I, Poutanen M. Mouse cysteine-rich secretory protein 4 (CRISP4): a member of the crisp family exclusively expressed in the epididymis in an androgen-dependent manner. *Biol Reprod*. 2005;72:1268–1274.

Jamsai D, Bianco DM, Smith SJ, Merriner DJ, Ly-Huynh JD, Herlihy A, Niranjan B, Gibbs GM, O'Bryan MK. Characterization of gametogenetin 1 (GGN1) and its potential role in male fertility through the interaction with the ion channel regulator, cysteine-rich secretory protein 2 (CRISP2) in the sperm tail. *Reproduction*. 2008;135:751–759.

Jamsai D, Rijal S, Bianco DM, O'Connor AE, Merriner DJ, Smith SJ, Gibbs GM, O'Bryan MK. A novel protein, sperm head and tail associated protein (SHTAP), interacts with cysteine-rich secretory protein 2 (CRISP2) during spermatogenesis in the mouse. *Biol Cell*. 2010;102:93–106.

Jing XW, Xing RW, Zhou QZ, Yu QF, Guo WB, Chen SM, Chu QJ, Feng CQ, Mao XM. Expressions of cysteine-rich secretory protein 2 in asthenospermia [in Chinese]. *Zhonghua Nan Ke Xue*. 2011;17:203–207.

Kaplan R, Kraicer PF. Effect of elevated calcium concentration on fertilization of rat oocytes in vitro. *Gamete Res*. 1978;1:281–285.

Kasahara M, Gutknecht J, Brew K, Spurr N, Goodfellow PN. Cloning and mapping of a testis-specific gene with sequence similarity to a sperm-coating glycoprotein gene. *Genomics*. 1989;5:527–534.

Kierszenbaum AL, Rivkin E, Tres LL. Acroplaxome, an F-actin-keratin–containing plate, anchors the acrosome to the nucleus during shaping of the spermatid head. *Mol Biol Cell*. 2003;14:4628–4640.

Kjeldsen L, Cowland JB, Johnsen AH, Borregaard N. SGP28, a novel matrix glycoprotein in specific granules of human neutrophils with similarity to a human testis-specific gene product and a rodent sperm-coating glycoprotein. *FEBS Lett*. 1996;380:246–250.

Laemmli UK. Cleavege of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227:680–685.

Lv ZM, Wang M, Xu C. Antifertility characteristics of the N-terminal region of mouse equatorial segment protein. *Anat Rec (Hoboken)*. 2010;293:171–181.

Maeda T, Nishida J, Nakanishi Y. Expression pattern, subcellular localization and structure-function relationship of rat Tpx-1, a spermatogenic cell adhesion molecule responsible for association with Sertoli cells. *Dev Growth Differ*. 1999;41:715–722.

Maeda T, Sakashita M, Ohba Y, Nakanishi Y. Molecular cloning of the rat Tpx-1 responsible for the interaction between spermatogenic and Sertoli cells. *Biochem Biophys Res Commun*. 1998;248:140–146.

Martineau P, Leclerc C, Hofnung M. Modulating the immunological properties of a linear B-cell epitope by insertion into permissive sites of the MalE protein. *Mol Immunol*. 1996;33:1345–1358.

National Institutes of Health, *Guide for Care and Use of Laboratory Animals*. 8th ed. Washington, DC: National Academy Press; 2011.

O'Bryan MK, Loveland KL, Herszfeld D, McFarlane JR, Hearn MT, De Kretser DM. Identification of a rat testis–specific gene encoding a potential rat outer dense fibre protein. *Mol Reprod Dev*. 1998;50:313–322.

O'Bryan MK, Sebire K, Meinhardt A, Edgar K, Keah H, Hearn MT, De Kretser DM. Tpx-1 is a component of the outer dense fibers and acosome of rat spermatozoa. *Mol Reprod Dev*. 2001;58:116–125.

Perez Martinez S, Conesa D, Cuasnicú PS. Potential contraceptive use of epididymal proteins: evidence for the participation of specific antibodies against rat epididymal protein DE in male and female fertility inhibition. *J Reprod Immunol*. 1995;29:31–45.

Reddy T, Gibbs GM, Merriner DJ, Kerr JB, O'Bryan MK. Cysteine-rich secretory proteins are not exclusively expressed in the male reproductive tract. *Dev Dyn*. 2008;237:3313–3323.

Rivkin E, Kierszenbaum AL, Gil M, Tres LL. Rnf19a, a ubiquitin protein ligase, and Psmc3, a component of the 26S proteasome, tether to the acrosome membranes and the head-tail coupling apparatus during rat spermatid development. *Dev Dyn*. 2009;238:1851–1861.

Rochwerger L, Cohen DJ, Cuasnicú PS. Mammalian sperm-egg fusion: the rat egg has complementary sites for a sperm protein that mediates gamete fusion. *Dev Biol*. 1992;153:83–90.

Rochwerger L, Cuasnicu PS. Redistribution of a rat sperm epididymal glycoprotein after in vivo and in vitro capacitation. *Mol Reprod Dev*. 1992;31:34–41.

Takano H, Yanagimachi R, Urch UA. Evidence that acrosin activity is important for the development of fusibility of mammalian spermatozoa with the oolemma: inhibitor studies using the golden hamster. *Zygote*. 1993;1:79–91.

Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrilamyde gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci U S A*. 1979;76:4350–4360.

Udby L, Bjartell A, Malm J, Egesten A, Lundwall A, Cowland JB, Borregaard N, Kjeldsen L. Characterization and localization of cysteine-rich secretory protein 3 (CRISP-3) in the human male reproductive tract. *J Androl*. 2005;26:333–342.

Wang DG, Huang TH, Xie QD, An G. Investigation of recombinant mouse sperm protein Izumo as a potential immunocontraceptive antigen. *Am J Reprod Immunol*. 2008;59:225–234.

Wheeler K, Tardif S, Rival C, Luu B, Bui E, Del Rio R, Teuscher C, Sparwasser T, Hardy D, Tung KS. Regulatory T cells control tolerogenic versus autoimmune response to sperm in vasectomy. *Proc Natl Acad Sci U S A*. 2011;108:7511–7516.

Yamazaki Y, Morita T. Structure and function of snake venom cysteine-rich secretory proteins. *Toxicon*. 2004;44:227–231.