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ORIGINAL RESEARCH

Fertilization defects in sperm from *Cysteine-rich secretory protein 2 (Crisp2)* knockout mice: implications for fertility disorders

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STUDY HYPOTHESIS: We hypothesize that fertility disorders in patients with aberrant expression of Cysteine-RIch Secretory Protein 2 (CRISP2) could be linked to the proposed functional role of this protein in fertilization.

STUDY FINDING: Our *in vivo* and *in vitro* observations reveal that *Crisp2*-knockout mice exhibit significant defects in fertility-associated parameters under demanding conditions, as well as deficiencies in sperm fertilizing ability, hyperactivation development and intracellular Ca^{2+} regulation.

WHAT IS KNOWN ALREADY: Testicular CRISP2 is present in mature sperm and has been proposed to participate in gamete fusion in both humans and rodents. Interestingly, evidence in humans shows that aberrant expression of CRISP2 is associated with male infertility.

STUDY DESIGN, SAMPLES/MATERIALS, METHODS: A mouse line carrying a deletion in the sixth exon of the *Crisp2* gene was generated. The analyses of the reproductive phenotype of *Crisp2*$^{-/-}$ adult males included the evaluation of their fertility before and after being subjected to unilateral vasectomy, *in vivo* fertilization rates obtained after mating with either estrus or superovulated females, *in vitro* sperm fertilizing ability and different sperm functional parameters associated with capacitation such as tyrosine phosphorylation (by western blot), acrosome reaction (by Coomassie Blue staining), hyperactivation (by computer-assisted sperm analysis) and intracellular Ca^{2+} levels (by flow cytometry).

MAIN RESULTS AND THE ROLE OF CHANCE: *Crisp2*$^{-/-}$ males presented normal fertility and *in vivo* fertilization rates when mated with estrus females. However, the mutant mice showed clear defects in those reproductive parameters compared with controls under more demanding conditions, i.e. when subjected to unilateral vasectomy to reduce the number of ejaculated sperm ($n = 5$; $P < 0.05$), or when mated with hormone-treated females containing a high number of eggs in the *ampulla* ($n \geq 5$; $P < 0.01$). *In vitro* fertilization studies revealed that *Crisp2*$^{-/-}$ sperm exhibited deficiencies to penetrate the egg vestments (i.e. *cumulus oophorus* and *zona pellucida*) and to fuse with the egg ($n \geq 6$; $P < 0.01$). Consistent with this, *Crisp2*-null sperm showed lower levels of hyperactivation ($n = 7$; $P < 0.05$), a vigorous motility required for penetration of the egg coats, as well as a dysregulation in intracellular Ca^{2+} levels associated with capacitation ($n = 5$; $P < 0.001$).

LIMITATIONS, REASONS FOR CAUTION: The analysis of the possible mechanisms involved in fertility disorders in men with abnormal expression of CRISP2 was carried out in *Crisp2* knockout mice due to the ethical and technical problems inherent to the use of human gametes for fertilization studies.

WIDER IMPLICATIONS OF THE FINDINGS: Our findings in mice showing that *Crisp2*$^{-/-}$ males exhibit fertility and fertilization defects under demanding conditions support fertilization defects in sperm as a mechanism underlying infertility in men with aberrant expression of CRISP2. Moreover, our observations in mice resemble the situation in humans where fertility disorders can or cannot be detected depending on the accumulation of own individual defects or the fertility status of the partner. Finally, the fact that reproductive defects in mice are masked by conventional mating highlights the need of using different experimental approaches to analyze male fertility.

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Key words: CRISP / testes / fertility / sperm / motility / fertilization / hyperactivation / knockout mice

Introduction

Fertility disorders, likely underestimated, affect 10–15% of couples worldwide. Approximately half of these cases are assigned to male factors; however, generally the genetic basis underlying the disease remains largely unknown. In this sense, studies aimed at the identification of infertility-causing genes revealed testicular *Cysteine-rich secretory protein 2* (*Crisp2*) as a potential candidate. Evidence showed that translocation or breakpoints in the chromosomal region encompassing *Crisp2* gene were associated with infertility in three brothers and in three other independent men (Paoloni-Giacobino *et al.*, 2000; Olesen *et al.*, 2001). Moreover, a reduced CRISP2 protein level was found in infertile patients with azoospermia or oligoasthenoteratospermia (Du *et al.*, 2006) or with asthenospermia (Jing *et al.*, 2011) syndromes. Recently, a retrospective follow-up study showed that a decrease in CRISP2 expression significantly correlated with low sperm progressive motility, abnormal sperm morphology and infertility (Zhou *et al.*, 2015), suggesting that fertility problems occur as a consequence of sperm function deficiencies in cells with lower levels of CRISP2.

CRISP2, formerly known as TPX-1, is expressed in the testes independently of androgens (Haendler *et al.*, 1997) and more specifically in germ cells of guinea pig (Hardy *et al.*, 1988), rat (Maeda *et al.*, 1998; O'Bryan *et al.*, 1998), mouse (Kasahara *et al.*, 1987; Mizuki *et al.*, 1992), human (Kasahara *et al.*, 1989) and horse (Giese *et al.*, 2002). Localization studies in mature sperm revealed that this protein exists as a component of the acrosome (Hardy *et al.*, 1988; Foster and Gerton, 1996; Kim *et al.*, 2001; O'Bryan *et al.*, 2001; Busso *et al.*, 2005, 2007; Nimlamool *et al.*, 2013), the connecting piece of the neck and the outer dense fibers of the tail (O'Bryan *et al.*, 1998, 2001). Based on its structure, CRISP2 is considered a member of the CRISP family (Foster and Gerton, 1996; Kratzschmar *et al.*, 1996), characterized by the presence of 16 conserved cysteines, 10 of which are located in the C-terminal region containing both a Cysteine-Rich Domain (CRD) and a hinge that connects to the plant Pathogenesis Related-1 (PR-1) domain located in the N-terminus (Gibbs *et al.*, 2008). Besides CRISP2, of testicular origin, mammalian members of the CRISP family include CRISP1, mainly expressed in the epididymis (Cameo and Blaquier, 1976; Eberspaecher *et al.*, 1995), CRISP3, with a wider tissue distribution that includes reproductive (i.e. seminal vesicles, prostate) and non-reproductive (i.e. salivary gland, thymus) organs (Haendler *et al.*, 1993; Udby *et al.*, 2005; Reddy *et al.*, 2008), and CRISP4, specifically synthesized in the epididymis (Jalkanen *et al.*, 2005; Nolan *et al.*, 2006).

As CRISP proteins are mainly expressed in the male reproductive tract, they have been proposed to be relevant for sperm physiology. However, the exact role of each CRISP protein is still under investigation. Evidence suggests that the two functional domains present in CRISP molecules display distinct biological roles (Gibbs *et al.*, 2008). In this sense, the PR-1 domain of CRISP2 was shown to exhibit Sertoli-germ cell adhesion activity (Maeda *et al.*, 1999) whereas the C-terminal domain was reported to possess ion channel regulatory activity (Gibbs *et al.*, 2006). In addition to these roles, studies by our group supported the participation of rodent and human CRISP2 in the fertilization process at the sperm-egg fusion step (Busso *et al.*, 2005, 2007; Da Ros *et al.*, 2008; Munoz *et al.*, 2012). According to these observations, it is likely that fertility problems observed in patients with aberrant expression of CRISP2 are associated with the proposed role of this protein in gamete interaction. With the aim of investigating this possibility, in the present work we generated CRISP2-deficient mice and analyzed their reproductive phenotype.

Materials and Methods

Generation of CRISP2-deficient mice

The targeting vector for *Crisp2* (PG00061_Y_6_A09) was obtained from the International Knockout Mouse Consortium (https://www.mousephenotype.org/imits/targ_rep/targeted_alleles/19162) and electroporated into C57BL/6N EGR-G101 embryonic stem cells (Fujihara *et al.*, 2013) after linearization with AsiSI. Clones were then selected using G418. The correct targeting events in the cells were confirmed by PCR analysis using primers for the 5′ arm (5′-GCGTTGCTGCTGCGATCACCACTC-3′/5′-CACAACGGGTTCTTCTGTTAGTCC-3′) and for loxP (5′-GTGCACATAAGTGTTATTCCTGCTATCTTG-3′/5′-ACTGATGGCGAGCTCAGACC-3′). Nested PCR analysis was performed to confirm targeting events for the 3′ arm using primers (5′-ATCCGGGGGTACCGCGTCGAG-3′/5′-CTCGGCAATTCATGAAACCTACTC-3′) followed by primers (5′-TATAGGAACTTCGTCGAGATAACTTCG-3′/5′-CTTCTACTCGGCAATTCATGAAACCTACTC-3′). The correctly targeted embryonic stem cells were injected to ICR 8-cell embryos to obtain chimeric mice. These chimeric males were mated with B6D2F1 females and germ-line transmission was confirmed by PCR analysis using the same primers as for the cells. To remove the floxed-exon, F1 mice were mated with B6;D2 CAG-Cre Transgenic animals that ubiquitously express Cre (Matsumura *et al.*, 2004). The obtained *Crisp2*$^{+/-}$ animals were intercrossed to obtain *Crisp2*$^{-/-}$ homozygotes. Genotyping was performed by PCR using genomic DNA and primers (5′-GTCTTCTGTAAGCATGTGTCAAGC-3′/ 5′-CATGTGGGAGATATGCACTTGAATAAGC-3′ and 5′-CACAACGGGTTCTTCTGTTAGTCC-3′). This combination of primers amplifies fragments of 380 bp and 540 bp for the wild type and null allele, respectively.

Animals were housed at 23°C under a 12/12 h light/dark cycle with *ad libitum* access to food and water. Approval for the study protocol was obtained from the Animal Care and Use Committee of the Research Institute for Microbial Diseases (Osaka University, Osaka, Japan) and the Bioethics Committee of the Institute of Biology and Experimental Medicine (CONICET, Buenos Aires, Argentina). All protocols were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (NIH).

Sperm collection and *in vitro* capacitation

Mouse sperm were recovered by incising the cauda epididymides in a 300 μl drop of capacitation media (Fraser and Drury, 1975) supplemented with

0.3% (w/v) of bovine serum albumin (BSA, Sigma), covered with paraffin oil (Ewe, Sanitas SA, Buenos Aires, Argentina). Aliquots of the suspension were added to 300 μl of fresh medium, previously placed in tissue culture dishes under paraffin oil, to give a final concentration of 1–10 × 10^6 cells/ml, and incubation was allowed to proceed for 90 min at 37°C in an atmosphere of 5% (v/v) CO_2 in air.

Immunoblot analysis

Testes were homogenized in 200–400 μl ice-cold lysis buffer (phosphate-buffered saline containing 5 mM EDTA, 1% (w/v) IGEPAL CA360 (Sigma), 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 5 mM $MgCl_2$, and 10 mM HEPES, pH 7.2) with 0.2 mM phenylmethylsulfonyl fluoride (Sigma). After 30 min incubation on ice, samples were centrifuged at 15 000*g* for 20 min at 4°C and an aliquot of the supernatant diluted in Laemmli sample buffer (Laemmli, 1970). Sperm aliquots obtained before or after capacitation (1 × 10^6 spermatozoa) were washed with phosphate-buffered saline and resuspended in Laemmli sample buffer (Laemmli, 1970). In all cases, after a 5 min-incubation, samples were boiled, centrifuged at 5000*g* for 5 min and the supernatants were recovered. For tyrosine phosphorylation assessment, the supernatants were boiled again in the presence of 70 mM 2-mercaptoethanol (Sigma). In all cases, solubilized proteins were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and immunoblotted as previously described (Da Ros *et al.*, 2008) with the anti-mouse CRISP1 (1:1000; #AF4675; R&D Systems, Minneapolis, MN, USA) or anti-human CRISP2 (1:1000; #AF2575; Proteintech, Chicago, IL, USA) or anti-mouse CRISP4 (1:1000; #AF5017; R&D Systems) polyclonal antibodies, or anti-phosphotyrosine monoclonal antibody (1:10 000; clone 4G10; Upstate, Lake Placid, NY, USA). Protein loading was analyzed by β-tubulin immunoblot (anti-β-tubulin antibody; 1:5000; clone D66; Sigma). The immunoreactive proteins were detected by enhanced chemiluminescence (GE Healthcare, Piscataway, NJ, USA) and images captured with G:BOX GENI (Syngene, Synoptics Ltd, Cambridge, England) according to the manufacturer's instructions. The densitometric analyses were performed using the ImageJ 1.44p software (Wayne Rasband, National Institutes of Health, USA).

Unilateral vasectomy

Males (2 months old) were anesthetized for surgery by i.p. injection of xylazine/ketamine (10:100 mg/kg body weight). On one side of the animal, a lower midabdominal incision was performed and the vas deferens was exposed without causing injury to the adherent blood vessels. The proximal end was tied and a portion (~1 cm) of the vas deferens was removed. Then, the tissues were placed back in the abdominal cavity and the incision was closed in two layers. Fertility trials and *in vivo* fertilization assays were carried out no earlier than 2 weeks after the surgery to allow fully recovery of the animals and no later than 8 weeks from the surgery to avoid production of antisperm antibodies (Judd *et al.*, 1997).

Assessment of fertility

Sexually mature (2–6 months old) littermate males of *Crisp2*$^{+/-}$ and *Crisp2*$^{-/-}$ genotypes were individually caged with 2 adult (2–6 months old) wild-type females for a 3-week period. Each male was subjected to one or two rounds of matings and their fertility was analyzed for percentage of pregnant females, litter size and time since the beginning of mating to the delivery of the pups.

Assessment of estrous cycle and hormone treatment

Estrous cycle stage of sexually mature females was determined every morning by the analysis of vaginal smears (Caligioni, 2009). Briefly, vaginal epithelial cell smears were collected in 30 μl of phosphate-buffered saline with a pipette, transferred to glass slides and examined unstained under a light microscope (×100). The different stages (pro-estrus, estrus, metestrus and diestrus) were classified based on the proportion of the three cell types, i.e. leukocytes and both cornified and nucleated epithelial cells. For ovulation induction, adult females were treated with an i.p. injection of equine chorionic gonadotrophin (eCG; 5 IU; Syntex SA, Buenos Aires, Argentina) either on the day of the pro-estrus phase (Zhu *et al.*, 2012) (for *in vivo* studies) or at any stage of the cycle (for *in vitro* studies), followed by an i.p. injection of human chorionic gonadotrophin (hCG; 5 IU, Sigma) 48 h later.

In vivo fertilization

Each sexually mature *Crisp2*$^{+/-}$ or *Crisp2*$^{-/-}$ male was individually caged for 18 h with one female on the late afternoon of pro-estrus (Nakamura *et al.*, 2011) or with one hormone-stimulated female immediately after the hCG injection. In both approaches, each male was subjected to two rounds of mating. In all cases, females were checked for the presence of vaginal plug as evidence of successful mating. Eggs were then recovered from the oviducts, fixed with 2% paraformaldehyde, washed, stained with 10 μg/ml Hoechst 33342 (Sigma), mounted on slides, and finally analyzed under a Nikon Optiphot microscope (Nikon, Tokyo, Japan) equipped with epifluorescence optics (×200). Eggs were considered fertilized when at least one decondensing sperm nucleus or two *pronuclei* were observed in the egg cytoplasm.

In vitro fertilization

Cumulus-oocyte complexes (COC) were collected from superovulated females 12–15 h post-hCG administration. Cumulus cells were removed by incubating the COC for 3–5 min in 0.3 mg/ml hyaluronidase (type IV; Sigma). The *zona pellucida* (ZP) was dissolved by treating the eggs with acid Tyrode solution (pH: 2.5) for 10–20 s (Nicolson *et al.*, 1975). COC and zona pellucida-intact eggs were inseminated with capacitated sperm (final concentration: 1–5 × 10^5 cells/ml) and the gametes co-incubated in capacitation media for 3 h at 37°C in an atmosphere of 5% (v/v) CO_2 in air. For fusion assays, zona pellucida-free eggs were inseminated with capacitated sperm (final concentration: 1–5 × 10^4 cells/ml) and the gametes co-incubated for 1 h. In all cases, eggs were then evaluated for evidence of fertilization as described above. In some experiments, the co-incubation of COC with sperm was allowed to continue for 24 h and fertilization was assessed by scoring 2-cell embryos under a stereoscopic microscope.

Cumulus penetration assay

COC were inseminated with capacitated sperm (final concentration: 1–2.5 × 10^4 sperm/ml) previously stained with 3 μg/ml Hoechst 33342 and gametes were co-incubated for 15 min at 37°C in an atmosphere of 5% (v/v) CO_2 in air. COC were washed and fixed as described above and the number of sperm within the cumulus was determined under the Nikon Optiphot microscope equipped with epifluorescence optics (×200).

Sperm-zona pellucida binding assay

Zona pellucida-intact eggs and 2-cell embryos (used as control) were co-incubated with capacitated sperm (final concentration: 1 × 10^5 cells/ml) for 30 min at 37°C in an atmosphere of 5% (v/v) CO_2 in air. Eggs and embryos were washed thoroughly until no sperm remained bound to the embryos. Finally, eggs were fixed and the number of sperm bound to the zona pellucida was counted under a light microscope (×400).

Evaluation of acrosome reaction

Sperm were exposed to vehicle alone (dimethyl sulfoxide; Sigma) or progesterone (30 μM; Sigma) during the last 15 min of capacitation, and then subjected to Coomassie Brilliant Blue staining as previously described (Busso *et al.*, 2007). Four hundred spermatozoa were evaluated in each treatment under a light microscope (×400). Sperm were scored as acrosome-intact

when a bright blue staining was observed in the dorsal region of the head or as acrosome-reacted when no labeling was observed.

Computer-assisted sperm analysis (CASA)

Sperm aliquots (15 μl) were placed between pre-warmed slides and cover slips (22 × 22 mm) to create a chamber with ~30 μm depth, and were examined at 37°C using the ISAS® (Integrated Semen Analysis System) v1.2 CASA system (Proiser R&D, S.L., Valencia, Spain). For each sample, a minimum of two hundred cells distributed in at least twenty different microscope fields were scored (30 frames acquired at 60 Hz for each measurement). The following parameters were evaluated: average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), linearity (LIN, %), amplitude of lateral head displacement (ALH, μm), straightness (STR, %) and beat cross frequency (BCF, Hz). Sperm were considered motile when showing VSL > 0, and hyperactivated when presenting VCL ≥ 271, LIN < 23.6 and ALH ≥ 5. These custom cutoffs were selected for our experimental conditions based on previously reported recommendations (Bray *et al.*, 2005).

Intracellular Ca^{2+} measurement

Intracellular Ca^{2+} levels were measured by flow cytometry as previously described (Mata-Martinez *et al.*, 2013). Sperm aliquots were loaded with 2 μM of Fluo-4, AM (Invitrogen, Carlsbad, CA, USA), diluted in 10% (w/v) Pluronic F-127 (Invitrogen), before or after incubation under capacitating or non-capacitating (without bicarbonate and BSA) conditions. Samples were washed to remove the excess of probe, suspended in BSA-free medium, and exposed to 2.5 μg/ml propidium iodide (PI; Sigma). Fluorescence was detected using a BD FACSCanto™ II analyzer following the manufacturer's indications. Data analysis was performed by FlowJo 7.6 software (FlowJo LLC, Ashland, OR, USA). Results are shown as mean fluorescence intensity (MFI) for Fluo-4 AM obtained from at least 10 000 live (PI negative) sperm.

Statistical analysis

Data represent the mean ± SEM from at least three independent experiments. Calculations were performed using the Prism 3.0 software (GraphPad Software, La Jolla, CA, USA). In all cases, comparisons were made by the Student's *t*-test except for the acrosome reaction (AR) and Ca^{2+} measurements, which were evaluated by two-way ANOVA, and for Mendelian frequency, which was analyzed by chi-square test. Pearson's correlation analysis was used to assess the correlations between the number of ovulated eggs and the percentage of fertilization. Differences were considered significant at a level of $P < 0.05$.

Results

Generation of *Crisp2*$^{-/-}$ mice

As an approach to investigate the functional relevance of CRISP2, a mouse line carrying a deletion in the *Crisp2* gene was established (Fig. 1A). The mutation in the null animals was evidenced by PCR from genomic DNA (Fig. 1B) whereas the absence of the protein was confirmed by both western blotting of testicular (Fig. 1C) and sperm (Fig. 1D) extracts and indirect inmunofluorescence of epididymal sperm (Supplementary Fig. S1). Breeding of heterozygotes yielded the predicted number of mice from each genotype at Mendelian frequency (*Crisp2*$^{+/+}$: 25.2%; *Crisp2*$^{+/-}$: 48.6%; *Crisp2*$^{-/-}$: 25.2%; $n = 258$; n.s.), indicating that the mutation is neither embryonic nor fetal lethal. In addition, littermates of all genotypes were indistinguishable among groups in terms of development, behavior and general health condition.

Analysis of the testis weight of *Crisp2*$^{-/-}$ males showed no significant differences with that of their heterozygous littermates (Supplementary Fig. S2A), and histological examination of *Crisp2*$^{-/-}$ testes revealed

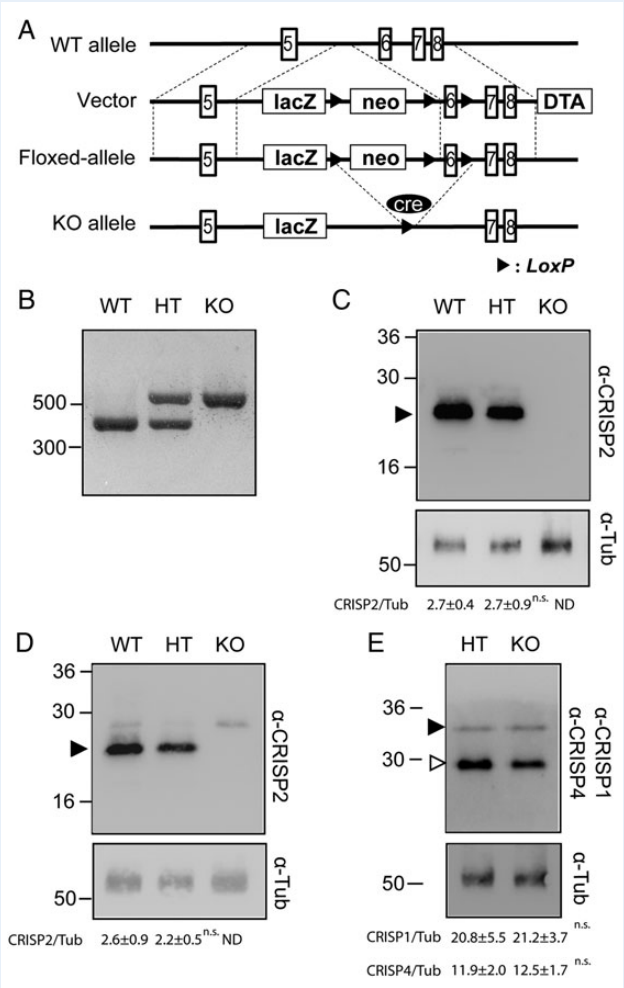

Figure 1 Generation of *Cysteine-rich secretory protein 2* (*Crisp2*) knockout mice. (**A**) Schematic representation of a fragment of the *Crisp2* wild type (WT) allele, the targeting vector, the floxed-allele with *LoxP* sequences flanking exon 6, and the null (KO) allele generated by *Cre*-mediated recombination. Exons are represented by numbered white boxes. The black triangle indicates LoxP sequence. lacZ:β-galactosidase gene; neo: neomycin resistance cassette; DTA: diphtheria toxin A chain. (**B**) Animals were genotyped by PCR using genomic DNA extracts obtaining bands of 380 bp (WT), 540 bp (KO) or both 380 bp and 540 bp (heterozygous, HT). (**C** and **D**) Total protein extracts from testes (C) or epididymal sperm (D) were evaluated for the presence of CRISP2 (filled arrowhead) by western blotting using an anti-CRISP2 antibody. β-tubulin was used as loading control (bottom panels). Densitometry results (mean ± SEM) are shown below each blot. ND: non-detected; $n = 5$; not statistically significant (n.s). Representative blots are shown. (**E**) Total protein extracts from epididymal sperm were evaluated for the presence of CRISP1 (open arrowhead) and CRISP4 (filled arrowhead) by western blotting using anti-CRISP1 and anti-CRISP4 antibodies, simultaneously. β-Tubulin was used as loading control (bottom panel). Densitometry results (mean ± SEM) are shown below the blot. $n = 5$; n.s. A representative blot is shown.

seminiferous tubules with normal diameters and abundant spermatogenic cells in the epithelium and sperm in the lumen (Supplementary Fig. S2B). In addition, the number, viability, morphology and motility of *Crisp2*$^{-/-}$ sperm did not differ from that of the controls (Supplementary Fig. S2C–F). As the expression of related proteins might be altered in knockout mice (Drabent *et al.*, 2000; Stein *et al.*, 2005), we next compared the abundance of epididymal CRISP1 and CRISP4 proteins in *Crisp2*$^{-/-}$ and *Crisp2*$^{+/-}$ sperm by western blotting followed by densitometric analysis. As shown in Fig. 1E, no differences in CRISP1 or CRISP4 levels were detected between the two genotypes.

In vivo fertilizing ability of *Crisp2*$^{-/-}$ sperm

Having confirmed the absence of CRISP2 in mutant sperm, the next step was to analyze the fertility of the *Crisp2* knockout males. The results of mating experiments using adult females revealed no differences between genotypes in several parameters indicative of fertility (Table I). These results led us to evaluate the fertility of *Crisp2*$^{-/-}$ males under more demanding conditions. For this purpose, young adult males of both genotypes were subjected to unilateral vasectomy to reduce the number of sperm in the ejaculate (Judd *et al.*, 1997). Under these conditions, *Crisp2*$^{-/-}$ males showed a significant decrease in both the percentage of pregnancy and the number of pups per litter compared with *Crisp2*$^{+/-}$ males (Table II). Consistent with this, when the mutant vasectomized males were mated with natural estrus females, a significant reduction in the percentage of fertilized eggs in the *ampulla* was observed compared with controls (Table II).

As another approach to evaluate the effect of the lack of CRISP2 on *in vivo* fertilization, *Crisp2*$^{-/-}$ and *Crisp2*$^{+/-}$ males were mated with both natural estrus and hormone-stimulated females as a way to challenge the mutant sperm to a high number of ovulated eggs. Whereas normal fertilization rates were obtained for *Crisp2*$^{-/-}$ males mated with estrus females, a marked reduction in fertilization levels was observed when the animals were mated with stimulated females (Fig. 2A), revealing defects in mutant sperm to deal with the changes imposed by the hormone treatment. Further analysis showed that although the total number of ovulated eggs (naturally or stimulated) did not differ between the two mating groups (Fig. 2B), the fertilization competence of *Crisp2*$^{-/-}$ but not *Crisp2*$^{+/-}$ sperm decreased significantly as a function of the number of ovulated eggs (Fig. 2C).

Table I Fertility of Cysteine-RIch Secretory Protein 2 (CRISP2)-deficient mice.

Genotype	Pregnancy (%)	Litter size (no. of pups)	Time to delivery (days)
Crisp2$^{+/-}$	90 ± 7.0	8.1 ± 0.6	24.9 ± 1.6
Crisp2$^{-/-}$	90 ± 5.2	7.8 ± 0.6	24.2 ± 1.4

Crisp2$^{+/-}$ or *Crisp2*$^{-/-}$ males were housed for 3 weeks with control females and the percentage of pregnant females, litter size and time to delivery were determined. Data are mean ± SEM, $n = 8$; n.s.

Table II Effect of unilateral vasectomy on fertility and *in vivo* fertilization of Cysteine-RIch Secretory Protein 2 (CRISP2)-deficient mice.

	Pregnancy (%)	Litter size (no. of pups)	Fertilization rate (%)
Crisp2$^{+/-}$	100.0	7.7 ± 0.9	99.0 ± 1.0
Crisp2$^{-/-}$	70.0 ± 13.3*	2.4 ± 0.6**	29.7 ± 11.6**

Crisp2$^{+/-}$ or *Crisp2*$^{-/-}$ males undergoing unilateral vasectomy were housed with control females and the percentage of pregnant females and litter size were determined. These males were then mated with natural estrus females and the percentage of fertilized eggs recovered from the *ampulla* evaluated the following day. Data are mean ± SEM, $n = 5$; *$P < 0.05$, **$P < 0.005$ versus control.

In vitro fertilizing ability of *Crisp2*$^{-/-}$ sperm

To analyze whether the lower *in vivo* fertilization rates corresponding to *Crisp2*$^{-/-}$ mice were due to a sperm deficiency to interact with the egg, *Crisp2*$^{+/-}$ and *Crisp2*$^{-/-}$ epididymal sperm collected from the same males that had been previously subjected to mating (see Fig. 2A), were capacitated and co-incubated *in vitro* with COC. Under these conditions, the percentages of eggs fertilized by *Crisp2*$^{-/-}$ epididymal sperm were markedly lower than those of the controls (Fig. 2A). Interestingly, the *in vitro* fertilization rates obtained with *Crisp2*$^{+/-}$ epididymal sperm did not differ from those observed for ejaculated sperm after mating with estrus or stimulated females. To evaluate whether a lower quality of the oocytes ovulated in response to stimulation could have contributed to the reduced fertilization rates obtained with the mutant cells, sperm of both genotypes were subjected to *in vitro* fertilization assays using the same number of either naturally or hormone-induced ovulated COC. Results showed that *Crisp2*$^{-/-}$ sperm behaved poorly compared with controls when exposed to both types of eggs (Table III), confirming that *Crisp2*$^{-/-}$ sperm exhibit specific defects in their *in vivo* and *in vitro* fertilizing ability. Based on this, we then analyzed the sperm behavior at different stages of the fertilization process. For this purpose, *Crisp2*$^{+/-}$ and *Crisp2*$^{-/-}$ capacitated sperm were co-incubated *in vitro* with either eggs surrounded by both the cumulus and the zona pellucida (COC), eggs coated only by the zona pellucida or cumulus- and zona pellucida-free eggs. Results showed significantly lower fertilization rates for *Crisp2*$^{-/-}$ than for *Crisp2*$^{+/-}$ sperm under the three conditions tested (Fig. 3A–C), suggesting sperm defects at different fertilization levels. To investigate whether these impairments could be due to a delayed fertilization, control COC were exposed to *Crisp2*$^{+/-}$ or *Crisp2*$^{-/-}$, and fertilized eggs were allowed to develop to 2-cell embryos. In this case, the fertilization rates corresponding to each genotype (Fig. 3D) were similar to those previously observed (see Fig. 3A), indicating that the fertilizing deficiencies of mutant sperm could not be overcome by longer gamete co-incubation periods.

The finding that the decrease in fertilization rates was greater for both cumulus- and zona pellucida-intact eggs than for zona pellucida-free eggs suggested a deficiency of null sperm to interact with the coats that surround the egg. To examine the ability of *Crisp2*$^{-/-}$ sperm to penetrate the *cumulus oophorus*, COC were co-incubated with capacitated sperm of each genotype and the number of sperm heads within the cumulus mass was determined 15 min later. As observed in Fig. 3E, a significantly lower number of *Crisp2*$^{-/-}$ sperm penetrated the cumulus matrix compared with controls. If mutant sperm had a normal ability to penetrate the zona pellucida, sperm would accumulate in the perivitelline space due to their lower ability to fuse with the egg plasma membrane (see Fig. 3C). As

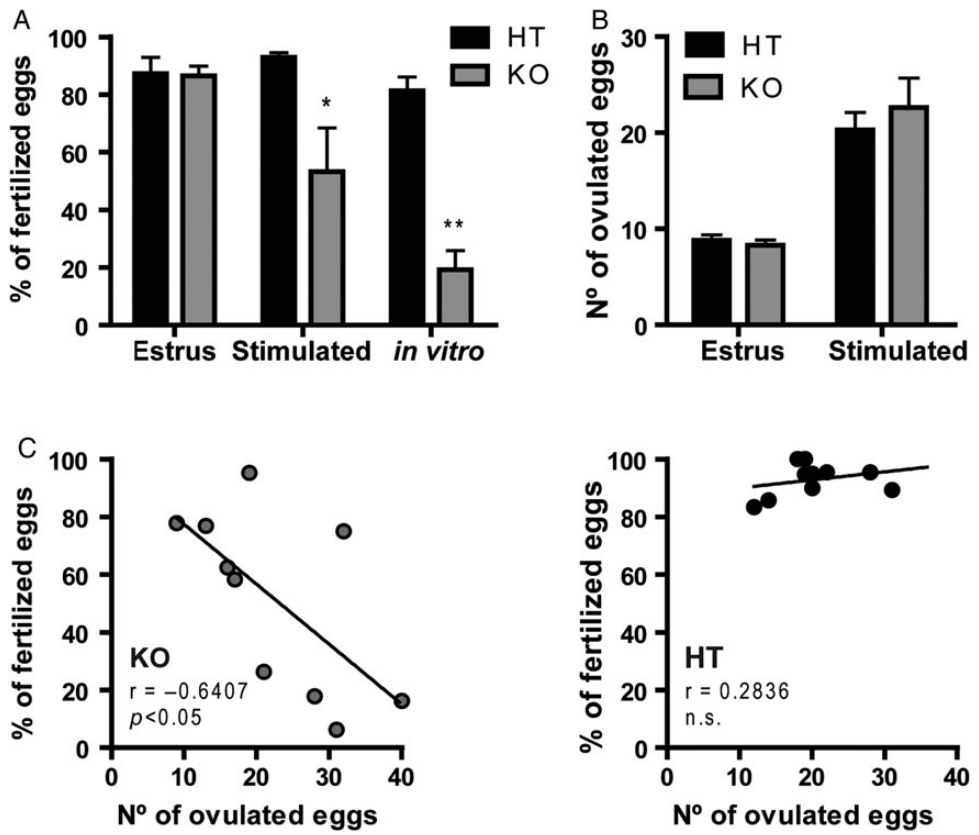

Figure 2 Fertilizing ability of Cysteine-RIch Secretory Protein 2 (CRISP2)-deficient sperm under *in vivo* and *in vitro* conditions. (**A**) Heterozygous (HT) and knockout (KO) males were mated with natural estrus and hormone-stimulated females and the percentage of fertilized eggs recovered from the *ampulla* evaluated the following day. Sperm recovered from the same males were subjected to *in vitro* fertilization. Data are mean + SEM, $n = 5$; *$P < 0.01$; **$P < 0.001$. (**B**) Average number of eggs from estrus or hormone-stimulated females corresponding to mating studies shown in (A). Data are mean + SEM, $n = 5$; not statistically significant (n.s.). (**C**) Fertilization rates as a function of the number of ovulated eggs corresponding to *Crisp2*$^{-/-}$ (left panel) and *Crisp2*$^{+/-}$ (right panel) mice. The Pearson's coefficient '*r*' and the significance are included in each panel.

Table III ***In vitro*** **sperm fertilizing ability using eggs from estrus or hormone-stimulated females.**

	Estrus (%)	Stimulated (%)
Crisp2$^{+/-}$	91.5 ± 6.5	82.5 ± 6.5
Crisp2$^{-/-}$	23.7 ± 13.1*	22.2 ± 8.3*

Crisp2$^{+/-}$ or *Crisp2*$^{-/-}$ capacitated sperm were subjected to *in vitro* fertilization with eggs from estrus or hormone-stimulated females. Data are mean ± SEM, $n = 5$; *$P < 0.01$ versus control. No statistical differences within each genotype were observed between stimulated and estrus females.

no accumulation of *Crisp2*$^{-/-}$ sperm was observed in either fertilized or non-fertilized zona pellucida intact eggs, mutant sperm may exhibit a defect in their ability to bind to and/or penetrate the zona pellucida. To investigate these possibilities, zona pellucida-intact eggs were co-incubated with either *Crisp2*$^{-/-}$ or control sperm and the number of sperm bound to the zona pellucida evaluated 30 min later. Results revealed no differences between genotypes in the number of zona pellucida-bound sperm (Fig. 3F), supporting a deficiency of null sperm at the zona pellucida-penetration level.

Molecular mechanisms underlying impaired fertilizing ability of *Crisp2*$^{-/-}$ sperm

To elucidate the mechanisms leading to the lower fertilizing ability of *Crisp2*$^{-/-}$ cells observed under both *in vivo* and *in vitro* conditions, *Crisp2*$^{-/-}$ and *Crisp2*$^{+/-}$ sperm were incubated under capacitating conditions, and different sperm capacitation-dependent events were examined. Western blot analysis of control and mutant sperm extracts revealed no differences between genotypes in the increase in protein tyrosine phosphorylation that takes place during capacitation (Fig. 4A). Differences between groups were neither observed in the percentage of spontaneous or progesterone-induced AR, an exocytotic event in the sperm head necessary for zona pellucida-penetration and gamete fusion (Fig. 4B). However, objective analysis of sperm motility by CASA revealed that, in spite of their normal total motility (*Crisp2*$^{-/-}$: 58 ± 5% versus *Crisp2*$^{+/-}$: 60 ± 6%; mean ± SEM; $n = 7$; n.s.), null sperm exhibited a significant reduction in most of the motility parameters associated with capacitation (Table IV) as well as in the percentage of cells that developed hyperactivation, a vigorous motility displayed by capacitated sperm and required for penetration of the egg vestments (Fig. 4C). Considering that a decrease in these motility parameters has been associated with abnormal fluctuations in Ca^{2+} levels (Ren *et al.*,

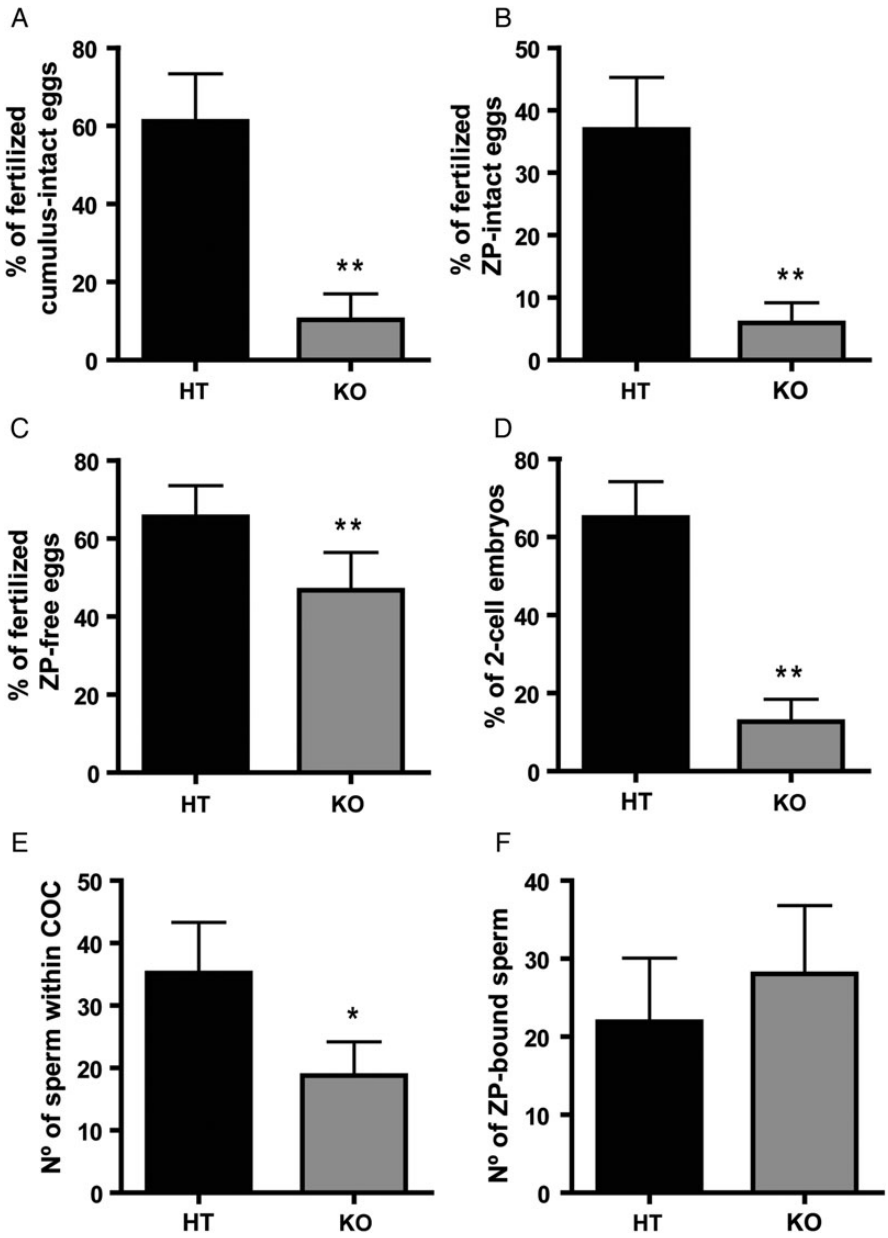

Figure 3 Cysteine-RIch Secretory Protein 2 (CRISP2)-deficient sperm behavior at different stages of the fertilization process. Heterozygous (HT) and knockout (KO) capacitated sperm were co-incubated with (**A**) Cumulus-intact eggs for 3 h, (**B**) Zona pellucida (ZP)-intact eggs for 3 h, (**C**) zona pellucida-free eggs for 1 h or (**D**) Cumulus-intact eggs for 18 h. At the end of all incubations, the percentage of fertilization was evaluated by the presence of decondensing sperm heads within the egg cytoplasm (A,B,C) or 2-cell embryo (D). Data are mean + SEM (A) $n = 6$; $^{**}P < 0.01$, (B) $n = 9$; $^{**}P < 0.01$, (C) $n = 7$; $^{**}P < 0.01$, (D) $n = 5$; $^{**}P < 0.01$. (**E**) Hoescht-stained capacitated sperm were co-incubated with COC for 15 min and the number of sperm within the cumulus matrix was determined. Data are mean + SEM, $n = 4$; $^{*}P < 0.05$. (**F**) Capacitated sperm were co-incubated with zona pellucida-intact eggs for 30 min and the number of sperm bound to the zona pellucida was evaluated. Data are mean + SEM, $n = 5$; not statistically significant.

2001; Schuh *et al.*, 2004), intracellular Ca^{2+} was measured by flow cytometry in *Crisp2*$^{-/-}$ and *Crisp2*$^{+/-}$ epididymal sperm incubated under capacitating and non-capacitating conditions. Results showed that fresh sperm as well as those incubated under non-capacitating conditions exhibited low fluorescence values which did not differ between genotypes (Fig. 4D). In addition, whereas both *Crisp2*$^{-/-}$ and *Crisp2*$^{+/-}$ sperm populations showed the reported increase in intracellular Ca^{2+} during capacitation (Ruknudin and Silver, 1990), null capacitated sperm exhibited significantly higher levels of fluorescence than *Crisp2*$^{+/-}$ capacitated cells (Fig. 4D). Together, these observations support a dysregulation in intracellular Ca^{2+} as a mechanism underlying the capacitation and fertilization defects of *Crisp2*$^{-/-}$ sperm.

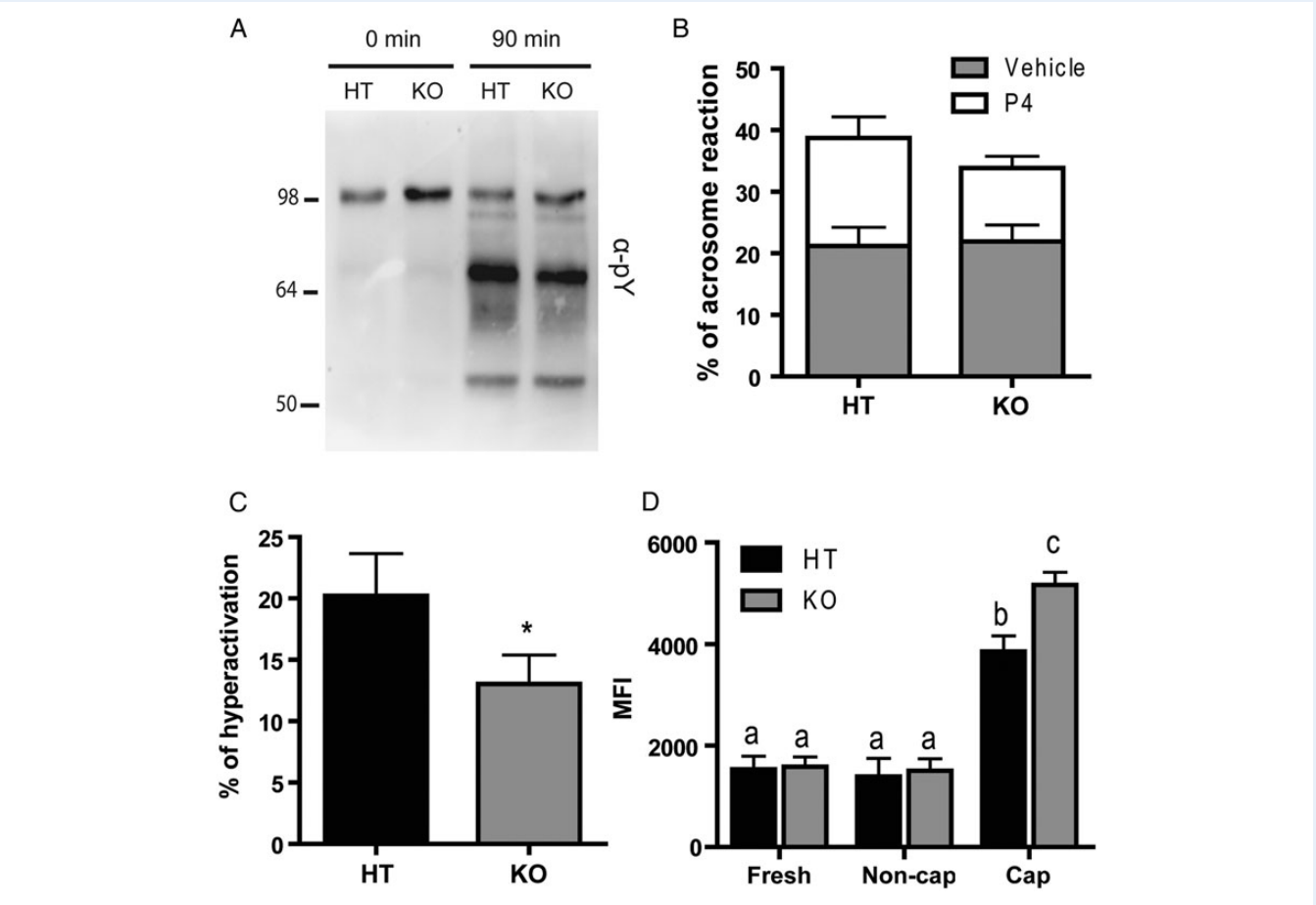

Figure 4 Capacitation-associated events in Cysteine-Rich Secretory Protein 2 (CRISP2)-deficient sperm. Heterozygous (HT) and knockout (KO) sperm were incubated under capacitating conditions for 90 min and different functional parameters evaluated. (**A**) Protein tyrosine phosphorylation analyzed by western blotting using an anti-phosphotyrosine antibody. A representative blot is shown. $n = 4$. (**B**) Percentage of acrosome reaction determined by Coomassie Brilliant Blue staining in sperm exposed to progesterone (P4) or dimethyl sulfoxide alone (vehicle) during the last 15 min of capacitation. Data are mean + SEM, $n = 4$; n.s. (**C**) Percentage of hyperactivation evaluated by CASA. Data are mean + SEM, $n = 7$; $^*P < 0.05$. (**D**) Intracellular Ca^{2+} levels evaluated by flow cytometry using Fluo-4, AM. Results are shown as mean fluorescence intensity (MFI) for fresh, non-capacitated and capacitated sperm. Data are mean + SEM, $n = 5$; (a) versus (b) $P < 0.001$; (b) versus (c) $P < 0.001$.

Table IV Effect of the lack of Cysteine-RIch Secretory Protein 2 (CRISP2) on motility of capacitated sperm.

	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	BCF (Hz)	STR (%)	LIN (%)
Crisp2$^{+/-}$	122.1 ± 5.3	52.6 ± 3.0	226.0 ± 12.4	4.7 ± 0.3	11.7 ± 0.3	43.6 ± 0.1	24.2 ± 0.7
Crisp2$^{-/-}$	110.7 ± 4.2*	46.7 ± 1.8*	202.9 ± 10.7**	4.2 ± 0.2***	12.0 ± 0.2	43.1 ± 1.6	24.4 ± 1.3

Crisp2$^{+/-}$ or *Crisp2*$^{-/-}$ capacitated sperm were subjected to computer-assisted semen analysis and different motility parameters analyzed.
VAP, average path velocity; VSL, straight line velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, straightness; LIN, linearity.
Data are mean ± SEM, $n = 7$; $^*P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$ versus control.

Discussion

In the present study, we generated *Crisp2* knockout mice as an approach to evaluate whether fertility disorders in patients with aberrant expression of CRISP2 could be linked to the proposed functional role of this protein in fertilization.

In spite of CRISP2 expression in germ cells (Kasahara *et al.*, 1987, 1989; Hardy *et al.*, 1988; Mizuki *et al.*, 1992; Maeda *et al.*, 1998; O'Bryan *et al.*, 1998; Giese *et al.*, 2002) and its proposed role in cell adhesion between spermatids and Sertoli cells (Maeda *et al.*, 1998, 1999), *Crisp2* knockout mice exhibited normal testes and epididymal sperm, indicating that the lack of CRISP2 does not affect the progression of

spermatogenesis. As previously observed for both CRISP1 and CRISP4 deficient animals (Da Ros *et al.*, 2008; Gibbs *et al.*, 2011; Turunen *et al.*, 2012), *Crisp2*-null mice are fertile, suggesting that the lack of one of these CRISP homologues might be compensated by the others. Thus, it is likely that the simultaneous absence of more than one CRISP protein (i.e. double or triple knockouts) may lead to defects in mouse fertility. Our results are also consistent with the normal fertility levels observed in most knockout mice for sperm proteins with demonstrated roles in *in vitro* fertilization (Okabe and Cummins, 2007). In this regard, it is important to note that the female tract has an extremely efficient mechanism to select those few but high quality fertilizing sperm required for fertilization in the *ampulla*. This efficient selection system may lead to a sufficient number of fertilizing sperm in the *ampulla* even in males with reproductive deficiencies, thus, masking sperm defects that become evident only under more demanding conditions. Supporting this notion, whereas the 50% reduction in the number of ejaculated sperm in unilaterally vasectomized males did not affect the fertility outcome of *Crisp2*$^{+/-}$ mice, it produced a significant impact on fertility parameters in the mutant mice. The fact that unilaterally vasectomized *Crisp2*$^{-/-}$ mice also showed lower levels of fertilization in the *ampulla* confirmed fertilization defects as responsible for the lower fertility rates observed for these mice. In another approach, we found that *Crisp2*$^{-/-}$ males exhibited lower *in vivo* fertilization rates than controls when mated with hormone-stimulated females that ovulate a higher number of eggs and may exhibit other reproductive changes compared with the natural estrus females. According to our results, it is clear that whereas *Crisp2*$^{+/-}$ males could deal with different *in vivo* modifications, *Crisp2*$^{-/-}$ males could not. Interestingly, these observations may be extrapolated to humans where the subfertility of an individual can or cannot be detected depending on the combination of own defects or on the fertility status of the partner.

In vitro fertilization studies supported the idea that the lower levels of *in vivo* fertilization observed could be due to defects in the ability of mutant sperm to interact with the COC. As it has been shown that the hormone stimulation protocols may have detrimental effects on the eggs (Edgar *et al.*, 1987), it is possible that a lower egg quality could have contributed to the lower fertilization rates obtained for *Crisp2*$^{-/-}$ sperm under *in vivo* (*Crisp2*$^{-/-}$ males mated with stimulated females) and *in vitro* conditions. The finding that the mutant sperm behaved poorly when co-incubated with either naturally or hormone-induced ovulated COC argues against this possibility. Subsequent studies exposing sperm to eggs surrounded or denuded of their vestments revealed that *Crisp2*$^{-/-}$ sperm defects were detected at different stages of the fertilization process. The reduced penetration of zona pellucida-free eggs by null sperm which exhibited normal levels of spontaneous AR supports our previous observations proposing the involvement of CRISP2 in gamete fusion through its interaction with egg complementary sites in both rodents and humans (Busso *et al.*, 2005, 2007; Da Ros *et al.*, 2008; Munoz *et al.*, 2012). Consistent with this, CRISP2 relocalizes during capacitation from the acrosomal compartment to the equatorial segment, the region through which the sperm fuses with the egg plasma membrane (Busso *et al.*, 2005, 2007; Nimlamool *et al.*, 2013). Interestingly, CRISP1 also localizes in the equatorial segment of capacitated sperm and has been proposed to participate in gamete fusion (Rochwerger and Cuasnicu, 1992; Rochwerger *et al.*, 1992; Cohen *et al.*, 2000, 2001; Da Ros *et al.*, 2008) through its interaction with the same egg binding sites that CRISP2 (Ellerman *et al.*, 2006; Busso *et al.*, 2007), supporting CRISP1 as a candidate to compensate for the lack of CRISP2 during gamete fusion.

The differences between the fertilizing ability of *Crisp2*$^{+/-}$ and *Crisp2*$^{-/-}$ sperm were more clearly shown when sperm were exposed to COC or zona pellucida-intact eggs. These functional sperm defects could not be overcome by longer incubation periods, indicating that the lower fertilization rates obtained for *Crisp2*$^{-/-}$ sperm were not merely due to a delayed fertilization as previously described for acrosin-deficient sperm (Adham *et al.*, 1997). The fact that, in spite of their lower fusion ability, *Crisp2*$^{-/-}$ sperm did not accumulate in the perivitelline space of fertilized or non-fertilized zona pellucida-intact eggs indicates a failure of the mutant sperm to bind to and/or to penetrate the zona pellucida. In this regard, differently from *Crisp1*$^{-/-}$ (Cohen *et al.*, 2011) and *Crisp4*$^{-/-}$ (Turunen *et al.*, 2012) cells, *Crisp2*$^{-/-}$ sperm did not exhibit defects in their zona pellucida-binding ability, supporting that these cells may have defects in their ability to penetrate the zona pellucida. Exposure of sperm to anti-CRISP2, however, does not affect zona pellucida-penetration and results in the accumulation of perivitelline sperm by blocking the function of the intra-acrosomal CRISP2 protein exposed after the AR (Busso *et al.*, 2007). Based on this, it can be proposed that *Crisp2*$^{-/-}$ sperm fail to penetrate the zona pellucida due to defects associated with the intracellular CRISP2 protein present in the neck and tail and which is not accessible to the antibody blockage in control sperm. This functional deficiency could also be responsible for the lower ability of *Crisp2*$^{-/-}$ sperm to penetrate the *cumulus oophorus* in our assays. Together, our *in vitro* studies support the participation of CRISP2 in gamete fusion and reveal a new potential role for the protein in the penetration of both the cumulus and zona pellucida vestments.

To explore the molecular mechanisms leading to the reduced fertilizing ability of *Crisp2*$^{-/-}$ sperm, different functional parameters associated with sperm capacitation were analyzed. Protein tyrosine phosphorylation levels, which increase during capacitation (Visconti *et al.*, 1995), were similar for sperm of both genotypes, suggesting no major defects in the signaling pathways associated with sperm capacitation. Similarly, no differences in the levels of spontaneous or progesterone-induced AR for *Crisp2*$^{-/-}$ and *Crisp2*$^{+/-}$ sperm were observed, excluding the possibility that the lower fertilizing ability of mutant sperm was due to defects in this exocytotic event. In this regard, it is possible that CRISP4, reported to be involved in the progesterone-induced AR (Gibbs *et al.*, 2011; Turunen *et al.*, 2012), compensates for the lack of CRISP2 in the knockout sperm. Objective analysis of motility by CASA revealed a significant decrease in most of the kinematic parameters associated with the development of hyperactivation as well as in the percentage of hyperactivated cells. These motility defects in *Crisp2*$^{-/-}$ sperm are consistent with their reduced ability to fertilize cumulus- and zona pellucida-intact eggs as hyperactivation is a requisite for penetration of the egg coats (Suarez and Dai, 1992; Yanagimachi, 1994; Stauss *et al.*, 1995; Ren *et al.*, 2001). As CRISP2 has been identified as a component of the outer dense fibers of the tail (O'Bryan *et al.*, 2001), the lower hyperactivation levels in *Crisp2*$^{-/-}$ sperm could be attributed to structural alterations in the flagella due to the lack of the protein in these cells. CRISP2-deficient sperm, however, do not exhibit defects in either their morphology at a light microscopic level or initial motility, as have been reported for two different knockout models for outer dense fiber proteins (Tarnasky *et al.*, 2010; Yang *et al.*, 2012). Alternatively, as Ca^{2+} is critical for hyperactivation development (Yanagimachi,

1994), the role of CRISP2 in motility could be linked to its reported *in vitro* capacity to regulate ryanodine receptor Ca^{2+} channels in other cells (Gibbs *et al.*, 2006). This possibility is supported by both the high intracellular Ca^{2+} observed in *Crisp2*$^{-/-}$ sperm after capacitation and the reported hyperactivation defects found in sperm with increased levels of the cation (Schuh *et al.*, 2004). Moreover, considering the localization of both ryanodine receptors (Harper *et al.*, 2004) and CRISP2 (O'Bryan *et al.*, 2001) in the neck, and that Ca^{2+} released from intracellular stores at the neck is involved in sperm hyperactivation (Chang and Suarez, 2011), it is possible that CRISP2 modulates sperm motility through the regulation of ion channels controlling intracellular Ca^{2+} stores. Finally, based on the essential role of CatSper channel for hyperactivation development (Ren *et al.*, 2001; Carlson *et al.*, 2003), the possibility that CRISP2 regulates the CatSper-mediated Ca^{2+} influx cannot be excluded (Ernesto *et al.*, 2015). The finding that the higher intracellular Ca^{2+} did not affect the occurrence of the AR in *Crisp2*$^{-/-}$ sperm is in agreement with previous reports in mice showing that an increase in Ca^{2+} is not necessarily associated with higher AR levels (Chang and Suarez, 2011) and that different Ca^{2+} signaling pathways may lead to different sperm behaviors (Tateno *et al.*, 2013).

Collectively, our *in vivo* and *in vitro* results reveal that *Crisp2*$^{-/-}$ sperm exhibit clear fertilization deficiencies, likely linked to defects in hyperactivation development and intracellular Ca^{2+} regulation. These observations support the idea that a defective sperm fertilizing ability may be underlying the fertility disorders observed in men with aberrant expression of CRISP2 (Du *et al.*, 2006; Jing *et al.*, 2011; Zhou *et al.*, 2015). Of note, whereas the normal fertilization rates observed for CRISP2-deficient males after conventional mating confirm the critical role of the female reproductive tract in selecting the best functional sperm, the finding that sperm defects in these mice became evident under more demanding conditions highlights the relevance of using different experimental approaches to analyze male fertility.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors' roles

P.S.C. conceived the study. N.G.B., V.G.D. and P.S.C. designed the experiments, analyzed the results and wrote the manuscript. N.G.B. performed most experiments assisted by V.G.D. H.M. and M.I. generated the mutant mice. P.T. and D.L. collaborated with motility evaluation. J.J.C. contributed to the Ca^{2+} determinations. All authors read and approved the final manuscript.

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Conflict of interest

None declared.

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