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Function of the Acrosomal Matrix: Zona Pellucida 3 Receptor (ZP3R/sp56) Is Not Essential for Mouse Fertilization[1]

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ABSTRACT

In mammalian fertilization, sperm-zona pellucida binding is considered to be a critical aspect of gamete interaction. In this study, we examine the mouse sperm acrosomal matrix protein zona pellucida 3 receptor (ZP3R; formerly called sp56) because of our interest in defining the function of the acrosomal matrix, the particulate compartment within the sperm secretory acrosome. Using targeted deletion of the *Zp3r* gene by homologous recombination, we examined the fertility of nullizygous animals. Our experiments showed that males and females homozygous for the affected gene exhibited no differences in litter sizes compared to wild-type and heterozygous animals. Testis weights of nullizygous males were equivalent to those of wild-type and heterozygous males, and no differences in the number of sperm produced by mice of three genotypes were found. In vitro fertilization rates using cumulus-intact and cumulus-free oocytes were also equivalent. Examination of sperm-binding zonae of unfertilized eggs and the ability of the sperm to undergo acrosomal exocytosis in response to calcium ionophore A23187 displayed no differences between wild-type, heterozygous, and nullizygous mouse sperm. These results provide further evidence that either ZP3R is not involved in sperm-zona pellucida binding or this process might be functionally redundant, involving multiple proteins for gamete interactions.

acrosome, acrosome reaction, fertilization, sperm, zona pellucida

INTRODUCTION

The molecular and cellular mechanisms of fertilization are subjects of intense scrutiny because of their importance in the treatment of infertility and in the development of new contraceptive approaches. In particular, binding of sperm and zona pellucida (ZP) is a critical step of gamete interaction in many mammalian species. Studies of fertilization using bovine, porcine, and murine systems suggest that gamete adhesion occurs by protein-carbohydrate interactions between a binding protein on the sperm and one or more carbohydrate ligands on the ZP [1, 2]. Whereas the number of ZP sulfoglycoproteins involved in these binding events is limited [3], many sperm molecules have been shown to possess ZP-binding activity. In mice, virtually none of the proteins reported to be important in sperm-ZP interaction have proven to be essential for fertilization when the genes encoding these proteins have been disrupted by homologous recombination. This is the case for proacrosin [4], β-1,4-galactosyltransferase [5], MFGE8 (SED-1) [6], SPAM1 (PH-20) [7], and zonadhesin [8]. The binding of sperm to the ZP appears to involve multiple sperm proteins, providing an element of redundancy to a process that is a very critical step in the maintenance of a given species.

In the present work, we studied the mouse sperm protein zona pellucida 3 receptor (ZP3R; formerly called sp56 [9]) because of our interest in defining the function of the acrosomal matrix, the particulate compartment within the acrosome. ZP3R was initially discovered by photoaffinity cross-linking with zona pellucida 3 (ZP3) [10] and was later characterized as having affinity for mouse ZP3 oligosaccharides involved in sperm binding [9, 11]. The protein was initially claimed to be a sperm surface (plasma membrane), ZP-binding protein [9, 10]. However, one of our laboratories subsequently demonstrated that ZP3R is a component of the acrosomal matrix, because it is actually located within the acrosomal compartment, it cannot be detected on the plasma membrane of live uncapacitated sperm, and it is associated with a Triton X-100-resistant structure within the acrosome [12, 13]. When sperm are incubated under conditions that mimic capacitation, the maturational process that normally occurs within the female reproductive tract and enables sperm to be capable of fertilization, ZP3R becomes exposed to the external environment and remains transiently associated with the sperm surface [14]. During acrosomal exocytosis, the secretory process that eventually leads to the release of the acrosomal contents from the sperm, soluble acrosomal components are readily released, but the loss of the particulate acrosomal matrix components is delayed because of the need for the complex to be dissolved by proteases, resulting in the cleavage of ZP3R and its release into the surrounding environment [15]. It should be noted that most studies of the function of ZP3R have been performed in the mouse. An orthologue has been identified in the guinea pig (AM67) [12] and the rat [16]. An inference has been made to a putative human orthologue, but

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no sequence data were provided or any repository accession information cited [17].

In the present study, we examined the role of ZP3R in the fertilization process using targeted deletion of the *Zp3r* gene by homologous recombination. We assessed its participation in sperm-ZP binding, and in fertilization in general, using in vivo and in vitro approaches. The resulting nullizygous male mice were completely fertile and displayed no obvious reproductive phenotype. Thus, these findings demonstrate that ZP3R is not an essential player in the process of binding and/or penetration of the ZP and provide further evidence that sperm-ZP binding is a very redundant process.

MATERIALS AND METHODS

Antibodies

Mouse anti-mouse ZP3R monoclonal antibody 7C5 was purchased from QED Bioscience, Inc. Rat anti-mouse IZUMO1 monoclonal antibody (mAb#125) was previously generated in the Okabe laboratory according to the standard method [18].

Construction of the Zp3r Gene Disruption Vector

The mouse *Zp3r* gene consists of 12 exons and maps to chromosome 1. To generate the targeting vector, the bacterial artificial chromosome (BAC) recombineering system was used [19]. The targeting vector was designed to replace exon 4 of the *Zp3r* gene with a Neo cassette. First, pML104 plasmid was transduced to *Escherichia coli* carrying a BAC encoding the *Zp3r* sequence. The pML104 plasmid expresses *Red*E and *Rec*T, which cause homologous recombination. Then, a BAC targeting vector was obtained by PCR using as a template the FRT-PGK-gb2-neo-FRT-loxP Neomycin Selection Cassette that contains the neomycin-resistance gene (*neo*) as a positive selection marker. The PCR forward and reverse primers used were as follows: 5′-ATA GAG TGG CAG TTG ATG TAT TTG TGC AAG TGT TTC TGT AGT TAG ATA GGA TAA CTT CGT-3′ and 5′-TTA TCA CAT GAG ATT AGG AGC ATA CAG AAG TTT GTT CTC AGG AAC GAT TGA TAT CAA GT-3′. These primers were used to add the 50-mer fragment of *Zp3r* sequences around exon 4 to the BAC targeting vector. The BAC targeting vector was electroporated into *E. coli* containing BAC and pML104 to cause the homologous recombination. The targeting vector was checked by Neo selection and PCR.

Generation of Zp3r-Disrupted Mice

The targeting vector was linearized with PI-*Sce*I digestion and electroporated into embryonic stem (ES) cells [129SV(D3)-Tg(NCAG-EGFP)CZ-001-FM260Osb]. ES cell clones that were candidates for possessing the targeted allele were isolated by positive selection with G418. Correct targeting of the *Zp3r* allele in ES cell clones by homologous recombination was confirmed by PCR and quantitative PCR at both ends. Positive ES clones were subsequently used to generate chimeras by injection into blastocysts from C57BL/6 Cr mice (age, >2 mo; Japan SLC, Inc.). Injected blastocysts were transferred to ICR pseudopregnant foster mothers, resulting in the birth of chimeric mice. Male chimeric mice were crossed with C57BL/6 to obtain F1 heterozygous offspring. *Zp3r*-deficient mice were generated by intercrossing F1 offspring mice. Mice used in the present study were of B6/129 mixed background. All experiments with mice were performed with the consent of the Animal Care and Use Committee of Osaka University.

Reverse Transcription-Polymerase Chain Reaction

Mouse cDNA was prepared from various adult mouse tissues. RT-PCR was performed using 10 ng of cDNA and the following forward and reverse primers: for *Zp3r*, 5′-CTC AAC CGA GCA TGC CAT GAA-3′ and 5′-CTA ATT GCA AAG TCT AAA TGA CCT ATT AAT TTT CAT AGA C-3′, respectively; for the glyceraldehyde-3-phosphatate dehydrogenase (*Gapdh*) gene, 5′-AGT GGA GAT TGT TGC CAT CAA CGA C-3′ and 5′-GGG AGT TGC TGT TGA AGT CGC AGG A-3′, respectively.

PCR Analysis

Genomic DNA preparations derived from tail biopsy specimens of $Zp3r^{+/+}$, $Zp3r^{+/-}$, and $Zp3r^{-/-}$ mice were used as templates for PCR analysis. The forward and reverse primers used were as follows: for the mutant allele, 5′-CTT TAC GGT ATC GCC GCT CCC GAT T-3′ and 5′-GCC ATA TGG CAT CCA CAT TTG G-3′; for the wild-type allele, 5′-GCC ATA TGG CAT CCA CAT TTG G-3′ and 5′-CCA TCT GAC CTT TTG GTA GGC TC-3′.

Northern Blot Analysis

Northern hybridization was performed using 10 μg of total RNA extracted from testes of $Zp3r^{+/+}$, $Zp3r^{+/-}$, and $Zp3r^{-/-}$ male mice. The RNAs were separated by electrophoresis on agarose gels, transferred to Hybond-N+ membranes (GE Healthcare Bio-Sciences Corp.), and hybridized to ^{32}P-labeled probes at 60°C overnight. Mouse *Zp3r* and cDNAs were used as probes. The *Zp3r* probe consisted of an entire cDNA amplified from mouse testis total RNA by RT-PCR using 5′-TGA ATT CTT CAA AGT GGC CTG CAG ACT GTC-3′ and 5′-TGA ATT CGC CAT TGG CTG TCA ACT TTA GCC-3′ as primers. For *Gapdh*, the probes were 5′-AGT GGA GAT TGT TGC CAT CAA CGA C-3′ and 5′-GGG AGT TGC TGT TGA AGT CGC AGG A-3′.

Immunoblotting Analysis

Immunoblot analysis was performed as described previously [20]. Briefly, sperm from the epididymis and vas deferens were collected and incubated in lysis buffer containing 1% Triton X-100 for 1 h on ice. The sperm were then centrifuged at 15 300 × *g* for 20 min at 4°C, and the supernatants were collected. Proteins were separated by SDS-PAGE under nonreducing conditions and transferred electrophoretically to Immobilon-P (Millipore). After blocking, blots were incubated with anti-ZP3R or anti-IZUMO antibody overnight at 4°C, then incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Ig) G and goat anti-rat IgG (GE Healthcare Bio-Sciences Corp.). The detection was performed using the Enhanced Chemiluminescence Plus Western blotting detection system (GE Healthcare Bio-Sciences Corp.).

Immunofluorescence Staining

Sperm from caudae epididymides were allowed to swim out into Toyoda, Yokoyama, and Hoshi (TYH) medium and resuspended in PBS [21]. Sperm suspensions were mounted on glass slides and dried at 37°C. Slides were blocked with 10% newborn calf serum (NBCS) in PBS for 1 h and fixed with 4% paraformaldehyde in PBS. After washing with 10% NBCS/PBS containing 0.05% Tween-20, the cells were incubated with mouse anti-ZP3R antibody in 10% NBCS/PBS at 4°C overnight. After washing with 10% NBCS/PBS containing 0.05% Tween-20, the slides were incubated with anti-mouse IgG labeled with Alexa Fluor 488 (Invitrogen) in 10% NBCS/PBS for 1 h. The slides were then washed with PBS containing 0.05% Tween-20 and observed under an Olympus IX-70 fluorescence microscope.

Assessment of the Fertilizing Ability of ZP3R-Deficient Mice

Sexually mature male mice of $Zp3r^{+/+}$, $Zp3r^{+/-}$, and $Zp3r^{-/-}$ genotypes were caged with (C57BL/6J 3 DBA/2N)F1 (also known as B6D2F1) female mice (age, >2 mo; CLEA Japan, Inc.), and the number of pups in each cage was counted within 1 wk of birth.

In Vitro Fertilization Assay

Female mice were superovulated by injection of 5 IU of equine chorionic gonadotropin followed 48 h later by 5 IU of human chorionic gonadotropin (hCG). Ovulated egg masses were collected from the oviducts 14 h after hCG injection. Eggs were placed in 100-μl drops of modified HTF (mHTF) medium from Irvine Scientific covered with paraffin oil. For the cumulus-free condition, the cumulus cells were removed by pretreatment with hyaluronidase for 5 min. Male mice of each genotype were subjected to an in vitro fertilization test. Sperm collected as a clot from mechanically dissected caudae epididymides were introduced into a 100-μl drop of mHTF medium. After 2 h of incubation, each sperm suspension was added to a drop containing eggs at final concentration of 5×10^4 sperm/ml. Twenty-four hrs after insemination, the numbers of 2-cell embryos were counted.

Sperm-ZP Binding Assay

The in vitro assays for sperm binding to ZP were performed with eggs from which the cumulus cells had been removed by treatment for 5 min with hyaluronidase (1 mg/ml; Wako). In brief, cumulus-free eggs from female B6D2F1 mice (age, >2 mo; Japan SLC, Inc.) were placed in a 200-μl drop of mHTF and inseminated with an aliquot of capacitated sperm (5×10^4 sperm/ml) from $Zp3r^{+/+}$, $Zp3r^{+/-}$, and $Zp3r^{-/-}$ males. After incubation for 30 min at 37°C under 5%

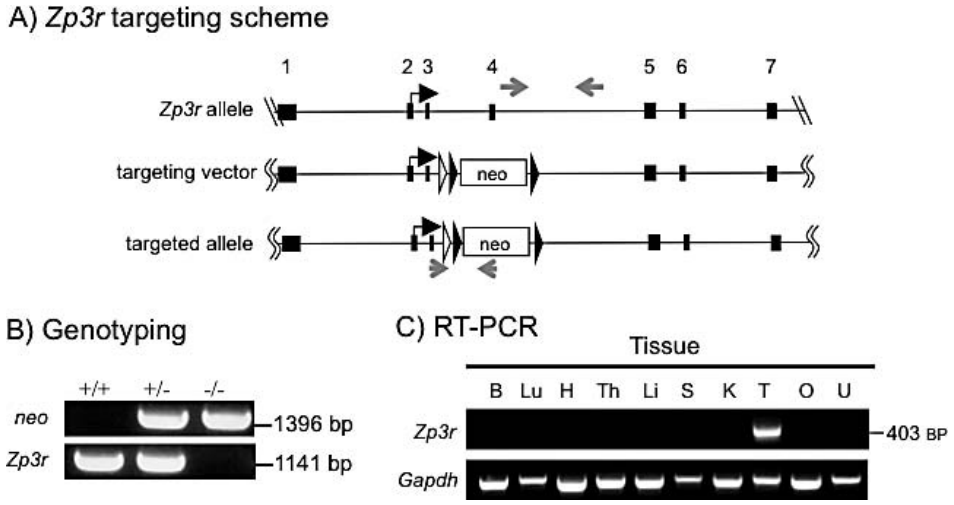

FIG. 1. Targeted deletion of *Zp3r* gene. **A**) Diagram of the vector construct. **B**) Genotyping results. **C**) Expression of *Zp3r* gene in different tissues. B, brain; H, heart; K, kidney; Li, liver; Lu, lung; O, ovary; S, spleen; T, testis; Th, thyroid gland; U, uterus.

CO_2 in air, the egg-sperm mixtures were fixed with 4% paraformaldehyde in PBS for 5 min and stained with Hoechst 33258. The sperm bound to eggs were observed with an Olympus IX-70 fluorescence microscope.

Analysis of Acrosomal Exocytosis and Sperm Motility

To investigate the influence of *Zp3r* disruption on sperm acrosomal exocytosis, we took advantage of a transgenic mouse line Tg(Acr-Egfp), the males of which express enhanced green fluorescent protein (EGFP) in the sperm acrosome under the control of the acrosin (*Acr*) promoter and secretory signal sequence [22]. Tg(Acr-Egfp) females were crossed with $Zp3r^{-/-}$ males. $Zp3r^{+/-}$ and Tg(Acr-Egfp) double-transgenic F1 offspring were intercrossed to generate $Zp3r^{+/+}$,Tg(Acr-Egfp) as well as $Zp3r^{+/-}$,Tg(Acr-Egfp) and $Zp3r^{-/-}$, Tg(Acr-Egfp). Sperm from these mice were assayed as described previously [18]. Briefly, sperm were squeezed out from the incisions made in caudae epididymides or vasa deferentia and were suspended and incubated in TYH medium for 180 min. Then, calcium ionophore A23187 (final concentration, 1 μM) was added, and the cells were incubated for an additional 15 min. Cells were then washed with PBS and observed under the fluorescent microscope for the presence of soluble green flourescent protein in their acrosomes. The status of individual sperm acrosomes was determined from the fluorescence detected by flow cytometry at 0, 30, 60, 120, and 180 min after insemination. The viable sperm were selected by staining with propidium iodide (final concentration, 10 μg/ml), and their acrosomal integrity was determined by the presence of acrosomal EGFP as indicated earlier. Additionally, sperm motilities were observed and videotaped after 15 min and 2 h of incubation.

Statistical Analysis

Results are expressed as the mean ± SD. Statistical differences between groups were evaluated by one-way ANOVA followed by Bonferroni multiple-comparison test. All tests were two-tailed, with statistical significance assessed at the $P < 0.05$ level. Statistical analyses were performed using the Prism 4.0 program (GraphPad Software).

RESULTS

Targeted Disruption of the Zp3r *Gene*

The *Zp3r* gene was disrupted by homologous recombination as described in Figure 1. The targeting vector replaced exon 4 with a *neo* cassette in the mutated allele, resulting in the complete disruption of the *Zp3r* gene. This replacement altered the 1327 bp of the *Zp3r* sequence, resulting in a frame shift that creates an early termination codon, which is predicted to cause nonsense-mediated decay of the *Zp3r* mRNA. Genotyping with the primer sets depicted in Figure 1A detected a band of 1141 bp in wild-type DNA and 1396 bp in nullizygous mouse DNA; as expected, heterozygous mice displayed approximately equal amounts of both bands. RT-PCR confirmed that the expression of *Zp3r* was restricted to the testis (Fig. 1C).

Comparison of Testes and Sperm from Wild-Type and Mutated Mice

To assess the effect of the mutation on males, we examined their testes and sperm. As shown in Figure 2A, the weights of testes from $Zp3r^{+/-}$ and $Zp3r^{-/-}$ mice were comparable to those of wild-type males. However, the level of *Zp3r* mRNA was lower in heterozygous animals and the expression of the $Zp3r^{-}$ allele was dramatically decreased in nullizygous animals (Fig. 2B). The slightly smaller band detected reflects the predicted alteration in the size of mRNA produced from the mutated allele. Most importantly, we were unable to detect ZP3R protein by immunoblotting and indirect immunofluorescence using specific anti-ZP3R antibodies (Fig. 2, C and D). Morphologically, sperm from $Zp3r^{-/-}$ males looked normal (Fig. 2D) and displayed a normal forward motility (Fig. 2, E–H, and Supplemental Movies 1–4 available at www.biolreprod.org). Similarly, no differences were observed in the number of sperm obtained from $Zp3r^{-/-}$ mice compared with those from wild-type mice (not shown).

Fertilizing Ability of ZP3R-Deficient Mice

Adult males of the three genotypes were bred with wild-type females for 3 mo. Neither $Zp3r^{+/-}$ nor $Zp3r^{-/-}$ males showed statistically significantly fewer offspring than wild-type males (Table 1). Next, we examined the fertilization in vitro using cumulus-intact or cumulus-free, metaphase II-arrested eggs (Fig. 3). In both conditions, sperm from $Zp3r^{+/-}$ nor $Zp3r^{-/-}$ males showed normal fertilization rates compared with wild-type males. We also examined the ability of sperm from $Zp3r^{+/-}$ or $Zp3r^{-/-}$ males to bind to ZP of mature eggs. The number of bound sperm in the three conditions did not show statistically significant differences (Fig. 4).

ZP3R-Deficient Mice Undergo Acrosomal Exocytosis Normally

Acrosomal exocytosis is a critical step in the mammalian fertilization process. We examined the ability of ZP3R-

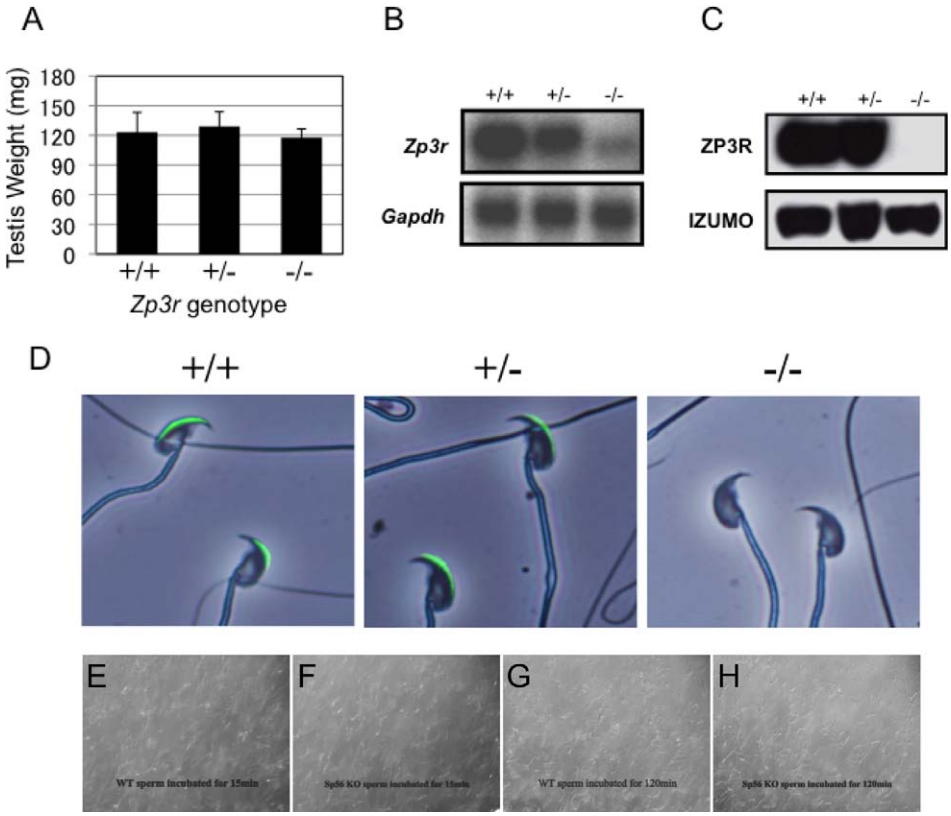

FIG. 2. Expression of ZP3R in wild-type, heterozygous, and nullizygous male mice. **A**) Testis weight (n = 8) and sperm count and motility. **B**) Northern blot analysis of *Zp3r* mRNA from testis of wild-type and *Zp3r*-null mice. **C**) Immunoblotting analysis of ZP3R protein in wild-type and *Zp3r*-null mice. **D**) Indirect immunofluorescence analysis of ZP3R protein in wild-type versus targeted-deletion mice. Still frames from Supplemental Movies 1–4 illustrating sperm motility from $Zp3r^{+/+}$ and $Zp3r^{-/-}$ males after the incubation under capacitating conditions for 15 min (**E** and **F**, respectively [Movies 1 and 2]) and 120 min (**G** and **H**, respectively [Movies 3 and 4]) are shown. Original magnification ×400 (**D**) and ×40 (**E**–**H**).

deficient sperm to undergo acrosomal exocytosis when stimulated with calcium ionophore A23187. The loss of the soluble acrosomal contents was assessed by direct fluorescence of the soluble acrosomal EGFP using the transgenic mouse line with enhanced green fluorescent protein in the sperm acrosome (ACR-EGFP) crossed to $Zp3r^{-/-}$ males [22]. Sperm from *Zp3r*-deficient and $Zp3r^{+/-}$ mice underwent acrosomal exocytosis normally (Fig. 5). Also, they did not exhibit differences in the percentages of spontaneous acrosomal exocytosis at different time points of the incubation in capacitating conditions.

DISCUSSION

Sperm-ZP binding is a fundamental step of gamete interaction in many species. Mutant sperm that fail to bind to the ZP cannot penetrate this structure, thus precluding them from reaching the plasma membrane of the egg [23]. Studies of the molecular mechanisms of sperm-ZP binding have strongly implicated ZP3, one of the three sulfated glycoproteins that comprise the ZP, as the primary ligand in the mouse ZP (for review, see Wassarman [3]). The physicochemical nature of the ligand moiety is still unresolved. Strong evidence exists that a carbohydrate structure of ZP3 is bound by the sperm, but other studies suggest that the true nature of binding may be quite complex [2, 24–26].

Many studies to resolve this issue in the mouse have focused on identifying the sperm protein(s) with the ability to bind ZP3. Several ZP-binding proteins of sperm have been identified, but the most widely studied ZP3-binding protein has been ZP3R [9, 10, 12, 13, 15, 27, 28]. ZP3R was initially identified (as sp56) by cross-linking ZP3 to mouse sperm using the [^{125}I]Denny-Jaffe reagent [10]. Subsequently, the protein was purified by affinity chromatography on a ZP3 column and its cDNA cloned and characterized [27]. Following these original studies, ZP3R was demonstrated to be an intra-acrosomal protein and a component of the acrosomal matrix [12]. Its monomeric molecular weight is 67 000, and the native protein is found in a disulfide-bonded homomeric complex, likely composed of six to eight monomers [28]. Based on these properties, we felt that ZP3R would likely form a multivalent, ZP3-binding protein. As such, deletion of the protein from the sperm acrosomal matrix was predicted to result in a modification of acrosomal function and potential disruption of sperm-ZP binding.

Our study was designed to delete exon 4 of the *Zp3r* gene and cause a frame-shift mutation that resulted in an early termination codon, leading to the absence of the protein in nullizygous mouse sperm. However, the mutation did not cause infertility in male mice carrying two copies of the affected

TABLE 1. Breeding success as assessed by the number of pups and litter size.

Genotype		Total no. of females	Total no. of offspring	Mean litter size
Male	Female			
+/–	+/+	11	95	8.6 ± 1.6
–/–	+/+	10	94	9.4 ± 0.7

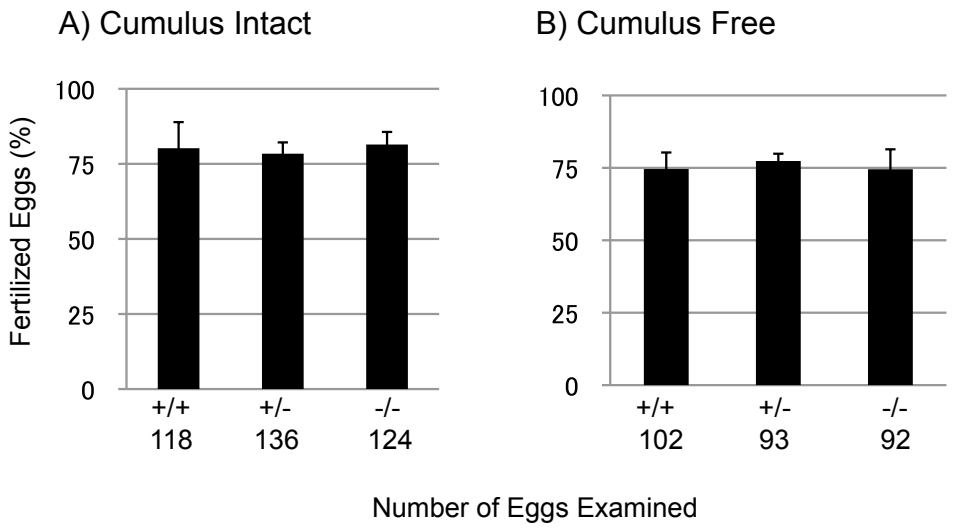

FIG. 3. Fertilizing capacity of ZP3R-deficient sperm. Sperm from $Zp3r^{+/+}$, $Zp3r^{+/-}$, and $Zp3r^{-/-}$ were used to inseminate cumulus-intact (**A**) or cumulus-free (**B**), metaphase II-arrested eggs using in vitro fertilization. Results are expressed as the mean ± SD of four independent experiments. No statistical differences were observed between groups ($P > 0.05$).

allele. That ablation of a protein involved in sperm-ZP binding does not create an obvious effect on fertility is not new. Other acrosomal matrix proteins with ZP-binding properties, such as proacrosin and zonadhesin, have also been deleted by homologous recombination, and the resulting mutant mice have been completely fertile in vivo and in vitro [4, 8]. In addition, sperm surface proteins, such as β-1,4-galactosyltransferase, have also been eliminated by gene targeting, with nullizygous males retaining their fertility [5]. MFGE8, a member of a small group of secreted cell-matrix adhesive proteins that contain Notch-like EGF repeats and discoidin/F5/8 type C domains, localizes on the sperm plasma membrane overlying the acrosome and binds specifically to the ZP of unfertilized oocytes but not to the ZP of fertilized eggs [6]. MFGE8-null males are subfertile, and their sperm are unable to bind to the egg coat in vitro [6]. These findings suggest a functional redundancy in the ZP-binding steps. From an evolutionary point of view, this feature is consistent with the conservation of any given species. In this way, a point mutation of one of the ZP-binding proteins would not eliminate the fertilizing capacity of the affected individual. However, we cannot rule out the possibility that this is not the case, because further evidence that sperm-ZP binding is a functionally redundant process needs to be presented. For example, the combination of multiple mice with targeted deletion of proteins

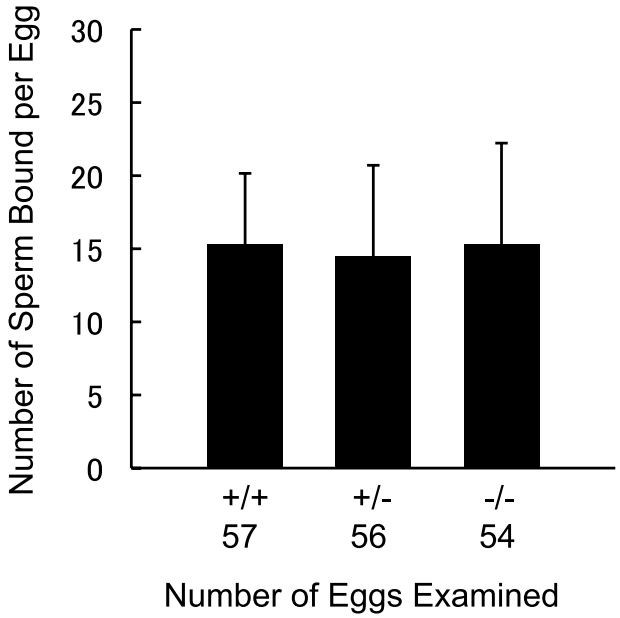

FIG. 4. Binding of wild-type, heterozygous, and nullizygous mouse sperm to ZP. Mouse sperm from $Zp3r^{+/+}$, $Zp3r^{+/-}$, and $Zp3r^{-/-}$ males were incubated with metaphase II-arrested eggs for 30 min to promote adhesion to the ZP. Four independent experiments with more than 200 oocytes were scored per antibody. No statistical differences were observed between groups ($P > 0.05$).

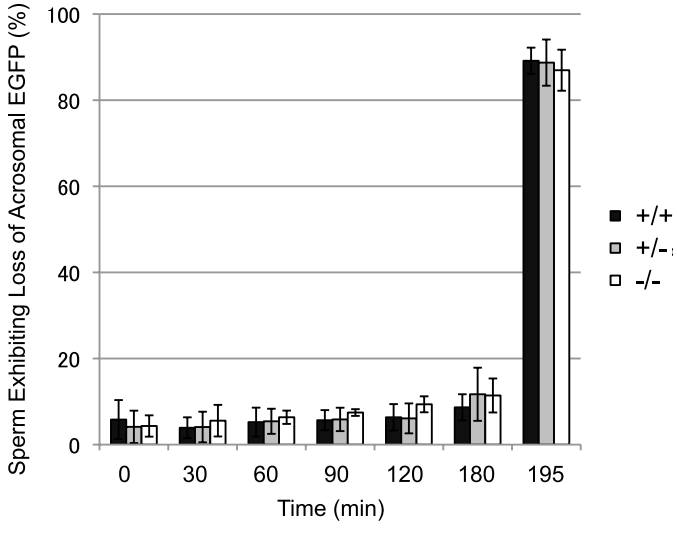

FIG. 5. Acrosomal exocytosis in ZP3R-deficient sperm. Mouse sperm from $Zp3r^{+/+}$, $Zp3r^{+/-}$, and $Zp3r^{-/-}$ males were incubated under capacitating conditions for 180 min. Then, calcium ionophore A23187 (final concentration, 1 μM) was added, and the cells were incubated for an additional 15 min. Next, cells were washed with PBS and observed under the fluorescence microscope for the presence of soluble green fluorescent protein in their acrosomes. Results are expressed as the mean ± SD of four independent experiments. No statistical differences were observed between groups ($P > 0.05$).

that resulted in ''fertile'' phenotypes could address this possibility.

Although the loss of one of the several ZP-binding proteins may not result in a case of sterility or even subfertility, there still may be a subtle phenotype that could place an affected individual at a competitive disadvantage. Despite the deletion of a gene encoding a ZP-binding protein not having an impact on fertility measured experimentally, cases are known in which mutated animals with gene deletions are not completely normal when examined more closely. For example, proacrosin-deficient mice display a delay of almost 30 min in both ZP penetration and fertilization compared with wild-type and heterozygous counterparts [4]. Zonadhesin-deficient mice exhibit increased adhesion of mouse spermatozoa to pig, cow, and rabbit ZP but not to mouse ZP [8]. Also, β-1,4-galactosyltransferase-deficient sperm experience a lower rate of acrosomal exocytosis in response to ZP proteins [5], whereas mouse sperm deficient in inner acrosomal membrane protein CD46 (membrane cofactor protein) exhibit a more rapid rate of acrosomal exocytosis [29]. These differences may not be important to animal husbandry in a controlled laboratory setting, but they could have profound effects in the wild, where sperm competition and species specificity may have more significant roles.

With these considerations, it remains possible that ZP3R plays an important role in the fertilization process. Although it was beyond the scope of the present study to examine rates of acrosomal exocytosis, the sperm-ZP binding process, and ZP penetration, absence of the multivalent, multimeric ZP3R could affect these processes in some yet-to-be-identified manner. We have previously demonstrated that ZP3R is processed to a lower molecular weight coincident with its loss from the sperm during the course of acrosomal exocytosis [15]. If the ZP3R multimer is part of a multiprotein complex, it is possible that loss of ZP3R expression could impact the release of other acrosomal proteins during fertilization. These concepts—and the evolving paradigm that the mouse sperm initiates acrosomal exocytosis before binding to the ZP of cumulus-enclosed eggs [30]—must be incorporated into considerations of the role of ZP3R in fertilization. It is evident that recreating the tubal environment where sperm-ZP interactions take place in natural matings is difficult. Because most of the biochemical and mechanistic studies of sperm-ZP interactions in mammals have employed cumulus-free oocytes, future studies should examine sperm exposed to the full effects of cumulus cells and their associated extracellular milieu, including the complex fluid bathing the oocyte-cumulus mass in vivo.

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