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Osaka University
Sperm equatorial segment protein 1, SPESP1, is required for fully fertile sperm in mouse

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Summary

Mammalian fertilization is a multistep process that culminates in the fusion of the sperm and egg plasma membrane. It is widely accepted that the equatorial segment of the acrosome-reacted sperm is important in initiating fusion with the egg plasma membrane during fertilization. There are various proteins known to be distributed only in the equatorial segment of sperm. The role of these proteins must be clarified to understand the membrane fusion process. We produced a mouse line that lacked SPESP1 (sperm equatorial segment protein 1) and analyzed the fertilizing ability of the sperm. The average number of pups that were fathered by Spesp1+/+ and Spesp1+/− males was significantly lower than that of wild-type fathers. In these mouse lines, fewer sperm were found to migrate into oviducts and fewer eggs were fertilized. The Spesp1+/+ and Spesp1−/− sperm showed a lower fusing ability compared with the wild-type sperm. The disruption of Spesp1 was shown to cause an aberrant distribution of various sperm proteins. Moreover, scanning electron microscopy revealed that the membrane in the equatorial segment area, which usually forms an acrosomal sheath, disappears after acrosome reaction in Spesp1-deficient mice. It was demonstrated that SPESP1 is necessary to produce the fully "fusion competent" sperm.

Key words: Equatorial segment, Fertilization, Knockout mouse, Sperm-egg fusion

Introduction

After meiosis, terminally differentiated gametes are no longer able to divide and need to fuse with gametes from the other sex to survive and continue the species. Thus, cell-cell fusion is a very important process in species that adopt sexual reproduction. However, until recently, the precise mechanism of sperm-egg fusion has remained unclear. At the molecular level, the only factors that have been proved to be essential are IZUMO1 on the sperm (Inoue et al., 2005) and CD9 on the egg (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000). Electron microscopy indicated that the equatorial segment is the place where fusion initiates in mammals (Yanagimachi, 1994). This suggests that the fusion protein has to be localized in the equatorial segment. However, the fusion protein should not be available in the equatorial segment of fresh sperm because sperm are not able to fuse with eggs before acrosome reaction. IZUMO1 is not expressed on the sperm surface, and appears on the sperm surface only after acrosome reaction. However, it localizes not only in the equatorial segment, but also spreads into the post-acrosomal region (Kawai et al., 1989). Therefore, the localization of IZUMO1 does not perfectly match the fusion competent area observed using electron microscopy. This led us to speculate that there might be a protein that localizes only to the equatorial segment and thereby facilitates fusion.

In search of an equatorial segment-localized protein, SPESP1 (sperm equatorial segment protein 1) (Wolkowicz et al., 2003) caught our attention. It was reported that the anti-SPESP1 antibody inhibited sperm-egg fusion in the human sperm-hamster egg system (Wolkowicz et al., 2008) and also in the mouse in vitro fertilization system (Lv et al., 2009). In the work reported here, we produced, by homologous recombination, a mouse line that lacks Spesp1 and we analyzed the fertilizing ability of their sperm. Here we describe a haplo-insufficient phenotype of Spesp1-disrupted sperm, probably resulting from the aberrant localization of membrane proteins, accompanied by a destabilization of the equatorial segment membrane.

Results

Expression and localization of mouse SPESP1

Examination of a number of different mouse tissues by RT-PCR showed that Spesp1 was expressed specifically in testis (Fig. 1A). Haplotype-specific expression was confirmed by the appearance of SPESP1 in tissue from mice aged between 2 and 3 weeks (faint and clear bands, respectively in supplementary material Fig. S1). The onset of Spesp1 expression occurred along with the expression of the gene for the putative fusion protein IZUMO1. SPESP1 was detected by immunostaining over a range of developmental stages, from round spermatid to cauda epididymal sperm. The localization was noticeably different from that of IZUMO1 (Fig. 1B,C). Since SPESP1 resides in the acrosomal vesicle, the SPESP1 antigen became reactive to the antibody after membrane permeabilization. When sperm underwent the acrosome reaction (estimated by the spreading of IZUMO1 over the entire head), the SPESP1 protein was no longer detectable in the equatorial segment (Fig. 1C). When SPESP1 was extracted with Triton X-114 and subjected to a phase separation, the protein was distributed in the detergent-depleted phase, indicating that SPESP1 is a soluble protein (Fig. 1D). By contrast, the transmembrane protein IZUMO1 was distributed in the detergent-enriched phase.

Fertilizing ability of Spesp1-deficient mice

The Spesp1 gene was disrupted by homologous recombination as described in supplementary material Fig. S2. Adult males of the...
Interestingly, sperm from Spesp1–/– males showed a delayed onset of fertilization ratio against the time after coitus (Fig. 2A, Spesp1+/–: 90.9±5.1%, Spesp1–/–: 90.9±5.1%, P<0.005). Moreover, when we examined the fusing ability using zona-free eggs, sperm from Spesp1–/– males had significantly less fusion ability compared with wild-type males (Fig. 2B: Spesp1+/–: 44.1±2.8%, Spesp1–/–: 35.1±6.5%, P<0.05; Student t-test). Values in A and B are means ± standard deviations. (C) The sperm from Spesp1–/– deficient males, which migrated into the oviduct, were visualized using GFP-tagged acrosomes and RFP-tagged mitochondria ‘RBGS sperm’ (Hasuwa et al., 2010) 8 hours after of coitus. The number of sperm in oviducts of females mated with Spesp1–/– deficient males was significantly less compared to that of wild-type males.

In vitro sperm analysis in Spesp1-deficient mice

There was no difference observed in the number, motility and swimming patterns of sperm obtained from Spesp1-disrupted mice compared with those of wild-type mice, as shown in the supplementary material Movies 1 and 2. When added to the eggs, we found no defect in zona binding ability in Spesp1-disrupted mouse sperm (supplementary material Fig. S4). However, both Spesp1+/+ and Spesp1–/– sperm were less fertile in the in vitro fertilization system compared with wild-type sperm (Fig. 3A: Spesp1+/+: 37.2±24.5%, Spesp1–/–: 36.1±17.5%, *significantly different from wild-type, P<0.005). Moreover, when we examined the fusing ability using zona-free eggs, sperm from both Spesp1+/+ and Spesp1–/– had significantly less fusion ability compared with wild-type sperm (Fig. 3B: Spesp1+/+: 4.2±0.4, Spesp1–/–: 3.5±0.4, *significantly different from wild-type, P<0.05). Three genotypes were bred with wild-type females for 3 months. Although Spesp1+/+ females show normal fertility, both Spesp1+/+ and Spesp1–/– males had statistically significant fewer offspring than wild-type males (Fig. 3C, n=162): Spesp1+/+: 94.8±6.0%, Spesp1–/–: 89.8±6.0%, P=0.05). Values in A and B are means ± standard deviations. (C) The sperm from Spesp1–/– deficient males, which migrated into the oviduct, were visualized using GFP-tagged acrosomes and RFP-tagged mitochondria ‘RBGS sperm’ (Hasuwa et al., 2010) 8 hours after of coitus. The number of sperm in oviducts of females mated with Spesp1–/– deficient males was significantly less compared to that of wild-type males.

In vivo sperm analysis in Spesp1-deficient mice

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Proteins were extracted from wild-type, Spesp1+/– and Spesp1–/– animals with 1% Triton X-114 and analyzed by immunoblot using antibodies to IZUMO1, MN9 antigen, ADAM2, ADAM3, basigin, MC101 and SPESP1. The most prominent difference in the three genotypes was found in MN9 antigen, while basigin and SPESP1 showed no difference. The localization pattern of MN9 antigen was significantly affected by the disruption of SPESP1. Normally, MN9 antigen localizes to the acrosomal cap area and spreads to the equatorial segment after acrosome reaction, but in Spesp1 disrupted sperm, MN9 antigen showed an acrosome-reacted pattern before acrosome reaction.

Electron microscopy of Spesp1-deficient sperm
The offspring of Spesp1–/– mice crossed with a transgenic mouse line producing sperm with GFP in the acrosome was subjected to fluorescence-activated cell sorting (supplementary material Fig. S5) to separate acrosome-intact and acrosome-reacted sperm, followed by fixation and observation by scanning electron microscopy (SEM). The acrosome-intact, Spesp1+/– sperm were morphologically similar to wild-type sperm (Fig. 4A,B). However, when acrosome-reacted sperm were examined, it was found that in almost all of the sperm, the equatorial segment membrane was completely lost in Spesp1 null sperm (Fig. 4C,D). The electron microscopy results demonstrated that SPESP1 is required for the correct architecture of the equatorial segment.

Discussion
We re-confirmed that the SPESP1 protein was localized in the equatorial segment of mouse sperm, using a newly available anti-SPESP1 peptide antibody (Fig. 1). No nonspecific reactivity was observed with our antibody because the immunostaining band in western blotting and the immunostaining of sperm was completely absent in Spesp1+/– sperm (supplementary material Fig. S2E). However, unlike the findings from previous studies (Lv et al., 2009; Wolkowicz et al., 2008), SPESP1 disappeared from sperm when acrosome-reacted sperm were examined, it was found that in almost all of the sperm, the equatorial segment membrane was completely lost in Spesp1 null sperm (Fig. 4C,D). The electron microscopy results demonstrated that SPESP1 is required for the correct architecture of the equatorial segment.

Analysis of membrane proteins from Spesp1-deficient sperm
The extraction of proteins using Triton X-114 and subsequent phase separation divides proteins into either a detergent-depleted or detergent-enriched phase, depending on their conformation (Bordier, 1981). An immunoglobulin superfamily protein basigin (BSG) (Igakura et al., 1998) was found exclusively in the detergent-enriched phase, and the amount was the same in Spesp1+/– and wild-type sperm (Fig. 3C). By contrast, the soluble acrosomal protein MC101 (Toshimori et al., 1995) was lower in Spesp1–/– sperm. ADAM family proteins implicated in sperm–zona binding were also affected by the disruption of Spesp1 (less in the detergent-depleted phase). The amount of IZUMO1 (Inoue et al., 2005) in Spesp1+/– sperm was the same as in wild-type sperm (supplementary material Fig. S2E). Another potential candidate for a fusion protein, MN9 antigen (Toshimori et al., 1992), was much higher in the detergent-depleted phase from Spesp1–/– and Spesp1+/– sperm (Fig. 3C). As a whole, it was found that the disruption of the equatorial segment protein SPESP1 affected the amount and distribution of some proteins specifically.

Concerning the localization of IZUMO1, it was found that IZUMO1 spread in a punctate manner in many of the Spesp1+/– sperm (Fig. 3D). The most evident difference was in the staining of MN9 antigen. The normal characteristic pattern of MN9 antigen is to migrate to the plasma membrane covering the equatorial segment after acrosome reaction (Toshimori et al., 1992). However, in Spesp1–/– sperm, MN9 antigen was found in the equatorial segment before acrosome reaction (Fig. 3E).
that the migration was not impaired by a decrease in number of ejaculated sperm or by decreased sperm motility. A sufficient number of sperm were observed in the uterus of animals mated with Spesp1-disrupted males, and the epididymal sperm suspension obtained from Spesp1-disrupted mice showed no difference in concentration and in motility (supplementary material Movies 1 and 2). There are at least five genes known to affect sperm migration into the oviduct and all are reported to relate to each other, resulting in sperm with aberrant ADAM3 behavior (Cho et al., 1998; Ikawa et al., 1997; Krege et al., 1995; Nishimura et al., 2004; Shamsadin et al., 1999; Yamagata et al., 2002; Yamaguchi et al., 2009; Yamaguchi et al., 2006). Therefore, various sperm antigens, including ADAM3, have been examined in Spesp1-disrupted sperm. Although a slight difference was observed in ADAM3 from Spesp1 disrupted sperm, it was not clear if this difference caused the impaired sperm migration into oviduct. However, we found a significant increase in the amount of MN9 antigen (Toshimori et al., 1998), which has been reported to function in sperm-egg fusion and in motility (supplementary material Movies 1 and 2). RT-PCR

Mouse cDNA was prepared from various tissues of adult ICR, and testes from 1- to 5-week-old ICR males. RT-PCR was performed using 10 ng of cDNA and the following forward and reverse primers: 5'-GCTTCCTCGTGTAAGTGCTAA-3' and 5'-TGTAAGGGCCAGTCTTCACAG-3' for Spesp1, 5'-AAGTGACGTTGAGC-ATCCG-3' and 5'-GATCCACGCTGTAAG-3' for the β-actin gene. 

Immunofluorescent staining

Immunofluorescent staining was performed as described previously (Kotaja et al., 2004) for round spermatid, and for epididymal sperm (Yamaguchi et al., 2006). Mouse monoclonal antibodies MN9 and MC101, were gifts from Dr Kiyotaka Toshimori (Chiba University, Chiba, Japan). Monoclonal antibodies against mouse ADAM2 (fertilin- b; 9D2.1) and ADAM3 (cyritestin; 7C1.2) were purchased from Chemicon International Inc. (Temecula, CA). Immunoglobulin superfamily protein basigin (CD147) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Antibodies

Rabbit anti-mouse SPESP1 polyclonal antisera was produced by immunization with mouse SPESP1 polyprotein (MYGVSDEVAGRTSD). Anti-IZumo1 monoclonal antibody (mAb125) was previously generated in our laboratory according to the standard method (Yamaguchi et al., 2006). Mouse monoclonal antibodies MN9 and MC101, were gifts from Dr Kiyotaka Toshimori (Chiba University, Chiba, Japan). Monoclonal antibodies against mouse ADAM2 (fertilin-b; 9D2.1) and ADAM3 (cyritestin; 7C1.2) were purchased from Chemicon International Inc. (Temecula, CA). Immunoglobulin superfamily protein basigin (CD147) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

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As shown in the Results, the ability of Spesp1+/− sperm to fuse with eggs was diminished to less than half that of wild-type sperm (Fig. 3B). This might be caused by a combination of downstream effects on various sperm proteins. However, the infertility caused by the Spesp1 disruption may derive not only from the aberrant placement of membrane proteins (Fig. 3C) but also by the altered physical nature of the sperm membrane, demonstrated by the loss of equatorial membrane after acrosome reaction using SEM in Spesp1-disrupted sperm (Fig. 4). This drastic change was observed in almost all of the Spesp1 KO sperm examined. How SPESP1 maintains the integrity of the equatorial segment membrane will have to be clarified in the future.

As the past gene disruption research history indicates, it is very difficult to predict the role of certain factors in fertilization (Okabe and Cummins, 2007); however, defects of more than 100 genes are known to cause infertility (http://www.informatics.jax.org/searches/Phat.cgi?id=MP:0001925;top). This indicates that the best way to elucidate the mechanism of fertilization is to accumulate information on genes that relate to infertility by gene disruption experiments. In this context, the relationships between sperm proteins that we have clarified in the Spesp1−/− mouse are valuable for understanding the fertilization process. In turn, the information described here will assist the development of new treatments for infertility and/or new methods for contraception in the future.

Materials and Methods

RT-PCR

Mouse cDNA was prepared from various tissues of adult ICR, and testes from 1- to 5-week-old ICR males. RT-PCR was performed using 10 ng of cDNA and the following forward and reverse primers: 5'-GCTTCCTCGTGTAAGTGCTAA-3' and 5'-TGTAAGGGCCAGTCTTCACAG-3' for Spesp1, 5'-AAGTGACGTTGAGC-ATCCG-3' and 5'-GATCCACGCTGTAAG-3' for the β-actin gene.

Phase separation of Triton X-114 extracts of sperm

Phase separation of Triton X-114 extracts of sperm were performed as described previously (Kotaja et al., 2004) for round spermatid, and for epididymal sperm (Yamaguchi et al., 2006). The amplification conditions were 1 minute at 96°C, followed by 36 cycles for the experiments shown in Fig. 1A and 27 cycles for the experiments shown in supplementary material Fig. S1. Cycles were 96°C for 15 seconds, 65°C for 30 seconds, and 72°C for 30 seconds, with a final 1-minute extension at 72°C.

Antibodies

Rabbit anti-mouse SPESP1 polyclonal antisera was produced by immunization with mouse SPESP1 polyprotein (MYGVSDEVAGRTSD). Anti-IZumo1 monoclonal antibody (mAb125) was previously generated in our laboratory according to the standard method (Yamaguchi et al., 2006). Mouse monoclonal antibodies MN9 and MC101, were gifts from Dr Kiyotaka Toshimori (Chiba University, Chiba, Japan). Monoclonal antibodies against mouse ADAM2 (fertilin-b; 9D2.1) and ADAM3 (cyritestin; 7C1.2) were purchased from Chemicon International Inc. (Temecula, CA). Immunoglobulin superfamily protein basigin (CD147) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

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Construction of the Spesp1 gene disruption vector

A targeting vector was constructed using pNT1.1 containing the Neo-resistance gene (Neo) as a positive selection marker and a herpes simplex virus thymidine kinase as a negative selection marker. A 0.9-kb Pmel-NotI fragment as a short arm and a
6.1-kb 

Fragment as a long arm were obtained by PCR using genomic DNA in D3 ethylenimine (ES) cells as a template. The PCR-amplified were as follows: 5'-CCCTTTAAAACCCGGTGACCGTGTCCTAGTTGAGG-3' and 5'-CCCGGCGGCGCTATGCTGTTATCCCATGACGAGAAG-3' for the short arm: 5'-CCCGGCGGCGCAATGTTACCTGGATGTCGGGACT-3' and 5'-CCCCCCAGTTAGAAACGCGGCTCGTACCACTTCTGGTTT-3' for the long arm. These two fragments were inserted into a pNT1 vector and the targeting construct was linearized with Pmel digestion. ES cells were electroporated and colonies were screened.

**Generation of Spesp1-disrupted mice**

The mouse Spesp1 gene consists of two exons and maps to chromosome 9. The targeting vector was designed to remove a part of the second exon of the Spesp1 gene (supplementary material Fig. S2A) and was electroporated into D3 ES cells after linearization. Potentially targeted ES cell clones were separated by positive-negative selection with G418 and ganciclovir. Correct targeting of the Spesp1 allele in ES cell clones was determined by PCR and Southern blot analysis for homologous recombination at both ends (supplementary material Fig. S2B,C). Mating between heterozygous mutant mice yielded the expected mendelian ratios: Spesp1+/−, 24.6%; Spesp1−/−, 50.8%; Spesp1+/-: 24.6% of offspring (n=65). Northern and western blot analysis showed that Spesp1 mRNA expression was undetectable in the Spesp1−/− testis (supplementary material Fig. S2D). The Spesp1 protein was also not detected in sperm from the cauda epididymis and was deficient in Spesp1−/− mice (supplementary material Fig. S2E). Spesp1−/− mice were healthy and showed no overt developmental abnormalities. Mice used in this study were of B6; 129 mixed background and male mice of three genotypes, and Spesp1−/− littermates were used as controls.

**Southern and northern blot and PCR analysis**

Scul-digested and AffII-digested genomic DNA (20 μg) were electrophoresed though a 0.8% agarose gel, transferred to a Hybond N+ membrane (Amersham Bioscience), and hybridized with the 32P-labeled 5′- and 3′- probes. These probes amplified by the primers used were as follows: 5′-CTCTGAGAAGAGAACTTCTTG-3′ and 5′-ATCCAAAGTGAGTTGATGACCGG-3′ for 5′- probe; 5′-GAGCCTAGAAGACGAATGCAG-3′ and 5′-GCTTCGATAGAACGACCTTGCTG-3′ for 3′- probe.

Northern hybridization was performed using 10 μg of total RNA extracted from various tissues of adult ICR mice. RNAs were separated by electrophoresis on a 0.8% agarose gel, transferred to a Hybond N+ membrane (Amersham Bioscience), and hybridized with the 32P-labeled 5′- and 3′- probes. These probes amplified by the primers used were as follows: 5′-CCCTTTAAAACCCGGTGACCGTGTCCTAGTTGAGG-3′ and 5′-CCCGGCGGCGCTATGCTGTTATCCCATGACGAGAAG-3′ for the short arm: 5′-CCCGGCGGCGCAATGTTACCTGGATGTCGGGACT-3′ and 5′-CCCCCCAGTTAGAAACGCGGCTCGTACCACTTCTGGTTT-3′ for the long arm.

**References**


Fig. S1. Spesp1 gene expression pattern during spermatogenesis. RT-PCR was performed using 10 ng of total RNA extracted from testes from 1- to 5-week-old ICR males. Scp3 was used as a marker for the meiotic spermatocyte stage, and Prm1 was used as a marker for the spermatid stage.
Fig. S2. Generation of Spesp1 knockout mice.
Strategy of Spesp1 gene disruption. (A) Confirmation of Spesp1 knockout mice. (B-E) PCR analysis detected the mutant allele of Spesp1 knockout mice (B). Southern blot results for wild-type and mutant alleles of the Spesp1 gene (C). Northern blot analysis of total testis RNA confirmed the absence of gene-specific transcripts in KO testis (D). Western blot analysis also confirmed the absence of SPESP1 proteins in KO sperm (E).
Fig. S4 (Y. Fujihara et al.)

**Fig. S4.** A histogram of sperm GFP expression from Spesp1-deficient bearing Acr-EGFP transgenic mice. In the peaks on the left, acrosome-reacted (GFP negative) sperm were distributed, while acrosome-intact sperm were contained in peaks on the right.
Fig. S3. Observation of oviducts from females mated with wild-type and Spesp1 -/- males with mitochondria tagged RFP 8 hrs after coitus. Red fluorescent illumination indicate the existence of sperm within the uterus and the oviductal isthmus. When Spesp1 -/- males were mated, there were significantly lower number of migrated sperm into the oviductal isthmus compared to wild-type males.
**Fig. S4.** Sperm-ZP binding assay in *Spesp1*-deficient sperm. *Spesp1*-deficient sperm showed a normal ability to bind to the zona pellucida compared to wild-type sperm (A). A sperm-ZP binding assay was performed as described in (Yamaguchi et al., 2006)
Fig. S5 (Y. Fujihara et al.)

**Fig. S5.** A histogram of sperm GFP expression from Spesp1-deficient bearing Acr-EGFP transgenic mice. In the peaks on the left, acrosome-reacted (GFP negative) sperm were distributed, while acrosome-intact sperm were contained in peaks on the right.