Mice expressing aberrant sperm-specific protein PMIS2 produce normal-looking but fertilization-incompetent spermatozoa

Ryo Yamaguchi\textsuperscript{a,b}, Yoshitaka Fujihara\textsuperscript{a}, Masahito Ikawa\textsuperscript{a}, and Masaru Okabe\textsuperscript{a,b}
\textsuperscript{a}Research Institute for Microbial Diseases and \textsuperscript{b}Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan

\section*{ABSTRACT}
Eight kinds of gene-disrupted mice (Clgn, Calr3, Pdilt, Tpst2, Ace, Adam1a, Adam2, and Adam3) show impaired sperm transition into the oviducts and defective sperm binding to the zona pellucida. All of these knockout strains are reported to lack or show aberrant expression of a disintegrin and metallopeptidase domain 3 (ADAM3) on the sperm membrane. We performed proteomic analyses of the proteins of these infertile spermatozoa to clarify whether the abnormal function is caused exclusively by a deficiency in ADAM3 expression. Two proteins, named PMIS1 and PMIS2, were missing in spermatozoa from Clgn-deficient mice. To study their roles, we generated two gene-disrupted mouse lines. Pmis1-knockout mice were fertile, but Pmis2-knockout males were sterile because of a failure of sperm transport into the oviducts. Pmis2-deficient spermatozoa also failed to bind to the zona pellucida. However, they showed normal fertilizing ability when eggs surrounded with cumulus cells were used for in vitro fertilization. Further analysis revealed that these spermatozoa lacked the ADAM3 protein, but the amount of PMIS2 was also severely reduced in Adam3-deficient spermatozoa. These results suggest that PMIS2 might function both as the ultimate factor regulating sperm transport into the oviducts and in modulating sperm-zona binding.

\section*{INTRODUCTION}
The mammalian fertilization process starts with the deposition of spermatozoa into the female reproductive tract (in the mouse, directly into the uterus, and in the human, into the vagina). The spermatozoa are then required to migrate to the oviduct, where the ovulated eggs and spermatozoa meet (Suarez, 2008). Gene disruption experiments have been used to produce at least eight lines of specific gene-disrupted mouse lines with knockouts for Clgn (Ikawa et al., 1997), Calr3 (Ikawa et al., 2011), Pdilt (Tokuhiro et al., 2012), Tpst2 (Marcello et al., 2011), Ace (Krege et al., 1995), Adam1a (Nishimura et al., 2004), Adam2 (Cho et al., 1998), and Adam3 (Shamsadin et al., 1999). In these mice, the spermatozoa show impaired migration into the oviduct, whereas their motility remains unaffected. The spermatozoa from all of these gene-knockout (KO) mouse lines were shown to have lost their zona-binding ability when mixed with cumulus-free eggs in vitro. The mechanisms were not clarified, but as reported for Adam1a, Adam3, and Pdilt KO lines (Nishimura et al., 2004; Tokuhiro et al., 2012), these mutant spermatozoa could fertilize cumulus-surrounded eggs. This indicates that the primary cause for infertility in these mouse strains is not a defect in sperm–zona binding but the inability of spermatozoa to migrate from the uterus into the oviduct.

Zamboni (1972) reported that in the mouse the uterine junction (UTJ) is patent for only a short time after mating. However, as just indicated, spermatozoa from various gene-disrupted mouse strains failed to migrate into the oviducts at the normal time. This indicates that UTJ patency is not controlled solely by the female's physiology after mating. If the UTJ barrier opens in response to
wild-type (WT) spermatozoa or to some male factors, those spermatozoa that cannot pass through the UTJ might have their transport “rescued” with the coexistence of WT spermatozoa. Previously, we examined this possibility by producing a chimeric strain in which Clnn−/− mice were crossed with a transgenic mouse in which the sperm acrosome was tagged using enhanced green fluorescent protein (GFP; Nakanishi et al., 1999). When these chimeras were mated with females, only the WT spermatozoa migrated into the oviduct, whereas the concomitantly ejaculated Clnn−/− spermatozoa remained in the uterus (Nakanishi et al., 2004). From these results, it appears that correct interaction with the UTJ plays an important role in the migration of spermatozoa into the oviducts.

A disintegrin and metalloproteinase domain 3 (ADAM3) requires assistance from at least seven other genes to be delivered properly onto the sperm surface, and in all of the reported gene-disrupted mouse lines indicated earlier (except for Ace, in which the microlocalization of ADAM3 on spermatozoa is impaired), ADAM3 is always missing from the sperm membrane (Ikawa et al., 2010; Tokuiro et al., 2012). Thus the lack of ADAM3 on the sperm surface could be the major reason for the infertility of these gene-disrupted mouse lines. However, there is no concrete evidence that ADAM3 is the element that is ultimately responsible for sperm migration through the UTJ. To examine whether elements other than ADAM3 might be responsible for sperm–UTJ interaction, we performed two-dimensional (2D) gel analyses on sperm extracts and found that there were two proteins missing in the spermatozoa from Clnn-disrupted mice. In the present article, we describe the identification of these proteins, which we named Protein Missing in Infertile Sperm 1 and 2 (Pmis1 and Pmis2, respectively). After cloning of the genes for these proteins (Pmis1 and Pmis2), we produced two gene-KO mouse lines. Pmis1-deficient males showed no apparent phenotype, but Pmis2 disruption resulted in male infertility with a similar phenotype as in the Clnn−/− line. Thus Pmis2 could be the factor responsible for normal sperm migration through the UTJ.

RESULTS
Search for proteins missing in Clnn-KO mouse spermatozoa
To examine whether there might be a factor other than ADAM3 that regulates the ability of spermatozoa to pass through the UTJ into the oviducts, we compared protein extracts from Clnn-KO mice spermatozoa with WT spermatozoa on 2D gels. Assuming that the responsible factor is a membrane protein, we analyzed the detergent-enriched fraction after the extraction of proteins using Triton X-114 and subsequent phase separation. The 2D gel patterns were almost the same in Clnn−/− and WT spermatozoa as seen by silver staining. However, we found two spots that disappeared in Clnn−/− spermatozoa at a PI of 6.7, M, 24 kDa, and a pl of 4.9, M, 18 kDa (Figure 1A and Supplemental Figure S1A). We named these proteins Pmis1 and Pmis2, respectively. We also observed that Pmis2 was missing in spermatozoa from Calr−/− mice (Figure 1A, c and f).

These spots were isolated from WT spermatozoa after Sypro-Ruby staining. After digestion of the proteins with trypsin, the peptides were subjected to analysis by liquid chromatography–tandem mass spectrometry (LC-MS/MS). We identified peptide fragments derived from the testis-specific expressed sequence tag (EST) data (predicted gene 128 [Gm128; NM_001024841] and RIKEN cDNA 4930479M11 [NR_027848]). We searched the National Center for Biotechnology Information database using these EST data and estimated the full coding regions (Figure 1B and Supplemental Figure S2). Northern blot analysis with a probe to full-length cDNA revealed that both of these genes are expressed specifically in the testis (Figure 1C and Supplemental Figure S1B). The estimated sizes of Pmis1 and Pmis2 mRNAs obtained by Northern blotting were 0.98 and 0.68 kb, respectively. Moreover, when we performed reverse transcription PCR (RT-PCR) analysis using mRNA prepared from testes of 1- to 5-wk-old mice, we detected the Pmis2 mRNA only from mice of age 3 wk or older, suggesting that this gene is expressed in germ cells later than the spermatid stage of spermatogenesis (Figure 1D).

Pmis1 consists of 350 amino acids with a calculated PI of 8.17 and a molecular mass of 38 kDa. However, these values were significantly different from the results obtained by the 2D gel. The Pmis1 gene has putative splicing sites, and the hypothetical isoform consists of 190 amino acids with a PI of 6.75 and 21-kDa molecular mass (Supplemental Figure S2). The size of the RNA obtained by Northern blot was 0.98 kb, and this matched the calculated size of the spliced isoform. We estimate that some modifications must have taken place to the putative spliced form of Pmis1 because the molecular mass calculated from the position of Pmis1 on the 2D gel was less than that calculated from the amino acid sequence but greater than the mass calculated after taking into account the putative splicing sites.

Pmis2 consists of 96 amino acids with two hydrophobic regions bearing a single N-glycosylation site. The pl value of this protein was around 5.0 on the 2D gel, and this matched the calculated value of 5.04 based on the amino acid sequence. The apparent molecular mass on the 2D gel (18 kDa) was slightly greater than that of the calculated value (11 kDa), indicating that Pmis2 is probably modified in some manner that might include glycosylation.

Generation of Pmis1- and Pmis2-deficient mice and assessment of fertility
To test the functions of Pmis1 and Pmis2 in sperm migration into the oviduct, we generated Pmis1 and Pmis2 gene–disrupted mouse lines by homologous recombination (Figure 2A and Supplemental Figure S3). Pmis1−/− male mice showed normal mating behavior and vaginal plug formation. Wild-type eggs recovered from the oviducts of superovulated female mice mated with Pmis1−/− males (n = 3) showed slightly lower fertilization rates than controls (Pmis1+/-, 89.8 ± 10.9%; Pmis1−/−, 61.7 ± 8.0%; p < 0.01); however, in natural mating conditions (n = 4), we found no difference in their litter sizes (Pmis1+/-, 9.5 ± 2.4; Pmis1−/−, 9.4 ± 2.3; p = 0.85; Supplemental Figure S4, A and B).

Concerning Pmis2, by mating heterozygous mice, we obtained the expected Mendelian ratios of pups (Pmis2+/+, 31, or 28%; Pmis2−/−, 58, or 52%; Pmis2−/−, 23, or 20%; n = 112). We confirmed that the Pmis2 mRNA was eliminated in the Pmis2−/− testes (Figure 2C), and the Pmis2 protein was also undetectable in the testes and spermatozoa by Western blotting (Figure 2D). To examine the effect of Pmis2−/− on fertilization, we placed the Pmis2-disrupted mice with WT mice for 3 mo. Pups were obtained from Pmis2−/− male and Pmis2−/− female mice with superovulated WT females. Eggs were collected from the oviducts after examining for the formation of a vaginal plug, and the fertilization rate was examined. We could obtain no fertilized eggs from the females that mated with Pmis2−/− males, whereas almost 100% of the eggs were fertilized in the females mated with Pmis2+/− males (Figure 3B). These results suggest that Pmis2 is essential for fertilization by natural mating.

Gene-KO experiments occasionally reveal phenotypes derived from unexpected side effects of the gene manipulation, such as

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silencing a neighboring gene (Yoon et al., 1997) or exon jumping to an adjacent gene (Rossi et al., 2001). Therefore we generated transgenic mouse lines harboring hexahistidine (His6)-tagged Pmis2 cDNA under the regulation of the Clgn promoter and performed a gene rescue experiment (Figure 3C). Although the protein was not recognized by anti-PMIS2 antibody, the expression of recombinant PMIS2 protein was detected using an anti-His antibody. Both of these transgenic mouse lines expressing His6-tagged PMIS2 protein were shown to rescue the infertile phenotype by being incorporated into the Pmis2−/− genetic background (Figure 3, D–F), indicating that the recombinant PMIS2 with His6 tag could function normally and that infertility in the Pmis2−/− mice was caused directly by the absence of Pmis2 from spermatozoa.

In the next experiment, we examined the fertilizing ability of spermatozoa from Pmis2−/− males in vitro. These spermatozoa exhibited normal fusing ability when added to zona-free eggs (unpublished data). However, when the spermatozoa were mixed with zona-intact eggs, the zona-binding ability was significantly decreased compared with WT spermatozoa (Figure 4A). This phenotype was similar to those of spermatozoa lacking ADAM3. However, as reported previously (Nishimura et al., 2004) for an Adam1a-disrupted mouse line, the loss of zona-binding ability did not result in the failure of in vitro fertilization (IVF) when cumulus-intact eggs were used (Figure 4B).

**Impaired migration through the UTJ by PMIS2-deficient spermatozoa**

Because the Pmis2−/− spermatozoa were assumed to have a defect in oviduct migration, we crossed Pmis2−/− mice with Acr-EGFP+/− transgenic mice and examined the ability of their spermatozoa to penetrate the UTJ. This enabled us to visualize the migration of spermatozoa into the oviduct using a live-imaging system. A normal amount of spermatozoa was found in the uterus of females mated with GFP-tagged spermatozoa from Pmis2+/− males together with many spermatozoa in the oviducts of these females (Figure 5, A–C). On the other hand, when the females were mated with Pmis2−/− males producing GFP-tagged spermatozoa, no spermatozoa were found in the oviducts of female mice, whereas many spermatozoa were observed in the uterus (Figure 5, D–F). Thus Pmis2−/− spermatozoa are unable to cross the UTJ.
Fertilization-related proteins in PMIS2-deficient spermatozoa

To clarify the molecular nature of infertility observed in PMis2−/− males, we examined the fertilization-related proteins ADAM1a, ADAM2, ADAM3, testicular ACE (tACE), CLGN, and CALR3 in testes and spermatozoa. The last two are testis-specific molecular chaperones and act to fold ADAM family proteins. Both of these testis-specific chaperones remained unaffected in the PMis2−/− testis (Figure 6A). Although it is assumed that CLGN and CALR3 are involved in the folding of PMIS2, we were not able to detect the interaction of these chaperones with PMIS2 by immunoprecipitation studies.

The levels of CLGN, CALR3, ADAM1a, ADAM2, and ADAM3 were found to be normal in the testes of PMis2−/− males. The formation of ADAM1a/ADAM2 and ADAM1b/ADAM2 heterodimers was also found to be normal (Figure 6A). ADAM1b and ADAM2 expression levels also remained normal in the spermatozoa (Figure 6B). The expression of tACE was also found to be unaffected in PMis2−/− spermatozoa, indicating that PMIS2 must be the factor responsible for infertility. However, we were unable to verify this, as ADAM3 was absent in PMis2−/− spermatozoa. For a further analysis of the link between PMIS2 and ADAM3, we tested for the presence of the PMIS2 protein in Adam3−/− spermatozoa. As shown in Figure 6C, PMIS2 become undetectable in spermatozoa after disruption of Adam3. Thus PMIS2 and ADAM3 are both involved in sperm migration into the oviduct.

DISCUSSION

As a result of ongoing research into the mechanism of fertilization using gene-manipulated animals, a new scheme is emerging to replace the old models (Ikawa et al., 2010). Detailed analysis revealed that in at least seven gene-KO mouse lines (for Clgn, Calr3, Pdilt, Tpst2, Ace, Adam1a, and Adam2), ADAM3 became undetectable (or showed aberrant localization) in spermatozoa and the male mice were infertile (Cho et al., 1998; Nishimura et al., 2004; Yamaguchi et al., 2006; Ikawa et al., 2011; Marcello et al., 2011; Tokuhiro et al., 2012). Therefore ADAM3 is considered to be a key molecule for ensuring fertility.

Even though they show vigorous movement, when they come into contact with zona pellucida WT spermatozoa stick to it very efficiently. All of the various kinds of ADAM3-deficient spermatozoa were shown to fail in binding to the zona pellucida of cumulus-free eggs. If we assume that ADAM3 is directly involved in sperm–zona binding, then quantifying ADAM3 on the sperm surface is of interest. The presence of ADAM3 on spermatozoa has been verified by Western blotting and immunostaining (Kim et al., 2004). However, as far as we know, ADAM3 has never been detected by a direct
spite of multiple trials (e.g., the use of whole recombinant PMIS2 protein expressed in mammalian cells as an antigen), we were not able to obtain an antibody that worked for immunostaining of native PMIS2 or recombinant PMIS2 on spermatozoa. Therefore the interactions of PMIS2 with other molecules and the localization of PMIS2 on the spermatozoon need to be clarified to understand the function of PMIS2 in fertilization.

Spermatozoa from Pmis2−/− mice showed normal fertilizing ability in an IVF system with cumulus-enclosed eggs, despite their impaired zona-binding ability. We do not know why; however, this phenomenon was already reported in spermatozoa lacking ADAM3 (Nishimura et al., 2004; Tokuhiro et al., 2012). In spite of the normal fertilizing ability in IVF, Pmis2−/−-disrupted males were infertile in natural mating. The spermatozoa from Pmis2−/− mice had a deficiency in sperm migration into the oviduct. This indicates that the immediate cause of Pmis2−/− male infertility is the inability of spermatozoa to reach the oviduct rather than impaired zona-binding ability.

staining method on gels such as by silver staining or by proteomic analysis using mass spectrometry. We hypothesized that another molecule(s) might be more abundant than ADAM3 and function directly in sperm–egg binding. On the basis of this hypothesis, we performed a proteomic analysis of mouse sperm extracts and discovered the possible involvement of Pmis1 and Pmis2 in the fertilization process. Here we described the cloning of these genes and their disruption by homologous recombination. Pmis1 was shown not to be essential for fertilization (Supplemental Figure S4), but Pmis2 (RIKEN cDNA 4930479M11), registered as a noncoding gene, was found to be producing mRNA, and the PMIS2 protein was essential for fertilization.

Because PMIS2 became undetectable in sperm by disruption of Clgn or Calr3, we assumed that these chaperones are necessary to fold PMIS2 properly, but we could not demonstrate the interaction of PMIS2 with these molecules by immunoprecipitation. We also tried to identify the localization of PMIS2 on spermatozoa, but in spite of multiple trials (e.g., the use of whole recombinant PMIS2 protein expressed in mammalian cells as an antigen), we were not able to obtain an antibody that worked for immunostaining of native PMIS2 or recombinant PMIS2 on spermatozoa. Therefore the interactions of PMIS2 with other molecules and the localization of PMIS2 on the spermatozoon need to be clarified to understand the function of PMIS2 in fertilization.

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FIGURE 3: Defect of fertilization in Pmis2−/− mice and transgenic rescue. (A) Pmis2+/− males and Pmis2−/− females delivered normal numbers of pups when mated with WT mice, whereas the combination of WT females with Pmis2−/− males produced no pups. (B) The defect was at the fertilization level, because no fertilized eggs were recovered from the superovulated females mated with Pmis2−/− males. Parentheses indicate the numbers of eggs examined. (C) Top, the targeting vector used to disrupt the mouse Pmis2 allele. Bottom, the transgene used for rescue experiments to express Pmis2 under the control of the Clgn promoter. The arrows indicate the positions of primer sets to detect the targeted alleles. (D) Genotyping of tail-tip DNA from various groups of mice by PCR amplification with the primers indicated in Materials and Methods. (E) Protein extracts from Pmis2+/− and Pmis2−/− and spermatozoa from transgenically rescued Pmis2−/− mice were subjected to immunoblot analysis using anti-PMIS2 and anti–His-tag antibodies. ADAM2 was indicated as a reference marker for the successful extraction of proteins. (F) Litter sizes obtained by mating Pmis2+/− transgenically rescued Pmis2−/− males with WT female mice. The sterile phenotype of Pmis2−/− mice was restored by the insertion of the Pmis2 transgene.
Therefore the sperm–zona binding ability measured in vitro using cumulus-free eggs might not be a good indicator of the ultimate fertilizing ability of spermatozoa.

PMIS2 consists of two hydrophobic regions and a putative N-glycosylation site and shows homology to CD225 (also known as interferon-induced transmembrane protein 1) superfamily, based on motif searching. CD225 has been reported to mediate antiproliferative activity and cell–cell adhesion in human B cells and leukocytes (Deblandre et al., 1995). If PMIS2 is related to the interaction of spermatozoa with the zona pellucida or with the epithelial cells of the UTJ, a common motif (amino acid sequence GAP to ILV) might be the responsible domain.

We found that PMIS2 was absent from spermatozoa in three independent mouse KO lines, for Adam3, Clgn, and Calr3. From its speculated abundance on spermatozoa, we assume that PMIS2 could be the ultimate factor involved in sperm migration into the oviduct, possibly based on an interaction of the sperm surface with the UTJ epithelium. However, when we disrupted Pmis2, ADAM3 became undetectable in the spermatozoa. On the other hand, the disruption of Pmis2 did not affect any of the remaining four gene products (Clgn, Calr3, Adam1a, and Adam2). Because both PMIS2 and ADAM3 become undetectable in spermatozoa if the other is not expressed, we assume that these proteins might interact closely. However, we have not been able to demonstrate the formation of heterodimers or direct interactions between the two proteins. The identification of PMIS2 as a novel candidate factor for the ultimate sperm–UTJ interaction (and also sperm–zona binding) will help clarify the general mechanism of mammalian fertilization.

MATERIALS AND METHODS

Two-dimensional gel electrophoresis

Cauda epididymal and vas deferens spermatozoa were collected in phosphate-buffered saline and resuspended in lysis buffer containing 1% Triton X-114. Sperm extracts were separated into two phases as described (Yamaguchi et al., 2006). Detergent-enriched fractions were resuspended in 8 M urea, 2% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, and 10 mM dithiothreitol (DTT). After centrifugation, samples were desalted using MicroSpin G-25 columns (GE Healthcare, Little Chalfont, United Kingdom). Isoelectric focusing was performed in Immobiline DryStrip gels (pH 4–7) with IPG-phor (GE Healthcare UK) at 21°C, 50 μA/strip, and 500 V (1 h), 1000 V (1 h), and 8000 V (4 h). Strips were equilibrated for 20 min in buffer containing 50 mM Tris/HCl (pH 6.8), 6 M urea, 30% glycerol, 1% SDS, and 25 mM DTT and then subjected to SDS-PAGE under reducing conditions. The gels were viewed after silver staining, and digital images were acquired by scanning. Unique spots were detected by visual judgment. Following SyproRuby staining, the spots were isolated and subjected to trypsin digestion. The resultant peptides were subjected to analysis by LC-MS/MS. The analysis was conducted in the APRO Life Science Institute (Tokushima, Japan). MS/MS spectra were searched using the Mascot search engine (Matrix Science, Boston, MA).

Northern blot and RT-PCR analysis

Northern hybridization and RT-PCR were performed as described (Yamaguchi et al., 2008; Fujihara et al., 2010). Briefly, 10-μg aliquots of total RNA extracted from various tissues of adult ICR strain mice (>2 mo old; Japan SLC, Shizuoka, Japan) were hybridized with the cDNAs for mouse Pmis2 and glyceraldehyde-3-phosphate dehydrogenase (Gapdh). The Pmis2 probe consisting of a cDNA fragment was amplified from mouse testis total RNA by PCR using 5′-GGAATTCGACACCCAGATCTGCTAGTTGATGTTTCTCTGG-3′ (Pr-B) and 5′-AGAAGAAGCACGCTAAGCTGGC-3′ (Pr-A) as primers.

These primers were used also for detection of the Pmis2 WT allele.

For the Gapdh gene we used 5′-AGTGGAGATTGTGGCCATCAACGAC-3′ and 5′-GGGAGTTGCTGTTGAAGTCGCAGGATGTTTCTCTGG-3′. RT-PCR was performed using 10 ng of testicular cDNA from 1- to 5-wk-old ICR male mice using the forward and reverse primers 5′-AAGTGTGACGGATGACATCGC-3′ and 5′-GATCCACATCTGCTGGGAAGG-3′, respectively, for the β-actin gene. Primers for Pmis2 were the same as for the Northern blot analysis.

Generation of Pmis2-knockout mice

A targeting vector for the Pmis2 gene disruption was constructed using the pPNT vector containing the Neo-resistance gene (Neo) as a positive selection marker and a herpes simplex virus thymidine kinase (βk) gene as a negative selection marker (Tybulewicz et al., 1991). A 1.5-kb NotI–Xhol fragment as a short arm and a 6.0-kb MfeI–KpnI as a long arm were obtained by PCR using genomic DNA of D3 embryonic stem (ES) cells as a template. The PCR primers used were 5′-AGCGGACCCGGTAGGGAGTCTGCTTTACACCACTAC-3′ and 5′-TCTCGAGGTGTCTGCTGGTAGATGTTCCTG-3′ for the short arm and 5′-CCGTCGAGTCCGCTTCTACATACTGCTGG-3′ and 5′-CCAAAGGACCCAGATCAGTCCACTGGC-3′ for the long arm.

FIGURE 4: Assessment of the fertilizing ability of Pmis2−/− spermatozoa by IVF. (A) Pmis2−/− spermatozoa showed impaired zona-binding ability. (B) However, the spermatozoa were able to fertilize eggs, as well as WT spermatozoa, when added to cumulus-enclosed eggs.
Production of Pmis2 transgenic mice

His6-tagged mouse Pmis2 cDNA with a rabbit polyA signal was inserted downstream of the Clgn promoter. The transgene cassette was injected into the pronuclei of zygotes from B6D2F1 mice (>2 mo old; CLEA Japan). Injected eggs were transferred to pseudo-pregnant females at the two-cell stage. Pups carrying the transgene were screened by PCR using the primers 5′-CCTTCCTGCGGCTTGGTTCTC-3′ (Pr-G) and 5′-CGAATTCTCATAGGACTAGGACAAG-3′ (Pr-H).

IVF assay

Female B6D2F1 mice were superovulated after intraperitoneal injections of equine chorionic gonadotropin (eCG; Teikoku Zoki, Tokyo, Japan) and human chorionic gonadotropin (hCG; Teikoku Zoki) at 48-h intervals. Ovulated egg masses were recovered 13 h after the hCG injection and placed in a 200-μl drop of modified Krebs–Ringer bicarbonate solution (TYH medium; Toyoda et al., 1971) containing glucose, sodium pyruvate, bovine serum albumin, and antibiotics (0.05 mg/ml penicillin, 100 IU/ml streptomycin). Fresh spermatozoa from the cauda epididymis were dispersed in...
the pronuclear stage and cultured as described. Fertilization rates were assessed by pronuclear formation and subsequent two-cell formation.

Sperm–UTJ transition assay

Females from a transgenic mouse line that expressed enhanced GFP in the acrosomes of spermatozoa (Acr-EGFP; Nakashita et al., 1999) were crossed with Pmis2+/− males. Double-transgenic F1 offspring were intercrossed to generate Pmis2+/− and Acr-Egfp+/− strains. These doubly transgenic mice were used for sperm migration analysis as described (Yamaguchi et al., 2009). Briefly, 1–2 h after copulation, the uteri and oviducts were excised and straightened by cutting the mesosalpinx. They were transferred to slides and examined for the presence of spermatozoa expressing the acrosomal GFP marker, using a fluorescence microscope (BZ-8000; Keyence, Osaka, Japan). The experiments were performed twice, and we examined at least six different oviducts in each genotype. The number of mating pairs was five, using five different males. In each of two trials, three males made a vaginal plug. Thus we observed at least three and at most five different males.

Antibodies

A rabbit anti-mouse Pmis2 polyclonal antibody was produced by immunization of rabbits with the mouse Pmis2 peptide–KLH conjugate (CSNWEDAYRNSRTMWF-NML). Rat monoclonal antibodies (mAbs) against mouse IZUMO1 (MAB#125) and ADAM1b (MAB#158) were produced by immunization of rats with whole spermatozoa. Monoclonal antibodies against mouse ADAM2 (fertilin β; 9D2.2) and ADAM3 (cyritestin; 7C1.2) were purchased from Chemicon International (Temecula, CA). Rabbit polyclonal antibodies against CLGN and CALR3 and a mouse mAb against ACE were obtained as described (Yamaguchi et al., 2006; Ikawa et al., 2011).

Immunoblot analysis

Immunoblot analysis was performed as described (Ueda et al., 2007). Briefly, spermatozoa from the epididymis and vas deferens were collected and incubated in lysis buffer containing 1% Triton X-100 for 20 min on ice. The testes were excised and minced, and homogenized in lysis buffer and then placed on ice for 1 h. The spermatozoa and testis extracts were centrifuged, and the supernatants were collected. Proteins were separated by SDS–PAGE under reducing conditions and transferred electrophoretically to polyvinylidene fluoride membranes. After blocking, blots were incubated with primary antibodies overnight at 4°C and then incubated with horseradish peroxidase–conjugated secondary antibodies. The detection was performed using an ECL Western blotting detection kit (GE Healthcare UK). To detect ADAM1/2 heterodimers, testis extracts were separated by SDS–PAGE under nonreducing conditions at 4°C. Detection was performed using a mouse anti-ADAM2 mAb.

Fertility testing

Sexually mature male mice of the Pmis2+/− and Pmis2−/− genotypes were caged with B6D2F1 female mice for 3 mo, and the number of pups in each cage was counted within a week of birth. Female B6D2F1 mice were superovulated after intraperitoneal injections of eCG and hCG. Pmis2+/− and Pmis2−/− males were mated with superovulated females 7 h after hCG injection. Eggs were recovered from females at the pronuclear stage and cultured as described. Fertilization rates were assessed by pronuclear formation and subsequent two-cell formation.

FIGURE 6: ADAM-family proteins in the various gene-disrupted male testes and spermatozoa. Protein extracts from Clgn−/−, Calr3−/−, and Pmis2−/− testes (A) and spermatozoa (B) were separated by electrophoresis and subjected to immunoblot analysis. (A) Molecular chaperones and ADAM-family proteins were unaffected in Pmis2−/− testes, including the normal formation of ADAM1/ADAM2 heterodimers. (B) Pmis2 disappeared in Clgn−/− and Calr3−/− spermatozoa, consistent with the result of 2D electrophoresis. ADAM1b and ADAM2 were found in Pmis2−/− spermatozoa, but ADAM3—present in the testis—was not detected in spermatozoa. (C) Protein extracts from Adam3−/− and Pmis2−/− spermatozoa were subjected to immunoblot analysis. Both ADAM3 and Pmis2 proteins disappeared in spermatozoa from Adam3−/− and Pmis2−/− mice.
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